Mechanism of Assembly of the Outer Membrane of Salmonella typhimurium

SITE OF SYNTHESIS OF LIPOPOLYSACCHARIDE*

(Received for publication, February 11, 1972)

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SUMMARY

The site of synthesis of lipopolysaccharide has been investigated in cytoplasmic and outer membrane fractions isolated by isopycnic sucrose gradient centrifugation of the total membrane fraction from lysozyme-EDTA spheroplasts. Evidence that synthesis of O-antigen occurs exclusively in cytoplasmic membrane was obtained by pulse-chase experiments in vivo and by assay of biosynthetic enzymes in isolated membrane fractions. O-Antigen chains pulse-labeled in vivo with [14C]mannose appeared initially in cytoplasmic membrane, but were rapidly transferred to outer membrane during a subsequent chase with nonradioactive mannose. In accord, the specific activities of enzymes of O-antigen synthesis in isolated cytoplasmic membrane fractions were 15 to 30-fold greater than in outer membrane. The cytoplasmic membrane fractions were also enriched for glycosyltransferase activities involved in biosynthesis of the core region of lipopolysaccharide. However, unequivocal localization of core transferase enzymes in the isolated membranes was not possible since these activities were also found with soluble fraction, and secondary binding of soluble enzyme to both cytoplasmic and outer membrane was shown to occur during the isolation procedure. Evidence that the cytoplasmic membrane is the site of synthesis of the core region was derived from pulse-chase experiments in mutants in which [14C]galactose is incorporated exclusively into the core portion of the polysaccharide. Over 90% of the [14C]galactose incorporated into lipopolysaccharide during a 1-min pulse was recovered in the cytoplasmic membrane fraction.

The mechanism of translocation of lipopolysaccharide from the cytoplasmic to the outer membrane is unknown. Evidence suggesting that the process is not readily reversible was obtained in experiments in mutants with conditional defects in lipopolysaccharide synthesis. Incomplete lipopolysaccharides incorporated into outer membrane during growth under nonpermissive conditions could not be completed following subsequent shift to permissive growth conditions.

The external layer of the cell envelope of Salmonella and related gram-negative enteric bacteria is a membranous structure which contains the lipopolysaccharide of the envelope in addition to phospholipid and protein (1-4). The recent development of techniques for separation of this outer membrane from the underlying cytoplasmic membrane (1-3) has opened a new approach to investigation of the mechanism of assembly of the outer membrane and the role of the cytoplasmic membrane in this process. The present studies were undertaken to determine whether synthesis of lipopolysaccharide occurs in situ in the outer membrane, or whether the polymer is synthesized in the cytoplasmic membrane and only subsequently inserted into the outer membrane structure. The results of pulse-chase experiments in vivo and localization of biosynthetic enzyme activities in isolated membrane fractions strongly support the latter hypothesis. The data indicate that synthesis of the internal core region of the polysaccharide and the O-antigen, as well as attachment of O-antigen chains to the core, occurs exclusively in the cytoplasmic membrane, and that newly synthesized lipopolysaccharide is then rapidly translocated into the outer membrane.

The mechanism of the translocation process from cytoplasmic to outer membrane remains to be established. Evidence suggesting that translocation is not readily reversible was obtained from studies with conditional mutants in lipopolysaccharide synthesis, which showed that the incomplete lipopolysaccharides synthesized under nonpermissive growth conditions could not be completed during subsequent growth under permissive conditions. The results indicate that lipopolysaccharide which is integrated into the outer membrane is not available to biosynthetic enzymes in the cytoplasmic membrane, and suggest that outer membrane lipopolysaccharide is not in equilibrium with the nascent polymer at the sites of synthesis.

* This work was supported by United States Public Health Service Grant AI-08650 and a research grant from The American Heart Association. Paper 1 in this series is Reference 1.
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EXPERIMENTAL PROCEDURES

Bacteria and Media—The following mutants of Salmonella typhimurium LT-2 were isolated in this laboratory: G-30, UDP-galactose-4-epimerase-negative (epi-) (5); GB-1, a derivative of G-30 blocked in the first step of O-antigen synthesis (epi-rfb); GRA-5, a rfa derivative of G-30 (6); and M3D11, phosphomannose isomerase negative (pmi-) (7). SL 3656, an amber mutant in UDP-glucose-lipopolysaccharide glycosyltransferase I and SL 3657, an amber mutant in UDP-galactose-lipopolysaccharide α,3-galactosyltransferase (galactosyltransferase I) were obtained from Drs. T. T. Kuo and B. A. D. Stocker, Department of Medical Microbiology, Stanford University School of Medicine. TV119 (rfb) (8) and SL 1032 (glucosyltransferase I negative) (9) were also from the collection of Dr. Stocker.

Bacteria were grown in proteose-peptone-beef extract medium (Medium A) (10), with supplements as indicated. Growth was at 37°C with vigorous aeration.

Chemicals—All radiochemicals were purchased from New England Nuclear. Other reagents were obtained from standard commercial sources. Phosphatidylethanolamine purified from S. typhimurium was the kind gift of Mr. A. Hinckley of this department.

Lipopolysaccharides—These were isolated by aqueous phenol extraction as previously described (5) and purified by the procedures of Romeo et al. (11).

Preparation of Glycosyl Carrier Lipid and O-Antigen Intermediates

Glycosyl Carrier Lipid (Undecaprenyl Phosphate)—Details of the purification procedure will be presented elsewhere. Briefly, the method involves alkaline hydrolysis of a crude lipid extract from strain G-30 and chromatography of the alkali-stable fraction on DEAE-cellulose. Cells (200 g wet weight) were extracted with 3 liters of CHCl₃-methanol (2:1). The extract was washed with CHCl₃-methanol-H₂O (3:48:47) containing 0.05 M KCl (12), evaporated to dryness under reduced pressure at 0°C, and taken up in 60 ml of CHCl₃-methanol (2:1). Alkaline hydrolysis was carried out on 20-ml aliquots by the method of Brockenhoff (13), and the organic phases pooled and concentrated for chromatography. The sample was applied in CHCl₃-methanol (1:1) to a 2.5 × 20 cm column of DEAE-acetate, prepared by the method of Roussel et al. (14). The column was washed with 200 ml of CHCl₃-methanol (1:1) and 600 ml of 99% methanol and P-GCL was then eluted with 600 to 800 ml of 50 mM ammonium acetate-5 mM acetic acid in 99% methanol (15). Peak fractions were pooled and washed with H₂O to remove salt. Purity at this stage was approximately 50% on the basis of total phosphate, as judged by thin layer chromatography and enzymatic assay in the galactose-PP-GCL synthetase system. After rechromatography on a column (0.9 × 10 cm) of DEAE-cellulose under the same conditions, the product gave a single spot on thin layer chromatography in three systems, and was the preparation employed in the present studies. Enzymatic conversion to galactose-PP-GCL was 75 to 80% based on total phosphate.

