sulfhydryl group prior to its transfer to ACP. It is of interest that a different fatty acyl transacylase which catalyzes the transfer of longer-chain-length acyl groups from CoA to ACP now has been identified.[4] The significance of this enzyme has not yet been determined, but it is possible that it is involved in the metabolism of long-chain fatty acids, i.e., in elongation, desaturation, oxidation, or esterification reactions.

The availability of substrate quantities of acyl-ACP derivatives has permitted the study of β-ketoacyl-ACP reductase and enoyl-ACP hydrase activities in the *E. coli* system. Similar reactions have been studied previously in fatty acid synthetase preparations from yeast (Lynen, 1961, 1962), rat brain (Robinson *et al.*, 1963b), rat adipose tissue (Robinson *et al.*, 1963a; Martin and Vagelos, 1964), and avian liver (Wakil and Bressler, 1962). In those cases acyl thiolesters of CoA, pantetheine, and *N*-acetyl cysteamine were used as substrates which apparently acted as model compounds for the protein-bound intermediates.

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O-Phosphorylethanolamine: A Component of Lipopolysaccharide in Certain Gram-negative Bacteria*

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O-Phosphorylethanolamine has been isolated from acid hydrolysates of polysaccharide prepared from the cell-wall lipopolysaccharide of a UDP-D-galactose-4-epimeraseless mutant of *Salmonella typhimurium*. The material was obtained in crystalline form and identified by melting point, elemental analysis, and infrared spectrum, and was detected by paper chromatography in hydrolysates of the lipopolysaccharide of certain other Gram-negative bacteria. Together with L-glycero-D-mannoheptose, 3-deoxyoctulosonate, and phosphate, *O*-phosphorylethanolamine appears to be an integral component of the phosphorylated polysaccharide to which glucose and other sugars composing the antigenic side chains are attached. It contains 50% of the total phosphate in the polysaccharide and appears to be linked to the polymer through phosphodiester bridges.

Glucose, glucosamine, galactose, mannose, rhamnose, and abequose are well-known components of the lipopolysaccharide *O*-antigen of *Salmonella typhimurium* (Kauffmann *et al.*, 1960). Recently, 3-deoxyoctulosonate (Heath and Ghalambor, 1963; Osborn, 1963) and L-glycero-D-mannoheptose (Kauffman *et al.*, 1960; M. J. Osborn and B. L. Horecker, unpublished observations) were identified as carbohydrate components of the *O*-antigen, and a mutant strain of *S. typhimurium* was isolated whose lipopolysaccharide contained only these two sugars in addition to glucose (Nikaido, 1962; Osborn *et al.*, 1962). It has been suggested that heptose,

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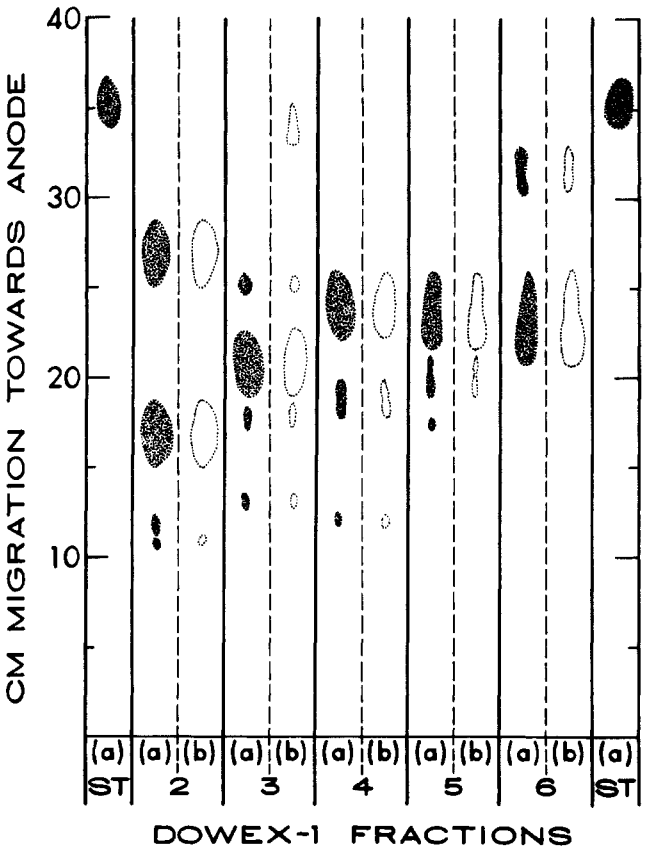

