Further Studies on Enzymatic Synthesis of O-Antigen in *Salmonella typhimurium**

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ABSTRACT: Enzymatic synthesis of O-specific polymers, both haptenic and lipopolysaccharide bound, by *Salmonella typhimurium* correlates well with the results of *in vivo* studies. Mutant strains blocked in the synthesis of the core moiety accumulate the unattached hapten both *in vivo* and *in vitro*, whereas strains which form a complete core and can synthesize wild-type lipopolysaccharide during growth on exogenous substrates can also attach O-specific polymers to the endogenous lipopolysaccharide of cell envelope preparations. An apparent exception is a nonleaky mutant which lacks uridine-5′-diphosphogalactose-4-epimerase and whose lipopolysaccharide therefore contains only the proximal core residue; cell envelope preparations, however, incorporate O-specific sugars into lipopolysaccharide very efficiently without concomitant completion of endogenous core. Relative rates of synthesis and accumulation for each O-specific polymer are widely variable and strongly dependent upon the physiological state of the cells from which the cell envelope fraction is prepared. Polymerization of the oligosaccharide precursor is the rate-limiting step in cell-free preparations. Under conditions of most efficient polymerization, the initial rate of incorporation of [^{14}C]abequose into each polymer is approximately the same, and the hapten formed initially is highly polymerized. The results are consistent with the hypothesis that the O-specific hapten is a precursor of O-antigenic chains in wild-type lipopolysaccharide.

Previous studies (Weiner *et al.*, 1965; Robbins *et al.*, 1967) on the role of lipid-linked intermediates in biosynthesis of the O-antigen of *Salmonella* have demonstrated enzymatic formation of an O-specific polymer linked to the intermediate antigen-carrier lipid, and the preceding paper (Kent and Osborn, 1968a) presented evidence that this product is also accumulated by mutant strains which are blocked in attachment of O-antigenic side chains to lipopolysaccharide. This paper describes further studies on the kinetics of O-antigen synthesis in cell-free systems, and presents additional characterization of the several O-specific products formed by enzyme preparations obtained from wild-type and mutant strains of *Salmonella typhimurium*. In general, a good correlation was observed between the patterns of biosynthesis *in vitro* and *in vivo;* enzyme preparations derived from strains capable of synthesizing wild-type lipopolysaccharide catalyzed incorporation of O-specific sugars into both lipopolysaccharide and hapten, but the relative rates of synthesis or accumulation of the two products varied widely according to experimental conditions. The haptenic polymer was the only product observed in cell envelope preparations from mutants which accumulate this product *in vivo*. However, unexpected results were obtained with a mutant lacking UDP-galactose-4-epimerase; in this case, substantial incorporation of O-antigen into lipopolysaccharide was observed despite the incomplete core present in the endogenous lipopolysaccharide, thus raising the question of the site of attachment of O-specific chains in the acceptor core lipopolysaccharide.

Materials and Methods

A. Growth of Bacteria and Preparation of Cell Envelope. The bacterial strains used are derivatives of *S. typhimurium LT2* and are described in Table I. *SL1032* and *TV161* were kindly supplied by Dr. B. A. D. Stocker, Stanford University School of Medicine.

Growth on proteose peptone–beef extract and preparation of the cell envelope fraction have been described (Osborn *et al.*, 1962; Rothfield *et al.*, 1964); using the galactose oxidase system, we were unable to detect any galactose in concentrated aliquots of this medium, even after acid hydrolysis. The cell envelope fraction was prepared either from exponentially growing cell cultures which had been stored overnight at −20° as washed cell pellets or from aliquots of cell pastes harvested after growth in a 100-l. fermentor.

B. Incubation Procedures. The standard incubation mixture for incorporation of sugar residues into O-anti-

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TABLE I: Strains of *S. typhimurium* Employed.

Strain	Genotype[a]	Lipopolysaccharide Chemotype[b]	Product of O-Antigen Synthesis *in Vivo*	References
LT-2	*Wild type*	S	Lipopolysaccharide	
M2	*pmi*$^-$	Ra (−Man) S (+Man)	None (−Man) Lipopolysaccharide (+Man)	Zeleznick *et al.* (1965)
TV161	*rfa*	Rb	Hapten	Subbaiah and Stocker (1964); Beckmann *et al.* (1964)
SL1032	*Glu-transferase I*$^-$	Rd	Hapten	Osborn (1968)
G30	*epi*$^-$	Rc (−Gal) S (+Gal)	None (−Gal) Lipopolysaccharide (+Gal)	Osborn *et al.* (1962)

[a] The genotype designations are as follows: *pmi*, phosphomannose isomerase; *epi*, UDP-galactose-4-epimerase; *rfa*, locus controlling synthesis of the core region of the polysaccharide; *Glu-transferase I*, the enzyme catalyzing incorporation of the proximal glucose residue of the core. Abbreviations used in all tables: Man, D-mannose; Gal, D-galactose; and Abe, abequose. [b] Chemotype designations are those employed by Lüderitz *et al.* (1966). *S* corresponds to wild-type lipopolysaccharide containing the normal complement of O-specific side chains; *Ra* to the complete core structure; and Rb, Rc, and Rd to incomplete core structures; see Figure 1, Kent and Osborn (1968a), for structural diagrams. In *M2* and *G30*, O-antigen synthesis is dependent upon addition to the medium of exogenous mannose and galactose, respectively; the structures formed in the presence and absence of the exogenous sugar are indicated in the table.

gen contained 0.5 mg of cell envelope protein, 80 μmoles of Tris-acetate buffer (pH 8.5), 2 μmoles of $MgCl_2$, 30 mμmoles of UDP-galactose, 20 mμmoles of TDP-rhamnose, and 5 mμmoles of GDP-mannose in a total volume of 0.25 ml. CDP-abequose (3.3 mμmoles) was added as indicated. Reactions were stopped by addition of 2 ml of 0.1 N acetic acid and the cell envelope was washed and counted as previously described (Weiner *et al.*, 1965).

C. Materials and Analytical Procedures. CDP-[^{14}C]-abequose, CDP-abequose, UDP-galactose, and TDP-rhamnose were prepared as described earlier (Rosen and Zeleznick, 1966; Zeleznick *et al.*, 1965; Osborn *et al.*, 1962). UDP-Glucose, UDP-*N*-acetylglucosamine, and GDP-mannose were obtained from Calbiochem, and UDP-[^{14}C]galactose from the International Chemical and Nuclear Corp. [^{14}C]Galactose and [^{14}C]mannose were purchased from New England Nuclear Corp. Optical grade CsCl was purchased from the Harshaw Corp. Alkaline phosphatase was obtained from Worthington, and coffee bean α-galactosidase was purified as described previously (Zeleznick *et al.*, 1965). Carrier polysaccharide from *G30* was prepared as previously described (Osborn, 1963); wild-type lipopolysaccharide used as carrier was purchased from Difco. Preparation of wild-type lipopolysaccharide labeled with [^{14}C]mannose or [^{14}C]galactose has been described (Osborn and Weiner, 1968; Kent and Osborn, 1968a).