Man-Rha-Gal-PP-GCL—Enzyme synthesis of the trisaccharide-lipid intermediate (Fig. 1) was carried out using a soluble enzyme preparation obtained by extraction of cell envelope (5) from strain G-30 with Poly-Tergent S-305-LF (Olin Chemicals). The cell envelope (approximately 30 mg per ml protein) in 50 mM Tris, pH 8.5 was stirred with 0.07 volume of a 10% (v/v) solution of the detergent for 5 min at 0°C and centrifuged at 105,000 × g for 1 hour at 2-5°C. The supernatant fraction contained over 50% of the total activity of the enzymes leading to

1. L. Müller, J. M. Gilbert, and M. J. Osborn, manuscript in preparation.
2. The abbreviations used are: P-GCL, phosphoglycosyl carrier lipid (undecaprenyl phosphate); PP-GCL, pyrophosphoryl glycosyl carrier lipid; DTT, dithiothreitol; KDO, 2-keto-3-deoxyoctonate (3-deoxy-d-manno-2-octulosonate).
trisaccharide-PP-GCL synthesis. The soluble enzyme preparation was diluted with glycerol (20% (v/v) final concentration) and stored at -18°C. Synthesis of the intermediate was as follows. Purified P-GCL (150 nmol) in CHCl₃ was evaporated to dryness under a stream of N₂, taken up in 0.12 ml of methanol plus 0.42 ml of 0.5% (v/v) Alfonie 1012-6 (Conoco Petrochemicals, Rockville, Maryland), and dispersed by vigorous mixing on a Vortes mixer. To this were added 600 nmol of MgCl₂, 750 nmol each of UDP-galactose and TDP-rhamnose, 600 mmol of GDP-[¹⁴C]mannose (4,500 to 11,000 cpm per nmol) and 1.5 ml of enzyme (1.65 mg of protein). After incubation at 25°C for 2 hours, the reaction mixture was extracted with 18 ml of CHCl₃-methanol (2:1). The product was recovered in the organic phase, which was washed three times with 5 ml of methanol-0.1 M KCl (1:1). The washed organic phase was evaporated to dryness under a stream of N₂ in the cold, and dissolved in 2 ml of 0.1% (v/v) Alfonie 1012-6. The yield of Man-Rha-Gal-PP-GCL was approximately 110 nmol. The product was used as substrate for O-antigen polymerase without further purification, and could be stored at -70°C for up to 10 days without significant degradation.

**Polysaccharide-PP-GCL—**Enzymatic synthesis of the GCL-linked polymer of trisaccharide repeating units was carried out as follows: ⁴ The incubation mixture contained 0.18 M Tris-maleate buffer, pH 6.0, 12.5 mM MgCl₂, 0.8 mM of [¹⁴C]mannose-labeled trisaccharide-PP-GCL in 0.1% Alfonie 1012-6 (40 to 50 nmol) and cell envelope (18 mg of protein) in a total volume of 3.25 ml. The cell envelope fraction was obtained from strain B 69 (rif, esp) in order to prevent transfer of the polymeric product to endogenous lipopolysaccharide during the course of the reaction (16). After incubation for 2 hours at 25°C, the reaction mixture was extracted with 12 ml of CHCl₃-methanol (2:1), and the phases were separated by centrifugation. The product was recovered in the insoluble material at the interface. The aqueous and organic phases were carefully removed and the residue was suspended in 2 ml of 0.25% (v/v) Alfonie 1012-6. The suspension was sonicated in an ice bath for 3-5 min periods, keeping the temperature below 15°C. Insoluble material was removed by centrifugation at 105,000 × g for 1 hour at 2–5°C. Recovery of polysaccharide-PP-GCL in the soluble fraction was 60 to 80%. The preparation was free of P-GCL and trisaccharide-PP-GCL and was used as substrate for O-antigen ligase without further purification. The preparation was stable to storage at -70°C for several days.

**Enzyme Assays**

**DPNH Oxidase—**Assays were carried out as previously described (1).

**UDP-sugar Hydrolyase—**Hydrolysis of UDP-glucose was determined by the method of Glaser et al. (17).

**Galactose-PP-GCL Synthetase—**Transfer of galactose from UDP-[¹⁴C]galactose to purified P-GCL was measured. ¹ Incubation mixtures contained in a volume of 0.10 ml: 50 mM Tris-acetate, pH 5.5, 10 mM MgCl₂, 0.2 mM UDP-[¹⁴C]galactose (approximately 6000 cpm per nmole), purified P-GCL (25 μg based on phosphate), 10% methanol, 0.075% Alfonie 1012-6, and membrane fraction (2 to 50 μg of protein). After incubation for 15 min at 25°C, reaction was stopped by addition of 2 ml of CHCl₃-methanol (2:1). The mixture, which gave a single phase, was permitted to stand at room temperature for 5 min and then extracted three times with 0.5 ml of CHCl₃-methanol-H₂O (3:48:47) containing 0.05 M KCl. Phases were separated by brief centrifugation, and the upper aqueous layer discarded. The organic phase was transferred to a scintillation vial, dried under a heat lamp, and counted in toluene Liquifluor-Bisolvon scintillation fluid (1). Values were corrected for blank tubes in each of which CHCl₃-methanol was added before enzyme.

**O-Antigen Polymerase—**The assay was based on conversion of isolated trisaccharide-lipid intermediate, [¹⁴C]Man-Rha-Gal-PP-GCL, to a polymeric product. ⁵ Although the trisaccharide intermediate is not the normal substrate for polymerization in vivo (Fig. 1), previous studies have shown (18) that it is an effective substrate in vitro. Incubation mixtures contained in a volume of 45 μl: 0.22 M Tris-maleate, pH 6.6, 11 mM MgCl₂, 10 μl of [¹⁴C]Man-Rha-Gal-PP-GCL in 0.1% Alfonie 1012-6 as described above (approximately 0.5 nmole, 11,000 cpm per nmole), and membrane fraction (5 to 30 μg of protein). After incubation for 90 min at 25°C, the reaction was stopped by heating in a boiling H₂O bath for 3 min. The entire reaction mixture was streaked on a sheet of Whatman No. 40 paper and developed in Solvent V for 18 to 24 hours. Polymeric products remained within the first 2 inches of the chromatogram. This portion of the paper was cut out and counted as described under "Counting Procedures." Values obtained were corrected for blanks containing boiled enzyme.

**O-Antigen Ligase—**Transfer of O-antigen chains to lipopolysaccharide was measured by conversion of isolated [¹⁴C]-polysaccharide PP-GCL to an alkali stable product. ⁶ Acceptor lipopolysaccharide was prepared by incubating a mixture of 20 μl of TV119 lipopolysaccharide (11.2 nmol of heptose per ml), 10 μl of 0.5 M Tris-maleate, pH 6.0 and 10 μl of 0.5% (v/v) Alfonie 1012-6 for 30 min at 37°C. Fifty microliters of [¹⁴C]-polysaccharide-PP-GCL (2500 cpm) and 10 μl of membrane fraction (5 to 30 μg of protein) were then added, and the mixture incubated for 1 hour at 25°C. At the end of the incubation period 1 ml of 2 N NH₄OH-5 mM MgCl₂ was added and the mixture was incubated for 2 hours at 37°C in order to hydrolyze the alkali-labile glycosyl-PP-GCL linkage (19). After this treatment lipopolysaccharide is retained by a Millipore filter, while the hydrolyzed substrate passes through the filter. Samples were collected on a Millipore filter (type HA), washed three times with 5 ml of 0.1 N acetic acid, dried, and counted. Values were corrected for blanks containing heat-inactivated enzyme.

