JOURNAL OF BACTERIOLOGY, Feb. 1980, p. 635-644
0021-9193/80/02-0635/10$02.00/0

Vol. 141, No. 2

Purification and Characterization of 3-Deoxy-D-*manno*-Octulosonate 8-Phosphate Synthetase from *Escherichia coli*

PAUL H. RAY

Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

3-Deoxy-D-*manno*-octulosonate (KDO)-8-phosphate synthetase has been purified 450-fold from frozen *Escherichia coli* B cells. The purified enzyme catalyzed the stoichiometric formation of KDO-8-phosphate and Pi from phosphoenolpyruvate (PEP) and D-arabinose-5-phosphate. The enzyme showed no metal requirement for activity and was inhibited by 1 mM Cd^{2+}, Cu^{2+}, Zn^{2+}, and Hg^{2+}. The inhibition by Hg^{2+} could be reversed by dithiothreitol. The optimum temperature for enzyme activity was determined to be 45°C, and the energy of activation calculated by the Arrhenius equation was 15,000 calories (ca. 3,585 J) per mol. The enzyme activity was shown to be pH and buffer dependent, showing two pH optima, one at pH 4.0 to 6.0 in succinate buffer and one at pH 9.0 in glycine buffer. The isoelectric point of the enzyme was 5.1. KDO-8-phosphate synthetase had a molecular weight of 90,000 ± 6,000 as determined by molecular sieving through G-200 Sephadex and by Ferguson analysis using polyacrylamide gels. Based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the 90,000-molecular-weight native enzyme was composed of three identical subunits, each with an apparent molecular weight of 32,000 ± 4,000. The enzyme had an apparent K_m for D-arabinose-5-phosphate of 2×10^{-5} M and an apparent K_m for PEP of 6×10^{-6} M. No other sugar or sugar-phosphate could substitute for D-arabinose-5-phosphate. D-Ribose-5-phosphate was a competitive inhibitor of D-arabinose-5-phosphate, with an apparent K_i of 1×10^{-3} M. The purified enzyme has been utilized to synthesize millimole quantities of pure KDO-8-phosphate.

KDO (3-deoxy-D-*manno*-octulosonic acid), an unusual eight-carbon acidic sugar, first detected by Levin and Racker (20), was shown to be an integral part of the lipopolysaccharide (LPS) region in *Escherichia coli* by Heath and Ghalambor (13) and in *Salmonella typhimurium* by Osborn (26). Other laboratories, including those of Lüderitz et al. (23), Vincent and Cameron (32), and Ellwood (7) have since demonstrated the occurrence of KDO in the LPS region of most gram-negative bacteria. The innermost region of the LPS, containing lipid A and KDO, appears to be required, for the growth of bacteria and mutants defective in the biosynthesis of this region have only recently been isolated (28, 29). Lehmann et al., Munson et al., and Rick et al. (19, 24, 27-29) have subsequently indicated by using a mutant defective in KDO biosynthesis that, in the absence of KDO synthesis, LPS synthesis stops, and this is immediately followed after one generation by the cessation of DNA, RNA, and protein syntheses.

Even though the biosynthesis and utilization of KDO are known to involve at least five sequential reactions, shown below, the enzymes involved have not been studied in detail. These reactions are: (i) D-ribulose-5-phosphate $\rightleftharpoons$ D-arabinose-5-phosphate; (ii) D-arabinose-5-phosphate + phosphoenolpyruvate $\rightarrow$ KDO-8-phosphate + Pi; (iii) KDO-8-phosphate $\rightarrow$ KDO + Pi; (iv) KDO + CTP $\rightleftharpoons$ CMP-KDO + PPi; and (v) 2 CMP-KDO + lipid A precursor $\rightarrow$ KDO-KDO-lipid A precursor + 2 CMP. Reactions i through v, respectively, are catalyzed by D-arabinose-5-phosphate isomerase (21, 33), KDO-8-phosphate synthetase (20), KDO-8-phosphate phosphatase (4), CMP-KDO synthetase (11), and KDO transferase(s) (24).

In this paper we describe the purification and some of the properties of KDO-8-phosphate synthetase isolated from *E. coli* B. Previous work on this enzyme by Levin and Racker (20) utilized an ammonium sulfate precipitate from *Pseudomonas aeruginosa* to show that the enzyme catalyzes the condensation of D-arabinose-5-phosphate and phosphoenolpyruvate (PEP) to form KDO-8-phosphate and Pi. Ghalambor and Heath (11) and others (19, 24) have utilized crude extracts to synthesize KDO as a substrate for subsequent work. Rick and Osborn (28, 29) have isolated a temperature-sensitive mutant that contains an altered KDO-8-phosphate synthetase, have documented the consequences of the inhibition of KDO biosynthesis, and have

mapped the region of the *Salmonella typhimurium* chromosome that codes for KDO-8-phosphate synthetase at 57 min.

MATERIALS AND METHODS

Bacteria. *E. coli* B cells (ATCC 11303) grown to mid-logarithmic phase on glucose minimal medium were purchased as a frozen cell paste from Grain Processing Inc., Muscatine, Iowa. *E. coli* B and *E. coli* ML-30 were stored at −90°C in 20% glycerol and grown in glucose minimal medium (12) or in nutrient broth (Difco) at 37°C with vigorous aeration.

Chemicals. D-Arabinose-5-phosphate was either purchased from Sigma Chemical Co. or synthesized from D-glucosamine-6-phosphate by the method of Volk (33) and purified after butanol extraction of the buffer solution to remove the reacted ninhydrin (buffer solution extracted with an equal volume of butanol until colorless) by Bio-Rad AG-1 (Cl) column chromatography. D-Arabinose-5-phosphate was eluted with a LiCl gradient (0 to 0.4 M) and desalted by Bio-Gel P-2 (Bio-Rad Laboratories) column chromatography. PEP, thiobarbituric acid, *E. coli* alkaline phosphatase, sugars, and sugar phosphates were obtained from Sigma Chemical Co. Molecular weight standards for electrophoresis and G-200 Sephadex column chromatography were obtained from Schwarz-Mann, Sigma, or Pharmacia Fine Chemicals. DEAE-Sephadex (A-50), G-200 Sephadex, and carrier ampholytes (Pharmalyte, pH 4.0 to 6.5) were obtained from Pharmacia Fine Chemicals. Bio-Gel HT (hydroxylapatite) and Dowex AG-1 (Cl form) were obtained from Bio-Rad Laboratories. To increase the flow rate of commercial hydroxylapatite, cellulose (Whatman CF-1 cellulose powder) was added to a final content estimated to be 25% (wt/wt). All other chemicals were reagent grade.