Total carbohydrate and protein were measured by the phenol–sulfuric acid (Dubois *et al.*, 1951) and Lowry (Lowry *et al.*, 1951) methods, respectively. Lipopolysaccharide and O-specific hapten were isolated by phenol extraction and separated by ethanol fractionation as described in the preceding paper (Kent and Osborn, 1968a). Polysaccharides were released from lipopolysaccharides by hydrolysis at pH 3–3.5 as described previously (Osborn, 1963). For gel filtration, a column of Sephadex G-50 coarse (1 × 20 cm) was equilibrated in 0.02 M ammonium acetate, and one of Sephadex G-200 medium (0.8 × 51 cm) was equilibrated with 0.05 M ammonium carbonate. Sedimentation of polysaccharides at 105,000*g* was for 3 hr at 0°.

The following solvents were used for chromatographic and electrophoretic analyses: solvent A, ethyl acetate–acetic acid–water (3:3:1); solvent B, 1-butanol–pyridine–H_2O (6:4:3); solvent C, 95% acetone; and solvent D, pyridine–acetic acid–water (1:10:89) (pH 3.5). Whatman No. 1 or 40 paper was used for descending chromatography, and Whatman No. 1 paper for electrophoresis. Electrophoretic separations were carried out at 50–100 V/cm for 1–2 hr.

Radioactivity on paper strips was located with a Baird-Atomic 4π windowless scanogram. Alternatively, the strips were cut into 0.25-in. segments and placed in vials with 15 ml of toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene for scintillation counting. Bray's solution with 2% thixotropic gel (Cabosil) was used as solvent for scintillation counting of fractions from CsCl density gradients.

Results

A. Characterization of Enzymatically Synthesized O-Specific Products. Separation of O-Specific Hapten and Lipopolysaccharide. 1. PATTERNS OF SYNTHESIS IN WILD-TYPE AND MUTANT PREPARATIONS. In order to compare the properties of O-specific products synthesized *in vitro*

with those formed *in vivo*, enzymatic products prepared from mutant strains which accumulate O-specific hapten during growth were compared with those obtained from organisms capable of attaching these chains to lipopolysaccharide. The organisms used and their properties are summarized in Table I. Cell envelope fractions prepared from freshly grown cells, harvested during exponential growth, were incubated with mixtures of UDP-galactose, TDP-rhamnose, and GDP-[^{14}C]mannose. Since addition of CDP-abequose had little effect on the size or amount of O-specific polymer formed (see below, section B), this nucleotide sugar was usually omitted from reaction mixtures. Radioactive O-specific products were isolated from the cell envelope by mild acid hydrolysis (0.5 N acetic acid for 30 min at 100°). Under these conditions, the polysaccharide–lipid linkages of both lipopolysaccharide and O-specific hapten are quantitatively split and the corresponding polysaccharides released into solution (Osborn, 1963; Zeleznick *et al.*, 1965). In every case over 98% of the radioactivity initially incorporated into the cell envelope fraction was recovered in the soluble lipid-free polysaccharide fraction.

The polysaccharides obtained from lipopolysaccharides, which contain phosphodiester groups associated with the backbone region of the polymer, were distinguished from O-specific haptenic products by paper electrophoresis and by differential adsorption to DEAE-cellulose columns. The acetic acid hydrolysates were first treated with *Escherichia coli* alkaline phosphatase in order to remove residual phosphomonoester at the reducing terminus of the O-specific hapten (Weiner *et al.*, 1965); the phosphodiester groups of the polysaccharides obtained from lipopolysaccharide are resistant to monoesterase (Osborn, 1963). Paper electrophoresis of the enzymatic products containing [^{14}C]mannose is shown in Figure 1. As anticipated, preparations from *SL1032* and *TV161*, mutants which accumulate O-specific hapten *in vivo*, showed only a single neutral peak of radioactivity corresponding to the dephosphorylated haptenic polymer.[1] This component was also present in the enzymatic products obtained from the wild type. In addition, however, 20–50% of the radioactivity of the wild-type product appeared in a polydisperse fraction migrating anionically, as expected for the phosphorylated polysaccharide derived from lipopolysaccharide. Similar results have previously been reported for a mutant (*M2*) lacking phosphomannose isomerase (Zeleznick *et al.*, 1965).

Equivalent results were obtained by chromatography of the enzymatic products on DEAE-cellulose. The results obtained with *SL1032* and the wild type are illustrated in Figure 2a. The acetic acid extracts from cell envelope were treated with alkaline phosphatase prior

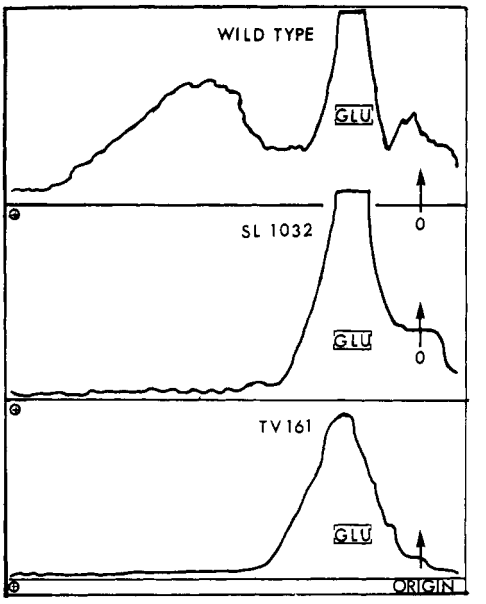

FIGURE 1: Electrophoretic mobility of enzymatically synthesized products labeled with [^{14}C]mannose. Cell envelope preparations of each strain were incubated at 37° for 30 min with UDP-galactose, TDP-rhamnose, and GDP-[^{14}C]-mannose at 8 × 10^4 cpm/mμmole under standard assay conditions. The reaction was stopped with cold 0.1 N acetic acid, and the precipitates were collected by centrifugation, washed twice, and resuspended in 0.5 N acetic acid for hydrolysis at 100° for 30 min. The sample was centrifuged and rehydrolyzed once, and the combined supernatant solutions were evaporated several times to dryness under reduced pressure to remove the acid. Each sample was then resuspended in 20 μl of 0.02 M Tris buffer (pH 8), and the sample was incubated overnight at 37° with 0.1 μg of alkaline phosphatase. The entire mixture was then spotted on paper for electrophoresis in solvent D. Radioactivity was located by strip counting.

to chromatography on DEAE-cellulose; the product obtained from *SL1032* was not adsorbed but was quantitatively recovered in the H_2O wash, whereas approximately 40% of the radioactivity of the wild-type preparation was strongly adsorbed to the column, and its elution pattern in the gradient (Figure 2a, to right of arrow) was like that of polysaccharides obtained by mild acid hydrolysis of purified wild-type lipopolysaccharide labeled with [^{14}C]mannose (Figure 2b). Less than 3% of the radioactivity of the lipid-free polysaccharide derived from authentic lipopolysaccharide was recovered in the water wash (Figure 2b). These results show that the enzymatic products recovered in the water wash and designated "hapten" (Figure 2a) are not degradation artifacts of the acid extraction procedure.

