Studies of a Phospholipid-Requiring Bacterial Enzyme. I. Purification and Properties of Uridine Diphosphate Galactose:Lipopolysaccharide α-3-Galactosyl Transferase*

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ABSTRACT: The uridine diphosphate galactose:lipopolysaccharide α-3-galactosyl transferase of *Salmonella typhimurium* requires phospholipid for activity and catalyzes one of the reactions involved in biosynthesis of the cell envelope lipopolysaccharide of the organism. The enzyme has now been purified approximately 6000-fold. The purified enzyme required phospholipid for activity and was inhibited in a competitive manner by closely related nonsubstrate lipopolysaccharides. Extraction of the purified enzyme with organic solvents removed a bound lipid-soluble component, which contained no phosphorus and is still unidentified. This component (lipid Y) caused aggregation of the enzyme, but no role of the lipid in the enzyme reaction itself has yet been established.

Several enzymes are now known which require phospholipid cofactors for activity. Many of these are located in the lipid-rich environment of biological membranes (Rothfield and Finkelstein, 1968) and the involvement of phospholipids in their activity may reflect a general property of membrane-bound enzymes. However, in most cases these enzymes have been difficult to purify and therefore have not been available for detailed study. Several enzymes involved in biosynthesis of bacterial lipopolysaccharides fall into this group and the present paper describes the purification and properties of one such enzyme, the UDP-galactose:lipopolysaccharide α-3-galactosyl transferase of *Salmonella typhimurium*.

Lipopolysaccharides of gram-negative enteric bacteria consist of a lipid portion (lipid A) covalently linked to a complex polysaccharide (Figure 1) (Westphal, 1960). Evidence from various sources indicates that the lipopolysaccharides are located in the outer membrane of the cell envelope (Mergenhagen *et al.*, 1966; Shands, 1966; Knox *et al.*, 1966; DePetris, 1967) although it is possible that lipopolysaccharide molecules are also present elsewhere in the cell envelope.

The enzymes responsible for the biosynthesis of the polysaccharide portion of the molecule are also located in the cell envelope fraction, in close association with their lipopolysaccharide substrates (Osborn *et al.*, 1964). However, in the case of the galactosyl transferase of *S. typhimurium* 20–40% of the total enzyme activity can be recovered from the supernatant fraction obtained by high-speed centrifugation after disruption of the cells by sonication. The remainder of the activity is present in the particulate cell envelope fraction (Rothfield *et al.*, 1964). We have assumed that the supernatant and particulate activities represent the same enzyme since both activities disappear together in a mutant strain deficient in the transferase enzyme (Osborn, 1968).

The enzyme catalyzes the transfer of galactose from UDP-galactose to galactose-deficient lipopolysaccharides obtained from mutant strains deficient in UDP-galactose. We have previously shown that phospholipids are required as a cofactor in the reaction catalyzed by the crude enzyme (Rothfield and Horecker, 1964), as shown in the following scheme

$$\text{UDP-galactose} + \text{galactose-deficient LPS}^{1} \xrightarrow[\text{phospholipid}]{\text{enzyme}} \text{galactosyl-LPS} (+\text{UDP})$$

More recently, a variety of evidence has indicated that the phospholipid first interacts with an acceptor lipopolysaccharide, and that a lipopolysaccharide–phospholipid complex is the active substrate in the reaction (Weiser and Rothfield, 1968). The following sequence has been suggested previously on the basis of experiments with a crude enzyme preparation (Rothfield and Takeshita, 1965)

$$\text{galactose-deficient LPS} + \text{phospholipid} \longrightarrow \text{LPS}\cdot\text{phospholipid} \quad (1)$$

$$\text{LPS}\cdot\text{phospholipid} + \text{enzyme} \longrightarrow \text{enzyme–LPS}\cdot\text{phospholipid} \quad (2)$$

$$\text{enzyme–LPS}\cdot\text{phospholipid} + \text{UDP-galactose} \longrightarrow \text{galactosyl–LPS}\cdot\text{phospholipid} (+\text{UDP} + \text{enzyme}) \quad (3)$$

This paper describes the purification and properties of the enzyme. The following paper deals with the role of phospholipid in the reaction.

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[1] Abbreviations used are: LPS, lipopolysaccharide; KDO, 2-keto-3-deoxyoctonate; PE, phosphatidylethanolamine.

Materials and Methods

Bacterial Strains. The following strains of *S. typhimurium* were obtained from Dr. M. J. Osborn of this department: LT2 (wild type), TV119 (rough B) (Beckmann *et al.*, 1964), G-30, EI-1, G-30A, G-30F, and G-30G. Strain G-30 is a mutant of the parent LT2 strain and is deficient in UDP-galactose 4-epimerase (Osborn *et al.*, 1962); strain EI-1 is a double mutant deficient in UDP-galactose 4-epimerase and phosphohexose isomerase (Fraenkel *et al.*, 1963); strains G-30A, G-30F, and G-30G are derivatives of strain G-30 and are defective in biosynthesis of the heptose-containing backbone of the lipopolysaccharide. The lipopolysaccharide of G-30G contains no detectable heptose, and the lipopolysaccharides of G-30A and G-30F contain approximately 10% of the normal content of heptose (M. J. Osborn, 1968, personal communication). Dr. Bruce Stocker kindly supplied cultures of *S. typhimurium* SL1032 and SL1060. Strain SL1032 lacks UDP-glucose:lipopolysaccharide glucosyl transferase and SL1060 has greatly reduced levels of UDP-galactose:lipopolysaccharide α-3-galactosyl transferase (Osborn, 1968). The structures of the lipopolysaccharides of these strains are diagramatically illustrated in Figure 1. *E. coli* J5 is a mutant of *E. coli* 0111 deficient in UDP-galactose 4-epimerase, isolated in the laboratory of Dr. E. Heath (Edstrom and Heath, 1964).

