Properties of the O-Specific Hapten Formed *in Vivo* by Mutant Strains of *Salmonella typhimurium**

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ABSTRACT: The isolation and separation of two polymers each containing sugars characteristic of O-antigen in *Salmonella typhimurium* have shown that mutant strains blocked in synthesis of the core moiety of lipopolysaccharide accumulate significant amounts of O-specific hapten. In contrast, cultures of wild-type cells or wild-type phenocopies incorporate the bulk of O-specific moieties into lipopolysaccharide and contain only minor amounts of hapten. Although both O-specific polymers are firmly bound to the particulate cell envelope fraction and, in general, are similarly distributed among the subcellular fractions, experiments with intact mutant cells show that the haptenic polymer is not on the outer surface of the cell. Treatment with EDTA or with EDTA plus lysozyme exposes the hapten as judged by a significant increase in adsorption of O-specific phage by these cells. After phenol extraction of intact cells, the hapten was separated from lipopolysaccharide by differential ethanol precipitation, by chromatography on DEAE-cellulose columns, by centrifugation at 105,000*g*, and by equilibrium sedimentation in CsCl density gradients.

Its properties are identical with those previously described for the O-specific hapten synthesized enzymatically: (1) galactose 1-phosphate is the reducing terminus; (2) average degree of polymerization is about 30; (3) characteristic oligosaccharides are recovered after partial acid hydrolysis. Both polymers are precipitated by anti-O antiserum and each competes with the other for precipitation.

The lipopolysaccharide of *Salmonella typhimurium* is a complex heteropolysaccharide covalently linked to a specific lipid; it is responsible for the major antigenic properties of the cell and for many of its phage sensitivity patterns. The polysaccharide moiety can be subdivided into three distinct structural regions as shown schematically in Figure 1. Long O-antigenic polymer chains, composed of tetrasaccharide repeating units, are linked to an inner core of at least five hexose residues which in turn is attached to a polyanionic backbone of unusual composition. The core region is generated by sequential addition of individual hexose residues to the growing acceptor molecule (Osborn *et al.*, 1964). In contrast, studies on enzymatic synthesis of the O-antigen chains have demonstrated initial formation of the repeating unit as an oligosaccharide–phosphate intermediate covalently linked to a specific lipid coenzyme: antigen-carrier lipid (Weiner *et al.*, 1965; Wright *et al.*, 1965; Osborn and Weiner, 1968). The carrier lipid has recently been identified by Wright *et al.* (1967) as the phosphomonoester of a C_{55}-polyisoprenoid alcohol. Subsequent reactions then result in polymerization of repeating units to form O-specific polysaccharide chains. However, previous work in this laboratory (Weiner *et al.*, 1965) has shown that enzymatic synthesis of O-antigen by isolated cell envelope fractions leads to accumulation of O-specific polysaccharide chains which are not attached to lipopolysaccharide but remain linked to the intermediate carrier lipid. An O-specific haptenic polysaccharide separate from lipopolysaccharide is also accumulated *in vivo* by certain mutants of *Salmonella;* Beckmann *et al.* (1964) first encountered this haptenic polymer in *rfa* mutants, which are blocked in a late step in synthesis of the core region of lipopolysaccharide, and its presence has since been confirmed in mutant classes blocked in earlier stages of core formation (Osborn, 1966a, 1968). However, the relationship of the O-specific hapten formed by these mutants to the lipid-linked biosynthetic product has not previously been clarified.

These investigations have been concerned with the role of the haptenic (O-specific) polysaccharide as an intermediate in the biosynthesis of the complete wild-type lipopolysaccharide. Transfer of the polymeric hapten onto the preformed core of lipopolysaccharide would represent a unique biochemical reaction whereby two polymers, each synthesized from similar monomers but by a different mechanism, are subsequently linked by a covalent bond.

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TABLE 1: Summary of Bacterial Strains Employed.

Strain	Genotype[a]	Lipopolysaccharide Chemotype[b,c]	O-Specific Hapten *in Vivo*[c]	Reference
LT-2	*Wild type*	S	0	Osborn *et al.* (1962)
M2	*pmi*$^-$	Ra (−Man)	0	Rosen *et al.* (1965)
		S (+Man)	0	
G30	*epi*$^-$	Rc (−Gal)	0	Osborn *et al.* (1962)
		S (+Gal)	0	
GRA-5	*epi*$^-$, *rfa*	Rc (−Gal)	0 (−Gal)	New strain
		Rb (+Gal)	+ (+Gal)	
TV227, TV161	*rfa*	Rb	+	Subbaiah and Stocker (1964)
SL1060	*pmi*$^-$, *α-3-Gal-transferase*$^-$	Rb_3	0 (−Man)	Wilkinson and Stocker (1968);
			+ (+Man)	Osborn (1968)
SL1032	*Glu-transferase I*$^-$	Rd	+	Wilkinson and Stocker (1968); Osborn (1968)

[a] Gentotype designations are as follows: *pmi*, phosphomannose isomerase; *epi*, UDP-galactose-4-epimerase; *rfa*, complex locus governing synthesis of the core region (Subbaiah and Stocker, 1964); α-3-Gal-transferase (Gal-transferase I) and Glu-transferase I catalyze early steps in synthesis of the core (Rosen *et al.*, 1964; Rothfield *et al.*, 1964). [b] See Figure 1 for corresponding lipopolysaccharide structure. S signifies complete wild-type structure. [c] In mutants blocked in biosynthesis of nucleotide sugar precursors (*pmi*$^-$, *epi*$^-$), addition of the appropriate sugar (mannose or galactose, respectively) to the medium results in restoration of the parental phenotype. The relevant properties in the presence (+Man; +Gal) and absence (−Man; −Gal) of the exogenous hexose are indicated.

This communication describes the isolation and characterization of the O-specific hapten formed *in vivo* by core mutants of *S. typhimurium* and presents evidence for the identity of this polymer with the previously described enzymatic product. In the following papers, we report further studies in enzymatic synthesis of haptenic and lipopolysaccharide-bound O-antigen polymers (Kent and Osborn, 1968a), and describe the results of pulse-chase experiments indicating that the haptenic polymer can act *in vivo* as precursor of the O-antigen chains of lipopolysaccharide (Kent and Osborn, 1968b).

Materials and Methods

A. Organisms. The genotypes and relevant properties of strains employed are summarized in Table I. Strains *G30* and *M2* have been previously described. *GRA-5*, an *rfa* derivative of *G30*, was isolated after mutagenesis with ethyl methanesulfonate by selection for simultaneous resistance to phages P22, FO, and 6SR during growth in the presence of galactose (Osborn, 1966b). The formation of *rfa* type lipopolysaccharides during growth in the presence of galactose was confirmed by isolation and quantitative analysis of the sugar composition of the polysaccharide portion of the lipopolysaccharide. Wild-type *Salmonella LT2* and phage P22 were obtained from Dr. Norton Zinder of the Rockefeller University, and all other bacterial mutants and phages from Dr. B. A. D. Stocker, Stanford University School of Medicine.

Bacteria were grown at 37° with vigorous aeration in proteose peptone–beef extract (Difco) and harvested in mid to late log phase.

B. Materials. Wild-type lipopolysaccharide specifically labeled with [^{14}C]mannose was prepared from strain *M2* (phosphomannose isomerase negative) following growth in the presence of [^{14}C]mannose as previously described (Osborn and Weiner, 1968). The lipopolysaccharide was isolated by phenol extraction and purified by gel filtration and ethanol precipitation.

[^{14}C]Galactose and [^{14}C]mannose were obtained from New England Nuclear Corp., and optical grade CsCl from Harshaw Corp. Alkaline phosphatase was purchased from Worthington. Carrier-free [^{32}P]$_i$ was obtained from Tracerlab.