Assays. Protein concentrations were determined by the method of Lowry et al. (22) with bovine serum albumin as a standard. Organic and inorganic phosphate concentrations were determined by the method of Ames (1), except that the absorbance was determined at 725 nm rather than 810 nm; KH_2PO_4 was used as a standard. Pentose concentrations were measured by the orcinol method of Kerr and Seraidarian (16). D-Arabinose-5-phosphate isomerase was measured by the formation of the keto sugar D-ribulose-5-phosphate, by the method of Dische and Borenfreud (6). KDO-8-phosphate phosphatase was measured by the release of Pi; details of the assay will be published later (P. H. Ray and C. D. Benedict, manuscript in preparation). KDO concentrations were measured by the method of Weissbach and Hurwitz (34) as modified by Karkhanis et al. (15). KDO-8-phosphate synthetase activity was measured in a total reaction volume of 1 ml containing: 0.1 M buffer (pH 7.3), 3.0 mM D-arabinose-5-phosphate, 3.0 mM PEP (cyclohexylamine salt), and enzyme. After incubation for 2 min at 37°C, the reaction was initiated either by the addition of enzyme or D-arabinose-5-phosphate. At specified times, 0.15 ml of the reaction mixture was withdrawn, mixed with 0.15 ml of cold 10% trichloroacetic acid, and centrifuged at 10,000 × *g* for 2 min in an Eppendorf (5413) microcentrifuge to precipitate the protein. Portions of the supernatants were diluted up to 0.2 ml and mixed with 0.2 ml of $NaIO_4$. After being mixed at room temperature for 10 min, the excess $NaIO_4$ was removed by the addition of 0.4 ml of $NaAsO_2$. After the samples were mixed, 2.0 ml of the thiobarbituric acid agent was added, and the samples were heated in a boiling water bath for 10 min. Immediately after heating, 2.0 ml of dimethyl sulfoxide (DMSO) was added to each sample to stabilize the chromophore, and the samples were mixed and allowed to cool to room temperature. The optical densities of the samples were determined at 549 nm. All optical density measurements were determined with either a Gilford 300 N or Gilford Stasar III rapid sampling spectrophotometer. Under the conditions of assay, 1 μmol of KDO-8-phosphate gave a change in optical density (OD) of 21.4. The molar extinction coefficient of the β-formylpyruvate thiobarbituric acid chromophore under these conditions was 103 (± 7) × 10^3 (purified KDO-8-phosphate was used as a standard, and the concentration was determined by organic phosphate analysis). Alternatively with the purified enzyme, the synthesis of KDO-8-phosphate can be monitored by the release of inorganic phosphate measured by the method of Ames (1) after precipitation of the protein with cold 5% trichloroacetic acid, followed by centrifugation. PEP concentrations were determined enzymatically in a coupled assay system by using pyruvate kinase and lactate dehydrogenase (Worthington) as described by Levin and Racker (20). KDO-8-phosphate was prepared enzymatically and purified as described in the text. KDO was prepared from KDO-8-phosphate by treatment with alkaline phosphatase (Sigma) and purified by P-2 column chromatography (1 by 120 cm) in distilled water. Alkaline phosphatase activity was determined by the method of Garen and Levinthal (10), using 1.0 mM *p*-nitrophenylphosphate in 1.0 M Tris (pH 8.0) as a substrate.

Enzyme purification. All buffers used during the purification contained 0.2 mM dithiothreitol; the pH of the buffers was determined at 23°C and the buffers were used at 4°C unless otherwise stated.

Steps 1 and 2: cell disruption. Frozen *E. coli* B cells (454 g) were suspended in 200 ml of 20 mM Tris-chloride (pH 7.4) containing 10 mg each of RNase and DNase (DN-100, Sigma). The cells were thawed at 37°C and mixed in an Omnimixer (Sorvall). The cell suspension was subjected to sonic oscillation 10 times for 30 s; the temperature was held below 6°C during the sonication. The specific activity of the broken cells was determined at this step as described under Table 1. Whole cells and cell debris were removed by centrifugation at 10,000 × *g* for 20 min. The supernatant was decanted and placed in an ice bath. The cell pellet was suspended in 150 ml of the same buffer and again sonicated and centrifuged as before. The pellet was discarded, and the two supernatants were combined, giving a total volume of 610 ml.

Step 3: acid precipitation. Protamine sulfate (2.2%, pH 7.2) was added dropwise to the combined supernatants to a final concentration of 0.26%. After stirring for an additional 15 min, the suspension was centrifuged at 48,000 × *g* for 20 min, and the pellet was discarded. The pH of the supernatant was adjusted to pH 5.0 with the addition of 5 N acetic acid.

TABLE 1. *Purification of KDO-8-phosphate synthetase*

Step and fraction	Total vol (ml)	Total protein (g)	Total U	Sp act (U/mg)	% Recovery	Fold purification	Isomerase activity (IU)[a]	Phosphatase activity (IU)[b]
1: Broken cells[c]	600	42	372	0.008	100			
2: Crude extract	610	21.4	372	0.017	100	2.1	150	1,200
3: Acid precipitation	660	10.6	317	0.030	85	3.7	50	334
4: DEAE-Sephadex	101	1.1	172	0.156	46	19.5	16	0
5: Hydroxylapatite	14.5	0.160	146	0.78	37	97.5	0	0
6: Isoelectric focusing	10	ND	117	ND	ND	ND	0	0
7: G-200 Sephadex	29.5	0.008	30	3.7	8	462	0	0

[a] D-Arabinose-5-phosphate isomerase activity was determined after dialysis of a 1-ml sample against 10 mM Tris-chloride (pH 7.5) containing 1 mM EDTA + dithiothreitol.

[b] KDO-8-phosphate phosphatase activity was determined after dialysis in 10 mM Tris-chloride (pH 7.3) containing 1×10^{-4} M dithiothreitol.

[c] Initial specific activity was determined after first sonication before centrifugation in the presence of 0.2% toluene.

The suspension was stirred slowly for 20 min, the precipitated protein was removed by centrifugation at 17,000 × *g* for 30 min, and the supernatant (660 ml) was dialyzed overnight against 4 liters (×4) of 5 mM potassium phosphate buffer containing 75 mM KCl (pH 7.4).