Materials. Bacteria were grown and lipopolysaccharides were extracted and purified as previously described (Osborn *et al.*, 1962). Lipopolysaccharides of *E. coli* J5 (galactose grown) and wild-type *E. coli* 0111 were gifts from Dr. E. Heath of Johns Hopkins University. Phosphatidylethanolamine from *Azobacter agilis* was purified as previously described (Weiser and Rothfield, 1968).

Hydroxylapatite (Clarkson Chemical Co.), protamine sulfate (Eli Lilly and Co.), and DEAE-cellulose (Bio-Rad Laboratories) were obtained commercially. DEAE-cellulose was washed successively with 0.5 N NaOH and 0.5 N HCl prior to use. Galactose-^{14}C 1-phosphate was prepared by treatment of UDP-galactose-^{14}C with venom phosphodiesterase (Worthington Biochemical Corp.) and was purified by paper electrophoresis on Whatman No. 3MM paper in pyridine–acetic acid–water (10:1:89, v/v, pH 6.0) at 5000 V for 60 min. Other radioactive compounds were obtained from the International Chemical and Nuclear Corp. (UDP-galactose-^{14}C, ADP-galactose-^{14}C, and TDP-glucose-^{14}C) or from New England Nuclear Corp. (UDP-galactose-^{14}C and UDP-glucose-^{14}C). Radioactive compounds were randomly labeled in the hexose moiety and were characterized in the manner previously described (Weiser and Rothfield, 1968). Myoglobin and chymotrypsinogen were purchased from Mann Research Laboratories.

Methods. ENZYME ASSAY. Galactosyl transferase activity was determined by measuring the incorporation of galactose-^{14}C into an acid-insoluble product. A stock solution of acceptor was prepared by mixing 12 ml of 0.2 M Tris-HCl buffer (pH 8.5), 10 μmoles of EDTA, 7 mg of galactose-deficient lipopolysaccharide from strain G-30 (containing 4 μmoles of heptose), and 30 μmoles of *A. agilis* phosphatidylethanolamine in 2 ml of methanol. The mixture was heated at 60° for 30 min, cooled to room temperature, and stored at room temperature (Rothfield and Horecker, 1964). The enzyme assay mixture contained 0.14 ml of acceptor solution, 30 mμmoles of UDP-galactose-^{14}C (5000 cpm/mμmole), 1.5 μmoles of $MgCl_2$, and enzyme, in a total volume of 0.25 ml. After incubation at 37° for 10 min, 2 ml of cold 5% trichloroacetic acid was added and acid-precipitable radioactivity was determined by membrane filtration. One unit of enzyme activity is defined as 1.0 mμmole of galactose incorporated in 10 min. Appropriate experiments have demonstrated that this represents an accurate measure of the initial rate of the reaction in the range of enzyme concentrations used.

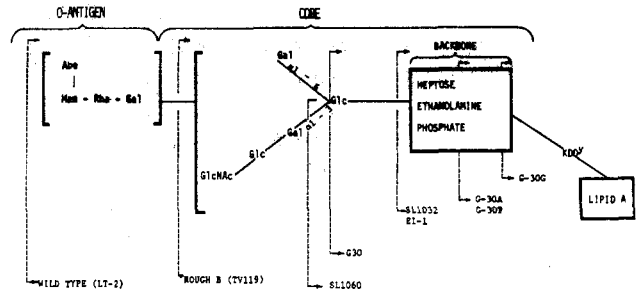

FIGURE 1: Schematic diagram of *S. typhimurium* lipopolysaccharides. The lipopolysaccharides used in the present study and the strains from which they were derived are indicated by the dotted lines. Abe, abequosyl; Man, mannosyl; Rha, rhamnosyl; GlcNAc, *N*-acetylglucosaminyl; Glc, glucosyl; Gal, galactosyl.

Sucrose Density Gradient Centrifugation. The density gradients consisted of 4.4 ml of a continuous gradient of 10–30% sucrose layered over 0.4 ml of 60% sucrose. All solutions contained 0.1 M Tris-HCl buffer (pH 8.5) and 0.001 M EDTA. Centrifugation was performed at 4° in a Spinco SW39 swinging-bucket rotor at 100,000*g* for 18 hr. Samples were collected from above using a thin polyethylene tube and a peristaltic pump.

Analytical Techniques. Thin-layer chromatography for nucleotides was performed on Polygram Gel PE-I anion exchanger (Brinkman Co.). The developing solvent was 0.5 M LiCl and the components were visualized under ultraviolet light (Verachtert *et al.*, 1965). Protein, phosphate, and heptose were measured as previously described (Weiser and Rothfield, 1968). Florisil column chromatography was performed as described by Carroll (1961), and UDP concentration was determined by the method of Leloir and Goldemberg (1960). High-resolution mass spectrometry was performed by the Sadtler Research Laboratories, Philadelphia, Pa.