C. Analytical Procedures. Total carbohydrate was determined by the phenol–H_2SO_4 procedure (Dubois *et al.*, 1951), heptose by a modification (Osborn, 1963) of the cysteine–H_2SO_4 reaction (Dische, 1953), and KDO[1] by the semicarbazide method (Umbarger and Magasanik, 1952). Abequose was liberated from the polysaccharide by hydrolysis in 0.025 N H_2SO_4 for 15 min at 100°, and measured by the procedures of Cynkin and Ashwell (1960); the final reaction mixture was made alkaline with NaOH before determination of absorbance at 532 mμ in order to minimize interference by KDO. Glucose and galactose were determined with glucose and galactose oxidase (Worthington), respectively, and glucosamine by the method of Reissig *et al.* (1955). Sam-

[1] The following abbreviations are employed: Abe, abequose; Man, D-mannose; Rha, L-rhamnose; Gal, D-galactose; Glu, D-glucose; GlcN, D-glucosamine; Hep, L-*glycero*-D-*manno*-heptose; KDO, 3-deoxy-D-*manno*-octulosonate.

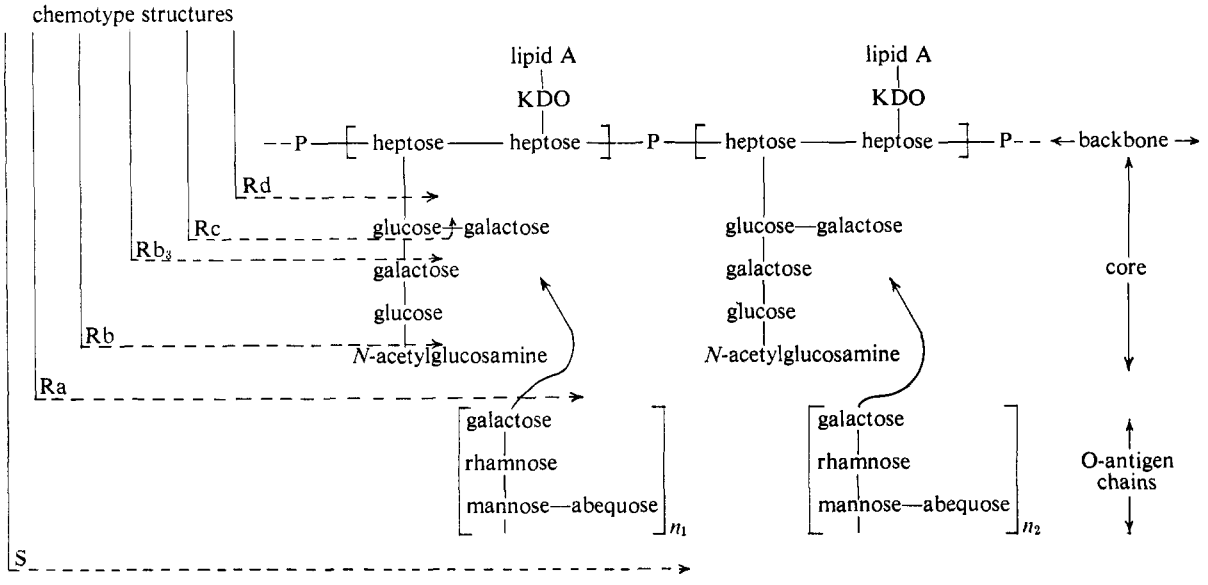


FIGURE 1: Schematic structure of *S. typhimurium* lipopolysaccharide. Chemotype designations (S, for complete lipopolysaccharide, Ra, Rb, etc., for mutant types) are those employed by Lüderitz *et al.* (1966). Note that Rx refers to lipopolysaccharide chemotype and *rfx* to the genotype. The backbone also contains phosphorylethanolamine.

ples were hydrolyzed in 1 N HCl for 3 hr at 100° prior to these determinations.

D. Chromatographic and Electrophoretic Analysis. The following solvents and buffers were used: solvent A, 1-butanol–pyridine–water (6:4:3); solvent B, ethyl acetate–acetic acid–water (3:3:1); solvent C, pyridine–acetic acid–water (1:10:89) (pH 3.5); solvent D, pyridine–acetic acid–water (10:1:89) (pH 6); and solvent E, 1-butanol–pyridine–0.05 M morpholinium borate (6:4:3) (Bray and Robbins, 1967). 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.

E. Counting Procedures. Radioactivity on paper strips was located with a Baird-Atomic 4π windowless Scanogram. Alternatively, the strips were cut in 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% Packard

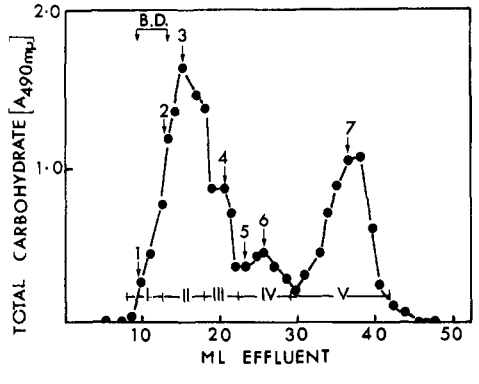


FIGURE 2: Purification of trichloroacetic acid extracted hapten by gel filtration on Sephadex G-100. The cell envelope fraction obtained from 50 g wet weight of cells was stirred with 500 ml of 5% trichloroacetic acid for 1 hr at 0°; the residue was collected by centrifugation and reextracted once. The crude trichloroacetic acid extract (110 mg of carbohydrate) was freed of trichloroacetic acid by ether extraction and concentrated under reduced pressure at 30° to 5 ml of viscous, yellowish solution. In order to remove the viscous material, two volumes of ethanol was added, and the mixture was chilled for 30 min at 0°. The resulting precipitate was removed by centrifugation and found to contain no carbohydrate. The supernatant fraction, which contained all of the original abequose, was neutralized, concentrated to 2 ml, and applied to a 1 × 40 cm column of Sephadex G-100. Elution was carried out with H_2O. Aliquots of the fractions indicated by arrows were taken for hydrolysis and chromatography. BD = blue dextran.

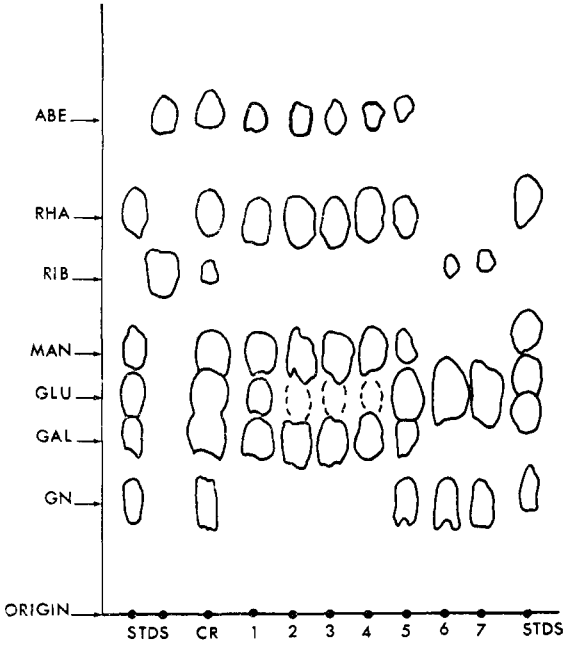


FIGURE 3: Chromatography of Sephadex G-100 fractions. Aliquots of the fractions indicated in Figure 2 were subjected to complete acid hydrolysis and chromatographed in solvent A for 20 hr.

TABLE II: Analysis of Sephadex G-100 Fractions.[a]

Fraction	Molar Ratio						Total Carbohydrate	
	Abe	Gal	Glu	GlcN	Hep	KDO	mg	% O-Specific
Crude	1.17	1.0	3.1	0.81	<0.03	<0.03	105.0	45
G-100 I	0.91	1.0	0.25	<0.03	<0.03	<0.03	4.5	92
II	1.08	1.0	0.07	<0.03	<0.03	<0.03	38.5	95
III	1.12	1.0	0.22	0.15	<0.03	<0.03	14.7	87
IV	0	0	1.0	0.7	<0.03	<0.03	10.4	0
V	0	0	1.0	0.4	<0.03	<0.03	31.7	0

[a] Fractions were pooled as shown in Figure 2. Determinations of heptose and total carbohydrate were carried out prior to acid hydrolysis, KDO and abequose after mild acid hydrolysis, and glucose, galactose, and glucosamine after total acid hydrolysis, as described in Materials and Methods. Mannose and rhamnose were present in the abequose-containing fractions, as shown in Figure 3.

thixotropic gel (Cab-O-Sil) was used as solvent for counting fractions from CsCl density gradients. Bray's solution was employed for scintillation counting of doubly labeled materials and for samples containing ^{3}H; ^{14}C was determined either by this method or by use of a windowless gas-flow counter.