Step 4: DEAE-Sephadex. The pH of the dialyzed enzyme preparation was adjusted to pH 7.4 with 1 M Tris base and adsorbed onto a column (5.0 by 60 cm) of DEAE-Sephadex A-50 at a flow rate of 90 ml h^{-1}. After adsorption of the enzyme solution, the column was washed with 300 ml of starting buffer (20 mM Tris-chloride, 75 mM KCl, pH 7.4), and the protein was eluted with a linear KCl gradient (0.075 to 0.4 M, 6000 ml total). The flow rate was 90 ml h^{-1}, and 22-ml fractions were collected, including those from the loading and wash. KDO-8-phosphate synthetase was eluted between 0.25 and 0.3 M KCl. The fractions containing the highest specific activity (tubes 245 to 305) were pooled (950 ml), frozen in a Dry Ice-ethanol bath, and concentrated to 70 ml by lyophilization. The concentrated enzyme solution was dialyzed (three times) for 4 h against 4 liters of 0.005 M potassium phosphate, (pH 7.3; final volume, 101 ml).

Step 5: hydroxylapatite. A column (2.5 by 30 cm) of hydroxylapatite containing 25% (wt/wt) CF-1 cellulose (Whatman) was packed and washed with 2 volumes of 0.005 M potassium phosphate buffer (pH 7.3). The dialyzed enzyme preparation was adsorbed onto the column and washed with 200 ml of 0.005 M phosphate buffer. The enzymatic activity was eluted with a linear gradient 0.005 to 0.4 M potassium phosphate, pH 7.2 (total volume, 2,000 ml), at a flow rate of 60 ml h^{-1}. Fractions (14 ml) containing the highest specific activity were pooled (430 ml) and concentrated overnight by Amicon Ultrafiltration to 14.5 ml by using a PM-10 filter and dialyzed (three times) against 5 mM Tris-chloride buffer (pH 7.4).

Step 6: isoelectric focusing. The dialyzed enzyme was added to 5% sucrose containing 2.8% ampholytes (Pharmalyte 4.5 to 6.0) in a final volume of 50 ml. The enzyme was mixed throughout a linear gradient of 5 to 50% sucrose containing 2.8% ampholytes in a 110-ml LKB isoelectric focusing column. The lower electrode buffer contained 57% sucrose in 1% sodium hydroxide, and the upper electrode buffer contained 1% phosphoric acid in distilled water. The enzyme was focused for 66 h at 4°C at 400 V, using constant voltage. After electrofocusing, fractions of 30 drops were collected from the gradient at 1.0 ml/min. Fractions were analyzed directly for enzyme activity (10 μl of the sucrose solution did not interfere with the thiobarbituric acid assay), pH, and 280-nm adsorption (see Fig. 4). The fractions containing the peak activity were pooled (10 ml), dialyzed twice against 1 liter of 0.1 M potassium phosphate buffer (pH 7.3), and concentrated to 1.5 ml by lyophilization.

Step 7: G-200 Sephadex. To remove the ampholytes the concentrated enzyme solution containing 5% glycerol was loaded onto a G-200 Sephadex column (1.5 by 150 cm) equilibrated with 0.1 M phosphate buffer (pH 7.2). Fractions of 1.5 ml were collected at a flow rate of 9 ml h^{-1}. Tubes containing the peak activity were pooled, dialyzed against 5 mM Tris-chloride (pH 7.43), and lyophilized to dryness.

Polyacrylamide slab gel electrophoresis. The concentrated enzyme was analyzed for purity by electrophoresis on 7.5% polyacrylamide slab gels (1.5 by 140 mm), using the Tris buffer system described by Davis (5). After electrophoresis a 1.0-cm longitudinal slice of the gel was stained by the method of Fairbanks et al. (8) Protein bands were recorded by scanning the 1.0-cm longitudinal slices at 540 nm with a Gilford 240 spectrophotometer equipped with a linear gel scanner operated at a rate of 2 cm min^{-1}. Densitometer tracings were made by using a Hewlett Packard 7015B X-Y recorder connected to a microcomputer as described by Woodward and Reilly (35) that was interfaced with the spectrophotometer. Another 0.5-cm longitudinal slice was divided into 2-mm slices and assayed for enzyme activity after soaking the individual gel slices in 200 μl of 0.1 M Tris-chloride buffer (pH 7.4) for 1 h. After location of enzyme activity, the remaining gel was sliced and broken with a glass rod, and the enzyme was eluted three times with 2 ml of the above buffer for 30 min. The enzyme from the combined washes was lyophilized and stored at −90°C for further analysis.

Molecular weight determination. The molecular weight of KDO-8-phosphate synthetase was estimated from G-200 gel filtration by the method of Andrews (3), using a column (1.0 by 100 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 7.3. The molecular weight was also estimated by Ferguson analysis (9) as described by Rodbard and Chrambach (30). The relative mobility of the enzyme and standards was measured in tube gels (0.5 by 12 cm) containing 5, 7, 9, 11, 13, and 15% polyacrylamide, using the buffer system of Davis (5). Standards were run separately, and their K_r values (slope of the line plotting the $\log_{10}$ of the relative mobility versus the polyacrylamide gel concentration) were determined. The proteins were stacked at 0.2 W per gel and electrophoresed at 0.5 W per gel to within 1 cm of the bottom of the tube. After measuring the migration of the dye front, the tube gels containing KDO-8-phosphate synthetase and alkaline phosphatase were sliced in 1-mm sections, using a Hoeffer electric gel slicer, and the activities were assayed directly in the reaction mixtures as described previously. The migration of the other standards was determined after staining with Coomassie brilliant blue (R-250) by the method of Fairbanks et al. (8).

Sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis was done by the method of Ames (2), using 12% polyacrylamide gels containing 0.1% SDS. Proteins were stained and scanned as described previously.

Units. A unit of activity equals 1 μmol of KDO-8-phosphate formed or 1 μmol of Pi released per minute at 37°C. Specific activity has the dimensions of units of protein per milligram.

RESULTS

KDO-8-phosphate synthetase was isolated and purified from frozen *E. coli* B cells. Initial experiments indicated the specific activity in these cells was comparable to that of freshly grown cultures of *E. coli* B and *E. coli* ML-30 grown on either glucose minimal medium or nutrient broth. By using the purification procedure summarized in Table 1, KDO-8-phosphate synthetase was purified approximately 500-fold with an 8% recovery of total enzyme units. The enzyme was stable when frozen at −90°C for up to 3 months: loss of activity occurs upon storage at 4°C or −20°C (50% loss in 14 days). The major steps in the purification procedure were hydroxylapatite column chromatography and isoelectric focusing.