To confirm that both radioactive products, the neutral hapten fraction and the anionic product derived from lipopolysaccharide, were indeed related to O-antigen, two further experiments were performed. Rhamnose and mannose were shown to be incorporated in equimolar amounts into the total product formed by cell envelope preparations of the wild type, and the distribution of the two radioactive sugars in the anionic (phosphatase resistant) and neutral fractions was found to be very similar. Partial formic acid hydrolysis of each fraction followed by reduction with $NaBH_4$ (Zeleznick *et al.*,

[1] If electrophoresis of these preparations were carried out prior to treatment with alkaline phosphatase, approximately 30% of the radioactivity migrated slowly toward the anode; treatment with phosphatase converted this material to a neutral form. Similar results were obtained with authentic hapten isolated in similar fashion from intact cells (Kent and Osborn, 1968a).

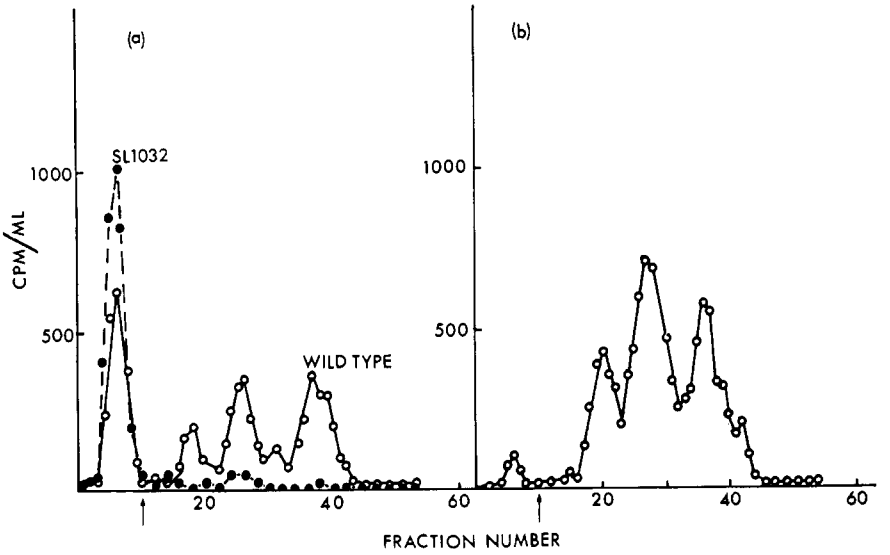

FIGURE 2: DEAE-cellulose chromatography of enzymatically synthesized products. (a) Enzymatic products from *SL1032* and wild type. (b) Mild acid hydrolysate of authentic wild-type lipopolysaccharide. Incubations were carried out with GDP-[^{14}C]mannose (8×10^4 cpm/mμmole) under standard conditions except that the reaction mixtures were scaled up 20-fold. Acetic acid extracts were prepared and treated with alkaline phosphatase as described in Figure 1. Authentic wild-type lipopolysaccharide labeled with [^{14}C]mannose was carried through the same procedure. Samples containing $4–5 \times 10^4$ cpm were diluted to 5 ml and applied to columns (1×20 cm) of DEAE- cellulose in the acetate form. The columns were washed with 30 ml of H_2O and eluted with a linear gradient of pyridinium acetate (pH 5.3) (100 ml of 0.3 M into 100 ml of 0.05 M). The arrow indicates application of the salt gradient (Osborn, 1963).

1965) yielded, in addition to mannitol, only the di- and trisaccharide products (mannosylrhamnitol and α-galactosylmannosylrhamnitol) characteristic of O-antigen. The oligosaccharides were isolated and characterized by the procedures described earlier (Zeleznick *et al.*, 1965). Furthermore, recovery of a significant proportion of label as the reduced trisaccharide, α-galactosylmannosylrhamnitol, after partial acid hydrolysis and reduction of the anionic fraction, indicated that the O-specific material incorporated into lipopolysaccharide consisted at least in part of polymeric chains rather than single repeating units (mannosylrhamnosylgalactose).

2. INCORPORATION OF O-SPECIFIC SUGARS INTO LIPOPOLYSACCHARIDE IN A MUTANT LACKING UDP-GALACTOSE-4-EPIMERASE. The results of the above experiments *in vitro* were consistent with the patterns of O-specific synthesis observed *in vivo*, and with the hypothesis that attachment of O-specific chains to lipopolysaccharide requires the presence of the complete core structure of the lipopolysaccharide. However, results were obtained with the mutant lacking UDP-galactose-4-epimerase (strain *G30*) which suggest that the entire core need not be required.

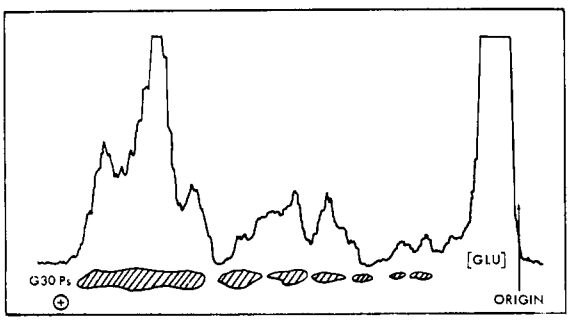

FIGURE 3: Electrophoretic migration of [^{14}C]mannose-polysaccharides formed enzymatically by strain *G30*. The experimental procedure detailed for Figure 1 was followed.

Since galactose is a component of both the O-antigen and the core region of lipopolysaccharide, this epimeraseless mutant produces an incomplete lipopolysaccharide containing only a portion of the normal core structure during growth without exogenous galactose. It was therefore anticipated that in cell envelope preparations from this mutant, O-specific hapten would be the sole product of O-antigen synthesis. However, electrophorograms of products extracted with hot acetic acid showed a pattern similar to that of the wild type (Figure 3). Approximately 25% of the total [^{14}C]mannose incorporated into cell envelope was recovered in a phosphatase-resistant, anionic fraction apparently corresponding to the polysaccharide portion of lipopolysaccharide. Chromatography of this fraction on DEAE-cellulose following elution from paper (Figure 4) was also consistent with the identification; the radioactivity was eluted by a pyridinium acetate gradient as a series of anionic peaks emerging between 0.05 and 0.2 M pyridinium acetate, in a pattern similar to that seen with the wild-type polysaccharide (*cf.* Figure 2) and also with the incomplete mutant polysaccharide of strain *G30*. Identification as O-specific polymer was confirmed by formic acid hydrolysis of both the haptenic fraction and presumptive lipopolysaccharide, each of which yielded the oligosaccharides characteristic of the O-specific polymer and indistinguishable from the products separated from wild-type preparations.