Results

Galactosyl Transferase Levels in Different Strains. The amount of soluble galactosyl transferase activity varied in extracts prepared from different strains of *S. typhimurium* (Table I). Highest activity was found in extracts of G-30A, a strain lacking the heptose-containing backbone of the lipopolysaccharide, and this strain was therefore used for purification of the enzyme.

Purification of Enzyme[2] (*Method A*). PREPARATION OF CELLS. *S. typhimurium* G-30A was grown in proteose peptone–beef extract–NaCl medium (Rothfield *et al.*, 1964) in a 100-l. fermentor to early stationary phase. Cells were collected by cen-

[2] All buffers contained 5 m*M* 2-mercaptoethanol and 1 m*M* EDTA and all procedures were performed at 4° unless otherwise stated. For large-scale enzyme preparations, the facilities of the New England Enzyme Center were used.

TABLE I: Galactosyl Transferase Activity in the Supernatant Fractions of Different Bacterial Strains.[a]

Strain	Enzyme Act. (units/ml)	Sp Act. (units/mg of protein)
G-30A	73.7	4.8
SL1032	34.5	2.3
EI-1	41.6	3.3
G-30	20.3	1.4
SL1060	5.1	0.3
TV119	26.8	2.0
LT2	23.5	1.9

[a] Bacterial strains were grown in proteose peptone–beef extract–NaCl medium in 2-l. flasks to early stationary phase. Cells were collected by centrifugation, washed twice with 0.9% NaCl, and then suspended in five volumes of 10 mM Tris-HCl buffer (pH 8.0). The cells were disrupted by sonication as described in the text and the 100,000*g* supernatant fraction was collected and assayed.

trifugation in a refrigerated Sharples centrifuge, washed twice with 0.9% NaCl, and frozen for 1–4 weeks before use.

PREPARATION OF EXTRACT. A total of 700 g of cells was suspended in 3500 ml of 10 mM Tris-HCl buffer (pH 8.0) and the cell suspension was sonicated in 200-ml portions for 4 min in a Branson sonicator with the temperature of the extract kept below 15° by use of an ice bath. After centrifugation at 30,000*g* for 120 min, the supernatant solution (3500 ml) was removed by decanting (Table II) (fraction I).

Protamine Precipitation and Elution. Protamine sulfate (420 ml of 1%) was added to fraction I with stirring and the precipitate was removed by centrifugation and discarded. The supernatant solution was mixed with an additional 420 ml of 1% protamine sulfate and the precipitate was collected by centrifugation. The precipitate was extracted twice by stirring for 2 hr with 500 ml of 0.3 M sodium succinate (pH 6.0), and the final supernatant solution was collected after centrifugation (fraction II, 1000 ml).

Solvent Extraction and Ammonium Sulfate Precipitation. Ammonium sulfate (226 g) was added to fraction II and the precipitate was collected by centrifugation. The precipitate was suspended in 20 volumes of ethanol–diethyl ether (3:1, v/v) and was shaken vigorously in a glass-stoppered bottle at

TABLE II: Summary of the Purification Procedure.

Fraction and Step	Total Act. (units)	Sp Act. (units/mg of protein)
I. Sonic extract	192,500	2.4
II. Protamine sulfate	495,000	41.6
III. Solvent extraction	336,000	76.1
IV. Ammonium sulfate	300,000	131
V. Hydroxylapatite	142,000	862
VI. DEAE-cellulose	35,200	15,000

TABLE III: Donor Specificity in the Galactosyl Transferase Reaction.[a]

Glycosyl Donor	^{14}C Sugar Incorp ($\mu\mu$moles/10 min)
UDP-galactose-^{14}C	337
ADP-galactose-^{14}C	<1
UDP-glucose-^{14}C	<1
TDP-glucose-^{14}C	<1
Galactose-^{14}C-1-P	<1

[a] Assays were performed as described in Methods with the appropriate glycosyl donor substituted for UDP-galactose.

−20° for 5 min until a homogeneous mixture was obtained. The suspension was filtered through Schleicher & Schuell No. 597 filter paper which previously had been washed in the same ethanol–ether mixture. The precipitated enzyme was removed by suspending the filter paper in 300 ml of 5 mM phosphate buffer (pH 6.8) and the solution was then dialyzed against the same buffer for 2 days at 4°. A precipitate which formed during dialysis was removed by centrifugation and discarded. The dialyzed enzyme (fraction III, 300 ml) was precipitated by addition of 132 g of ammonium sulfate. The precipitate was suspended in 30 ml of 50 mM phosphate buffer (pH 6.8) and the suspension dialyzed for 12 hr against the same buffer (fraction IV, 30 ml).