When a sample of enzyme eluted from the G-200 Sephadex column (Table 1) was analyzed by electrophoresis on a 7.5% polyacrylamide gel, only two polypeptide bands were observed (Fig. 1). The major band (A) coincided with KDO-8-phosphate synthetase activity. The lower band (B) had no detectable enzyme activity and is probably denatured enzyme, in that its mobility on this 8% gel indicates either a much lower molecular weight or a lower isoelectric point,

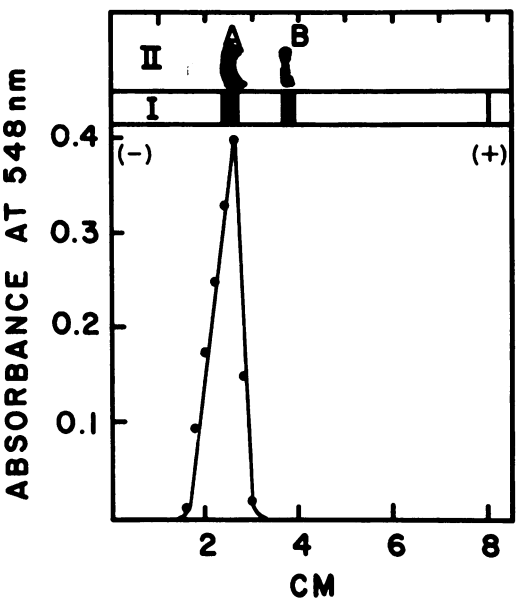

FIG. 1. *Assay of enzyme purity by electrophoresis on 7.5% polyacrylamide gels. The tubes containing the highest specific activity after G-200 Sephadex column chromatography (Table 1) were pooled, lyophilized to 5 ml, and dialyzed three times against 50 mM Tris-chloride (pH 7.4) containing 2 × 10^{-4} M dithiothreitol (1 liter) for 4 h. Portions (containing 6 to 12 μg of protein) were mixed with bromophenol blue, glycerol, mercaptoethanol, and Tris (pH 6.8) so that the final concentrations were 0.01, 10, 2%, and 10 mM, respectively. The sample was layered under the buffer and stacked at 50 V for 1 h and run at 100 V for 4 h. After electrophoresis, the gels were stained and assayed for enzyme activity as described in the text. I is a more distinct drawing of the actual gel labeled II.*

both of which should have been eliminated in the previous two purification steps. The recovery of electrophoretically pure KDO-8-phosphate synthetase (band A) after electrophoresis accounted for only 40% of the catalytic activity applied to the gel. Enzyme activity could not be recovered from band B by the addition of dithiothreitol, various cofactors, metals, or nucleotides or by elution with the substrates.

The formation of KDO-8-phosphate from the substrates was linear both with respect to time and protein concentration over a 20-fold range. Enzyme activity could be monitored with the purified enzyme either by the thiobarbituric acid assay or by the release of inorganic phosphate, as described in the previous section. The release of 1 mol of inorganic phosphate and the formation of 1 mol of KDO-8-phosphate corresponded to the stoichiometric utilization of 1 mol of D-arabinose-5-phosphate and 1 mol of PEP (Table

2). The enzyme preparation after DEAE-Sephadex column chromatography did not contain measurable KDO-8-phosphate phosphatase activity and could be utilized for the large-scale preparation of KDO-8-phosphate, the substrate for the next enzyme in the pathway. The substrates and the products of this reaction could be clearly separated by using a Dowex AG-1 column (Cl form) followed by elution with a linear LiCl gradient (Fig. 2). The product, KDO-8-phosphate, was freed of LiCl by Bio-Gel P-2 column chromatography in distilled water.

TABLE 2. *Stoichiometry of KDO-8-phosphate formation*[a]

Substrate or product	Concn (mM) at:			Change in volume (μmol) at:	
	Initial	30 min	60 min	30 min	60 min
D-Arabinose-5-phosphate	2.331	0.841	0.230	1.49	2.10
PEP	2.662	1.18	0.798	1.482	1.864
KDO-8-phosphate	0.000	1.531	2.03	1.531	2.030
Pi	0.067	1.495	2.04	1.428	1.973

[a] Reaction: D-arabinose-5-phosphate + PEP → KDO-8-phosphate + Pi. The reaction mixture contained, in a final volume of 1.0 ml: 2.5 μmol of D-arabinose-5-phosphate, 2.5 μmol of PEP, 60 μmol of Tris-chloride (pH 7.4), and 23.4 μg of enzyme having a specific activity of ~1.4. The reaction mixture was incubated at 30°C for 2 min and was initiated by the addition of 0.1 ml of enzyme. The reaction was terminated by the addition of 1.0 ml of cold 10% trichloroacetic acid. D-Arabinose-5-phosphate, Pi, PEP, and KDO-8-phosphate were assayed as described in the text.

Properties of KDO-8-phosphate synthetase. The effect of pH on enzyme activity is depicted in Fig. 3. The velocity of the reaction was both pH and buffer dependent; there was a low pH optimum in succinate buffer between pH 4.0 and 6.0 and a high pH optimum at 9.0 in glycine (NaOH) buffer. There was no detectable activity below pH 3.0 or above pH 10.0. The buffers giving the maximal velocity, however, are not suitable for enzyme purification or storage due to a loss of activity. The best storage buffer is 0.1 M potassium phosphate (pH 7.4), even though phosphate at this concentration slightly inhibits the catalytic activity. For routine assays 0.1 M Tris-chloride (pH 7.3) was utilized.

The temperature optimum for initial velocity studies was 45°C. However, incubation at this temperature for 15 min decreased the activity by 30% due to heat denaturation of the enzyme, and the activity could not be recovered after