Results

A. Purification and Properties of O-Specific Haptens from rfa and Related Core Mutants. 1. ISOLATION OF HAPTEN FROM *SL 1032* BY TRICHLOROACETIC ACID EXTRACTION AND PRELIMINARY CHARACTERIZATION. We have previously reported the presence of an O-specific haptenic fraction in aqueous phenol extracts of strain *SL 1032* (Osborn, 1966a, 1968). This mutant lacks the enzyme responsible for transfer of the first glucosyl residue into the core region of the lipopolysaccharide (glucosyl transferase I), produces an incomplete lipopolysaccharide lacking all sugars distal to heptose (*cf.* Figure 1), and accumulates hapten *in vivo*, presumably because the cell is unable to generate the proper sites for attachment of O-antigen chains to the lipopolysaccharide core. Preliminary experiments showed that extraction of whole cells or isolated cell envelope preparations with cold 5% trichloroacetic acid yielded a solubilized haptenic fraction containing all the O-specific residues of the cell; since the trichloroacetic acid extracts were essentially free of lipopolysaccharide and nucleic acid, they were advantageous for preliminary characterization of the hapten fraction. Cell envelope fractions were employed as starting material for preliminary purification studies because the amount of O-specific material obtained from cell envelope preparations was similar to that obtained from whole cells (see below), as judged quantitatively by abequose determination and qualitatively by paper chromatography of acid hydrolysates.

Acid hydrolysis and chromatography of concentrated crude trichloroacetic acid extracts revealed large amounts of the O-specific sugars, abequose, mannose, rhamnose, and galactose (*cf.* Figure 3, CR). Glucose and glucosamine were also present as major components, but little ribose or deoxyribose; examination of the ultraviolet spectrum showed minor nucleotide contamination. Filtration of the crude extract on Sephadex G-100 partially purified the hapten as illustrated in Figure 2. Hydrolysis and chromatography (Figure 3) of the fractions indicated by arrows in Figure 2 showed that the first peak contained all of the O-specific sugars, but little glucose and little or no glucosamine, while the later fractions, conversely, were rich in glucose and glucosamine, but lacked O-specific sugars. Quantitative analysis of these pooled fractions (Table II) showed that sugars characteristic of the core region of lipopolysaccharide (glucose, glucosamine, heptose, and KDO) were absent from fractions I and II; the small amount of glucosamine present in fraction III is attributed to contamination by fraction IV.

The results indicated that the O-specific polymer was not attached to core polysaccharide and were consistent with the biosynthetic evidence that the mechanism of synthesis of O-antigen is independent of that of the core polysaccharide. Although the small amount of glucose present in the major hapten fraction (fraction II) may indicate residual contamination by other polysaccharides, the O-antigen of *S. typhimurium* is known to contain variable amounts of glucose associated with the immunological determinant, 12_2 (Tinelli and Staub, 1960). Assuming equimolar ratios of the four major O-specific sugars, fraction II appeared to be approximately 95% pure with respect to total carbohydrate. The nonhapten fractions, IV and V, may correspond to the glucose–glucosamine polymer previously reported in *Escherichia coli* K 12 by Mayer *et al.* (1965).

2. ISOLATION OF HAPTEN BY PHENOL EXTRACTION AND SEPARATION FROM LIPOPOLYSACCHARIDE. In order to facilitate further studies on isolation and characterization of the O-specific hapten, preparations specifically labeled with [^{14}C]mannose were employed. *SL 1060*, a double mutant lacking phosphomannose isomerase and deficient in an enzyme of core biosynthesis (α-3-galactosyl transferase; *cf.* Figure 1), was grown in the presence

TABLE III: Isolation of O-Specific Hapten Labeled with [^{14}C]Mannose.[a]

Fraction	Radioactivity (cpm × 10^{-5})	Abequose (μmoles)	Heptose (μmoles)
1. Washed cells	50.0		
2. Aqueous phase of phenol extract	49.7	10.8	49.2
3. 105,000*g* pellet	0.15	<0.2	44.7
67% ethanol precipitable	0.15		45.2
67% ethanol soluble	<0.002		<0.5
4. 105,000*g* supernatant fraction	49.6		
5. G-50 excluded fraction of 4	3.1	9.7	3.4
67% ethanol precipitable	0.15	<0.2	2.9
67% ethanol soluble	3.0	9.2	<0.2

[a] An overnight culture of *SL 1060* (2 ml) was inoculated into 100 ml of medium containing [^{14}C]mannose (2×10^{-5} M; 4×10^{6} cpm/μmole), and the culture was grown for approximately four generations to late log phase. The cells were harvested by centrifugation, washed twice with 40 ml of cold 0.9% NaCl, and suspended in H_2O. The radioactive cell suspension was mixed with a suspension of nonradioactive bacteria of strain *SL 1032* (20 g in 60 ml of H_2O), sonicated at 20 kc for 3 min at 0°, and centrifuged at 30,000*g* for 30 min. The sediment was washed with 30 ml of H_2O by centrifugation and extracted with 140 ml of 45% phenol at 68° as previously described. The combined aqueous phases were freed of phenol by extraction with diethyl ether, concentrated to 20 ml under reduced pressure at 30°, and centrifuged at 105,000*g* for 3 hr. The pellet was dispersed in 20 ml of H_2O. Following addition of 1 M ammonium acetate, pH 5, to a final concentration of 0.02 M, two volumes of absolute ethanol were added and the mixture was chilled for 30 min and then centrifuged at 30,000*g* for 20 min at 4°. The 105,000*g* supernatant fraction was concentrated to 5 ml, chilled, and adjusted to pH 3 with 5 N acetic acid. The resultant precipitate, which contained nucleic acid but no significant [^{14}C]mannose, abequose, or heptose, was removed by centrifugation at 30,000*g* for 30 min in the cold. The supernatant solution was neutralized, concentrated to 2 ml, and passed through a column (1.5 × 40 cm) of Sephadex G-50 (coarse); elution was carried out with 0.05 M ammonium bicarbonate. The excluded fraction was concentrated to dryness under reduced pressure and taken up in 2 ml of H_2O, and ethanol precipitation was carried out as described above.

of [^{14}C]mannose and the labeled cells were mixed with nonradioactive, whole cells of strain *SL 1032* prior to extraction and fractionation. The results of the fractionation procedure are summarized in Table III. Following extraction with warm 45% phenol as described previously, the radioactivity was quantitatively recovered in the aqueous phase together with lipopolysaccharide (Osborn *et al.*, 1962).

A quantitative, rapid, and reproducible method for separation of haptenic material from lipopolysaccharide was needed. Preliminary fractionation obtained by centrifugation of the total extract at 105,000*g* yielded a waxy sediment with the bulk of the mutant lipopolysaccharide (as determined by heptose content) but little [^{14}C]mannose or abequose. The radioactive polysaccharide material in the supernatant solution was separated from low molecular weight materials by gel filtration on Sephadex G-50. Although over 90% of the extracted lipopolysaccharide heptose was sedimented at 105,000*g* in the present experiment, a significant contamination of the hapten fraction by lipopolysaccharide was observed; sedimentation gave variable results in our hands, particularly with wild-type lipopolysaccharides, where the fraction of lipopolysaccharide sedimented rarely exceeded 80%. In aqueous solution, lipopolysaccharides exist as aggregates of very high particle weight whose partition properties (*e.g.*, sedimentation characteristics) are not intrinsic to lipopolysaccharide but may vary with the specific purification history of the sample.