**UDP-galactose lipopolysaccharide α,3 Galactosyltransferase—**A modification of the procedure of Endo and Rothfield (20) was employed which permitted use of exogenous lipopolysaccharide acceptor for assay of membrane-bound enzyme activity. Acceptor was prepared according to Endo and Rothfield (20) and contained galactose-deficient lipopolysaccharide from strain G-30, S. typhimurium phosphatidylethanolamine and Tris buffer, pH 7.5. Incubation mixtures contained in a volume of 0.125 ml, 70 μl of acceptor, 8 mM MgCl₂, 0.12 mM UDP-[¹⁴C]galactose (5000 to 6000 cpm per nmole), 0.3% Alfonie 1012-6, and enzyme (2 to 20 μg of protein). After incubation for 20 min at 37°C, 2 ml of cold 5% trichloroacetic acid were added, and the product was collected on a Millipore filter (type HA, 0.45 μ), washed, dried, and counted (1). Addition of the nonionic detergent to the reaction mixture had no inhibitory effect on the activity of either
Site of Synthesis of Lipopolysaccharide in S. typhimurium

Vol. 247, No. 12

20  40  60
FRACTION NO.

Fig. 2. Transfer of pulse-labeled O-antigen from cytoplasmic mem- 
brane to outer membrane. Strain M23DJ3 was grown to mid-
log phase (A600 = 0.58) in 160 ml of Medium A containing 1 mM 
[2-H]glycerol (0.17 μCi pmol). The cells were harvested by 
centrifugation (3 min, 12,500 rpm, 25°C) and resuspended in 10 ml 
of fresh Medium A. [3H]Mannose (13 nmoles, 0.65 μCi) was added 
and the culture aerated vigorously for 1 min at 25°C. At this time 
5 ml were quickly pipetted into a mixture of 10 g of ice, 20 ml of 
Nediaun A and 1 ml of 0.1 M 2,4-dinitrophenol (pulse sample); the 
remainder was diluted with 20 ml Medium A containing 200 pmoles 
of nonradioactive mannose, incubated for an additional 2 min at 
25°C with rigorous aeration (chase sample), and poured into 10 g of 
ice plus 1 ml of 0.1 M dinitrophenol. The cells were collected im-
mediately by brief centrifugation (1 min at 12,500 rpm, 4°C), and 
quickness suspended in 10 ml of 0.75 M sucrose,10 mM Tris-acetate 
(pH 7.8):2.5 mM dinitrophenol containing 200 μg of lysozyme. 
Preparation of spheroplasts and isolation of membranes was 
carried out as previously described (1). Cytoplasmic and outer 
membranes were separated by isopycnic sucrose gradient centri-
fugation according to the standard procedure (1). a, sucrose gra-
dient pattern after 1 min pulse with [14C]mannose. b, sucrose gra-
dient pattern following 2 min chase. Approximately 40% of 
this sample was lost during transfer of the spheroplasts to the 
tube employed for sonication. [3H]Mannose, O; [2-H]glycerol, C. 
The tops of the gradients are at the right of the figures. L1 and L2 
designate cytoplasmic membrane fractions; and H, outer mem-
brane (1).
The values given represent total $^3$H and $^{14}$C in cytoplasmic and outer membrane fractions following a 1-min pulse of strain M2D3 with $[^14]$C mannose and subsequent 2-min chase with nonradioactive mannose. The data were obtained from the sucrose gradients shown in Fig. 2. The radioactivity in cytoplasmic membrane was obtained by summing Peaks L1 plus L2 as indicated in Fig. 2; Peak H was taken as outer membrane. The small amount of radioactivity in the region of the gradient between H and L0, which corresponds to unseparated envelope material, was ignored for purposes of these calculations. The data for the chase sample were corrected for loss of part of the sample (see legend to Fig. 2) using as correction factor the $^3$H ratio of the total membrane fractions prior to centrifugation ($^3$H of pulse sample to $^3$H of chase sample = 1.61). The observed recoveries of $^3$H and $^{14}$C in fractions from the chase samples (Line 2) were multiplied by this factor to yield the values given as corrected chase (Line 3).

| Sample         | Recovery of radioactivity (cpm X 10^{-3}) |
|----------------|------------------------------------------|
|                | $^{3}$H | $^{14}$C | $^{3}$H+$^{14}$C | $^{3}$H | $^{14}$C | $^{3}$H+$^{14}$C |
| Pulse (1 min)  | 15.8   | 29.3   | 1.9             | 15.3   | 16.9   | 1.1            |
| Chase (2 min)  | 9.9    | 3.1    | 0.3             | 9.4    | 35.5   | 3.9            |
| Corrected chase| 15.9   | 5.0    | 1.2             | 15.2   | 57.2   | 3.9            |
| Difference (corrected chase - pulse) | -24.3 | +40.3  | +16.0          |

**Fig. 3.** Distribution of galactose-PP-GCL synthetase activity following sucrose gradient centrifugation. The total membrane fraction was isolated from a 700-ml culture of strain G-30 by the usual procedure (1). Dithiothreitol (0.2 mM) was added prior to lysis of spheroplasts, and was present in all subsequent solutions. Isopycnic sucrose gradient centrifugation was carried out as described (1) in the SW 27 rotor for 20 hours at 25,000 rpm and 4°C. Fractions (0.4 ml) were collected in the cold with a tube puncturing device. Refractive indices were measured, and the fractions then diluted with 0.3 ml of 0.2 mM DTT to reduce viscosity. Determination of protein and assay of enzyme activities were carried out as described under "Experimental Procedures." a, protein (O---O) and buoyant density (X---X). b, enzyme activities: galactose-PP-GCL synthetase, ▲--▲; DPNH oxidase, ■--■; UDP-glucose hydrolase, ○--○.

The product of $[^14]$C mannose incorporation into cytoplasmic and outer membrane was characterized in a parallel experiment. Approximately 95% of the $[^14]$C mannose incorporated into the membranes was recovered in the cytoplasmic membrane fraction (L1 + L2); during the subsequent 2 min incubation with nonradioactive mannose over 80% of this radioactivity was chased out of the cytoplasmic membrane and into the outer membrane band. Although some increase in total $^{14}$C incorporation into membrane occurred during the chase, this accounted for less than one-half of the net flow of $^{14}$C into outer membrane during this time. It should be noted that the data for the chase sample in Table I have been corrected as described in the legend for loss of part of the sample during preparation of the spheroplast lysate.

The product of $[^14]$C mannose incorporation into cytoplasmic and outer membrane was characterized in a parallel experiment. Approximately 95% of the $[^14]$C was in both membranes was present as lipopolysaccharide as judged by phenol extraction, differential ethanol precipitation, and DEAE-chromatography (6). No significant accumulation of lipid-linked intermediates of O-antigen synthesis was observed under the pulse-labeling conditions employed in these experiments.

Under the conditions of time and temperature employed here, incorporation of $[^14]$C mannose into the outer membrane during the pulse was appreciable; however, by decreasing the time and temperature of the pulse over 90% of the mannose incorporated into the membranes could be recovered in the cytoplasmic membrane fraction.

**Distribution of Enzymes of O-Antigen Synthesis in Isolated Membrane Fractions.—**Further evidence for localization of the enzymes of O-antigen synthesis in the cytoplasmic membrane was obtained by assay of these activities in the isolated fractions. The distribution of the first enzyme of the pathway, galactose-PP-GCL synthetase (Reaction 1, Fig. 1), in the sucrose gradient is illustrated in Fig. 3. The assay system included purified P-GCL as exogenous acceptor in order to permit detection of enzyme activity independent of endogenous levels of the acceptor lipid. The distributions of DPNH oxidase, a cytoplasmic enzyme into outer membrane during the 2 min chase (Fig. 2b). The recoveries of radioactivity in cytoplasmic and outer membrane are summarized in Table I. At the end of the pulse period, 64% of the total $[^14]$C mannose incorporated into the membranes was recovered in the cytoplasmic membrane fraction (L1 + L2); during the subsequent 2 min incubation with nonradioactive mannose over 80% of this radioactivity was chased out of the cytoplasmic membrane and into the outer membrane band. Although some increase in total $^{14}$C incorporation into membrane occurred during the chase, this accounted for less than one-half of the net flow of $^{14}$C into outer membrane during this time. It should be noted that the data for the chase sample in Table I have been corrected as described in the legend for loss of part of the sample during preparation of the spheroplast lysate.