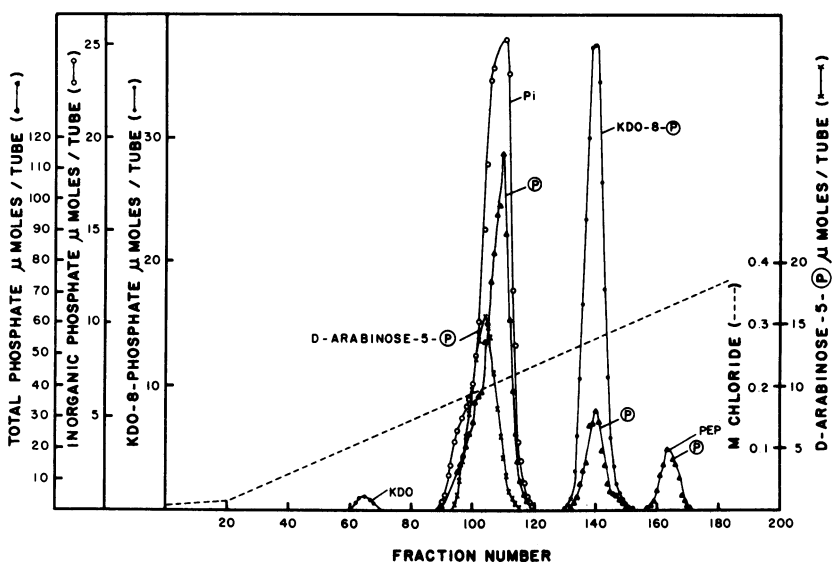

FIG. 2. *Separation of substrates and products of KDO-8-phosphate synthetase by Dowex AG-1 (Cl) column chromatography. The reaction mixture contained the following components in a final volume of 100 ml: 450 μmol of D-arabinose-5-phosphate, 450 μmol of PEP, 600 μmol of Tris-chloride (pH 7.5), 20 μmol of dithiothreitol, and 0.9 U of enzyme. The reaction was incubated at 35°C for 50 min and terminated by the addition of crushed ice to the equivalent of 250 ml of water. The mixture was adsorbed onto a column (2.5 by 20 cm) of Dowex AG-1 (Cl) at a flow rate of 180 ml h^{-1}. The column was washed with 250 ml of distilled water and the individual components were eluted with a linear gradient of LiCl composed of 500 ml of distilled water and 500 ml of 0.4 M LiCl. The column was eluted at a flow rate of 64 ml h^{-1}, and 6-min fractions were collected. Each tube was assayed for chloride, Pi, total phosphate, KDO (KDO-8-phosphate) and D-arabinose-5-phosphate. The results are presented as micromoles per tube.*

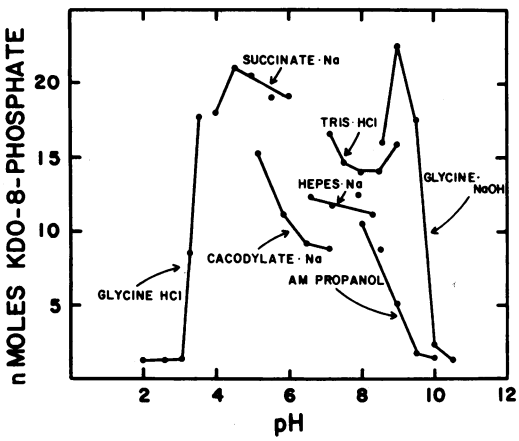

FIG. 3. *Effect of pH and buffers on KDO-8-phosphate synthetase activity. The following buffers were prepared at 200 mM at the pH indicated in the figure at 23°C: glycine (HCl); N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (NaOH), cacodylic acid (NaOH), 2-amino-2-methylpropanol (HCl), succinic acid (NaOH), Tris (HCl), and glycine (NaOH).*

cooling. For routine assays, a temperature of 37°C was utilized since at this temperature the specific activity remained constant for 30 min. The energy of activation, calculated by the Arrhenius equation, was 15,000 ± 600 calories (ca. 3,585 [±143] J) mol^{-1}, which is similar to the energy of hydrolysis of PEP. The enzyme showed no metal requirement and was not inhibited by 1 mM EDTA. The addition of 1 mM Li^+, Fe^{3+}, Mg^{2+}, Ba^{2+} and Mn^{2+} to the reaction mixture slightly stimulated the activity (5 to 10%). Overnight dialysis against 10 mM EDTA in 50 mM Tris-chloride buffer (pH 7.3) inactivated the enzyme, as did the addition of 1 mM Cd^{2+}, Cu^{2+}, Zn^{2+}, and Hg^{2+} to the reaction mixture. The inhibition by Hg^{2+} ions could be reversed by the addition of 1.0 mM dithiothreitol. Dithiothreitol was added to all buffers throughout the purification procedure, even though its omission from the reaction mixture had no effect on enzyme activity.

Physical properties of KDO-8-phosphate synthetase. Isoelectric focusing was used in the purification of KDO-8-phosphate synthetase. When a partially purified enzyme preparation was electrofocused for 60 h at 400 V, a single peak of enzyme activity was detected with an isoelectric point of 5.1 (Fig. 4). Refocusing, using a higher-specific-activity preparation, gave identical results. This method is suitable for large-scale preparation of the enzyme, since 90% of the activity is retained even after 60 h in an electrical field in dilute buffer. The molecular weight of KDO-8-phosphate synthetase was estimated to be 90,000 ± 6,000 (Fig. 5A and B) both by molecular sieving on G-200 Sephadex by the method of Andrews (3) and by electrophoretic mobility on non-SDS gels by the method of Ferguson (9). With both methods alkaline phosphatase was used as an internal standard, since it has a molecular weight of 86,000 and a pI of 4.5 (18), which are very similar to those of KDO-8-phosphate synthetase. With a precalibrated G-200 Sephadex column, KDO-8-phosphate synthetase had a V_e/V_o value of 1.45 compared with that of alkaline phosphatase of 1.5, indicating that the synthetase was slightly larger than 86,000. Similarly, the K_r values (Fig. 5B), calculated from the mobility of the enzyme in gels containing varying acrylamide concentrations, indicate that alkaline phosphatase and KDO-8-phosphate synthetase have similar molecular weights of approximately 90,000. To determine the subunit composition of KDO-8-phosphate synthetase, electrophoretically pure enzyme was prepared by preparative gel electrophoresis on 3-mm-thick, 7.5% acrylamide slab gels. The activity was determined, eluted, and again subjected to gel electrophoresis. A densitometer tracing of the purified enzyme electro-

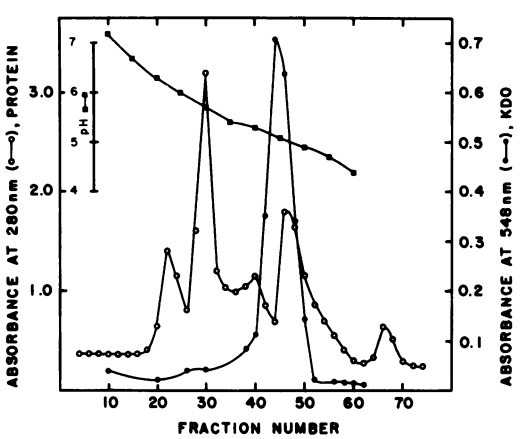

FIG. 4. *Purification of KDO-8-phosphate synthetase by isoelectric focusing. See purification section for experimental details. The enzyme preparation was layered throughout the 110-ml gradient by mixing the enzyme with sucrose and ampholytes, so that the final concentration of the ampholytes was 2.8%. After isoelectric focusing for 60 hours at 400 volts, the fractions (30 drops) were analyzed for pH (■—■), protein by absorbance at 280 nm (○—○) and enzyme activity (●—●). Enzyme activity was measured using 0.01 ml of each fraction in a total reaction mixture of 0.22 ml. The reaction was terminated after 30 min by the addition of 0.22 ml of cold 10% trichloroacetic acid. Portions (0.1 ml) were assayed by the thiobarbituric acid method. Under these conditions sucrose did not interfere with the measurement of KDO.*