Further fractionation with cold 67% ethanol showed that lipopolysaccharide was quantitatively precipitated while the haptenic material remained quantitatively soluble in ethanol (Table III). Ethanol fractionation proved to be extremely reproducible, and appears to provide a simple means of essentially complete separation of hapten and lipopolysaccharide. Further characterization of the 67% ethanol-soluble and -precipitable material, as described below, confirmed the effective fractionations achieved.

The phenol-extracted lipopolysaccharide was completely excluded from Sephadex G-200, but the ethanol-soluble haptenic fraction (of Table III) segregated on Sephadex G-200 (Figure 4) into a sharp peak, slightly retarded with respect to blue dextran, which contained approximately 33% of the total [^{14}C]mannose (fraction I) and a broad band (fraction II) partially or wholly included in the gel. The abequose of coextracted carrier *SL 1032* followed a similar pattern. It appeared unlikely that the radioactive material excluded on G-200 (fraction I) represented lipopolysaccharide contamination. The lipopolysaccharide of *SL 1032* appears to be entirely devoid of O-specific material, and no abequose (or mannose) has ever been detected in it. Although the galactose transferase defect of *SL 1060* is somewhat leaky,

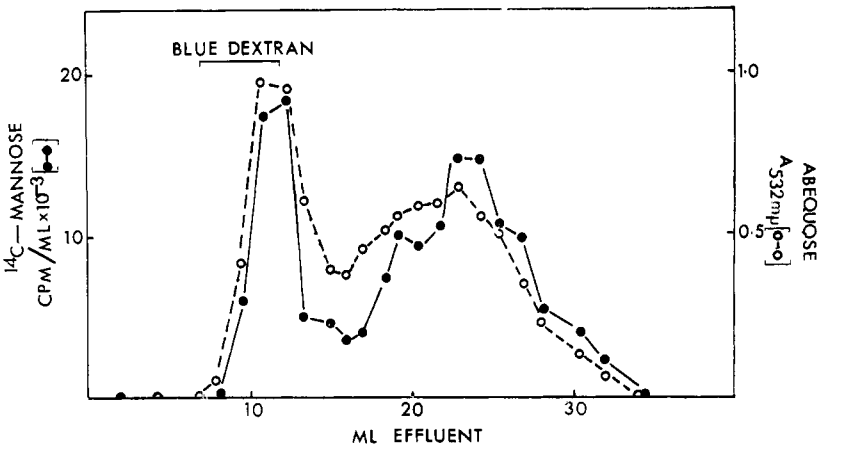


FIGURE 4: Gel filtration of [^{14}C]mannose-labeled hapten on Sephadex G-200. The haptenic fraction obtained by ultracentrifugation and ethanol fractionation (fraction 5b, Table III) was concentrated to 1.0 ml and passed through a 0.9 × 35 cm column of Sephadex G-200. Elution was carred out in 0.05 M ammonium bicarbonate.

TABLE IV: Chromatography of Wild-Type Lipopolysaccharide and Hapten on DEAE-Cellulose.[a]

Fraction	Per Cent Eluted								% Recovery	
	H_2O		Pyr-Ac (0.02 M)		Pyr-Ac (0.3 M)		NaOH (0.1 N)			
	[^{14}C]-Man	Abe	[^{14}C]-Man	Abe	[^{14}C]-Man	Abe	[^{14}C]-Man	Abe	[^{14}C]-Man	Abe
Lipopolysaccharide	0		0.5		0.2		8.5		9.2	
Hapten										
G-200 Fx I	22	44	55	43	5	0	2.1	1.7	84	89
G-200 Fx II	32	60	67	37	5	0	0	0	97	97
	27	55	68	42	2					
	22	50	64	47	3	0	0	0	94	97
Fx II Av	27	55	66	42	3	00	0	0	96	97

[a] Wild-type lipopolysaccharide labeled with [^{14}C]mannose was prepared from strain *M2* following growth in the presence of [^{14}C]mannose as described in Materials and Methods. The hapten fractions were those described in Figure 4. Samples containing 5 × 10^4 cpm of [^{14}C]mannose were diluted to 10 ml, adjusted to pH 8, and passed through columns (0.9 × 8 cm) of DEAE-cellulose (acetate form). The columns were washed successively with 50-ml portions of H_2O, 0.02 M pyridinium acetate (pH 5.3), 0.3 M pyridinium acetate (pH 5.3), and 0.1 N NaOH. Fractions (5 ml) were collected and analyzed for radioactivity and abequose.

and a small amount of [^{14}C]mannose was recovered in the lipopolysaccharide fraction of this mutant, the radioactivity associated with the fraction excluded from G-200 was defined as nonlipopolysaccharide O-specific hapten by additional evidence, as shown below.

Further characterization of the haptenic and lipopolysaccharide fractions was obtained by chromatography on DEAE-cellulose. As shown in Table IV, wild-type lipopolysaccharide labeled with [^{14}C]mannose was irreversibly adsorbed to the column under the conditions employed, while the haptenic fractions G-200-I and G-200-II were only partially adsorbed to DEAE-cellulose, and were readily eluted at 0.02 M pyridinium acetate. The recoveries of [^{14}C]mannose and abequose after DEAE chromatography of G-200-I indicated that no more than 10–15% of the material was irreversibly adsorbed, and this figure therefore represents the upper limit of contamination of this fraction by lipopolysaccharide.

The results of DEAE-cellulose chromatography not only provided an additional criterion for differentiation of O-specific hapten and lipopolysaccharide, but also indicated that a portion of the hapten isolated by phenol extraction was weakly anionic, as previously observed (Weiner *et al.*, 1965) for the O-specific polysaccharide synthesized enzymatically by isolated cell envelope fractions. Alkaline phosphatase treatment destroyed the anionic character of G-200 fraction I and II, exactly as for the enzymatically synthesized product. As shown in Figure 5, both G-200 fractions migrated slowly to-

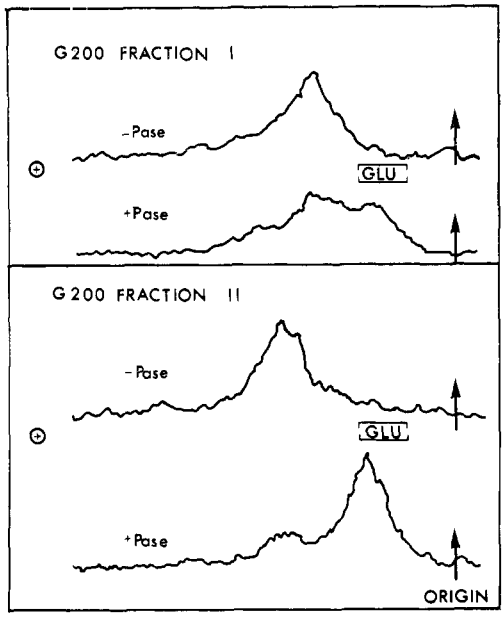


FIGURE 5: Effect of alkaline phosphatase on the electrophoretic mobility of haptenic fractions. Samples (1.5×10^4 cpm) of the Sephadex G-200 fractions described in Figure 4 were incubated for 4 hr at 37° in 0.1 ml of 0.02 M Tris-acetate (pH 8) containing 10 μg of *E. coli* alkaline phosphatase. Duplicate samples were incubated in the absence of phosphatase as control. The reaction mixtures were streaked on paper and subjected to electrophoresis in solvent D at pH 6.0 for 2 hr at 90 V/cm.