Hydroxylapatite Chromatography. Fraction IV was applied to a column of hydroxylapatite (11 cm^2 × 15 cm) which previously had been equilibrated with 50 mM phosphate buffer (pH 6.8) and the column was then washed with 600 ml of the same buffer. The enzyme was eluted with a linear gradient of 50 mM (400 ml) to 300 mM (400 ml) phosphate buffer (pH 6.8) at a flow rate of 60 ml/hr, and 19-ml fractions were collected. Enzyme activity was recovered between fractions 30 and 50. The most active fractions (260 ml) were pooled and precipitated by addition of 103 g of ammonium sulfate. The precipitate was resuspended in 10 ml of 5 mM phosphate buffer (pH 6.8) and dialyzed against the same buffer for 12 hr (fraction V, 12 ml).

DEAE-cellulose Chromatography. Fraction V was applied to a column of DEAE-cellulose (2.5 cm^2 × 20 cm) which previously had been equilibrated with 5 mM phosphate buffer (pH 6.8). After washing the column with 300 ml of the same buffer, a linear gradient of 5–50 mM phosphate buffer (pH 6.8, 150 ml of each) was applied, followed by a second linear gradient of 50–300 mM phosphate buffer (pH 6.8, 150 ml of each). Fractions of 10 ml were collected at a flow rate of 80 ml/hr and fractions with the highest specific activity (fractions 23–38) were collected and pooled (fraction VI, 160 ml).

Unsuccessful attempts were made to concentrate fraction VI by ammonium sulfate precipitation, ultrafiltration, and dialysis against polyethylene glycol. Major losses of activity resulted from all of these procedures and the enzyme was therefore kept in dilute solution (approximately 20 μg of protein/ml).

Purification of Enzyme[2] *(Method B).* In an alternate purification procedure the solvent extraction procedure was

TABLE IV: Acceptor Specificity in the Galactosyl Transferase Reaction.

Acceptor Lipopolysaccharide[a]	Galactose Incorp (mμmoles/10 min) Plus PE[b]
G-30F	<0.01
G-30G	<0.01
SL1032	<0.01
G-30	1.83 (0.21[c])
SL1060	1.49
TV119	<0.01
LT2	<0.01
E. coli J5	1.75
E. coli J5 (galactose grown)	<0.01
E. coli 0111	<0.01

[a] Lipopolysaccharides (0.07 mg) from the indicated strains of *S. typhimurium* and *E. coli* were substituted for G-30 lipopolysaccharide in the standard assay. In the case of SL1032, G-30, SL1060, and TV119, this amount of lipopolysaccharide contained 35–40 mμmoles of heptose. [b] PE was included in the assay mixture in the usual manner. [c] PE was omitted.

TABLE V: Effect of Nonsubstrate Lipopolysaccharides on the Galactosyl Transferase Reaction.

Addition[a] (μg)	Rate of Reaction[b] (% of control) Plus PE[c]	Minus PE[d]
None (control)	100	100
Lipopolysaccharides		
G-30F (30)	98	91
G-30G (30)	81	101
SL1032 (30)	48	8
(3)		53
TV119 (30)	47	8
(3)		58
LT2 (30)	<1	<1
(0.05)	58	
(0.005)		53
TV119 polysaccharide (30)[e]	62	31
TV119 lipid A (30)[e]	98	89

[a] The indicated compounds were added to the mixture of G-30 lipopolysaccharide and PE prior to the 60° heating procedure described in the Methods section. [b] The reaction rate (millimicromoles of galactose-^{14}C incorporated in 10 min) without any addition[a] was 0.8 in the complete assay and 0.128 when the assay was performed in the absence of PE. These rates were assigned a value of 100%. [c] PE was included in the assay mixture in the usual manner. [d] PE was omitted. [e] Lipopolysaccharide from strain TV119 was heated in 0.01 N HCl at 100° for 45 min, and then centrifuged for 10 min at 5000*g* at room temperature. The hydrolysis procedure was repeated twice more on the insoluble residue. After neutralization, the pooled supernatant fraction (polysaccharide) and precipitate (lipid A) obtained from 30 μg of lipopolysaccharide were assayed as described in footnote *a*.

omitted. The course of purification, the final specific activity, and the yield were approximately the same as with method A.

Properties of the Enzyme. GENERAL PROPERTIES (PURIFICATION METHOD A). A final purification of approximately 6000-fold was achieved with a yield of approximately 18%. The most purified fractions were stable in dilute solution (20–40 μg/ml) for at least 1 month when kept at 4° at pH 6.8. One cycle of freezing and thawing caused marked loss of activity, and storage at pH 8.5 for several days at 4° also resulted in inactivation. Enzyme prepared without the lipid extraction step (*i.e.*, method B) was much less stable (see below).

No activity was seen in the absence of divalent cations. Highest activity was observed with 8 mM $MgCl_2$ and approximately 20% of this level was observed when $MgCl_2$ was replaced by 4 mM $CaCl_2$ or 4 mM $MnCl_2$. No activity was detected in the presence of $ZnCl_2$.

The optimal pH of the reaction was 8.5–9.0 in Tris-HCl buffer and the K_m for UDP-galactose was 7.4×10^{-5} M as calculated by the method of Lineweaver and Burk (1934).

The enzyme had no detectable UDP-glucose:lipopolysaccharide glucosyl transferase activity, assayed with UDP-glucose-^{14}C and glucose-deficient lipopolysaccharide (from strain SL1032) in place of UDP-galactose-^{14}C and G-30 lipopolysaccharide (Rothfield and Horecker, 1964).