FIG. 1.—Electrophoresis of fractions obtained from column-chromatographic separation on Dowex-1 (acetate) of polysaccharide prepared from empimeraseless mutant of *S. typhimurium*. Two 100-μg samples of polysaccharide, purified as described in the text, were placed on adjacent spots and subjected to electrophoresis on Whatman 1 paper at 60 v/cm for 2 hours. Strips were cut out and developed by dipping (a) in a solution of 0.01 M periodic acid in 99% ethanol and allowing the paper to dry before developing with the alkaline silver nitrate reagents described by Anet and Reynolds (1954) or (b) in a 0.25% solution of ninhydrin in acetone and heating at 100° for 5 minutes. With the exception of two minor components, all polysaccharide subfractions that reacted with periodic acid–silver nitrate were also detected by the ninhydrin reagent. The standard compound (ST) shown is 3-deoxyoctulosonate.

3-deoxyoctulosonate, and phosphate constitute a phosphorylated polysaccharide "backbone" to which glucose and other sugars composing the antigenic side chains are attached (Osborn, 1963; Fraenkel *et al.*, 1963). In this communication we wish to report that *O*-phosphorylethanolamine is also present in the "backbone" polysaccharide of this mutant strain as well as in the lipopolysaccharide isolated from certain other Gram-negative bacteria.

EXPERIMENTAL PROCEDURE

Materials.—Chemically synthesized *O*-phosphorylethanolamine was obtained as a primary standard from the California Corp. for Biochemical Research and recrystallized from methanol-water. 1-Fluoro-2-4-dinitrobenzene (FDNB) was purchased from the Matheson Co., alkaline phosphatase was purified from *Escherichia coli* (Malamy, 1963), and the mutant of *S. typhimurium* lacking UDP-D-galactose-4-epimerase was grown commercially by the Grain Processing Corp. of Muscatine, Iowa.

Analytical Procedures.—Heptose was determined by the method of Dische (1953), glucose with glucose oxidase (Worthington Glucostat reagents), 3-deoxyoctulosonate by a modification (Osborn, 1963) of the thiobarbituric acid method (Weissbach and Hurwitz, 1959), and organic and inorganic phosphate by the Ames and Dubin (1960) modification of the method of Chen *et al.* (1956). Monoester phosphate was determined by measuring the increase of inorganic phosphate after treatment of the sample with alkaline phosphatase. Total carbohydrate was determined by the phenol–sulfuric acid reaction (Dubois *et al.*, 1951) and ethanolamine and *O*-phosphorylethanolamine with FDNB (Ghuysen and Strominger, 1963). Elemental analyses for carbon, nitrogen, and hydrogen were performed by the Schwarzkopf Microanalytical Laboratory of New York.

Purification of Polysaccharide.—Lipopolysaccharide was isolated from the UDP-D-galactose-4-epimeraseless mutant of *S. typhimurium* by a previously described modification (Osborn *et al.*, 1962) of the method of Westphal *et al.* (1952). This material, containing 100 mg of carbohydrate, was suspended in a small volume of water and precipitated with 20 volumes of chloroform-methanol (2:1, v/v) which served to extract any contaminating phospholipids. The chloroform-methanol extract was washed with 1/5 volume of water and the aqueous wash was combined with the precipitated lipopolysaccharide. The lipopolysaccharide suspension, in a volume of 24 ml, was adjusted to *p*H 3.4 with 1 M acetic acid and hydrolyzed for 1 hour at 100°, and the insoluble lipid material was removed by centrifugation. The supernatant solution, containing 98% of the total carbohydrate, was adjusted to *p*H 8.2, applied to a 2.5 × 30-cm column of Dowex-1 (acetate), and eluted with a linear gradient of pyridinium acetate, *p*H 5.3 (500 ml 0.3 M into 500 ml 0.02 M). The elution pattern was similar to that previously observed when the same polysaccharide was fractionated on DEAE-cellulose columns (Osborn, 1963), although the separation of components was somewhat better. Six fractions were separated by the column chromatography and the major Dowex fraction (fraction 3, Fig. 1), containing 23% of the total polysaccharide, was further purified by paper electrophoresis. The major electrophoretic component of fraction 3 was eluted from the paper with 0.1 N formic acid and passed through a 2.5 × 45-cm column of Sephadex G-50 to remove residual pyridine and colored impurities. After lyophilization and drying *in vacuo* over P_2O_5 at 56°, 19.4 mg of white powder was obtained which was used for further chemical studies. The composition of this material is shown in Table I.