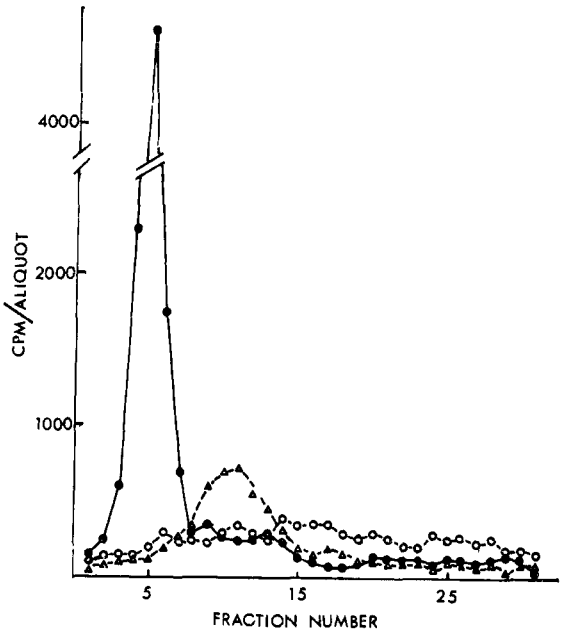


FIGURE 6: CsCl density gradient centrifugation of hapten and wild-type lipopolysaccharide. The preparation of samples is given in text; 2–4 $\times$ 10^4 cpm sample in 20 μl of H_2O was added to 4.0 ml of CsCl solution and centrifuged at 0° for 48 hr in a swinging-bucket SW-39 rotor at 35,000 rpm (Vinograd, 1963). About 35 fractions of 5 drops each were collected into iced tubes and 50 μl of each fraction was counted by scintillation as described in Materials and Methods. Direct refractometry at 0° on aliquots of six or seven spaced fractions from each gradient was done to check the linear gradients formed and to determine, by interpolation, the buoyant density of each radioactive species. (●—●) Lipopolysaccharide; (△—△) hapten, Sephadex G-200-I (excluded); (○—○) hapten, Sephadex G-200-II.

ward the anode during paper electrophoresis at pH 6 and most, but not all, of the radioactivity was converted to an electrophoretically neutral form by alkaline phosphomonoesterase (see section C).

Three criteria for distinguishing hapten from lipopolysaccharides emerged from the experiments described above: (1) sedimentation at 105,000*g;* (2) precipitation with cold 67% ethanol; and (3) differential elution from DEAE-cellulose columns. The haptenic fraction could also be differentiated from lipopolysaccharide by density gradient sedimentation in CsCl (Figure 6). Wild-type lipopolysaccharide specifically labeled with [^{14}C]-galactose was prepared by growth of an epimeraseless strain (*G30*) in the presence of exogenous [^{14}C]galactose, while the radioactive hapten fractions were Sephadex G-200 fractions I and II described above. The O-specific haptenic material included on Sephadex (G-200-II) formed no discrete band, but diffused as a smear throughout the gradient, whereas the larger excluded material (Sephadex G-200-I) produced a broad, asymmetrical band of average density 1.38, well separated from that of lipopolysaccharide and containing about 85% of the added radioactivity. Over 85% of the radioactivity added as lipopolysaccharide was recovered as a single sharp band at a reproducible buoyant density of 1.45 g/cc if the purification included ethanol precipitation as here, or reproducibly at 1.49 g/cc if no precipitation step were included. Falaschi and Kornberg (1965) have reported a value of 1.37 g/cc for an *E. coli* lipopolysaccharide purified by techniques not involving exposure to phenol or ethanol. When samples containing radioactive lipopolysaccharide were centrifuged at 105,000*g* in Tris-Cl buffer and $MgCl_2$ after ethanol precipitation, a greater percentage of the lipopolysaccharide was recovered in the pellet than for samples centrifuged without prior precipitation, again indicating the influence of aggregation state (and purification history) on partition properties of the sample.

3. PROPERTIES COMMON TO LIPOPOLYSACCHARIDE AND HAPTEN. Immune precipitation of wild-type lipopolysaccharide and of hapten by anti-O serum is shown in Figure 7. Purified antigen samples were: (1) wild-type lipopolysaccharide labeled with [^{14}C]galactose, an aliquot of the material used for CsCl centrifugation as described in Figure 6; and (2) hapten, labeled with [^{14}C]galactose, an aliquot of material isolated from strain *GRA-5*, as described in Table IV. Over 90% of the radioactive lipopolysaccharide was precipitable by anti-O serum but only half the haptenic counts were so precipitated. The supernatant fractions of hapten anti-O precipitates were tested for the presence of soluble antigen–antibody complexes by addition of $(NH_4)_2SO_4$ to 50% saturation, but considerable radioactivity was precipitated in tubes containing control sera and no further specific precipitation of radioactivity could be detected. The reasons for incomplete precipitability are unknown; haptenic material excluded from Sephadex G-200 (and hence having a larger average degree of polymerization) was no more effective than the present sample. That the same anti-

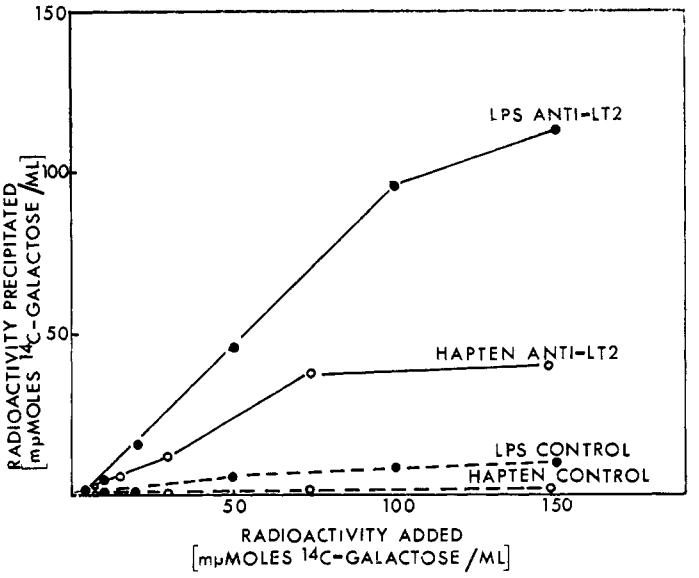


FIGURE 7: Immune precipitation of hapten and wild-type lipopolysaccharide. The preparations of lipopolysaccharide and hapten labeled with [^{14}C]galactose were those described in Table IV. Precipitations were carried out in the region of antibody excess by a microtechnique (Marcus and Grollman, 1966) using a commercial serum (Difco) against whole boiled cells. The tubes were incubated for 4 days at 2°. The washed precipitates and supernatant solution were counted on a liquid scintillation counter in Bray's solution.

genic determinants were involved in precipitation of hapten and of lipopolysaccharide was shown by competition experiments in which addition of the other unlabeled component to the mixture inhibited precipitation of either radioactive hapten or radioactive lipopolysaccharide. These results showed that common antigenic determinants are present in the haptenic and lipopolysaccharide fractions.

The presence of the characteristic O-antigen repeating unit in the purified haptenic polymer was confirmed by isolation of the expected oligosaccharides following partial acid hydrolysis. Samples of hapten and wild-type lipopolysaccharide containing [^{14}C]mannose were hydrolyzed with 60% formic acid and the products reduced with $NaBH_4$ as previously described (Zeleznick *et al.*, 1965). Chromatographic separation of the products in solvents A and B showed two radioactive oligosaccharides in addition to [^{14}C]mannitol. These were identified as α-galactosylmannosylrhamnitol and mannosylrhamnitol by techniques employed in earlier studies (Zeleznick *et al.*, 1965; Osborn and Weiner, 1968). The [^{14}C]mannose-labeled products obtained from the O-specific hapten fraction were identical qualitatively and quantitatively with those arising from the lipopolysaccharide.

Phage adsorption studies with modified *rfa* cells (see section B-2 below), which indicated that the hapten contains receptor sites for O-specific phage, provided further evidence for the structural relation of this polymer to the O-specific side chains of lipopolysaccharide.