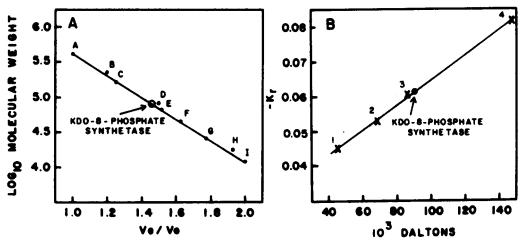

FIG. 5. *Estimation of the molecular weight of KDO-8-phosphate synthetase. (A) G-200 Sephadex column chromatography. A column (1.0 by 120 cm) was equilibrated with G-200 Sephadex by the method of Andrews (3) in 0.1 M phosphate buffer, pH 7.4. Standards applied at a concentration of 1 mg ml^{-1} included: A, ferritin (5.4 × 10^5); B, catalase (2.4 × 10^5); C, aldolase (1.58 × 10^5); D, alkaline phosphatase (8.6 × 10^4); E, bovine serum albumin (7 × 10^4); F, ovalbumin (4.5 × 10^4), G, chymotrypsinogen (2.5 × 10^4); H, myoglobin (1.7 × 10^4); and I, cytochrome c (1.25 × 10^4). Each V_e/V_o value represents an average of two determinations. (B) Ferguson analysis. Analytical gel electrophoresis was carried out by the method of Davis (5). Relative mobilities were calculated and slopes (K_r values) were determined by regression analysis of the $\log_{10} R_f$ versus gel concentration. The standards utilized were: (1) ovalbumin, molecular weight 45,000; (2) bovine serum albumin, molecular weight 67,000; (3) alkaline phosphatase, molecular weight 86,000; and (4) aldolase, molecular weight 158,000.*

phoresed through a 7.5% gel is shown in Fig. 6A. It can be seen that the enzyme preparation is 98% pure as determined by electrophoresis. A portion of this enzyme preparation was denatured by heating at 100°C for 5 min in the presence of 2% SDS and mercaptoethanol and subjected to electrophoresis on 12% SDS-acrylamide gels. Only one major protein band, comprising 82% of the total absorbance, was detected (Fig. 6B). The major band (designated 3) corresponds to a molecular weight of 32,000, as determined by comparing its relative mobility to those of the standards depicted in Fig. 6C. The minor bands, designated 1 and 2, correspond to proteins with molecular weights of 68,000 and 60,000, respectively, and may either be a dimer of the subunit that was not completely denatured, or a contaminant of the enzyme preparation shown in Fig. 6A. From the above data, it appears that the native enzyme, with a molecular weight of 90,000, is composed of three equal 32,000-molecular-weight subunits.

To determine the kinetic constants of the bireactant substrates, PEP and D-arabinose-5-phosphate, one of the reactant concentrations was varied, whereas the other was at a fixed high concentration (3 mM). All experiments were carried out by using measurements of initial velocities employing endpoint assays. By computer analysis of the data, the apparent K_m for D-arabinose-5-phosphate was estimated to be 1.95 (±0.13) × 10^{-5} M and 5.9 (±0.8) × 10^{-6} M for PEP. With both substrates the data fit a rectangular hyperbola, with no indication of a sigmoid dependence of reaction velocity on substrate concentration.

Possible inhibitors or substrates of the synthetase reaction. A number of sugars and sugar phosphates were tested both as inhibitors of the reaction and as possible alternate substrates for the enzyme. The sugars and sugar phosphates tested included: D-arabinose, L-arabinose, D-erythrose, D-galactose, D-glucose, D-arabinitol, D-erythritol, D-erythrose-4-phosphate, D-glucose-6-phosphate, D-ribulose-5-phosphate, D-ribose-5-phosphate, glycerol-3-phosphate, 2-phosphoglyceric acid, 3-phosphoglyceric acid, 2-deoxyglucose, maltose-1-phosphate, sedoheptulose-7-phosphate, 6-phosphogluconate, 2-deoxyribose-5-phosphate, 2-deoxy-6-phosphogluconate, D-xylulose-5-phosphate, sorbitol-6-phosphate, and fructose-1,6-diphosphate. KDO-8-phosphate synthetase exhibited an absolute specificity for PEP and D-arabinose-5-phosphate. No other triose, tetrose, pentose, or hexose sugar phosphate could substitute for PEP or D-arabinose-5-phosphate in the synthetase reaction. D-Ribose-5-phosphate, of the sugar phosphates tested, was the only effective inhibitor of the synthetase reaction. D-Ribose-5-phosphate was shown to be a weak competitive inhibitor, with an apparent K_i of 1.17 (±0.25) × 10^{-3} M. The end product of the reaction KDO-8-phosphate and its subsequent hydrolysis product KDO also appear to be very weak inhibitors of the reaction, with I_{50} values of 10 × 10^{-3} and 30 × 10^{-3} M, respectively. K_i values for KDO and KDO-8-phosphate were not determined, due to the sensitivity of the phosphate assay which had to be utilized in the presence of KDO-8-phosphate and KDO.

DISCUSSION

The purification and some of the properties of KDO-8-phosphate synthetase isolated from *E. coli* B are described. The enzyme appears to be completely cytoplasmic, in that no evidence was found indicating membrane attachment utilizing various methods of cell disruption in the early stages of purification. KDO does not become membrane bound until it is transferred from CMP-KDO to the lipid A precursor as shown by Munson et al. (24). We have seen no evidence in our work or that of others (11, 13, 20, 24) to indicate that D-arabinose-5-phosphate isomerase, KDO-8-phosphate synthetase, KDO-8-phosphatase or CMP-KDO synthetase, the enzymes involved in KDO synthesis and activa-