TABLE I

ANALYSIS OF THE MAJOR POLYSACCHARIDE FRACTION OF A UDP-D-GALACTOSE EPIMERASELESS MUTANT OF *S. typhimurium*

Elemental Analysis		Composition	
(element)	(% by wt)	(group)	(mole/mole P)
Carbon	35.10	Heptose	1.05
Hydrogen	5.92	Glucose	0.49
Nitrogen	1.22	3-Deoxyoctulosonate	0.12
Oxygen[a]	52.24	Amino nitrogen[b]	0.53
Phosphorus	5.52	Phosphate:	
		organic	1.00
		inorganic	0.00
		monoester	0.00

[a] Calculated by difference. [b] Determined by DNFB reaction.

TABLE II
CHROMATOGRAPHIC AND ELECTROPHORETIC PROPERTIES OF UNKNOWN NINHYDRIN-POSITIVE COMPOUNDS

	System[a]						
	1	2	3	4	5	6	7
	$R_{glucosamine}$						
Compound X	0.43	0.89	0.29	0.05	0.70	0.05	[b]
Crystalline material from *S. typhimurium*[c]	0.43	0.89	0.29	0.05	0.70	0.05	[b]
O-Phosphorylethanolamine	0.43	0.89	0.29	0.05	0.70	0.05	[b]
Compound X (treated with alkaline phosphatase)	1.1	1.5	1.4	1.0	1.6	1.8	1.2
Compound Y	1.1	1.5	1.4	1.0	1.6	1.8	1.2
Ethanolamine	1.1	1.5	1.4	1.0	1.6	1.8	1.2

[a] The following solvent and buffer systems were employed: (1) 95% ethanol–1 M ammonium acetate (7–3), (2) isobutyric acid–0.05 M ammonium hydroxide (5–3), (3) propanol–ethyl acetate–water (7–1–2), (4) butanol–pyridine–water (6–4–3), (5) butanol–acetic acid–water (62–15–25), (6) high-voltage electrophoresis for 2 hours in pyridinium acetate buffer, *p*H 3.4, 60 v/cm, (7) high voltage electrophoresis for 2 hours in pyridinium acetate buffer, *p*H 6.0 60 v/cm. [b] In each case the compound moved 8 cm toward the anode while glucosamine moved 15 cm toward the cathode. [c] Isolation of crystalline material described in the text.

RESULTS

Isolation and Identification of O-Phosphorylethanolamine.—The presence of a ninhydrin-positive compound containing phosphate was first detected in hydrolysates of the purified polysaccharide fraction. Hydrolysis in 1 N HCl for 5 hours at 100° released two ninhydrin-positive compounds which separated in paper chromatography and high-voltage electrophoresis (Table II). The slow-moving anionic component, compound X, contained phosphate and was the major hydrolysis product. The fast-moving cationic component, compound Y, was free of phosphate and present only in small quantities. Treatment of compound X with alkaline phosphatase resulted in a product with chromatographic properties identical with those of compound Y.