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*J. E. Gander and M. J. Osborn, manuscript in preparation.*
membrane marker (1), and UDP-glucose hydrolyase, which is present in both membranes (1), are included for comparison. Galactose-PP-GCL synthetase activity closely paralleled that of DPNH oxidase and was virtually absent from the outer membrane band, II.

The specific activities of galactose-PP-GCL synthetase, O-antigen polymerase (Reaction 5, Fig. 1) and O-antigen:lipopolysaccharide ligase (Reaction 6, Fig. 1) in the isolated fractions are summarized in Table II. The activities of the latter two enzymes were determined independently of preceding steps in the pathway by use of isolated triaccharide PP GCL as substrate for the polymerase reaction, and isolated polymer-PP-GCL plus exogenous acceptor lipopolysaccharide as substrates for the ligase reaction. The enzymes are firmly membrane-bound, and all the activity of the lyase was recovered in the total membrane fraction. The distribution of the three activities was similar, and all were clearly localized in the cytoplasmic membrane fractions, L1 and L2. The specific activities in L1 were somewhat higher than L2, as previously observed (1) with DPNH oxidase and other cytoplasmic membrane enzymes. The difference is thought to reflect a somewhat higher contamination of L2 by outer membrane material (1). Some activity was also present in the minor Fraction M, which has been identified as unseparated cell envelope fragments (1), but the residual activity in the outer membrane fraction, H, was less than 10% that of the cytoplasmic membrane. The values observed were consistent with the levels of residual DPNH oxidase activity in this fraction.

**Site of Synthesis of Core Region of Lipopolysaccharide**

**Distribution of Core Glycosyltransferase Activities**—The distal portion of the core region of the lipopolysaccharide of *S. typhimurium* consists of a branchel pentasaccharide unit containing glucose, galactose, and N-acetylgalcosamine (cf. Fig. 8). This region of the polysaccharide is formed by successive transfer of single sugar residues from the appropriate nucleotide sugar directly to the growing polysaccharide chain (25). Two of the glycosyltransferase enzymes have been characterized in detail. These are UDP-glucose-lipopolysaccharide glucosyltransferase I (21) and UDP-galactose-lipopolysaccharide α,3-galactosyltransferase (galactosyltransferase I) (20), which catalyze the sequential addition of glucose and galactose according to Reactions 1 and 2:

\[
\text{UDP Glc} + [\text{Hep Hep KDO . . .}] \rightarrow \text{Glc-}[\text{Hep-Hep-KDO . . .}] + \text{UDP} \\
\text{UDP-Gal} + \text{Glc-}[\text{Hep-Hep-KDO . . .}] \rightarrow \alpha,3\text{-Gal-Glc-}[\text{Hep-Hep-KDO . . .}] + \text{UDP} 
\]

[1] [Hep-Hep-KDO . . .] represents the internal backbone region of the lipopolysaccharide. The distribution of these two activities in isolated cytoplasmic and outer membrane was determined using a modification of the usual assay procedure which permits assay of membrane-bound transferase activity independently of the presence or nature of endogenous lipopolysaccharide. Reactions were carried out in the presence of exogenous acceptor lipopolysaccharide (added as a lipopolysaccharide-phosphatidyl-ethanolamine complex (20)) and nonionic detergent (see "Experimental Procedures" for details). In contrast to the enzymes of O-antigen synthesis which are firmly membrane-bound and were associated exclusively with cytoplasmic membrane, the core glycosyltransferase activities are partially soluble (20, 21), and significant activity was found in both cytoplasmic and outer membrane (Table III). However, the distribution of activity between soluble and total membrane fractions, and between cytoplasmic and outer membrane, was dependent on the method employed for preparation of the membranes. When spheroplasts were lysed by sonication, only 20% of the galactosyltransferase and 38% of the glucosyltransferase activity was membrane-bound, and in such preparations the specific activities of cytoplasmic and outer membrane were similar. After lysis by osmotic shock, 70% of each activity was recovered in the washed total membrane fraction, and in this case the specific activities in the cytoplasmic membrane fractions (L1 and L2) were 3 to 6-fold greater than that of outer membrane. The results ob-

**Table II**

| Fraction | Galactose-PP-GCL synthetase | O-Antigen polymerase | O-Antigen ligase |
|----------|-----------------------------|----------------------|-----------------|
| Total membranes | 7.9 | 1.5 | 0.64 |
| L1 | 35.5 | 8.8 | 1.57 |
| L2 | 17.0 | 5.6 | 1.24 |
| M | 1.8 | 2.0 | 0.30 |
| H | 1.1 | 0.6 | <0.06 |

**Table III**

| Enzyme | Method of lysis | Total activity on membrane bound | Specific activity |
|--------|-----------------|----------------------------------|-------------------|
| Glucosyltransferase I | Sonication | 37.5 | 0.41 |
| Osmotic shock | 74.1 | 0.70 | 1.77 |
| Galactosyltransferase I | Sonication | 20.7 | 0.65 |
| Osmotic shock | 69.2 | 2.00 | 2.82 |

* N.D., not determined.

Although little or no dissociation of enzyme activity from these membranes was encountered during the washing procedure prior to sucrose gradient centrifugation, partial dissociation of both activities occurred under the conditions of the sucrose gradient. As much as 33% of the galactosyltransferase activity of the total membrane fraction, and 15 to 20% of the glucosyltransferase, was found in the soluble enzyme fraction at the top of the gradient.
tained with gentle lysis suggested the cytoplasmic membrane as the major site of core synthesis, but the significance of the relatively high activity in the outer membrane, which was not consistent with the localization of enzymes of O-antigen synthesis, remained to be assessed.

Soluble galactosyltransferase has been shown by Rothfield and co-workers to bind to lipopolysaccharide-phospholipid complexes in bulk dispersion (27) and in monolayer films (28). This suggested that enzyme activity associated with isolated outer membrane might arise by secondary binding of soluble enzyme released during spheroplast lysis. This possibility was tested in experiments in which partially purified galactosyltransferase I was added during lysis of spheroplasts obtained from an amber mutant in galactosyltransferase I (SL 3657). Spheroplasts were lysed either by sonication or osmotic shock, and membrane fractions isolated by the usual procedure. Two concentrations of soluble enzyme were employed, the lower of which (14 units per 140 A₆₀₀ units of culture) corresponded to the total galactosyltransferase content of an equivalent lysate of strain G-30. The results (Table IV) showed that added soluble enzyme was indeed bound to the membranes, and the binding of the exogenous enzyme was very similar to that of endogenous galactosyltransferase in G-30. Activity was recovered in both cytoplasmic and outer membrane fractions, and the specific activities of the isolated fractions were comparable to those seen in G-30. Residual galactosyltransferase activity observed in the amber mutant in the absence of added enzyme is presumably due to the presence of galactosyltransferase II (α,6-transferase, see Fig. 8). Entirely comparable results were obtained in similar experiments employing partially purified glucosyltransferase I and an amber mutant in this enzyme (SL 3656). The results of these experiments indicated that secondary binding of soluble core transferases released during lysis could account for essentially all of the activity recovered in the membrane fractions, and the observed distribution of activity was therefore not interpretable in terms of localization in vivo.