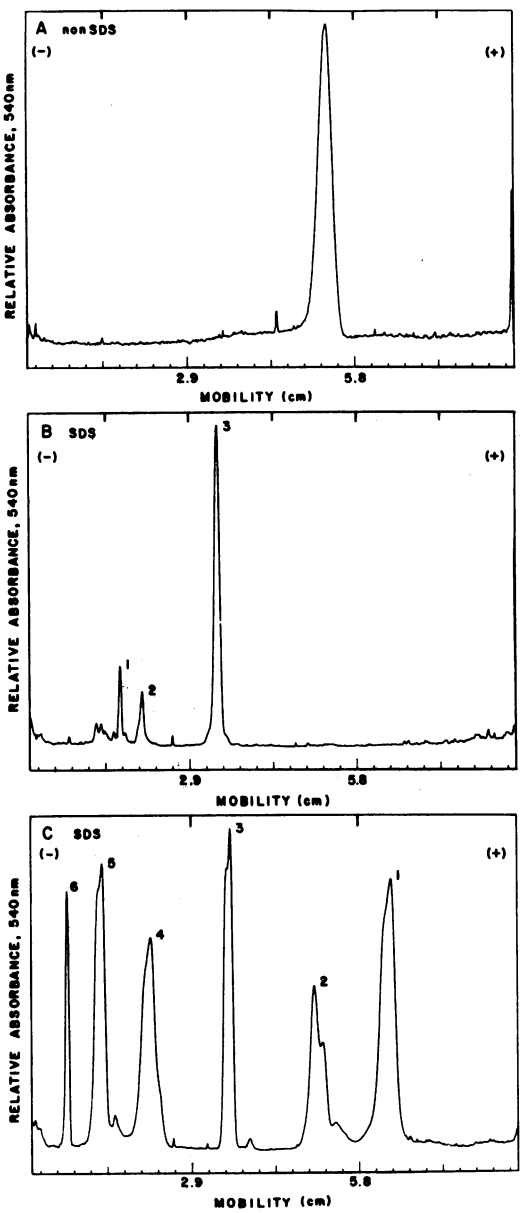

FIG. 6. *Densitometer tracings of KDO-8-phosphate synthetase electrophoresed on non-SDS and SDS acrylamide gels: estimation of subunit molecular weight. KDO-8-phosphate synthetase was prepared as shown in Table 1, and a portion was subjected to preparative 7.5% acrylamide slab gel electrophoresis, using a single well (3.5 cm wide and 3 mm thick) containing approximately 300 μg of protein. The enzyme was electrophoresed as described previously. After electrophoresis, the 3.5-cm-wide gel was sliced into 2-mm sections with a razor blade, and each slice was put in a tube and eluted with 0.3 ml of 10 mM Tris-chloride buffer, pH 7.4. (A) Non-SDS gel. A portion of the enzyme eluted from the 2-mm slice containing the highest enzyme activity was again electrophoresed on a 7.5% acrylamide slab gel. After staining by the method of Fairbanks et al. (8), the gel was scanned as described above. (B) SDS gel. Another portion of the enzyme, prepared as described above, was denatured by boiling in SDS and mercaptoethanol as described above and electrophoresed by the method of Laemmli (17) as described by Ames (2), using a 12% polyacrylamide gel containing 0.1% SDS in all buffers. (C) SDS gel. A scan of an SDS gel containing the Low Molecular Weight Calibration Kit proteins supplied by Pharmacia. The standards were treated in the same manner as the enzyme and electrophoresed under identical conditions. The standard kit contained: (1) α lactalbumin (14,400); (2) trypsin inhibitor (20,100); (3) carbonic anhydrase (30,000); (4) ovalbumin (43,000); (5) bovine serum albumin (67,000); and (6) phosphorylase b (94,000). The relative mobilities of these standards were used to calculate the subunit molecular weight of KDO-8-phosphate synthetase.*

tion, are even loosely membrane bound. KDO transferase(s) is, however, tightly membrane bound (24). These reactions are very similar to those involved in *N*-acetylneuraminic acid synthesis in mammalian cells (14, 31).

We are now in the process of purifying and characterizing D-arabinose-5-phosphate isomerase and CMP-KDO synthetase from *E. coli* B. The specific activities in crude extracts of the first four enzymes in the KDO pathway (see above) appear to be very similar and their K_m values are similar, which is to be expected in a sequential pathway. The controlling steps in this pathway are not obvious. From the data of Rick and Osborn (28, 29) concerning the temperature-sensitive mutant of *Salmonella typhimurium* that has an elevated K_m for D-arabinose-5-phosphate at 37°C, the concentration of D-arabinose-5-phosphate is suggested as one possible control. This is also suggested by the work of Volk (33) and Lim and Cohen (21) and our own work (unpublished data), whereby it has been shown that the D-arabinose-5-phosphate isomerase reaction favors the formation of D-arabinose-5-phosphate (80%) from D-ribulose-5-phosphate (20%) but it is an equilibrium reaction. Thus any excess D-ribulose-5-phosphate can be converted to other pentose-phosphates and not allow the D-arabinose-5-phosphate pool to increase beyond a certain concentration.

When both the release of inorganic phosphate and the formation of thiobarbituric acid reactive material were measured, none of the other sugar phosphates tested could replace D-arabinose-5-phosphate or PEP in the reaction. Of the sugar phosphates tested, only D-ribose-5-phosphate inhibited the reaction and it was shown to be a competitive inhibitor, with a K_i of 1×10^{-3} M,

whereas the K_m of the enzyme for D-arabinose-5-phosphate was determined to be 5×10^{-5} M. The K_m for D-arabinose-5-phosphate reported here is fourfold lower than that reported by Rick and Osborn (28) utilizing crude extracts of *Salmonella typhimurium*.

The data concerning the pH optima were different than those reported by Levin and Racker (20) in that the enzyme from *Pseudomonas aeruginosa* showed a clear optimum at pH 7.2 in both Tris-chloride and histidine buffers, whereas the data presented for *E. coli* show two pH optima, one at pH 4.0 to 6.0 in sodium succinate buffer and the other at pH 9.0 in glycine (NaOH) buffer. This difference can be explained by either (i) the buffers tested, including the counter ions, (ii) the instability of the enzyme at the high and low pH ranges, or (iii) the different sources of enzyme.

As reported by Ghalambor and Heath (11) and repeated in our laboratory (data not shown), the product of the synthetase reaction, KDO-8-phosphate, is not a substrate for the CMP-KDO synthetase. The purification of KDO-8-phosphate synthetase has allowed the large-scale purification of KDO-8-phosphate, the substrate for the next reaction in the pathway. It should be mentioned that KDO-8-phosphate has been isolated in 95% yield by using this enzyme (Fig. 3). Upon purification of KDO-8-phosphate directly from the reaction mixture by Dowex AG-1 column chromatography, a small amount of KDO (1 to 5%) is always noted. This product of KDO-8-phosphate is not due to KDO-8-phosphate phosphatase activity and has been attributed to localized acid degradation upon chromatography.

ACKNOWLEDGMENTS

I thank Teri Miller and Rietta Dampier for their excellent technical assistance.