Larger quantities of compound X were isolated from a crude polysaccharide fraction prepared from the same epimeraseless mutant of *S. typhimurium* by a modification of the procedure of Freeman (1942). A suspension of bacteria (100 g wet wt) was extracted twice with 200-ml portions of 5% trichloroacetic acid at room temperature in the Waring Blendor. The acid-insoluble residue was extracted six times with ether to remove trichloroacetic acid and lipid material, adjusted to *p*H 3.4 with 1 N acetic acid in a volume of 300 ml, and heated for 2 hours at 100°. After centrifugation the clear supernatant solution containing the crude polysaccharide was diluted to 1 N with HCl and refluxed for 6 hours at 100°. The hydrolysate was evaporated to dryness under reduced pressure and HCl was removed *in vacuo* over NaOH. The residue was dissolved in 2.5 ml water and adsorbed to a 2.5 × 30–cm column of Dowex-50 in the H^+ form. A single peak containing equimolar amounts of phosphate and ninhydrin-reactive material (*O*-phosphorylethanolamine standard) was eluted with 0.01 N HCl). Acid was removed by evaporation *in vacuo* and the residue was dissolved in water. An equal volume of ethanol was added and the solution was allowed to stand at 4° overnight. Long white needles crystallized readily from this solution and were recrystallized five times from ethanol-water and twice from methanol-water with a final yield of 23 mg. The crystals were identified as *O*-phosphorylethanolamine by the following properties: Melting point and mixed melting point were 236–238°, reported mp 237° (Cherbuliez and Rabinowitz, 1958). Elemental analysis calcd for $C_2H_8O_4NP$: C, 17.04; H, 5.67; N, 9.92; P, 22.0. Found: C, 17.62; H, 5.94; N, 9.97; P, 22.6. The infrared spectrum was similar to that of authentic material (Fig. 2). Chromatographic properties of the crystalline material isolated from the crude polysaccharide were identical with compound X isolated from the purified polysaccharide (Table II). Compound Y, produced by enzymatic dephosphorylation of compound X, is therefore ethanolamine.

Linkage of O-Phosphorylethanolamine to the Polysaccharide.—It appears from these studies that *O*-phosphorylethanolamine is linked to the polysaccharide moiety of the lipopolysaccharide of the epimeraseless mutant of *S. typhimurium*. In the intact polysaccharide, the phosphate of *O*-phosphorylethanolamine is not present in monoester form as judged by its resistance to the action of *E. coli* alkaline phosphatase. The amino group is presumably free as seen by the ninhydrin reactivity of the polysaccharide fractions shown in Figure 1 and by the quantitative reaction of the polysaccharide with FDNB (1.03 mole DNP-polysaccharide per mole *O*-phosphorylethanolamine). These observations, together with the elemental analysis and composition (Table I), are consistent with a tentative structure in which ethanolamine is linked through phosphodiester bridges to heptose or glucose in the backbone polysaccharide. However, as *O*-phosphorylethanolamine is also present in purified polysaccharide fractions from a phosphoglucose-isomeraseless mutant, *S. typhimurium* 797 (Fraenkel *et al.*, 1963), in which heptose and 3-deoxyoctulosonate are the only sugars present, heptose is the probable site of linkage. Further chemical studies will be required to elucidate the chemical nature and site of this linkage.

O-Phosphorylethanolamine in Lipopolysaccharide of Other Gram-negative Bacteria.—In addition to the epimeraseless and isomeraseless mutants of *S. typhimurium*, chloroform-methanol-extracted lipopolysaccharide preparations from other Gram-negative bacteria were examined for *O*-phosphorylethanolamine. After hydrolysis, a ninhydrin-positive compound with the same chromatographic and electrophoretic mobility as authentic *O*-phosphorylethanolamine (solvent and buffer systems 3, 4, 5, 6, 7, see Table II) was identified in *S. typhimurium* (wild type), *S. abortus equi*, *S. enteritidis*, *S. typhosa* 0901, *Shigella flexneri*, *E. coli* 0111:B4, *E. coli* 0111:B4, J5 (UDP-D-galactose-epimeraseless mutant kindly provided by Dr. Edward Heath), *E. coli* 026:B6, *E. coli* 055:B5, *E. coli* 0127:B8, and *E. coli* 0128:B12. These lipopolysaccharides, purified by the method of Westphal *et al.* (1952), were obtained from Difco Laboratories.