SPECIFICITY OF GLYCOSYL DONOR AND LIPOPOLYSACCHARIDE ACCEPTOR. The reaction was highly specific for UDP-galactose (Table III); other nucleotide sugars and galactose-1-P were inactive.

The requirement for acceptor activity was satisfied only by lipopolysaccharides lacking the α-3-galactosyl residue of the core lipopolysaccharide (lipopolysaccharides from strains G-30 and SL1060) (Table IV). It is significant that SL1060 lipopolysaccharide contains one of the two galactosyl residues (the α-1,6 residue) normally present in the complete lipopolysaccharide. Identical rates and yields were observed with this acceptor and with G-30 lipopolysaccharide, which lacks both the α-1,3- and α-1,6-galactosyl residues (Osborn, 1968). No activity was obtained with lipopolysaccharides lacking the glucosyl residue which provides the site of attachment for galactose (lipopolysaccharides from strains SL1032, G-30F, and G-30G) nor was there activity with those lipopolysaccharides in which the α-1,3-galactosylglucose group is already present (from strains TV119 and LT2).

Lipopolysaccharides from *E. coli* behaved identically with *S. typhimurium* lipopolysaccharides in the transferase reaction. Galactose-deficient lipopolysaccharide from an epimeraseless strain of *E. coli* (strain J5) replaced the galactose-deficient lipopolysaccharides from *S. typhimurium* as an acceptor. Both the rate and yield of the reaction were identical. No acceptor activity was obtained with lipopolysaccharides possessing the normal complement of wild-type sugars (*E. coli* 0111, *E. coli* J5 grown in the presence of galactose, or *S. typhimurium* LT2).

INHIBITION BY NONSUBSTRATE LIPOPOLYSACCHARIDES. The galactosyl transfer reaction was inhibited by several lipopolysaccharides which are not themselves substrates for the en-

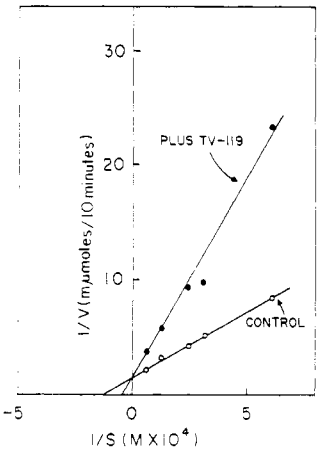

FIGURE 2: The effect of TV119 lipopolysaccharide on the double-reciprocal plot of reaction velocity *vs.* concentration of lipopolysaccharide in the galactosyl transferase reaction. Assays were performed using 1.6 units of enzyme. The concentration of G-30 lipopolysaccharide was varied as indicated and is expressed in terms of heptose concentration. The concentration of *A. agilis* PE was 0.25 μmole/ml of the reaction mixture. In the upper curve, 70 μg of TV119 lipopolysaccharide was added to the mixture of G-30 lipopolysaccharide and *A. agilis* PE prior to the 60° heating step.

zyme (SL1032, TV119, and LT2 lipopolysaccharides) (Table V). The small amount of activity normally seen in the absence of phosphatidylethanolamine was also inhibited, making it unlikely that the inhibition by nonsubstrate lipopolysaccharides was due solely to competition for available molecules of phosphatidylethanolamine. Detailed kinetic studies were performed with one of the inhibitor lipopolysaccharides (strain TV119), and analysis by the method of Lineweaver and Burk indicated that the inhibition was competitive with substrate lipopolysaccharide (Figure 2). Reproducible kinetic studies were not successfully obtained with other inhibitor lipopolysaccharides, possibly due to the marked differences in solubility of the different mutant lipopolysaccharides. Inhibition was also seen with the polysaccharide portion of the lipopolysaccharide molecule, but not with the lipid A fraction. Lipopolysaccharides which lacked the heptose-containing backbone of the polysaccharide (G-30F and G-30G) did not inhibit the reaction.

CHARACTERIZATION OF PRODUCTS. Evidence that the radioactive product was lipopolysaccharide was obtained by its isolation and purification from the reaction mixture (Table VI). Approximately 70% of the total acid-precipitable radioactivity was recovered in the purified lipopolysaccharide fraction after phenol extraction and Mg^{2+} precipitation. The radioactive polysaccharide fraction was released by mild acid hydrolysis and behaved identically with authentic lipopolysaccharide when examined by paper electrophoresis (Rothfield *et al.*, 1964). The radioactivity was identified as galactose by paper chromatography in butanol–pyridine–H_2O (6:4:3, v/v) after hydrolysis of the radioactive product in 1 N HCl at 100° for 90 min.