LITERATURE CITED

1. **Ames, B. N.** 1966. Assay of inorganic, total phosphate and phosphatases. Enzymologia **8**:116–118.
2. **Ames, G. F.** 1974. Resolution of bacterial protein by polyacrylamide gel electrophoresis on slabs. J. Biol. Chem. **249**:634–644.
3. **Andrews, P.** 1965. The gel-filtration behavior of proteins related to their molecular weights over a wide range. Biochem. J. **96**:595–606.
4. **Berger, H., and F. Hammerschmid.** 1975. Isolation of 3-deoxy-2-octulosonate-8-phosphate phosphatase, a new specific enzyme in the biosynthesis of lipopolysaccharide. Biochem. Soc. Trans. **3**:1096–1097.
5. **Davis, B. J.** 1964. Disc electrophoresis. II. Methods and application to human serum proteins. Ann. N.Y. Acad. Sci. **121**:404–427.
6. **Dische, Z., and E. Borenfreund.** 1951. A new spectrophotometric method for the detection and determination of keto sugars and trioses. J. Biol. Chem. **192**:583–587.
7. **Ellwood, D. C.** 1970. The distribution of 2-keto-3-deoxyoctonic acid in bacterial walls. J. Gen. Microbiol. **60**: 373–380.
8. **Fairbanks, G., T. L. Steck, and D. F. H. Wallach.** 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry **10**: 2606–2617.
9. **Ferguson, K. A.** 1967. Starch-gel electrophoresis-application to the classification of pituitary proteins and polypeptides. Metabolism **13**:985–1002.
10. **Garen, A., and C. Levinthal.** 1960. A fine structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. Biochim. Biophys. Acta **38**:470–483.
11. **Ghalambor, M. A., and E. C. Heath.** 1966. The biosynthesis of cell wall lipopolysaccharide in *Escherichia coli*. IV. Purification and properties of cytidine monophosphate-3-deoxy-D-manno-octulosonate synthetase. J. Biol. Chem. **241**:3216–3221.
12. **Harvey, R. J.** 1970. Metabolic regulation in glucose-limited chemostat cultures of *Escherichia coli*. J. Bacteriol. **104**:698–706.
13. **Heath, E. C., and M. A. Ghalambor.** 1963. 2-Keto-3-deoxyoctonate, a constituent of cell wall lipopolysaccharide preparations obtained from *Escherichia coli*. Biochem. Biophys. Res. Commun. **10**:340–345.
14. **Jourdian, G. W., A. L. Swanson, D. Watson, and S. Roseman.** 1964. Isolation of sialic acid-9-phosphatase from human erythrocytes. J. Biol. Chem. **239**:PC2714–2715.
15. **Karkhanis, Y. D., J. Y. Zeltner, J. J. Jackson, and D. J. Carlo.** 1978. A new and improved microassay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of Gram-negative bacteria. Anal. Biochem. **85**:596–601.
16. **Kerr, S. E., and K. Seraidarian.** 1945. The separation of purine nucleosides from free purines and the determination of the purines and ribose in these fractions. J. Biol. Chem. **159**:211–215.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T-4. Nature (London) **227**:680–685.
18. **Lazdunski, C., and M. Lazdunski.** 1969. Zn^{2+} and Co^{2+}-alkaline phosphatases of *E. coli*. A comparative kinetic study. Biochem. Biophys. Res. Commun. **37**:744–752.
19. **Lehmann, V., E. Rupprecht, and M. J. Osborn.** 1977. Isolation of mutants conditionally blocked in the biosynthesis of the 3-deoxy-D-manno-octulosonic lipid A portion of the lipopolysaccharide derived from *Salmonella typhimurium*. Eur. J. Biochem. **76**:41–49.
20. **Levin, D. H., and E. Racker.** 1959. Condensation of arabinose-5-phosphate and phosphorylenolpyruvate by 2-keto-3-deoxy-8-phosphooctonic acid synthetase. J. Biol. Chem. **234**:2532–2539.
21. **Lim, R., and S. S. Cohen.** 1966. D-phosphoarabinosisomerase and D-ribulokinase in *Escherichia coli*. J. Biol. Chem. **241**:4304–4315.
22. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
23. **Lüderitz, O., A. M. Staub, and O. Westphal.** 1966. Immunochemistry of O and R antigens of *Salmonella* and related *Enterobacteriaceae*. Bacteriol. Rev. **30**: 192–255.
24. **Munson, R. S., N. S. Rasmusson, and M. J. Osborn.** 1978. Biosynthesis of lipid A: enzymatic incorporation of 3-deoxy-D-mannooctulosonate into a precursor of Lipid A in *Salmonella typhimurium*. J. Biol. Chem. **253**:1503–1511.
25. **Nikaido, H.** 1973. Biosynthesis and assembly of lipopolysaccharide and the outer membrane layer of Gram-negative cell walls, p. 131–208. *In* L. Leive (ed.), Bacterial membranes and walls. M. Dekker, Inc., New York.
26. **Osborn, M. J.** 1963. Studies on the Gram-negative cell

wall, I. Evidence for the role of 2-keto-3-deoxyoctonate in lipopolysaccharide of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. U.S.A. **50**:499–506.

27. **Rick, P. D., L. W.-M. Fung, C. Ho, and M. J. Osborn.** 1977. Lipid A mutants of *Salmonella typhimurium*: purification and characterization of a lipid A precursor produced by a mutant in 3-deoxy-D-manno-octulosonate-8-phosphate synthetase. J. Biol. Chem. **252**:4904–4912.
28. **Rick, P. D., and M. J. Osborn.** 1972. Isolation of a mutant of *Salmonella typhimurium* dependent on D-arabinose-5-phosphate for growth and synthesis of 3-deoxy-D-mannoctulosonate (Ketodeoxyoctonate). Proc. Natl. Acad. Sci. U.S.A. **69**:3756–3760.
29. **Rick, P. D., and M. J. Osborn.** 1977. Lipid A mutants of *Salmonella typhimurium*: characterization of a conditional lethal mutant in 3-deoxy-mannooctulosonate-8-phosphate synthetase. J. Biol. Chem. **252**:4895–4903.
30. **Rodbard, D., and A. Chrambach.** 1971. Estimation of molecular radius, free mobility and valence using polyacrylamide gel electrophoresis. Anal. Biochem. **40**:95–134.
31. **Roseman, S.** 1962. Enzymatic synthesis of cytidine-5′-monophosphosialic acid. Proc. Natl. Acad. Sci. U.S.A. **48**:437–441.
32. **Vincent, W. F., and J. A. Cameron.** 1967. Thiobarbiturate-reacting materials in microorganisms. J. Bacteriol. **93**:156–158.
33. **Volk, W. A.** 1959. The enzymatic formation of D-arabinose-5-phosphate from L-arabinose and adenosine triphosphate by *Propionibacterium pentosaceum*. J. Biol. Chem. **234**:1931–1936.
34. **Weissbach, A., and J. Hurwitz.** 1958. The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* B. J. Biol. Chem. **234**:705–709.
35. **Woodward, W. S., and C. N. Reilly.** 1978. General purpose microcomputer in laboratory automation. Pure Appl. Chem. **50**:785–799.