The observed distribution of core transferases was similar to that previously found (1) for endonuclease I and RNAse I. The latter enzymes belong to the group of periplasmic proteins which are partially or totally released into the medium in the osmotic cold shock procedure of Heppel and co-workers (29). The possibility that the core transferases were also periplasmic was therefore tested (Table V). Although 90% of the known periplasmic enzyme, cyclic phosphodiesterase, and over 40% of the cytoplasmic enzyme, phosphoglucone isomerase were released under the conditions of the experiment, only 10% of the galactosyltransferase activity was found in the shock fluid.

If core transferase activities were normally present in the outer membrane of intact cells, it might be expected that the enzymes would be available to exogenous substrate. Incorporation of [³⁵S]galactose from externally added UDP-[³⁵S]galactose into endogenous galactose-deficient lipopolysaccharide was therefore measured in intact cells of a mutant lacking UDP-

| Experim. | Method of lysis | Enzyme added | Activity recovered |
|----------|----------------|--------------|--------------------|
| I        | Sonicaton      | units        | Total membrane | L₁ + L₂ | H | Top of gradient |
|          |                |              | units          |         |    |                |
| 1        | Sonicaton      | 1.02         | 0.59          | 0.21    | 0.10 |
| 14       | 2.53           | 1.14         | 0.56          | 0.51 |
| 25       | (0.80)         | (1.24)       | (0.32)        | 0.31 |
| II       | Osmotic shock  | 1.62         | 0.39          | 0.60    | 0.28 |
| 14       | 9.60           | 1.59         | 1.43          | 5.70 |
| 25       | (3.20)         | (1.96)       | (0.85)        | 7.80 |

Values in parentheses represent specific activity (nanomoles per min per mg of protein).

### Table IV

**Binding of added soluble galactosyltransferase I to membrane fractions**

A culture of SL 3657 in 550 ml of Medium A was harvested in mid-exponential phase (A₆₀₀ = 0.75). Spheroplasts were prepared as usual, and the spheroplast suspension divided into three equal parts. Partially purified soluble galactosyltransferase was added during lysis as follows. In Experiment I, the spheroplast suspensions were sonicated for 15 s, soluble enzyme was then added in the amounts indicated, and sonication continued for two additional 15-s periods. DTT (0.2 mM) was added prior to sonication. In Experiment II, the spheroplast suspensions were sonicated for 15 s, soluble enzyme was then added in the amounts indicated, and sonication continued for two additional 15-s periods. DTT (0.2 mM) was added prior to sonication. In Experiment II, the spheroplast suspensions were sonicated for 15 s, soluble enzyme was then added in the amounts indicated, and sonication continued for two additional 15-s periods. DTT (0.2 mM) was added prior to sonication. In Experiment II, the spheroplast suspensions were sonicated for 15 s, soluble enzyme was then added in the amounts indicated, and sonication continued for two additional 15-s periods. DTT (0.2 mM) was added prior to sonication.

### Table V

**Resistance of core galactosyltransferase I to release by osmotic cold shock**

A culture of strain G-30 in Medium A (200 ml) was harvested in mid-log phase (A₆₀₀ = 1.0), washed three times with 10 ml of 10 mM NaCl in the cold, and finally suspended in 10 ml of 20% sucrose (w/w)-33 mM Tris, pH 7.3-2 mM EDTA at room temperature. The suspension was stirred for 10 min at room temperature and centrifuged for 10 min at 12,000 rpm and 25°. The supernatant solution (sucrose supernate) was saved in the cold. The pellet was rapidly suspended in 10 ml of cold H₂O, stirred 10 min in the cold, and centrifuged at 4° for 10 min at 12,000 rpm. The supernatant solution (shock fluid) was saved in the cold. The residual cell pellet (shocked cells) was suspended in 10 ml of 50 mM Tris, pH 7.8-1 mM EDTA and sonicated for a total of 1 min in an ice-salt bath with intermittent cooling. Activity of galactosyltransferase I was measured as described under “Experimental Procedures.”

**Cyclic phosphodiesterase was determined by the method of Neu and Happler (29), using 2',3'-cyclic CMP as substrate. Phosphoglucone isomerase was measured spectrophotometrically; conversion of fructose-6-P to glucose-6-P was monitored in a coupled assay system using glucose-6-P dehydrogenase and TPN (30).**

| Fraction     | Protein | Galactosyltransferase | cyc-Photophosphodiesterase | Phosphoglucone isomerase |
|--------------|---------|----------------------|---------------------------|--------------------------|
| Sucrose supernate | 3.5     | 3.8                  | 7.5                       | 2.6                      |
| Shock fluid   | 24.5    | 10.0                 | 90.8                      | 46.3                     |
| Shocked cells | 71.0    | 86.2                 | 17                       | 51.1                     |
Incorporation of externally added UDP-[14C]galactose into lipopolysaccharide by intact cells. A culture of strain G-30 (UDP-galactose-4-epimerase negative) in Medium A (150 ml) was harvested in mid-log phase (A600 = 0.60) and resuspended in 5 ml of 20 mM PO4 buffer, pH 7.5. A portion of the suspension (2 ml) was sonicated for a total of 1 min with intermittent cooling to maintain the temperature below 10°. Incorporation of [14C]galactose from UDP-[14C]galactose into endogenous lipopolysaccharide was measured in incubation mixtures containing 0.1 M Tris-acetate, pH 8.5, 8 mM MgCl2, 0.08 mM UDP-[14C]galactose (5600 cpm per nmole) and 0.20 ml of cell suspension or sonicate in a total volume of 1.5 ml. Nonradioactive galactose-1-P (0.4 mM) was also present where indicated. Incubation was at 37°. At the times indicated 0.2-ml aliquots were removed into 2 ml of cold 5% trichloroacetic acid containing 10 mM EDTA and centrifuged in the cold for 5 min at 8000 rpm. Pellets were resuspended in trichloroacetic acid-EDTA, collected on a Millipore filter, washed three times with 5 ml of trichloroacetic acid-EDTA, dried, and counted as described under "Counting Procedures." Values were corrected for blanks in which substrate was added after trichloroacetic acid.

Galactose 4-epimerase (Fig. 4). Whereas a sonicated cell suspension catalyzed rapid transfer of [14C]galactose into the endogenous lipopolysaccharide of the cell envelope, only traces of incorporation were observed with intact cells. This marginal activity was abolished by addition of nonradioactive galactose-1-P, and probably resulted from hydrolysis of UDP-galactose by UDP-sugar hydrolase present in outer membrane (1) and subsequent uptake of galactose-1-P or free galactose. The results suggested, but did not prove, that functional core transference activity was absent from the outer membrane.