Uridine diphosphate was identified as the second product of the reaction by thin-layer chromatography. The entire reaction mixture from a 60-min incubation was applied directly to the plate and chromatography was performed as described in *Methods*. Only two ultraviolet-absorbing components were

TABLE VI: Characterization of the Radioactive Products of the Galactosyl Transferase Reaction.[a]

Stage of Purification	Radioactivity (cpm × 10^{-5})	Percentage of Total
1. Trichloroacetic acid precipitate	1.44	100
2. After phenol extraction	1.26	86
3. After Mg^{2+} precipitation and dialysis	1.01	70
4. After pH 2 hydrolysis (soluble polysaccharide)	0.92	65

[a] The transferase assay mixture described in Methods was scaled up tenfold (total volume 2.5 ml). The reaction mixture contained 40 units of enzyme (fraction VI, Table II) and the specific activity of UDP-galactose-^{14}C was 4800 cpm/mμmole. After incubation at 37° for 30 min, 20 ml of cold 5% trichloroacetic acid was added and the precipitate was collected by centrifugation and washed twice with 20 ml of cold 5% trichloroacetic acid. The final precipitate (fraction 1) was extracted with 45% phenol at 68° (Westphal *et al.*, 1952) and the phenol was removed by extraction with diethyl ether. Lipopolysaccharide was precipitated from the aqueous phase (fraction 2) by addition of 0.025 M $MgCl_2$ and the precipitate was dialyzed against water. The purified lipopolysaccharide (fraction 3) was hydrolyzed in 0.01 N H_2SO_4 to release the soluble polysaccharide as previously described (Rothfield *et al.*, 1964).

present and these migrated identically with authentic UDP-galactose and UDP.

Galactose-^{14}C incorporation and UDP production were also quantitatively determined in parallel reaction mixtures as described in *Methods*. In these experiments 1.24 moles of UDP was released for each mole of galactose-^{14}C transferred into lipopolysaccharide. Appropriate controls were incubated in the absence of enzyme.

HETEROGENEITY OF PURIFIED GALACTOSYL TRANSFERASE ENZYME. When the solvent extraction step was omitted from the standard purification procedure, the purified enzyme showed multiple peaks on density gradient centrifugation (Figure 3a) and in DEAE-cellulose column chromatography (Figure 4a). This heterogeneity appeared to be related to two factors: (1) aggregation caused by a bound lipid-soluble component, and (2) multiple forms of the enzyme.

BOUND LIPID-SOLUBLE COMPONENTS. When the enzyme was extracted with ethanol–ether (3:1, v/v) at −20° during the purification procedure (method A) there was a marked change in behavior on gradient centrifugation (Figure 3b). The rapidly sedimenting peaks of enzyme activity disappeared and were replaced by a more slowly sedimenting major peak, with recovery of approximately 80% of the original enzyme activity. The molecular weight of the material in this peak was estimated to be approximately 20,000 from cosedimentation studies with proteins of known molecular weight.

Two components (fractions X and Y) were seen when the

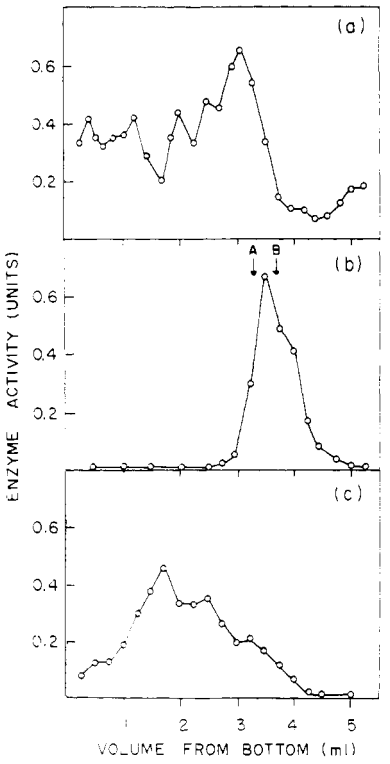

FIGURE 3: The effect of solvent extraction on the gradient centrifugation pattern of galactosyl transferase. (a) Enzyme was purified by method B and 0.2 ml, containing 50 units (4 μg) of enzyme, was subjected to sucrose gradient centrifugation as described in methods. Samples of 0.25 ml were collected and 0.01 ml of each fraction was assayed for enzyme activity. (b) Enzyme was purified by method A and subjected to gradient centrifugation as described above. Chymotrypsinogen A (mol wt 25,000) and myoglobin (mol wt 17,800) were applied to the gradient together with the transferase enzyme, and arrows labeled A and B indicate their respective positions in the tube. Concentrations of chymotrypsinogen A and myoglobin were determined by measuring absorbance at 280 and 420 mμ, respectively. (c) Lipid fraction Y was purified by Florisil column chromatography as described in the text and 0.2 μg of the purified material was mixed at 4° with 5 μg of enzyme purified by method A. The mixture was subjected to sucrose gradient centrifugation as described above.