Characterization of the products after isolation by phenol extraction provided further evidence for the incorporation of O-specific chains into lipopolysaccharides. The cell envelope fraction of the epimeraseless mu-

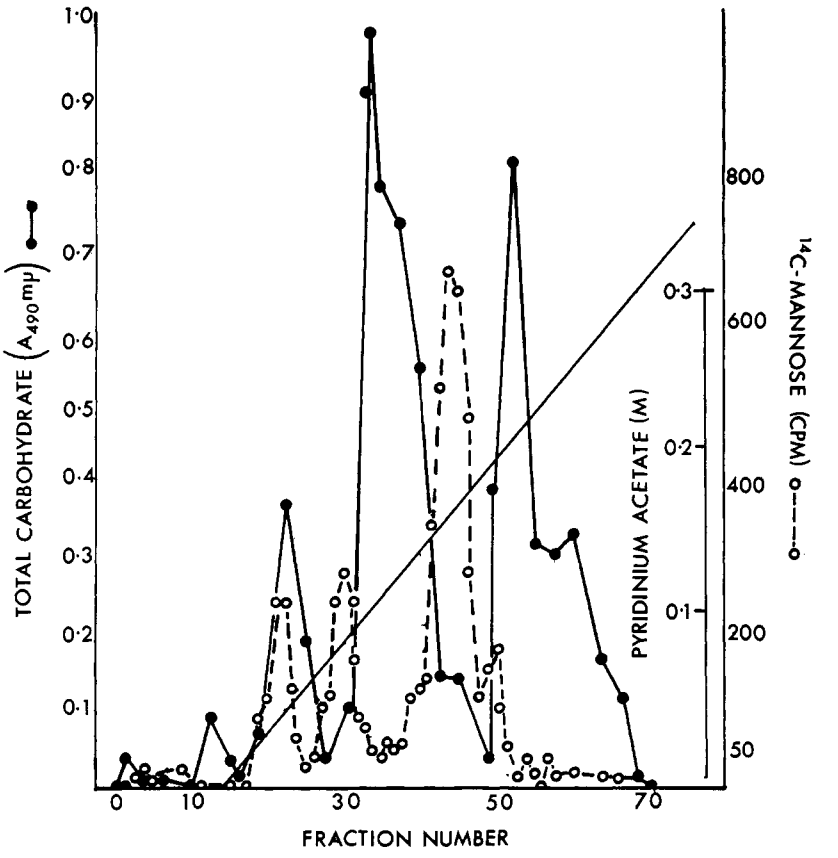

FIGURE 4: DEAE-cellulose chromatography of anionic [^{14}C]mannose-labeled polysaccharides formed by strain *G30*. The material which migrated anionically in the experiment described in Figure 3 was eluted from paper with 0.05 N acetic acid and evaporated repeatedly to dryness under reduced pressure. Endogenous polysaccharide (30 mg) from *G30* was added as carrier in a total volume of 5.0 ml of water (pH 8) containing the anionic enzymatic product (1.3×10^4 cpm), and the sample was applied to a DEAE-cellulose column (1×23 cm), which was washed with three volumes of water. Elution was carried out with a linear gradient of pyridinium acetate, pH 5.3, as described in Figure 2. Fractions (3.0 ml) were assayed for radioactivity and for carbohydrate.

tant was incubated with UDP-[^{14}C]galactose alone to allow incorporation of galactosyl residues into the core region of lipopolysaccharide and with GDP-[^{14}C]mannose plus TDP-rhamnose and UDP-galactose to permit synthesis of O-specific units. Radioactive products were extracted with 45% phenol at 68° and fractionated as shown in Table II. The aqueous phases of the phenol extracts were passed through Sephadex G-50 to remove nonpolymeric material; about two-thirds of the mannose-labeled product was excluded, the remainder behaving like oligosaccharide precursors. The polymeric material was further fractionated with ethanol in order to separate lipopolysaccharide from hapten as described in the preceding paper (Kent and Osborn, 1968a). Over 95% of the [^{14}C]galactose was recovered in the ethanol precipitate (which contains the lipopolysaccharide) and 65% of the mannose-labeled polymeric material. The ethanol-soluble fraction, which contains O-specific hapten, was further characterized in parallel to the precipitated material. The ethanol-soluble, mannose-labeled product formed a heterogeneous but partially included band on passage through Sephadex G-200, was not sedimented at 105,000*g*, and was only weakly adsorbed to DEAE-cellulose, all characteristics attributable to the unattached hapten. In contrast, essentially all the ethanol-precipitable counts of each product were excluded from a Sephadex G-200 column, could be sedimented at 105,000*g*, and were irreversibly adsorbed to a DEAE-cellulose column, all characteristics of lipopolysaccharide.

Density gradient centrifugation in CsCl (Kent and Osborn, 1968a) also indicated the presence of lipopolysaccharide containing [^{14}C]mannose. The phenol extracts were prepared for CsCl centrifugation by sequential filtration through Sephadex G-50 and G-200. Equilibrium centrifugation of the fractions excluded from G-200 and of a wild-type lipopolysaccharide marker (labeled *in vivo* with [^{14}C]galactose) gave buoyant densities of 1.495 for the marker, and 1.494 and 1.493 for the enzymatic products containing [^{14}C]mannose and [^{14}C]galactose, respectively (Figure 5). About 85% of the counts were recovered in the peak in each case. The width of the band formed by the wild-type marker and by the galactose-labeled product did not differ significantly, which suggests that the buoyant density of the lipopolysaccharide species (aggregate) is independent of its polysaccharide content, since the size of polysaccharide moiety in the incomplete galactose-labeled product is much smaller than that of the wild-type marker.

Efforts were made to assess the possibility that the

TABLE II: Characterization of O-Specific Products Formed by *G30*.[a]

	Reaction 1		Reaction 2	
	[^{14}C]Galactose (cpm)	% of Total	[^{14}C]Mannose (cpm)	% of Total
Total counts incorporated	6.5×10^4		5.0×10^4	
Aqueous phase of phenol extracted	6.5×10^4	100	5.1×10^4	100
G-50 Sephadex excluded	6.1×10^4	94	3.3×10^4	66
67% ethanol pellet	6.0×10^4	93	2.2×10^4	45
G-200 Sephadex excluded	5.9×10^4	91	2.2×10^4	45
105,000*g* pellet	5.8×10^4	90	2.2×10^4	45
DEAE-cellulose column				
cpm applied	930		1000	
cpm eluted	None		None	
Summary				
% lipopolysaccharide	100			67
% hapten	0			33

[a] A standard cell envelope preparation of strain *G30* was incubated at 37° for 30 min to contain in 250 μl: 0.5 mg of cell envelope protein, 80 μmoles of Tris-acetate (pH 8.5), and 2 μmoles of $MgCl_2$ as well as, in mixture 1, 30 mμmoles of UDP-[^{14}C]galactose (1.2×10^4 cpm/mμmole) and, in reaction 2, 30 mμmoles of UDP-galactose, 17 mμmoles of TDP-rhamnose, and 5 mμmoles of GDP-[^{14}C]mannose (5.8×10^4 cpm/mμmole). The reaction was stopped with cold 5% trichloroacetic acid. Relevant details of the purification procedures are described in the Methods section. Specific activity of total incorporation for labeled sugars was 10.8 mμmoles/mg of [^{14}C]galactose in reaction 1 and 2.0 mμmoles/mg of [^{14}C]mannose in reaction 2. Test aliquots of purified material were applied to columns of DEAE-cellulose (1 × 10 cm) as described in the legend to Figure 2, but elution was carried out sucessive 50-ml batches of H_2O, then with pyridinium acetate (pH 5.3) at 0.02, 0.03, and 0.3, and finally 0.1 N NaOH. The radioactive material (1.1×10^4 cpm) from reaction 2, soluble in 67% ethanol, was identified as hapten as described in the text.