Pulse-Chase Experiments in Vivo—More convincing evidence for localization of core biosynthesis in the cytoplasmic membrane was obtained by pulse-chase experiments in vivo. In order to monitor synthesis of the core region only, a double mutant, rfb and GDP-galactose-4-epimerase negative, was employed. The absence of exogenous galactose the double mutant produces the galactose-deficient, incompletely synthesized characteristic of epimeraseless mutants (Fig. 8). On addition of galactose to the medium, the full core region is synthesized, but O-antigen formation is prevented by the additional mutation at the rfb locus. The mutation was grown for several generations in the presence of [2-3H]glycerol as a general membrane marker, and then pulsed with [14C]galactose for 1 min at 25°. One-half of the culture was removed into ice-dinitrophenol, and the remainder subjected to a 2-min chase with nonradioactive galactose. Membranes were isolated and separated in the usual manner. The distribution of radioactivity in the sucrose gradient is shown in Fig. 5, and the recoveries in cytoplasmic and outer membrane are summarized in Table VI. At the end of the 1-min pulse virtually all of the [14C]galactose incorporated was recovered in cytoplasmic membrane; only 7% of the 14C was associated with the outer membrane band. The 14C originally present in cytoplasmic membrane was rapidly translocated to outer membrane during the chase. At the end of 2 min over 50% of the radioactivity had been chased into outer membrane, and after longer periods (not shown) over 90% of the pulse label could be recovered in the outer membrane fraction.

The product of [14C]galactose incorporation into cytoplasmic membrane was identified as lipopolysaccharide by phenol extraction, DEAE-chromatography and paper electrophoresis of the lipid-free polysaccharide fraction obtained by mild acid hydrolysis (6).
The experimental plan is summarized in Table VI. Pulse chase experiments were carried out to determine the ability of pre-existing incomplete core to be completed. Since O-antigen chains and the core region of lipopolysaccharide are assembled independently and by different mechanisms, separate experiments were carried out to determine the ability of pre-existing incomplete core lipopolysaccharide to accept newly synthesized O-antigen chains, and the ability of pre-existing incomplete core to be completed.

Addition of Newly Synthesized O-Antigen to Pre-existing Core Lipopolysaccharide—The mutant lacking phosphomannose isomerase was used for this experiment, and was shifted from conditions in which only core lipopolysaccharide was formed to conditions permitting O-antigen synthesis and attachment by addition of exogenous mannose to the medium. The design of the experiment was based on the difference in size between the core polysaccharide and the complete polysaccharide carrying O-antigen chains, which permits separation of the two polymers by gel filtration. The experimental plan is summarized in Fig. 6. A culture growing in the absence of mannose was exposed to [3H]glucose for one generation in order to introduce H into the core lipopolysaccharide. The culture was then harvested, and allowed to grow for one additional generation in fresh medium containing nonradioactive glucose (but no mannose) in order to minimize further incorporation of H into lipopolysaccharide during the subsequent shift to permissive conditions. The cells were recovered by centrifugation and split into three parts. Culture I served as zero time control, and was frozen without further incubation. Cultures II and III were suspended in fresh medium in the absence (Culture II) and presence (Culture III) of [4C]mannose and permitted to grow for one generation. Cultures I and II were expected to contain only core lipopolysaccharide, labeled with H, while culture III would also contain complete lipopolysaccharide, synthesized de novo, and labeled only with [3H]mannose. In addition, hybrid polysaccharide chains containing [4C]O-antigen attached to [3H]-labeled core would be present in III if, and only if, the [3H]-labeled core were completed by attachment of new O-antigen (Fig. 6). This species of polysaccharide chain could be recognized in gel filtration by a shift of [3H] from low molecular weight core polysaccharide into high molecular weight complete polysaccharide.

Accordingly, lipopolysaccharide was purified from the cell envelope fraction of each culture, and the polysaccharide chains liberated from the lipid portion by hydrolysis with dilute acetic acid (23). The lipid-free polysaccharides were purified by chromatography on DEAE-cellulose prior to gel filtration on Sephadex G 50. Recoveries of radioactivity at each step of the procedure are summarized in Table VII. Loss of [3H] during purification was due to nonspecific incorporation of radioactivity from [3H]glucose into nonlipopolysaccharide components. The purity of the [3H]-labeled polysaccharides from Cultures I and II was assessed by total acid hydrolysis and chromatography in Solvents I and III. Four major radioactive compounds were seen, with $P_F$ values corresponding to the major constituents of the core polysaccharide (glucose, galactose, glucosamine, and heptose). All of the [4C] of the polysaccharide from Culture III was recovered as [4C]mannose after total hydrolysis, and partial acid hydrolysis yielded [4C]oligosaccharides corresponding to the known O-antigen fragments, galactosyl-mannosyl-rhamnose and mannosyl-rhamnose (21).

Gel filtration of the polysaccharides on Sephadex G 50 is shown in Fig. 7. The bulk of the [4C] emerged in the excluded volume, although about 15% (Table VII) appeared in a second, partially included peak. The distribution of [4C] closely paralleled that of authentic complete polysaccharide isolated from wild type cells. The latter also showed the minor peak of partially included material, which may represent a population of polysaccharide chains containing abnormally short O-antigen...
Addition of newly synthesized O-antigen to old core lipopolysaccharide

Table VII

| Fraction | [3H]Glucose (cpm x 10^3) | [14C]-mannose (cpm x 10^3) |
|----------|--------------------------|---------------------------|
| Cell envelope | Culture I | 25.1 | 23.9 |
| Lipopolysaccharide | Culture II | 8.71 | 8.43 |
| Polysaccharide fractions | Culture III | 9.38 | 9.88 |
| Acetic acid hydrolysate | | 4.58 | 4.32 |
| DEAE-eluate | | 4.04 | 3.80 |
| Sephadex G-50 excluded fraction | | 3.00 | 2.91 |
| Sephadex G-50 included fraction | | 1.87 | 1.70 |

To determine the site of synthesis of lipopolysaccharide in S. typhimurium, pronase digestion as described by Romeo et al. (11). Carrier lipopolysaccharide (10 mg each of wild type and TV119) were added to an exponentially growing culture of M2D3L1 in Medium A (100 ml, A600 = 0.40). Incorporation of [3H]glucose into acid insoluble material was measured at 10 min intervals until no further incorporation was seen. The culture was immediately chilled, washed twice with 40 ml of cold 10 mM Tris, pH 7.8, and the cell pellet frozen. Cultures II and III were grown for one additional generation (35 min) in the absence of [3H]glucose (A600 = 1.08), the cells were washed with 40 ml of 10 mM Tris, pH 8.0 and sonicated for a total of 1 min with cooling. Potassium chloride was added to 0.3 M and the cell envelope fraction collected by centrifugation at 360,000 X g for 1 hour at 2-4°C. The particulate fraction was washed three times with 12 ml of 10 mM Tris (pH 8.0) - 0.3 M KCl and finally suspended in 5 ml of H2O. Lipopolysaccharide was isolated by aqueous phenol extraction (5) and purified by ethanolic precipitation and RNAse, DNase and pronase digestion as described by Romeo et al. (11). Carrier lipopolysaccharides (10 mg each of wild type and TV119) were added prior to phenol extraction. Lipid-free polysaccharides were obtained by hydrolysis of the purified lipopolysaccharides in 2 ml of 0.125 N acetic acid for 30 min at 100°C (23). The insoluble residues from the first hydrolysis were rehydrolyzed as before and the soluble fractions containing the lipid-free polysaccharides were combined, taken to dryness under reduced pressure, and dissolved in 5 ml of H2O, and the pH adjusted to 7.5 with NH4OH. The polysaccharides were purified by chromatography on 0.9 X 5 cm columns of DEAE-cellulose (acetate form) (23). Columns were washed successively with 10 ml each of H2O, 0.03 M, 0.25 m, and 0.5 m pyridinium acetate, pH 5.3. The 3H of the polysaccharide from Culture III was recovered in the 0.03 and 0.25 M eluates. These contained, respectively, 16 and 71% of the total 3H applied to the column. The 0.05 and 0.25 M eluates from each column were pooled, taken to dryness under reduced pressure, and dissolved in 1.5 ml of H2O. One milliliter of each sample was taken for gel filtration on Sephadex G-50 (Fig. 7). The remainder of polysaccharides I and II and 1 aliquot of III (10,000 cpm) was hydrolyzed in 1 N HCl for 5 hours at 100°C, and radioactive sugars identified by paper chromatography in Solvents I and III. Radioactive spots were located by cutting the chromatograms into 1-cm pieces and counting. The rest of polysaccharide III was subjected to partial acid hydrolysis and chromatography, as previously described (31) for identification of specific O-antigen oligosaccharide fragments.