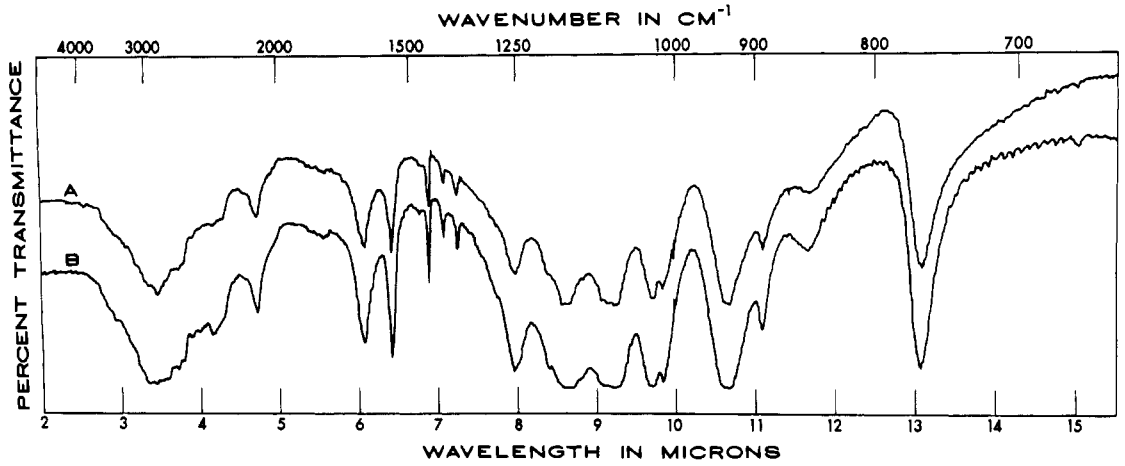

FIG. 2.—Infrared spectra of (A) chemically synthesized *O*-phosphorylethanolamine and (B) crystalline material isolated from acid hydrolysates of polysaccharide prepared from an epimeraseless mutant of *S. typhimurium*. Spectra were obtained, using a Perkin-Elmer infrared spectrophotometer, by dispersal of the samples in KBr.

DISCUSSION

O-Phosphorylethanolamine has been identified in Gram-negative bacteria only as a component of phosphatidylethanolamine (Geiger and Anderson, 1939); however, this phosphatide is not a component of lipopolysaccharides obtained from these organisms. It appears unlikely that *O*-phosphorylethanolamine isolated from the purified polysaccharide fraction represents phosphatidylethanolamine contamination for the following reasons: (1) The warm-phenol extraction procedure separates the bulk of the phosphatidylethanolamine-containing lipid from the lipopolysaccharide (Westphal *et al.*, 1952). (2) After extraction of a solution of polysaccharide with chloroform-methanol, all of the phosphate and *O*-phosphorylethanolamine were recovered in the aqueous phase. No lipids were detected in the organic phase by thin-layer chromatography or gas chromatographic analysis. (3) Hydrolysis of the polysaccharide in 1 N HCl for 5 hours at 100° yielded chiefly *O*-phosphorylethanolamine, while phosphatidylethanolamine subjected to the same conditions released predominantly free ethanolamine.

O-Phosphorylethanolamine contains 50% of the total phosphate in the "backbone" polysaccharide of *S. typhimurium* and has been identified in the lipopolysaccharide *O*-antigen of other enteric bacteria. It is of interest that free ethanolamine was previously detected by paper chromatography in lipopolysaccharide hydrolysates of strains of *Salmonella*, *E. coli*, and *Neisseria gonorrhea* (Tauber and Russell, 1960). Polysaccharide fractions isolated from lipopolysaccharides are heterogeneous in size, but *O*-Phosphorylethanolamine is probably present in all fractions as evidenced by the general ninhydrin reactivity of the polysaccharide fraction shown in Figure 1. Although the antigenic determinants of these organisms vary widely, there is a growing body of evidence (Osborn *et al.*, 1964) suggesting that the *O* antigen contains an internal "core" polysaccharide common to all enteric bacteria. *O*-Phosphorylethanolamine, as a component of the phosphorylated "backbone" polysaccharide, appears to be a major component of this "core."

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