enzymatic attachment of O-specific chains to lipopolysaccharide in *G30* could be attributed to leakiness in the UDP-galactose-4-epimerase mutation, and the resultant presence in the endogenous lipopolysaccharide of some complete core chains which could act as O-antigen acceptor sites. The presence of residual epimerase activity *in vivo* was tested by analysis of the products of metabolism of [^{14}C]galactose by growing cultures. A culture was exposed to [^{14}C]galactose (5×10^{-5} M) in proteose peptone–beef extract for three generations and the cells were washed and hydrolyzed in 1 N HCl for 6 hr at 100°. Chromatography of the hydrolysate (5×10^5 cpm) in solvents A–C showed only a single radioactive component with the R_F of galactose; less than 0.2% of the applied radioactivity was recovered in areas corresponding to L-*glycero*-D-*manno*-heptose, glucose, glucosamine, mannose, rhamnose, and ribose. The failure to convert galactose into other sugar components indicated essentially complete absence of epimerase activity. Attempts to detect the presence of sugars characteristic of the complete core region (galactose and glucosamine) or O-specific side chains in the incomplete *G30* lipopolysaccharide were also negative. Analysis of the lipopolysaccharide isolated after growth in the presence of [^{3}H]- or [^{14}C]glucose showed only traces of radioactivity (less than 0.5% of the total) in galactose, mannose, rhamnose, and abequose. Labeled glucosamine, a component of lipid A, was observed in hydrolysates obtained from intact lipopolysaccharide, but accounted for less than 2% of the radioactivity of the lipid-free polysaccharide isolated after mild acid hydrolysis of lipopolysaccharide.

Incorporation of O-specific chains into lipopolysaccharide by the cell envelope fraction of *G30* was not dependent upon enzymatic completion of the core region of the endogenous mutant lipopolysaccharide. These cell envelope preparations are able to carry out extension of the incomplete core upon incubation with UDP-glucose and UDP-*N*-acetylglucosamine, but addition of these nucleotide sugars had no detectable effect, qualitative or quantitative, on the incorporation of [^{14}C]-mannose into lipopolysaccharide. Wide fluctuation in the relative efficiency of O-specific sugar incorporation into hapten *vs.* lipopolysaccharide can be attributed, in large measure, to the variable physiological state of cells from which the cell envelope preparation was extracted; the present results, therefore, do not conflict with other, similar studies in which one or the other polymer was recovered as the major product (see section B and Discussion).

3. EFFECT OF ABEQUOSE ON THE SIZE DISTRIBUTION OF ENZYMATICALLY SYNTHESIZED O-SPECIFIC HAPTEN. To determine the size distribution of polymeric haptens synthesized from the two possible *in vitro* precursors, trisaccharide or tetrasaccharide A or B, the cell envelope was incubated with the appropriate nucleotide sugars, and

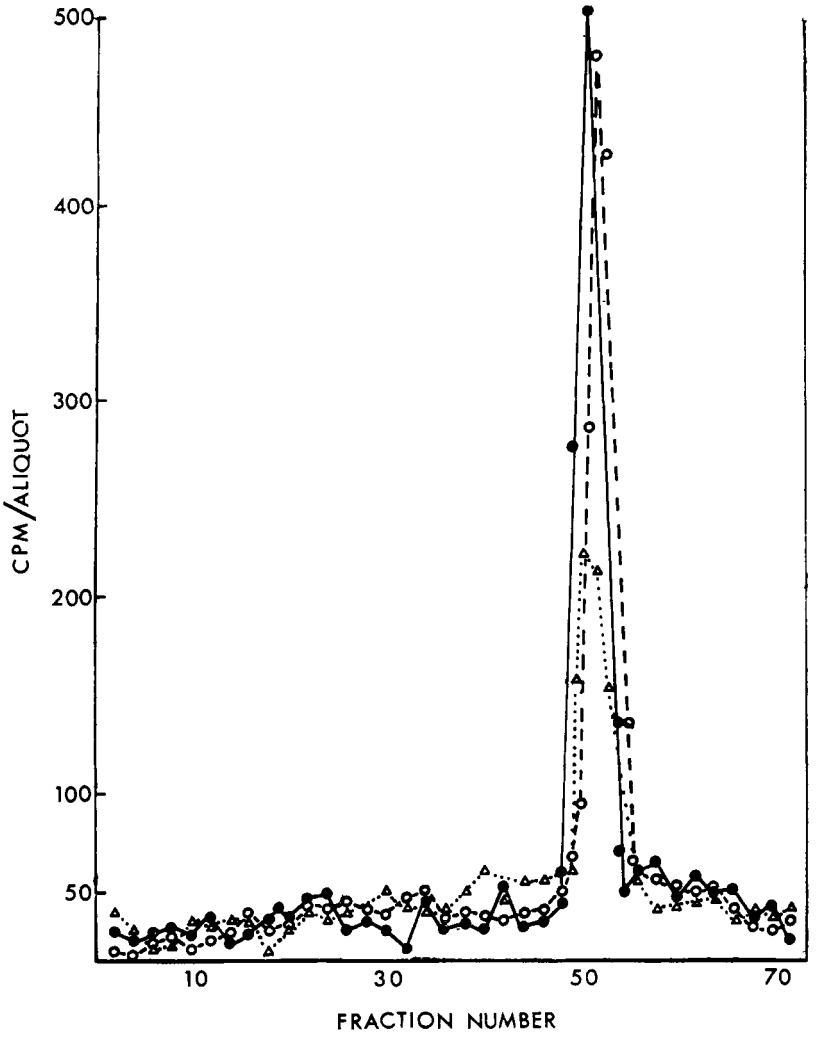

FIGURE 5: CsCl density gradient centrifugation of *in vitro* labeled lipopolysaccharides from *G30*. Two aliquots of a standard cell envelope preparation were incubated for 30 min at 37° to contain 2.9 mg of protein in 1.0 ml. One mixture contained 130 μmmoles of UDP-[^{14}C]galactose (2740 cpm/mμmole) and the other contained 125 mμmoles of GDP-[^{14}C]mannose (1.5×10^4 cpm/mμmole) plus 130 mμmoles of UDP-galactose and 67 mμmoles of TDP-rhamnose. The reaction was stopped with 0.1 N acetic acid, and the radioactivity was extracted from the washed precipitate with warm phenol. Polymeric products were separated by sequential filtration through Sephadex G-50 and G-200. Ethanol fractionation of a test aliquot from the G-200 excluded material showed less than 5% radioactive contamination of mannose-labeled lipopolysaccharide by hapten in this experiment. The samples were concentrated and sedimented in CsCl solutions for 48 hr at 0°. Aliquots of fractions collected from each gradient were counted by scintillation, and the buoyant density of lipopolysaccharide was interpolated on a graph constructed from refractometric measurements on about ten fractions per gradient. The *in vivo* marker was purified by the same procedures from a culture of *G30* grown as wild-type phenocopy on [^{14}C]galactose. (●—●) *In vivo* (lipopolysaccharide; (O---O) [^{14}C]galactose-lipopolysaccharide; (Δ···Δ) [^{14}C]mannose-lipopolysaccharide.

(mannosyl--rhamnosyl—galactosyl—PP—lipid)
A

abequosyl
|
(mannosyl—rhamnosyl—galactosyl—PP—lipid)
B

the size of phenol-extractable products was estimated from their filtration profiles on Sephadex gels. GDP-[^{14}C]mannose or CDP-[^{14}C]abequose was employed as radioactive substrate as indicated in Table III. The incubations were carried out at 25° rather than 37° since incorporation of abequose is markedly decreased at higher temperatures (Osborn and Weiner, 1968). The polymerization reaction also appears to be temperature sensitive. We have found the proportion of [^{14}C]mannose recovered in low molecular weight oligosaccharide intermediates (included by Sephadex G-50) to be reproducibly higher in products synthesized at 37° than at 25° (Table III).