Completion of Core Region—The double mutant, rfb rvi-, was used for this experiment. Under nonpermissive conditions (galactose-free medium) the lipopolysaccharide contains an incomplete, galactose-deficient core (Fig. 8); addition of galactose to the medium permits synthesis of the full core region. The experimental design (Fig. 8) was similar in principal to that described in the legend of Table VII, and filtered through a column (0.9 X 50 cm) of Sephadex G-50 (fine) in 50 mM NH4HCO3. Fractionation was with the same buffer. Portions of 0.5 ml were colleted, and 0.1-ml aliquots taken for counting. Culture II, 3H, • • • •. Culture III, 1C, O—O; 3H, • • • •. The elution profile of Culture I was indistinguishable from that of II. Elution positions of blue dextran (B.D.) and authentic core polysaccharide from the rfb mutant (CORE PS) are indicated.

![FIG. 7. Gel filtration of lipid-free polysaccharides.](http://www.jbc.org/)
FIG. 8. Completion of pre-existing core chains in strain GB-1. Summary of experimental design. The structure of the core polysaccharides formed in the absence and presence of galactose is illustrated at the top of the figure, which also shows the origin of the melibiose unit. The isotopic composition of the expected core structures and the melibiose derived from them is indicated schematically. See text for details. LPS, lipopolysaccharides.

described above. The culture was exposed to [3H]glucose for one generation, chased with nonradioactive glucose for one generation, and then divided into three parts. One part was harvested immediately (Culture I), one was grown for one generation in the absence of galactose (Culture II), and the third grown for one generation in the presence of [14C]galactose (Culture III). Lipopolysaccharides were isolated and the lipid-free polysaccharides prepared as described above. The 3H content of the full core polysaccharide obtained from Culture III was again employed as an index of the degree of completion of the pre-existing galactose-deficient lipopolysaccharide. In this case, however, gel filtration failed to separate adequately the full core polysaccharide from the galactose-deficient core. The 3H content of the completed core chains was therefore determined by isolation of melibiose after partial acid hydrolysis of the polysaccharide. The core region contains two galactosyl residues, linked α-1,3 and α-1,6 to the proximal glucose residue (Fig. 8). This glucose residue is present in the incomplete core produced under nonpermissive conditions, and was labeled during growth in [3H]glucose. Melibiosyl units (α-galactosyl-1,6-glucosyl) arising by galactosylation of the old 3H labeled lipopolysaccharide would therefore be expected to contain 3H glucose as well as 14C galactose, while those derived from de novo synthesis of lipopolysaccharide should contain only nonradioactive glucose. Thus, the ratio of [3H]glucose to [14C]galactose in the isolated melibiose provided a measure of the fraction of old chains which had been completed following addition of galactose.

Melibiose was isolated from the polysaccharide of Culture III as follows. The preparations from Cultures I and II were carried through the entire procedure as controls as described in the legend of Table VIII. The lipid-free polysaccharide was subjected to partial acid hydrolysis and the oligosaccharide fractions isolated by filtration through Sephadex G-10. Melibiose was purified from the crude oligosaccharide fraction by paper chromatography. Following chromatography in Solvent I (Fig. 9a), the melibiose area was eluted and rechromatographed in Solvent II (Fig. 9b). The recoveries of 3H and 14C at each step of the purification are given in Table VIII, and the theoretical and observed ratios of 3H:14C in the isolated melibiose are summarized in Table IX. The theoretical ratio for 100% completion of pre-existing lipopolysaccharide was calculated from determinations of the total [3H]glucose content of the polysaccharides (see legend, Table IX), assuming that 50% of the total [14C]galactose incorporated into polysaccharide III was present in α,6-linkage to glucose (cf. Fig. 8). The ratio of [3H]glucose to [14C]galactose in the isolated melibiose was determined after reduction, hydrolysis, and isolation of glucitol and galactose (see legend, Table IX). The final ratio of [3H]glucitol to [14C]galactose indicated that less than 4% of the pre-existing incomplete core had been galactosylated during growth for one generation under permissive conditions.
TABLE VIII
Addition of core galactose residues to old galactose-deficient lipopolysaccharide

Experimental conditions were similar to those described in Table VII except that strain GB-1 was used, and [14C]galactose was employed in the final period of growth under permissive conditions. An exponentially growing culture of GB-1 in Medium A was exposed first to [3H]glucose and then to nonradioactive glucose (see Table VII for conditions). The cells were then harvested and divided into three equal parts. Culture I was immediately washed and frozen; Culture II was grown for one additional generation in the absence of galactose, and Culture III was grown for one generation in the presence of 0.2 mM [14C]galactose (1.6 x 10⁶ cpm per µ mole). Isolation and purification of lipopolysaccharide and lipid-free polysaccharide were carried out as before (Table VII), except that a mixture of G-30 and TV19 lipopolysaccharide was added as carrier. A portion of each polysaccharide (20% of the total) was saved for determination of [3H]glucose content (Table IX), and the remainder subjected to partial acid hydrolysis (31). Samples were hydrolyzed in 1 ml of 60% HCOOH for 1 hour at 100°C and the hydrolysates taken to dryness under reduced pressure at 25°C. Residual HCOOH was removed by repeated evaporation to dryness from H₂O. Residues were dissolved in 1 ml of 20 mM NH₄HCO₃ and filtered through a 1.5 x 90 cm column of Sephadex G-10 in 20 mM NH₄HCO₃. Excluded fractions containing residual unhydrolyzed material were pooled, concentrated to dryness and rehydrolyzed as before. The hydrolysates were again filtered through Sephadex G-10, and the oligosaccharide fractions from the first and second hydrolyses were pooled and concentrated to dryness. Melibiose was isolated by paper chromatography in Solvent I (Fig. 9a) and purified by rechromatography in Solvent II (Fig. 9b).

| Fraction                          | Recovery of radioactivity | [%H]Glucose | [%C]Galactose |
|----------------------------------|---------------------------|-------------|---------------|
|                                  | Culture I | Culture II | Culture III |
|                                  | [cpm x 10⁶] | [cpm x 10⁴] | [cpm x 10³] |
| Cell envelope                    | 31.2      | 28.3      | 29.6         | 3.57         |
| Lipopolysaccharide               | 11.10     | 9.88      | 8.30         | 2.97         |
| Polysaccharide                   | 3.82      | 4.08      | 4.84         | 2.57         |
| Sephadex G-10 oligosaccharide    | 55.0      | 48.0      | 51.2         | 42.7         |
| Melibiose area, Solvent I        | 16.2      | 8.09      | 7.34         | 12.8         |
| Melibiose area, Solvent II       | 1.83      | 1.43      | 2.91         | 7.70         |