B. Subcellular Localization of Hapten. 1. ISOLATION FROM SUBCELLULAR FRACTIONS OF *rfa* AND WILD TYPE. We have already shown that the multienzyme system responsible for synthesis of O-antigen, as well as the products formed, are firmly bound to the particulate cell envelope fraction, and the experiments described above (section A-1) suggested a similar localization for O-specific haptens accumulated *in vivo* by mutant strains. Since extensive studies in Ribi's laboratory (Anacker *et al.*, 1964, 1966) on O-specific haptenic polysaccharides in smooth, wild-type strains of *E. coli* had suggested a cytoplasmic location for the haptenic polymer, we investigated its distribution in the major subcellular fractions of *S. typhimurium*. Two strains were employed: *G30*, a mutant lacking UDP-galactose-4-epimerase; and *GRA-5*, a *rfa* derivative of *G30*. In the absence of exogenous galactose, these mutants are blocked in synthesis of both O-antigen and the core region of the lipopolysaccharide due to their inability to synthesize the precursor, UDP-galactose (*cf.* Figure 1). When synthesis of the nucleotide sugar is initiated by addition of galactose to the medium, *G30* and *GRA-5* become phenocopies of wild type and *rfa*, respectively.[2]

Cultures of *G30* and *GRA-5* were grown in the presence of [^{14}C]galactose for approximately five generations. The labeled cells were disrupted by sonication and the sonic extracts were subjected to differential centrifugation for separation of three fractions (Table V): the cell envelope fraction (30,000*g* pellet), the small particle fraction (105,000*g* pellet), and the soluble fraction (105,000*g* supernatant solution). Each fraction was extracted with warm aqueous phenol and radioactive polymeric components were separated from low molecular weight radioactive constituents in the aqueous phase by filtration through Sephadex G-50. All of the radioactivity originally present in the two particulate fractions was polymeric (*i.e.*, excluded from Sephadex G-50), but the radioactivity recovered from the soluble fraction of sonicated cells consisted primarily of low molecular weight metabolites included on Sephadex G-50 columns. These were identified as free [^{14}C]galactose plus smaller amounts of [^{14}C]galactose-1-P and UDP-[^{14}C]galactose; about 10–20% of the radioactivity in the soluble fraction was polymeric an each case. Total incorporation of [^{14}C]galactose into polymer in *GRA-5* was about half that attained by growing cells of *G30*, but the distri-

[2] Mutants lacking UDP-galactose-4-epimerase were chosen for these experiments in preference to strains lacking phosphomannose isomerase, since the latter strains have shown a strong tendency for secondary mutations to semirough forms which produce reduced amounts of O-specific polymers.

TABLE V: Subcellular Localization of O-Specific Hapten.[a]

Cell Fractions		[14C]Galactose Incorporation into Polymers (μmoles)					
		GRA-5			*G30*		
		Total	Ethanol Soluble	% Ethanol Soluble	Total	Ethanol Soluble	% Ethanol Soluble
Cell envelope	30,000*g* sediment	4.15	1.39	33.5	10.0	0.33	3.3
Small particle	105,000*g* sediment	1.44	0.78	53.5	2.72	0.06	2.2
Soluble	105,000*g* supernatant solution	0.55	0.44	80	1.70	0.24	14.0
	Total	6.14	2.61	42.6	14.42	0.63	4.4

[a] Overnight cultures (10 ml) of strains *G30* and *GRA-5* were inoculated into 500 ml of medium containing 5×10^{-5} M [14C]galactose (1.73×10^6 cpm/μmole). After growth at 37° for approximately five generations, the cells were harvested, washed twice with 0.9% saline, and frozen overnight. The frozen pastes were suspended in 5 ml of iced 0.05 M Tris-acetate (pH 8.0) and disrupted by sonication (four periods of 10 sec at 0° in a Branson 20-kc sonic oscillator). The pellet obtained by centrifugation at 30,000*g* for 30 min at 4° was washed with 5 ml of buffer and again recovered by centrifugation. The original supernatant fraction and wash were pooled and centrifuged at 105,000*g* for 3 hr. The 30,000*g* and 105,000*g* sediments and the final supernatant solution were extracted with 45% phenol at 68°. Low molecular weight material was removed by gel filtration on Sephadex G-50 (1.5×40 cm column) in 0.05 M triethylammonium bicarbonate. Over 95% of the radioactivity of the two particulate fractions was recovered in the excluded volume, but 30–70% of the 14C of the 105,000*g* supernatant fraction appeared in the included fraction and was identified, as a mixture of [14C]galactose (paper chromatography in solvent A), [14C]galactose-1-P, and UDP-[14C]galactose (paper electrophoresis in solvent C). The excluded fractions were concentrated to 5 ml, 2.5 mg of non-radioactive wild-type lipopolysaccharide was added as carrier, and ethanol precipitation was carried out as described in Table III to separate hapten from lipopolysaccharide. The ethanol-insoluble material was dissolved in H_2O and the precipitation was repeated. Identification of the ethanol-soluble and -insoluble fractions as hapten and lipopolysaccharide, respectively, was confirmed by chromatography of aliquots of the fractions on DEAE-cellulose. The results indicated that less than 5% of the 14C in the haptenic fractions could represent contamination by lipopolysaccharide, and *vice versa*.

bution of counts incorporated into polymers was very similar; about 70% of the total polymeric [14C]galactose (*i.e.*, lipopolysaccharide plus hapten) was associated with the cell envelope fraction, 20% with the small particle fraction, and 10% with the soluble fraction.

The separation of the polymers into lipopolysaccharide and haptenic fractions by differential ethanol precipitation is summarized in Table V; since galactose is a constituent of the incomplete lipopolysaccharide produced by *rfa* mutants as well as of the O-specific polysaccharide (*cf.* Figure 1), a considerable amount of radioactivity was precipitated by 67% ethanol in the fractions obtained from *GRA-5*. The two classes were identified as follows. First, identification of the ethanol-insoluble fractions from *GRA-5* as mutant lipopolysaccharide devoid of O-specific side chains was confirmed by three different criteria: (a) the radioactivity was quantitatively adsorbed to DEAE-cellulose (less than 4% could be eluted by 0.02 or 0.3 M pyridinium acetate, pH 5.3), indicating that 97% of the [14C]galactose was associated with lipopolysaccharide (*cf.* Table IV); (b) less than 10% of the radioactivity was precipitated by anti-O serum in the region of antibody excess; and (c) the size distribution of the radioactive lipid-free polysaccharide obtained by mild acid hydrolysis was identical with that of authentic *rfa* polysaccharide (isolated from strain *TV161*) as estimated from their partial inclusion in Sephadex G-50 gels from which the wild-type material (containing O-antigenic chains) is excluded (Kent and Osborn, 1968a). Secondly, identification of the ethanol-soluble fractions from *GRA-5* as O-specific hapten was confirmed by: (a) DEAE-cellulose chromatography, with results similar to those shown in Table IV for hapten labeled with [14C]mannose; on this basis, no more than 5–6% of the ethanol-soluble radioactivity could represent lipopolysaccharide contamination; (b) immune precipitation of radioactive material by anti-O sera (shown in Figure 7); and (c) a filtration profile on Sephadex G-200 similar to that illustrated in Figure 4. Of the total [14C]galactose incorporated into polymer in strain *GRA-5*, about half was haptenic, and over 80% of this material was associated with the particulate fractions of the cell, principally with the cell envelope. Furthermore, the distribution of hapten between the two particulate fractions (65 and 35%) was similar to the distribution of activities observed for the enzymes of O-antigen synthesis (M. J. Osborn, unpublished observations). It is suggested that the small amount of hapten which appears in the cytoplasmic fraction arises as a result of secondary enzymatic or nonenzymatic degra-

dation of the primary lipid-linked haptenic product. The data strongly indicate that the O-specific polysaccharide chains accumulated by *rfa* and related mutants remain bound to membranous components at or near the site of biosynthesis.

In extracts of a wild-type phenocopy ([^{14}C]galactose-grown *G30*), ethanol fractionation of the isolated polymers yields a very different distribution of material among the cell fractions (Table V). The ethanol-insoluble fractions were characterized as wild-type lipopolysaccharide by their irreversible, quantitative adsorption to DEAE-cellulose columns and by their quantitative precipitation with anti-O antibody (Figure 7). Only about 5% of the total [^{14}C]galactose-labeled polymer was soluble in 67% ethanol, in marked contrast to the *rfa* mutant, but in good agreement with the value of 3–4% free, O-specific hapten found in the wild-type phenocopy of the phosphomannose isomeraseless mutant (*M2*) following growth in the presence of [^{14}C]mannose (Kent and Osborn, 1968b). Furthermore, the small amount of hapten recovered from strain *G30* was equally distributed between the soluble and particulate fractions. It should be noted that approximately the same amount of nonparticulate hapten was obtained from each strain; these results are consistent with the findings of Anacker *et al.* (1964, 1966) on the localization of hapten in wild-type strains of *E. coli*, and also confirm their observation that wild-type *Salmonella* contain only traces of O-specific hapten. Therefore, the bulk of unattached O-specific hapten accumulated by *rfa* mutants remains bound to membranous components, but the minor amounts of this product observed in nonaccumulating strains are found also in the cytoplasm.