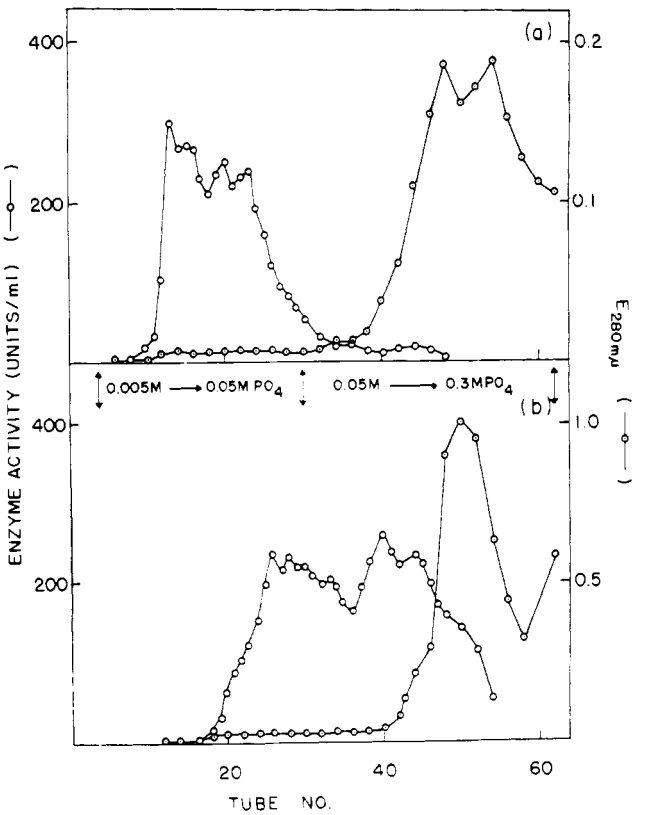

FIGURE 4: Behavior of the purified galactosyl transferase in DEAE-cellulose chromatography. This was the final column step described under Purification of Enzyme. Parts a and b represent purifications carried out by method B (without lipid extraction) and method A (with lipid extraction), respectively.

lipid extract of the enzyme was examined by thin-layer chromatography (Figure 5). Fractions X and Y were purified by column chromatography on Florisil and were eluted with 4% methanol in hexane and with 2% methanol in hexane, respectively. Approximately 0.2 μg of fraction Y was obtained from 100 units (7 μg) of purified enzyme. Readdition of fraction Y to the solvent-extracted enzyme resulted in reappearance of a pattern of multiple rapidly sedimenting peaks (Figure 3c) indicating that this component was the factor responsible for aggregation of the enzyme; readdition of fraction X had no effect on the sedimentation pattern. The presence or absence of fraction X or fraction Y had no detectable effect on enzyme activity.

The structure of fraction Y has not been determined. It was not phospholipid or lipopolysaccharide since it contained no detectable phosphate (<0.01%) or KDO (<0.05%). Authentic lipopolysaccharide from the same strain contained 8% KDO and 6% phosphate, while the lipid A portion of the lipopolysaccharide contained 0.6% phosphate. Fraction Y was eluted from Florisil with 2% methanol in hexane, unlike lipopolysaccharide, which is not eluted by this solvent.

Fraction X was identified as oxidized mercaptoethanol ($HOCH_2CH_2SSCH_2CH_2OH$) by high-resolution mass spectrometry, thin-layer chromatography, nuclear magnetic resonance studies, and by acetylation with acetic anhydride followed by mass spectrometry (unpublished data). We presume that fraction X originated by oxidation of the 2-mercaptoethanol added during the purification procedure.

In addition to affecting the state of aggregation of the enzyme, extraction of the bound lipid-soluble components markedly increased its stability so that activity was retained for longer than 1 month at 4°. In contrast, when the solvent extraction step was omitted from the procedure, the enzyme (purified by method B) lost 50% of its activity in 48 hr at 4° at pH 6.8 or 8.5.

MULTIPLE FORMS OF THE ENZYME. The DEAE chromatographic pattern continued to show multiple peaks despite removal of all lipid-soluble components (Figure 3c). Each of the peak fractions between tubes 20 and 40 of the DEAE column had the same specific activity when assayed at varying substrate concentrations and the fractions all showed identical heat stability. Amino acid compositions of these fractions were essentially identical within the range of experimental error (Table VII). Rechromatography of the DEAE fractions was unsuccessful because of poor recovery of the enzyme activity.

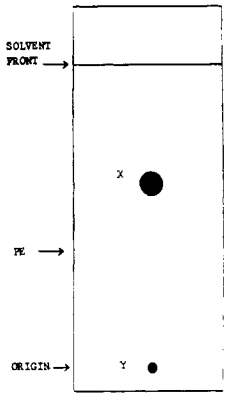

FIGURE 5: Thin-layer chromatography of lipid extract of the purified enzyme. Enzyme purified by method B (3 mg) was suspended in 20 volumes of ethanol–ether (3:1, v/v) and was extracted by shaking vigorously at −20° for 5 min. The insoluble residue was removed by centrifugation and the supernatant was evaporated to dryness and redissolved in a small volume of chloroform–methanol (19:1, v/v). Thin-layer chromatography was performed on silica gel G (Merck) using a solvent system of chloroform–methanol–water (65:25:4, v/v) and visualizing the components with iodine vapor. The arrow indicates the position of PE from *A. agilis* (PE), chromatographed on the same plate.

The DEAE peaks contained different amounts of glucosamine, ranging from <0.07 to >0.67 residue per histidine residue, and also showed significant differences in carbohydrate content as determined by the phenol–sulfuric acid method (Table VIII). It seemed unlikely that these findings reflected the presence of a contaminating glucosamine-containing polysaccharide since the peak of glucosamine content did not coincide with the tubes showing the highest concentrations of carbohydrate. The carbohydrate component did not appear to be lipopolysaccharide since the fractions contained no detectable phosphate or KDO (<0.005 mg of KDO per mg of carbohydrate) while authentic lipopolysaccharide contained 6% phosphate and 0.9 mg of KDO per mg of carbohydrate.