![Fig. 9. Purification of melibiose. The oligosaccharide fraction obtained from each hydrolysate by gel filtration (see text and the legend to Table VIII for details) was chromatographed in Solvent I, and spots containing ¹⁴C in the sample from Culture III were located by scanning a strip from the chromatogram (upper curve). The region corresponding to melibiose in each of the chromatograms was eluted with H₂O and rechromatographed in Solvent II. [¹⁴C]Melibiose was located by scanning the strip from Sample III (lower curve). This spot, and the corresponding regions of the chromatograms from Samples I and II were eluted with H₂O for counting and further analysis.](http://www.jbc.org/)

TABLE IX
Isotopic composition of purified melibiose

The theoretical ratio of [¹⁴C]glucose to [³H]galactose in melibiose, to be expected if 100% of the [³H]lipopolysaccharide were completed, was based on the assumption that all of the glucose residues of the incomplete, ³H-labeled lipopolysaccharide were capable of accepting α,6-galactosyl residues, and that 50% of the total [¹⁴C]galactose incorporated was in this linkage (Fig. 8). The total [¹⁴C]glucose content was determined by hydrolysis and paper chromatography of the lipid-free polysaccharides from control Cultures I and II. Aliquots of the polysaccharides (8 to 9 x 10⁶ cpm) were hydrolyzed in 1 ml of 1 N HCl for 5 hours at 100°C, and hydrochloride was removed by extraction with dioctylmethylamine in CHCl₃ (31). The hydrolysates were treated with E. coli alkaline phosphatase to hydrolyze residual heptose-phosphate and chromatographed in Solvent III. The chromatograms were cut into 1-cm strips and counted as described under "Counting Procedures." [³H]Glucose accounted for 40.0 and 43.1% of the total [³H]of polysaccharides I and II, respectively. The [³H]glucose content of polysaccharide III was calculated from the total [³H]content (Table VIII) and the average of the above determinations. For all calculations of [³H]/[¹⁴C] ratios, observed counts per min were converted to disintegrations per min. The [³H]glucose content of the melibiose was measured by reduction and hydrolysis of the isolated product, and determination of the amount of [¹³C] and [³H] in glucose and galactose, respectively. Reduction with NaBH₄ and hydrolysis were carried out as described earlier (31), and hexitols and hexoses separated by paper chromatography first in Solvent I followed by elution and rechromatography in Solvent IV according to Bray and Rohns (24). The chromatographic strip was cut into 1-cm pieces and counted as described under "Counting Procedures." Recoveries of radioactivity were ¹³C, 4790 cpm (8250 dpm) in galactose spot and 1024 cpm (1170 dpm) in hexitol region; ³H, 246 cpm (1022 dpm) in hexitol, 600 cpm (2580 dpm) in the hexose region.

| Melibiose fraction | [³H]/[¹⁴C] | Completion of old lipopolysaccharide |
|--------------------|------------|------------------------------------|
| Theoretical         | 3.64       | 100                                |
| Observed            | 0.124      | 3.4                                |
The data presented here provide strong evidence that the lipopolysaccharide of the outer membrane of S. typhimurium is synthesized in the cytoplasmic membrane and secondarily translocated to its final position in the outer membrane. The assignment of the site of synthesis and attachment of O-antigen chains is consistent both with the observed distribution of the biosynthetic enzymes in isolated membrane fractions and the results of pulse-chase experiments in vivo. Definitive localization of the site of synthesis of the internal core region of lipopolysaccharide proved to be more difficult, since in vitro distribution of core glycosyltransferase activities was found to be ambiguous as a criterion of in vivo localization of these enzymes. In contrast to the enzymes of O-antigen synthesis, the core glycosyltransferases were partially soluble, and activity was recovered in both outer and cytoplasmic membrane fractions. However, reconstruction experiments showed that exogenous soluble core transferases, added during spheroplast lysis, were bound to both membranes in a manner qualitatively and quantitatively similar to that of the endogenous activities. These findings raise some question as to the extent to which these activities are membrane-bound in the intact cell and the nature of their presumed association with membrane in vivo. It should be emphasized, however, that lysis of spheroplasts and isolation of membranes were carried out at low ionic strength and in the presence of EDTA. It is possible that these conditions favor solubilization of core glucosyl- and galactosyltransferase activities. Romeo et al. (28) have reported that Mg++ is required for interaction of purified galactosyltransferase I with monolayer films containing lipopolysaccharide plus phosphatidyl ethanolamine, and repeated washing at low ionic strength is known to result in release of the membrane ATPase and other proteins in Streptococcus faecalis (32). At present the conclusion that core synthesis is localized exclusively in the cytoplasmic membrane rests primarily on the results of pulse-chase experiments in vivo, which showed that over 80% of the galactose incorporated into the core region of lipopolysaccharide during a short pulse was recovered in the cytoplasmic membrane. The failure of the conditional mutant in core biosynthesis to complete the core region of pre-existing lipopolysaccharide after a shift to permissive conditions also argues that the outer membrane does not participate in lipopolysaccharide synthesis in vivo.

The mechanism of translocation of lipopolysaccharide to the outer membrane and the mechanism of assembly of this membrane are unknown. The data presented here, and kinetic studies to be described elsewhere indicate that translocation of lipopolysaccharide is rapid and unidirectional. Other studies have shown that the enzymes of phospholipid synthesis are also specifically localized in the cytoplasmic membrane in S. typhimurium (33) and E. coli (33, 34), and it is therefore probable that incorporation of phospholipids into the outer membrane requires translocation from sites of synthesis in cytoplasmic membrane. Although information is lacking on the site of origin of specific outer membrane proteins, it appears likely that some type of translocation occurs between synthesis of the polypeptides and assembly into the outer membrane structure. Two extreme types of model of translocation and outer membrane assembly might be envisioned: assembly of a lipopolysaccharide-phospholipid-protein subunit in or at the cytoplasmic membrane followed by obligatory translocation to outer membrane as a unit, or alternatively, translocation of individual components completely independently of each other followed by independent insertion of each into the outer membrane. The first hypothesis, in its extreme form, appears to be excluded by the findings of E. Work and her colleagues (33) and Rothfield and Pearlman-Kothesen (36), that synthesis of lipopolysaccharide and its incorporation into outer membrane continue for long periods of time following inhibition of protein synthesis by amino acid starvation or chloramphenicol. On the other hand, recent preliminary experiments in a glycerol auxotroph of strain G-30 have shown that starvation for glycerol results in rapid cessation of [14C]galactose incorporation into lipopolysaccharide. However, it is not yet clear whether the requirement for continued net synthesis of phospholipid is concerned with translocation of lipopolysaccharide or with early steps in its biosynthesis.

The presence of the peptidoglycan layer between the cytoplasmic and outer membranes would also appear to impose restrictions on possible mechanisms of translocation. If the cross-linked peptidoglycan network is continuous over the entire surface of the cell, the pore size of the meshwork becomes a consideration in terms of the dimensions of the macromolecules which might traverse the peptidoglycan. Romeo et al. (11) have estimated a cross-sectional area of about 250 A² for a lipopolysaccharide molecule in a mixed monolayer film with phosphatidylethanolamine, but experimental data on the pore size of peptidoglycan in gram-negative bacteria is not yet available. It is also possible that synthesis or translocation of outer membrane components or both are restricted to localized regions of relatively uncross-linked peptidoglycan, perhaps corresponding to areas of peptidoglycan synthesis. Bayer (37) has described zones of adhesion between cytoplasmic membrane and the external layers of the envelope, which would provide potential sites of contact between cytoplasmic and outer membrane. The speculation that such zones of adhesion may play a role in these processes is an attractive one.

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*J. Biol. Chem.* 1972, 247:3973-3986.

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