2. ADSORPTION OF O-SPECIFIC PHAGE BY *rfa* MUTANTS. Although the O-specific hapten of *rfa* mutants is predominantly localized in the cell envelope, the surface of the intact cell has no detectable O-antigen as determined by serological properties and resistance to O-specific phage P22. On the hypothesis that the hapten is present somewhere at or on the cell membrane but beneath the lipopolysaccharide layer of the outer surface, *rfa* mutants were treated with EDTA or with EDTA plus lysozyme, and assayed for increased ability to adsorb P22 as the criterion for exposed O-antigen on a newly accessible surface. Small (less than fivefold) but reproducible increases were observed over many experiments (Figure 8). Adsorption by normally sensitive wild-type cells was not affected to a significant extent in control experiments allowing 90% adsorption, showing that normal adsorption sites are not destroyed by this treatment. Nonspecific inactivation of plaque-forming units can be eliminated, since the cell-free fluid of treated, centrifuged cells did not inactivate phage to a significant extent. The inability of wild-type cells to adsorb a rough specific phage, 6SR, was not changed by these procedures. No infective center production by phage P22 on treated *rfa* bacteria could be detected under these conditions. Treatment of the cells with EDTA by the methods of Neu and Heppel (1965) or Leive (1965) did not result in release of detectable O-specific hapten or lipopolysaccharide into the medium, although Leive has reported that half

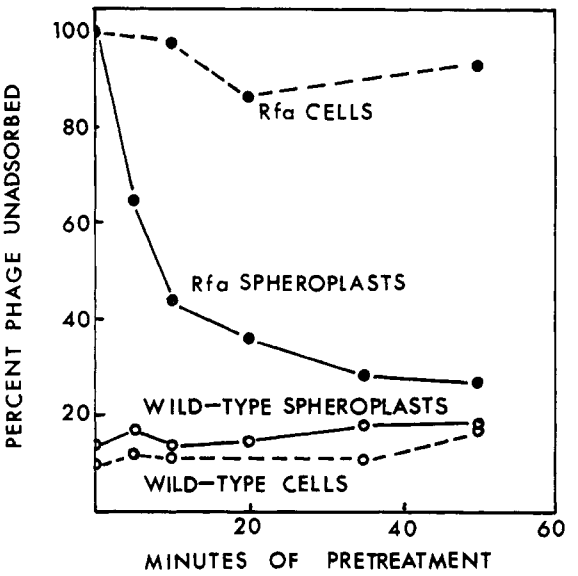
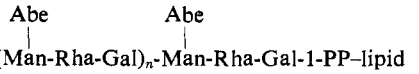


FIGURE 8: Adsorption of O-specific phage P22 by spheroplasts of *rfa* mutants. Two strains, wild-type and *rfa* mutant *TV227*, were used. Overnight cultures were diluted tenfold into 50 ml of fresh proteose–peptone beef extract and incubated at 37° with vigorous aeration for four generations to late log phase. Cells from each strain were harvested by centrifugation in the cold, washed once with 20 ml of 0.01 M Tris (pH 8), and resuspended with 10 ml of 20% sucrose–0.033 M Tris (pH 8); each sample was subdivided into control and experimental aliquots of 5 ml each and brought to 37° before addition to the experimental tubes of 12 mM EDTA at pH 8 and 5 μg/ml of freshly prepared lysozyme. At indicated intervals thereafter, aliquots of 0.3 ml were removed for analysis as follows: 0.1–0.9 ml of H_2O and 0.1–0.9 ml of the sucrose–Tris mixture for optical density measurements to determine efficiency of spheroplasting and 0.1–0.9 ml of phage mixture to assay ability to absorb phage. The phage mixture contained 20% sucrose–0.033 M Tris (pH 8) plus 0.05 M $MgCl_2$ plus P22 phage at 2.3×10^7 plaque-forming units/ml. The phage–cell mixture was incubated at 37° for 10 min and centrifuged for 5 min at 0° and 600*g* to remove cells with their adsorbed phage, and the supernatant was assayed by plaque counts on a lawn of wild-type cells. The multiplicity of infection was 0.02. About 75% of the optical density was conserved following dilution of treated cells into H_2O.

the lipopolysaccharide of *E. coli* O-111 can be released under the same conditions.

C. Evidence for Attachment of O-Specific Hapten Formed in Vivo to the Antigen-Carrier Lipid. We have previously presented evidence to suggest (Weiner *et al.* 1965) that enzymatic synthesis of O-antigen by isolated cell envelope fractions leads to accumulation of O-specific chains which are still attached through a galactose-1-P reducing terminus to the intermediate carrier lipid



The polysaccharide–lipid linkage is extremely labile, and it has not yet been possible to extract the lipid-linked polymer from the cell envelope in an undegraded form. Treatment of the cell envelope with 45% aqueous phenol at 68° was shown to result in cleavage of the pyrophosphate bridge and liberation of soluble polysaccharide

TABLE VI: Isolation of ^{32}P and ^{14}C Doubly Labeled Hapten.[a]

Fraction	Radioactivity (mμmoles)		Molar Ratio
	[^{32}P]	[^{14}C]Galactose	[^{14}C]:[^{32}P]
Aqueous phase of phenol extract	38,200	1,330	0.035
67% ethanol supernatant fraction	5,080	755	0.15
G-50-excluded fraction	178	501	2.8
DEAE-cellulose			
Fx I (unadsorbed)	7.3	208	28.6
Fx II (0.02 M Pyr-Ac eluate)	13.1	183	14.0

[a] An overnight culture of *GRA-5* (5 ml) was inoculated into 200 ml of peptone broth (Difco) containing [^{14}C]-galactose (1×10^{-4} M, 1.14×10^{6} cpm/μmole) and $^{32}P_i$ (7×10^{-4} M, 25 μCi/μmole). After growth at 37° for approximately four generations, the cells were harvested by centrifugation, washed twice with 0.9% saline, and extracted with 12 ml of 45% phenol at 68°. The aqueous phase was extracted with diethyl ether, concentrated to 5 ml, and precipitated with two volumes of ethanol as described in Table II. The supernatant fraction, which contained the hapten, was concentrated to 2 ml and passed through a column (0.9 × 50 cm) of Sephadex G-50 (coarse) in 0.05 M triethylammonium bicarbonate. The excluded fraction was lyophilized, taken up in 20 ml of H_2O, and applied to a column (0.9 × 10 cm) of DEAE-cellulose (acetate form). The column was washed with 50 ml of H_2O, followed by 60 ml of 0.02 M pyridinium acetate (pH 5.3).

chains containing a mixture of galactose-1-P and galactose 1,2-cyclic phosphate reducing termini. The presence of similar galactose phosphate reducing termini in the O-specific hapten accumulated *in vivo* was established by isolation of doubly labeled [^{32}P]- and [^{14}C]-galactose hapten from strain *GRA-5*. The organism was grown in the presence of $^{32}P_i$ and [^{14}C]galactose for five generations, and the haptenic fraction purified as summarized in Table VI. As with [^{14}C]mannose-labeled material, the [^{14}C]galactose-labeled hapten was separated into two fractions of approximately equal radioactivity by chromatography on DEAE-cellulose columns; fraction I, which was not adsorbed, and fraction II, which was readily eluted by 0.02 M pyridinium acetate, each contained small amounts of ^{32}P as well as [^{14}C]galactose. Paper electrophoresis at high voltage did not resolve the two isotopes in either fraction. The radioactive material migrated slowly toward the anode at pH 6 in a broad peak like that observed for [^{14}C]mannose-labeled hapten (*cf.* Figure 5), and the ratios of ^{32}P to ^{14}C were constant over the entire band.