Following extraction of the incorporation products with 45% phenol, the polymeric fractions were first separated from low molecular weight oligosaccharide intermediates by passage through Sephadex G-50. The fraction excluded by Sephadex G-50 was then passed through Sephadex G-200 (Figure 6). On Sephadex G-200 columns, each labeled product consisted of two discrete species, a minor excluded fraction and a major fraction partially or completely included. The patterns were qualitatively similar to those observed with O-specific hapten accumulated *in vivo* (Kent and Osborn,

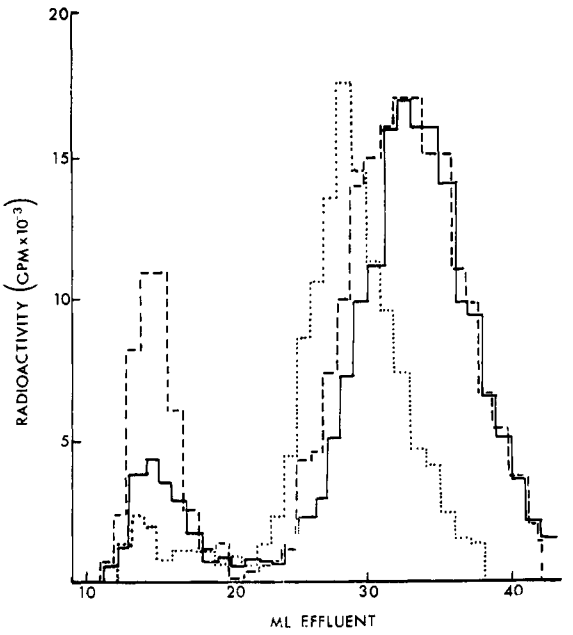

FIGURE 6: Gel filtration profiles of O-specific haptens formed *in vitro* from the trisaccharide or tetrasaccharide repeating unit. Elution profile on Sephadex G-200 of samples described in legend to Table III. The filtration was carried out at room temperature with 0.05 M ammonium carbonate, and 1.0-ml fractions were collected. (···) [^{14}C]Abequose-labeled product; (---) [^{14}C]mannose-labeled product, CDP-abequose omitted from incubation mixture; (——) [^{14}C]mannose-labeled product, CDP-abequose present.

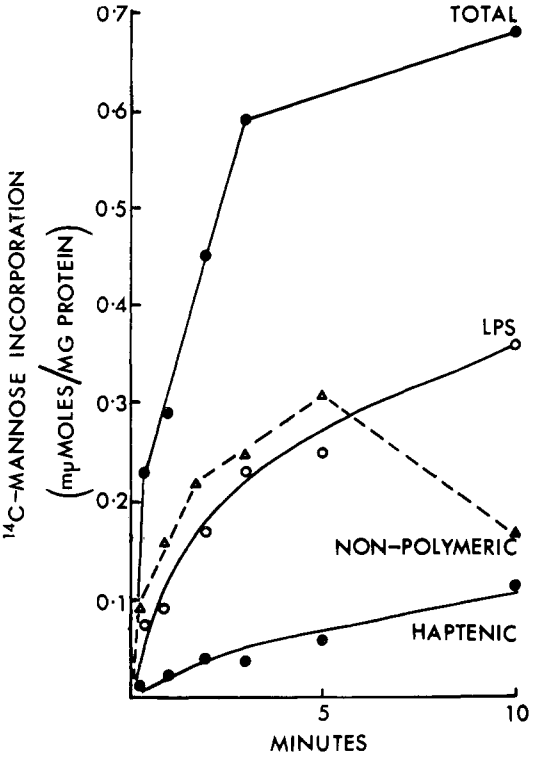

FIGURE 7: Incorporation of [^{14}C]mannose into polymers by wild-type cell envelope. The cell envelope fraction was prepared from an exponentially growing culture of wild-type cells after overnight storage of cell paste at −20°. The incubation mixture of 6.65 ml contained: 660 mμmoles of UDP-galactose, 440 mμmoles of TDP-rhamnose, 2.76 mg of protein, 52 μmoles of $MgCl_2$, 13 μmoles of EDTA, and 520 μmoles of Tris-acetate (pH 8.5). This mixture was incubated for 5 min at 37° and equilibrated to 23° in a water bath for 5 min before addition of 130 mμmoles of GDP-[^{14}C]-mannose (9240 cpm/mμmole) in 0.15 ml at zero time. At the indicated intervals thereafter, aliquots were removed to 0.1 N acetic acid, and the precipitated material was removed by centrifugation. The pellets were extracted with warm phenol, and the aqueous phases were concentrated under reduced pressure. The sample was passed through a column of Sephadex G-50 and collected as the excluded (polymeric), the partially included, and the included (nonpolymeric) fractions. The polymeric fraction was separated into hapten and lipopolysaccharides by ethanol precipitation.

1968a) except that the proportion of excluded material was smaller (Table III). In this experiment, only a small fraction of the radioactive products excluded by G-200 corresponded to lipopolysaccharide; the cell envelope fraction was prepared from fermentor-grown cells which had been stored at −20° for 2 weeks. We regard the data as representing a maximal estimate of lipopolysaccharide content in the G-200 excluded material. Each of the excluded mannose-labeled fractions failed to form a discrete band upon CsCl density gradient centrifugation. These results confirmed the absence of significant amounts of radioactive lipopolysaccharide, and suggested that *in vitro* haptenic material was smaller than the corresponding *in vivo* hapten, which formed a broad band at a density of 1.38 (Kent and Osborn, 1968a). Although the proportion of hapten excluded by Sephadex G-200 was not increased in the product containing [^{14}C]-abequose, the average size of the major, partially included fraction appeared to be somewhat larger than that observed with either mannose-labeled product (Figure 6). However, this difference could probably be accounted for by the increased size of the oligosaccharide repeating unit (tetrasaccharide as opposed to trisaccharide) rather than an increased chain length.

Hence, the chain length of the enzymatically synthesized haptenic polymer does not appear to be strongly dependent upon the nature of the repeating unit available for polymerization at 25°. The distribution of chain lengths was also relatively independent of the time of incubation. In a similar experiment carried out with abequose-labeled haptenic polymer formed in 30 sec at 23°, 44% of the counts incorporated into hapten was excluded from Sephadex G-200 in a sample in which lipopolysaccharide accounted for half the total polymer. Therefore, enzymatic synthesis of very long haptenic chains is extremely rapid, and can occur under conditions in which O-specific chains are also incorporated into lipopolysaccharide.