TABLE VII: Amino Acid Composition of DEAE-Cellulose Fractions.[a]

Amino Acid	Tube Number 24	27	30	33	36
Aspartic acid	3.93	4.19	3.63	4.00	4.03
Threonine	2.23	2.61	2.10	2.33	2.30
Serine	1.83	2.36	2.13	2.33	2.27
Glutamic acid	5.53	5.80	5.30	5.20	5.40
Proline	1.57	1.71	1.60	1.57	1.77
Glycine	3.47	4.19	3.47	3.40	3.80
Alanine	4.17	4.22	4.20	4.03	4.33
Half-cystine	0	0	0	0	0
Valine	2.70	2.97	2.87	2.83	2.83
Methionine	0.73	0.68	0.80	0.70	0.77
Isoleucine	2.67	3.01	2.97	2.77	2.80
Leucine	3.80	4.08	4.13	3.87	4.03
Tyrosine	1.03	1.04	0.97	0.97	1.03
Phenylalanine	1.33	1.36	1.30	1.43	1.33
Lysine	2.97	2.94		2.70	2.80
Histidine	1.00	1.00	1.00	1.00	1.00
Arginine	3.77	4.29	4.00	4.07	4.17
Glucosamine	<0.07	<0.07	>0.67	>0.33	<0.07

[a] The proteins in the indicated fractions from the DEAE-cellulose column (Figure 4b) were precipitated by addition of 5% trichloroacetic acid and were then hydrolyzed in 6 N HCl in evacuated sealed tubes at 100° for 24 hr. The hydrolysates were evaporated to dryness under reduced pressure. Amino acid analyses were performed on a Beckman amino acid analyzer through the kind cooperation of Dr. C. Y. Lai of this department. The results are expressed as the molar content relative to histidine.

Discussion

The galactosyl transferase was purified approximately 6000-fold by standard techniques, and contained no detectable phospholipid or lipopolysaccharide. However, the extensively purified enzyme appeared to be heterogeneous in size and composition. The size heterogeneity was shown by gradient centrifugation and was associated with the presence of a noncovalently bound lipid-soluble component (fraction Y). When this component was removed by solvent extraction, sedimentation studies indicated conversion of the enzyme into a smaller more homogeneous form, while the subsequent addition of fraction Y resulted in reaggregation with reappearance of multiple rapidly sedimenting peaks. Extensive studies failed to reveal any effect of fraction Y on the enzyme reaction. This unknown lipid does not appear to be related to lipopolysaccharide or to its lipid A component. Its structure has not yet been determined and nothing is known of its possible biological significance. The possibility that it may be an artifact has not been completely ruled out. The enzyme is thought to be located normally in the lipid-rich environment of the cell envelope and the tendency to form multimolecular complexes in the presence of fraction Y may reflect structural characteristics related to its presumed membrane localization.

A second type of heterogeneity was indicated by the presence of multiple peaks of enzyme activity on DEAE-cellulose chromatography both before and after lipid extraction. It seems likely that the multiple peaks contained enzyme fractions of similar structure, as indicated by their similar specific activities, heat stabilities, substrate saturation curves, and amino acid compositions. Sucrose gradient centrifugation indicated a molecular weight of approximately 20,000 for the purified enzyme. This relatively low molecular weight suggests that the enzyme is probably not composed of subunits and makes it unlikely that the heterogeneity reflects different combinations of peptide subunits.

The only difference seen among the DEAE fractions was in total carbohydrate, which ranged from 0.1 to 6.9%, and in glucosamine, which varied from <0.07 to 0.67 residue per histidine residue, suggesting that the multiplicity may reflect differences in carbohydrate constituents of the enzyme molecule. Further evidence that the sugars are covalently linked to the enzyme protein will be necessary to confirm this possibility.

It is unlikely that the multiple forms represent alterations in enzyme produced during the purification procedure since a

TABLE VIII: Carbohydrate Content of DEAE-Cellulose Fractions.[a]

Tube No.	Hexose (%)
23	2.6
25	1.4
26	3.5
28	6.9
29	1.8
31	0.7
32	0.1
34	1.4
35	<0.1

[a] The indicated fractions from the DEAE chromatography shown in Figure 3b were analyzed by phenol–H_2SO_4 (Dubois *et al.*, 1951), using glucose as a standard. The results are expressed relative to the protein content of each fraction.

similar pattern of enzyme activity was seen when DEAE chromatography was performed on the crude extract (fraction I) after lipid extraction.

The tranferase reaction was inhibited by several nonsubstrate lipopolysaccharides and by the lipid-free polysaccharide portions of these lipopolysaccharides. The inhibition appears to represent competition for a polysaccharide-binding site on the enzyme. No inhibition occurred with any of the mutant lipopolysaccharides which lack the heptose-containing backbone of lipopolysaccharide, while all lipopolysaccharides which contain this region were effective inhibitors (SL1032, TV119, and LT2). It is therefore likely that the backbone region is involved in the interaction of the lipopolysaccharide substrate with a polysaccharide binding site on the enzyme. There was no evidence of an analogous binding site for the lipid A portion of the molecule.

Detailed studies of the enzyme reaction and of the role of phospholipid are reported in the following paper (Endo and Rothfield, 1969).

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