In order to determine the end group and the average chain lengths of these haptenic fractions, each was treated with alkaline phosphatase. Over 95% of the ^{32}P in fraction II was released as inorganic phosphate, and the [^{14}C]galactose-containing hapten was quantitatively converted into an electrophoretically neutral form that was no longer adsorbed to DEAE-cellulose. Fraction I was resistant to monoesterase treatment; less than 15% of the ^{32}P was liberated as P_i, and the electrophoretic mobility of the fraction as well as the ^{32}P:^{14}C ratio was unchanged. The results were very similar to those previously reported (Weiner *et al.*, 1965) for the enzymatic product and indicated that the phosphate of fraction II was exclusively in monoester form while that of fraction I was predominantly diester, presumably as the 1,2-cyclic phosphate.

Reducing end groups were determined by reduction of the polysaccharide with sodium borohydride followed by acid hydrolysis and separation of the radioactive alditol by paper chromatography in solvent E (Table VII). No detectable reducible end groups were found unless the hapten had been pretreated with alkaline phosphatase. No reducible end groups were found in fraction I even after monoesterase treatment, in agreement with the postulated 1,2-cyclic phosphate structure. The end group of fraction II, unmasked by phosphatase treatment, was exclusively [^{14}C]galactitol. Other end groups were excluded in both I and II since reduction with [^{3}H]-sodium borohydride yielded no other [^{3}H]alditols. In phosphatase-treated fraction II, the ratio of [^{14}C]galactose (internal) to [^{14}C]galactitol (terminal reducing) was approximately 30, but the ratio ^{32}P:^{14}C of this fraction indicated a chain length of 15, half the expected value. The reason for this discrepancy is unknown but neither incomplete reduction of the dephosphorylated fraction nor the presence of pyrophosphate at the terminus is an adequate explanation. We do not believe fraction II is a polysaccharide pyrophosphate because it was unaffected by mild alkali which should degrade a pyrophosphate terminus to the cyclical monoester plus P_i. The presumptive cyclic phosphate form (resistant to monoesterase) of fraction I had a ratio of ^{32}P to [^{14}C]galactose corresponding to a chain length of 28, which agrees well with the chain length obtained upon reduction of dephosphorylated fraction II.

Discussion

We report here the isolation and separation of two

polymers, each containing specific sugars characteristic of the somatic O-antigen from *S. typhimurium*. O-Specific hapten, unattached to lipopolysaccharide, constitutes less than 5% of the total O-specific polymer in growing wild-type cells and in wild-type phenocopies. Mutants blocked in the synthesis of the core moiety, on the other hand, lack the normal attachment site and accumulate significant amounts of unattached O-specific hapten.

The O-specific polymers are each firmly bound to the particulate cell envelope fraction, and the distribution of accumulated hapten in the mutant organisms closely parallels that of lipopolysaccharide in subcellular fractions. Small amounts of unattached polymer are also found in the soluble cell fraction (105,000*g* supernatant) in both mutant and wild-type strains, perhaps reflecting degradation or turnover during growth and extraction; this material may correspond to that described by Anacker *et al.* (1964, 1966) in *E. coli*. Experiments with intact cells demonstrate that unattached O-specific hapten is located almost exclusively in the cell envelope fraction but not on the outer surface of the cell. Disruption of the integrity of the outer cell surface by treatment with EDTA plus lysozyme or with EDTA alone makes the haptenic polymer more accessible to the outside, since the treated *rfa* cells are now able to adsorb O-specific P22 phage two to five times more efficiently than control *rfa* cells.

Upon extraction of the cell envelope fraction with hot aqueous phenol the O-specific hapten is released in water-soluble form as a polysaccharide with terminal galactose 1-phosphate, and it can be separated from lipopolysaccharide by differential ethanol precipitation, by chromatography on DEAE-cellulose columns, or by CsCl density gradient centrifugation. Its properties are identical with those already described for the lipid-linked enzymatic products: (1) galactose 1-phosphate is the reducing terminus; (2) average degree of polymerization is about 30 both by double-label studies (^{32}P to [^{14}C]galactose) and also by direct determination of [^{14}C]galactose as galactitol *vs.* galactose after sodium borohydride reduction of dephosphorylated polymer; (3) characteristic oligosaccharides are recovered after partial hydrolysis with formic acid. From the average degree of polymerization (30) and the total content of hapten (2.4 μmoles of galactose/g wet weight of accumulating cells), the average number of chains can be estimated as 80-mμmoles chains/g wet weight of cells. This value is in reasonable agreement with the concentration of antigen-carrier lipid determined (by direct enzymatic assay) as approximately 40 mμmoles/g wet weight in these cells (M. J. Osborn, unpublished observations). The results suggest that most of the antigen-carrier lipid is present as the polysaccharide phosphate derivative in core mutants which are unable to transfer O-antigen to lipopolysaccharide. We have also observed that enzymatic incorporation of nucleotide sugars into O-specific products by cell envelope fractions obtained from hapten-producing mutants is usually five- to tenfold lower than in nonaccumulating strains, as expected for cells containing little available, unglycosylated carrier lipid.

TABLE VII: Reducing End-Group Determination of ^{32}P- and [^{14}C]Galactose-Labeled Hapten.[a]

Fraction	Radioactivity (cpm) Recovered as [^{14}C]-Galactitol	Radioactivity (cpm) Recovered as [^{14}C]-Galactose
Fraction I	34	49,270
Fraction II	56	51,520
Dephospho-fraction II	1,653	52,470

[a] Samples of DEAE fractions I and II (Table IV) containing approximately 5×10^4 cpm of [^{14}C]-galactose were treated with $NaBH_4$ (4 mg/ml; final volume 0.2 ml) for 16 hr at 4°. Excess $NaBH_4$ was discharged by addition of a small amount of AG-50-H^+ in the cold, and the resin was removed by filtration and washed. The filtrates were concentrated to dryness under reduced pressure, and borate was removed by repeated evaporation from methanol under reduced pressure. The residues were taken up in 0.2 ml of 1 N HCl and hydrolyzed for 5 hr at 100° and HCl was removed by extraction with *N,N*-dioctylmethylamine in $CHCl_3$, before chromatography of the hydrolysates in solvent E for 20 hr. The areas corresponding to galactose and galactitol were cut out, and the paper strips were counted by scintillation in toluene–Liquiflor. Dephospho-fraction II, which was carried through the same procedure in parallel, was prepared as follows. A sample of fraction II (6×10^4 cpm of ^{14}C was treated with 30 μg of *E. coli* alkaline phosphatase in 0.5 ml of 0.02 M Tris-acetate (pH 8), for 4 hr at 37°. The reaction mixture was then diluted to 5 ml and passed through a 0.9×4 cm column of DEAE-cellulose (acetate form). The column was washed first with 10 ml of H_2O, and then with 20 ml of 0.05 M pyridinium acetate (pH 5.3). The unadsorbed fraction (original effluent plus H_2O wash) contained 102% of the original ^{14}C and no detectable ^{32}P. All of the applied ^{32}P was recovered in the pyridinium acetate eluate, and was identified by paper electrophoresis as P_i. The unadsorbed fraction (dephospho-fraction II) was concentrated to dryness prior to reduction.

The properties of extracted lipopolysaccharide are variable and depend at least in part upon prior treatment of the sample. Thus, precipitation of lipopolysaccharide with 67% ethanol lowers its buoyant density in CsCl gradients from 1.49 to 1.45 g/cc without changing band width and increases the percentage of lipopolysaccharide which can subsequently be sedimented at 105,000*g*. Since phenol-extracted samples of lipopolysaccharide exist in solution as aggregates of high particle weight, it is reasonable to suppose that such an aggregated species containing about 50% lipid may exist in suspension as one of several alternative states, dependent upon solvents, etc. The change in buoyant density at constant band width suggests that the lipo-

polysaccharide aggregate is significantly altered in configuration but not in weight after ethanol precipitation. The altered species may bind Cs^+ less effectively or water more effectively which is manifested as a reduction of buoyant density in CsCl; such a change could occur, for example, if the accessibility of phosphate groups to aqueous regions were changed.

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