B. Kinetics of O-Specific Synthesis in Vitro. The relative rates of synthesis, or accumulation, of O-specific hapten and lipopolysaccharide-bound O-specific chains varied widely with different experimental conditions. The time course of incorporation of [^{14}C]mannose into oligosaccharide–lipid intermediate, haptenic polymer, and lipopolysaccharide in cell envelope preparations of wild-type cells is illustrated in Figure 7. The system was initially incubated for 5 min at 23° with UDP-galactose and TDP-rhamnose in order to form the disaccharide lipid intermediate. GDP-[^{14}C]mannose was then added

TABLE III: Effect of Abequose on Size Distribution of Enzymatically Synthesized Haptens.[a]

Radioactive Substrate	CDP-Abe Added	Incubation Temp (°C)	Total Incorp (mμmoles/mg)	% Polymeric	Polymer Excluded by Sephadex G-200		
					% as Hapten	% as Lipopolysaccharide	% of Total Sample
GDP-[^{14}C]Man	0	25	2.2	84	18	1.6	20
GDP-[^{14}C]Man	+	25	2.9	89	11	2.3	13
CDP-[^{14}C]Abe	+	25	1.3	88	6.4	2.1	8.5
GDP-[^{14}C]Man	0	37	1.8	55			

[a] A standard cell envelope fraction from *G30* was incubated in a total volume of 0.5 ml to contain: 1.5 mg of protein, 140 μmoles of Tris-acetate (pH 8.5), 5 μmoles of $MgCl_2$, 1 μmole of EDTA, and the following nucleotide sugars were included: 88 mμmoles of UDP-galactose, 40 mμmoles of TDP-rhamnose, 10 mμmoles of GDP-mannose (1.2×10^5 cpm/mμmole, if labeled), and 6.4 mμmoles of CDP-abequose (1.5×10^4 cpm/mμmole, if labeled). The last mixture was identical with the first but was incubated at 37°. After 30-min incubation, the reaction was stopped with 0.1 N acetic acid and the washed precipitates were extracted with warm phenol; each aqueous phase was concentrated under reduced pressure to 1.0 ml and applied to a column of Sephadex G-50 for elution. The excluded fraction (the polymeric material) was concentrated to 1.0 ml and applied to a column of Sephadex G-200 (see Figure 6 for filtration profiles). The fractions collected from G-200 were pooled as the excluded, partially included, and included components; each was concentrated and aliquots were tested for lipopolysaccharide content by (1) precipitation with 67% ethanol after addition of wild-type carrier lipopolysaccharide, and (2) elution of radioactivity from DEAE-cellulose columns as described in the legend to Figure 2.

and samples were removed for analysis thereafter. The rate of incorporation of [^{14}C]mannose into the trisaccharide intermediate was rapid relative to subsequent polymerization reactions and during the first 5-min incubation, oligosaccharide accounted for about 50% of the total radioactivity of the cell envelope. In this experiment, approximately 80% of the radioactivity present in polymer was recovered as lipopolysaccharide over the entire period of incubation. This ratio of lipopolysaccharide to haptenic polymer was unusually high; in other experiments, the rate of accumulation of O-specific hapten was approximately equal to that of lipopolysaccharide.

Rates of formation or accumulation of haptenic polymer much higher than that of lipopolysaccharide were observed under somewhat different experimental conditions. Such an experiment is illustrated in Figure 8. Cells of the epimeraseless strain (*G30*) were grown in a Casamino Acid medium and disrupted by freezing and thawing in 0.1 M EDTA previously adjusted to pH 8.6. Following incubation with UDP-galactose and TDP-rhamnose for 5 min, GDP-mannose and CDP-[^{14}C]-abequose were added at zero time. Immediately after removal of the 1-min sample, a rapid accumulation of hapten was initiated by increasing the concentration of Mg^{2+}. This had little effect on the rate of incorporation of [^{14}C]abequose into lipopolysaccharide. As previously reported (Osborn and Weiner, 1968), incorporation of abequose into polymers was extremely efficient, and no significant accumulation of [^{14}C]oligosaccharide precursors was observed at any time.

The results indicated that the rates of accumulation of hapten relative to lipopolysaccharide-bound O-antigen were subject to wide variation depending upon experimental conditions. However, it was not possible to determine from these data whether the haptenic polymer is an obligatory precursor of the O-specific chains incorporated into lipopolysaccharide.

Discussion

These studies extend previous observations (Zeleznick *et al.*, 1965; Weiner *et al.*, 1965; Osborn and Weiner, 1968) and provide definitive evidence for enzymatic incorporation of O-specific side chains into lipopolysaccharide in isolated cell envelope fractions and EDTA-treated cell preparations. Incorporation of O-specific sugars into lipopolysaccharide was dependent upon the nature of the endogenous acceptor lipopolysaccharide. Preparations obtained from both wild-type and phosphomannose isomeraseless strains, the lipopolysaccharides of which contain the complete core structure, catalyzed rapid incorporation of O-specific sugar into both the lipid-linked haptenic polymer and lipopolysaccharide. In contrast, mutants of the *rfa* and glucosyl transferase I types, which produce incomplete core structures, formed the lipid-linked O-specific polymer *in vitro*, but no significant incorporation into the mutant lipopolysaccharide was observed. The results obtained *in vitro* with these strains were closely correlated with the patterns of biosynthesis observed *in vivo*, in agreement with the currently accepted hypothesis that the complete core structure of the lipopolysaccharide is essential for attachment of O-antigen side chains.

The incorporation of O-antigen into lipopolysaccharide observed in the mutant lacking UDP-galactose-4-

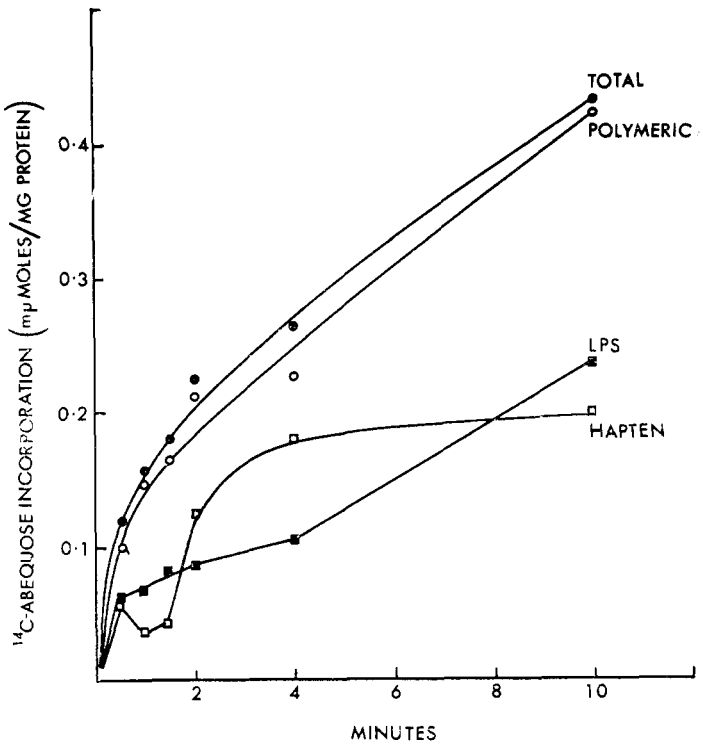

FIGURE 8: Time course of [^{14}C]abequose incorporation into polymers by cell-free preparations of strain *G30*. An exponentially growing culture of *G30* was harvested, washed once with 0.9% NaCl, resuspended in $^1/_{30}$ the original volume of 0.1 M EDTA (pH 8.6) (Wright *et al.*, 1965), and frozen overnight at −20°. The thawed extract (2.5 mg/ml of protein) was preincubated at 25° for 5 min in a total volume of 30 ml containing per ml: 150 mμmoles of UDP-galactose, 60 mμmoles of TDP-rhamnose, 80 μmoles of Tris-acetate buffer (pH 8.5), 1.38 mg of protein as cell extract (containing 5.5 μmoles of EDTA), and 6 μmoles of $MgCl_2$. At zero time, 20 mμmoles/ml of GDP-mannose and 1.1 mμmoles/ml of CDP-[^{14}C]-abequose (1.7×10^4 cpm/mμmole) were added simultaneously. Samples were removed at the indicated intervals thereafter to 0.1 N acetic acid. Immediately following removal of the 1-min sample, 1.5 μmoles/ml of $MgCl_2$ was added. The washed pellets were extracted with warm phenol, and the concentrated aqueous phases were filtered through Sephadex G-50. The polymeric (excluded) fraction was separated into lipopolysaccharide and hapten by ethanol precipitation.

epimerase (*G30*) was therefore unexpected, since the block in synthesis of the lipopolysaccharide core occurs at an earlier stage in this mutant than in the *rfa* type. The rate and extent of incorporation of O-specific sugars into lipopolysaccharide in enzyme preparations from *G30* were comparable with those observed in the wild type and *M2*. Although it is difficult to exclude definitively the possibility that the mutant contains sufficient residual epimerase activity to permit formation of some normal cores, no evidence of significant leakiness could be found. Final assessment of the results obtained with *G30* awaits further characterization of the enzyme catalyzing transfer of O-specific species to lipopolysaccharide and identification of the O-antigen attachment sites in the normal wild-type lipopolysaccharide and in the enzymatic products.

The rate of incorporation of O-specific sugars from nucleotide–sugar substrates into lipopolysaccharide and the rate of synthesis or accumulation of the hapten varied widely according to experimental conditions. To a large extent, this was dependent upon the physiological state of the cells from which the envelope fraction was prepared. Thus stationary-phase cells or frozen cells stored for several weeks at −20° tended to yield extracts in which the proportion of mannose incorporated into lipopolysaccharide relative to hapten was lowered. Since polymerization of the oligosaccharide–lipid intermediate appeared to be rate limiting (as judged by accumulation of $CHCl_3$–methanol-soluble intermediates), efforts were made to maximize the rate of polymerization. Advantage was taken of the fact that the tetrasaccharide unit tends to polymerize more quickly than the trisaccharide (Osborn and Weiner, 1968). In addition, disrupted cell preparations, obtained by freezing and thawing concentrated washed cells in dilute EDTA (Wright *et al.*, 1965), gave slightly better polymerization than the standard cell envelope fraction, and further improvement was obtained by lowering the incubation temperature from 37 to 23°. Combining all of these factors and preincubating with precursors of disaccharide, it was possible to obtain very good polymerization of tetrasaccharide by EDTA preparations. Half the polymeric [^{14}C]abequose was hapten associated at 23° in 30 sec after addition of label (analogous to the results obtained *in vivo* (Kent and Osborn, 1968b) and almost half of this was excluded from Sephadex G-200, showing thereby not only rapid but also extensive polymerization. Under optimal conditions, the rate of incorporation of [^{14}C] mannose or [^{14}C] abequose into lipopolysaccharide equaled or exceeded the rate of accumulation of the lipid-linked haptenic polymer. We were not successful in obtaining conditions permitting sequential incorporation into lipid-linked hapten and lipopolysaccharide *in vitro*, so that definitive conclusions as to the role of the lipid-linked polymer as an intermediate in the transfer of O-antigen to lipopolysaccharide could not be made. However, evidence that the haptenic polymer is indeed a precursor of the O-antigen chains of lipopolysaccharide has been obtained from further studies on the kinetics of O-antigen synthesis in intact cells (Kent and Osborn, 1968b).

References

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

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1951), *Anal. Chem. 28*, 350.

Kent, J. L., and Osborn, M. J. (1968a), *Biochemistry 7*, 4396 (this issue; preceding paper).
Kent, J. L., and Osborn, M. J. (1968b), *Biochemistry 7*, 4419 (this issue; following paper).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Lüderitz, O., Staub, A. M., and Westphal, O. (1966), *Bacteriol. Rev. 30*, 192.
Osborn, M. J. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 499.
Osborn, M. J. (1968), *Nature 217*, 957.
Osborn, M. J., Rosen, S. M., Rothfield, L., and Horecker, B. L. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1831.
Osborn, M. J., and Weiner, I. M. (1968), *J. Biol. Chem. 243*, 2631.
Robbins, P. W., Bray, D., Dankert, M., and Wright, A. (1967), *Science 158*, 1536.
Rosen, S. M., and Zeleznick, L. D. (1966), *Methods Enzymol. 8*, 145.
Rothfield, L., Osborn, M. J., and Horecker, B. L. (1964), *J. Biol. Chem. 239*, 2788.
Subbaiah, T. V., and Stocker, B. A. D. (1964), *Nature 201*, 1298.
Weiner, I. M., Higuchi, T., Rothfield, L. Saltmarsh-Andrew, M., Osborn, M. J., and Horecker, B. L. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 228.
Wright, A., Dankert, M., and Robbins, P. W. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 235.
Zeleznick, L. D., Rosen, S. M., Saltmarsh-Andrew, M., Osborn, M. J., and Horecker, B. L. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 207.

Haptenic O-Antigen as a Polymeric Intermediate of *in Vivo* Synthesis of Lipopolysaccharide by *Salmonella typhimurium**

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ABSTRACT: A mutant strain of *Salmonella typhimurium* deficient in phosphomannose isomerase was used to study the kinetics of O-antigen synthesis *in vivo*, these polysaccharides being the sole end products of [^{14}C]-mannose incorporation. The kinetics of uptake of radioactivity into haptenic O-antigen and lipopolysaccharide were consistent with the prediction of an intermediate with high turnover rate. Pulse-chase studies demonstrated rapid and efficient transfer of O-antigenic radioactivity from antigen-carrier lipid hapten to lipopolysaccharide; at least 80% of the label transferred to lipopolysaccharide during the initial chase period was derived from hapten. The addition of completed O-antigenic polymer to the preformed lipopolysaccharide acceptor represents a unique biochemical reaction whereby two different polymers are covalently joined.

Although the role of oligosaccharide intermediates linked to antigen-carrier lipid in biosynthesis of the O-antigen of *Salmonella* is now well established (Weiner *et al.*, 1965; Wright *et al.*, 1965; Osborn and Weiner, 1968), those steps of the pathway which result in attachment of O-antigen chains to lipopolysaccharide are still poorly understood. We have postulated that polymerization of O-antigen chains precedes transfer to lipopolysaccharide, and that the immediate precursor of the O-specific side chains of lipopolysaccharide is an antigen-carrier lipid-linked polysaccharide (O-specific hapten) which occurs as a product of O-antigen synthesis both *in vitro* and *in vivo*. This communication presents evidence in support of the proposed mechanism derived from studies on the kinetics of O-antigen synthesis *in vivo*. For this purpose we have employed a mutant of *Salmonella typhimurium* which lacks phosphomannose isomerase. Under the usual conditions of growth, no

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