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Purification and Characterization of Cytidine 5'-Triphosphate:Cytidine 5'-Monophosphate-3-Deoxy-D-*manno*-Octulosonate Cytidylyltransferase

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Cytidine 5'-triphosphate:cytidine 5'-monophosphate-3-deoxy-D-*manno*-octulosonate cytidylyltransferase (CMP-KDO synthetase) was purified 2,300-fold from frozen *Escherichia coli* B cells. The enzyme catalyzed the formation of CMP-KDO, a very labile product, from CTP and KDO. No other sugar tested could replace KDO as an alternate substrate. Uridine 5'-triphosphate at pH 9.5 and deoxycytidine 5'-triphosphate at pH 8.0 and 9.5 could be used as alternate substrates in place of CTP. CMP-KDO synthetase required Mg^{2+} at a concentration of 10.0 mM for optimal activity. The pH optimum was determined to be between 9.6 and 9.3 in tris(hydroxymethyl)aminomethane-acetate or sodium-glycine buffer. This enzyme had an isoelectric point between pH 4.15 and 4.4 and appeared to be a single polypeptide chain with a molecular weight of 36,000 to 40,000. The apparent K_m values for CTP and KDO in the presence of 10.0 mM Mg^{2+} were determined to be 2.0×10^{-4} and 2.9×10^{-4} M, respectively, at pH 9.5. Uridine 5'-triphosphate and deoxycytidine 5'-triphosphate had apparent K_m values of 8.8×10^{-4} and 3.4×10^{-4} M, respectively, at pH 9.5.

The lipopolysaccharide region of the gram-negative bacterial outer envelope is composed of a complex heteropolysaccharide chain linked covalently to a unique glucosamine-containing lipid, designated lipid A (14, 15). 3-Deoxy-D-*manno*-octulosonate (KDO), an eight-carbon keto sugar acid, is the direct link between lipid A and the growing polysaccharide chain (6, 14, 15). The innermost region of the lipopolysaccharide, containing KDO and lipid A, appears to be required for the growth of gram-negative bacteria since only mutants temperature sensitive in the biosynthesis of this region have been isolated (15, 19, 20). Osborn and co-workers have utilized these *Salmonella typhimurium* mutants to demonstrate the involvement of KDO in the maturation and translocation of the various lipid A precursors (15, 16, 22, 23).

The biosynthesis and metabolism of KDO involve at least four sequential reactions after the formation of D-arabinose-5-phosphate (10, 17):

D-arabinose-5-phosphate + phosphoenolpyruvate ⟶ KDO-8-phosphate + P_i (i)

KDO-8-phosphate ⟶ KDO + P_i (ii)

CTP + KDO ⟷ CMP-KDO + PP_i (iii)

2 CMP-KDO + lipid A precursor ⟶ KDO-KDO-lipid A precursor + 2 CMP (iv)

These four reactions are catalyzed by KDO-8-phosphate synthase (9, 17), KDO-8-phosphate phosphatase (3, 18), CMP-KDO synthetase (5), and KDO transferase(s) (13), respectively. In previous publications, we have reported the purification and characterization of KDO-8-phosphate synthase (17) and a specific KDO-8-phosphate phosphatase (18). In this paper, we describe the purification and characterization of CMP-KDO synthetase from *Escherichia coli* B. This enzyme catalyzes the formation of the nucleotide sugar CMP-KDO from CTP and KDO and was initially studied by Ghalambor and Heath (5).

MATERIALS AND METHODS

Bacteria. *E. coli* B (ATCC 11303), grown to the mid-logarithmic phase in glucose minimal medium, were purchased from Grain Processing Inc., Muscatine, Iowa, as a frozen cell paste and stored at −90°C until utilized.

Chemicals. Buffers, phosphorylated compounds, and molecular weight markers were obtained from Sigma Chemical Co., St. Louis, Mo. DEAE-Sephadex A-50, Sephadex G-200, Sephadex G-100, carrier am-

pholytes (Pharmalyte, pH 4.0 to 6.5) for isoelectric focusing, and molecular weight markers used in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Hydroxylapatite (Bio-Gel HT) was obtained from Bio-Rad Laboratories, Richmond, Calif., and prepared as previously described (17). All other chemicals were of reagent grade.

Assays. Protein was measured by the method of Lowry et al. (11), using bovine serum albumin as the standard. P_i was determined by the method of Ames (1), using KH_2PO_4 as the standard as described previously (17, 18). The formation of CMP-KDO was determined by a modification of the thiobarbituric acid assay utilized by Kean and Roseman (8) for the determination of CMP-sialic acid and by Ghalambor and Heath (5) for the determination of CMP-KDO. This assay is based on the fact that the carbonyl (C-2) group of KDO in CMP-KDO is resistant to reduction by $NaBH_4$, whereas free KDO, the substrate, is reduced and therefore will not react with thiobarbituric acid after periodate oxidation. KDO is released from CMP-KDO by mild acid hydrolysis, and the subsequent KDO is measured by the thiobarbituric acid assay. CMP-KDO synthetase activity was measured at 30°C in a final volume of 1.0 ml containing the following components: KDO, 2.0 μmol; CTP, 10.0 μmol; magnesium acetate, 10.0 μmol; Tris-acetate (pH 9.8), 200 μmol; and enzyme, 0.01 to 0.05 U. The reaction was initiated by the addition of enzyme and terminated (0 to 10 min) by withdrawal of 0.1 ml of the reaction mixture and dilution with 0.2 ml of cold ethanol. Portions were kept cold in ice until the completion of the experiment, at which time they were centrifuged at 10,000 × *g* for 3 min in an Eppendorf microcentrifuge. Portions of 0.1 ml from the supernatant were then transferred to tubes (13 by 100 mm) in an ice-water bath for determination of the amount of CMP-KDO formed. The free KDO remaining was reduced by the addition of 0.1 ml of cold 1.6 M $NaBH_4$ in 1.0 mM NaOH and incubation on ice for 10 min. The excess $NaBH_4$ was destroyed by the addition of 0.05 ml of cold 20.0 N H_3PO_4 followed by incubation on ice for 5 min. The acid-labile ketosidic bond of CMP-KDO was then hydrolyzed by incubation of the samples at 37°C for 10 min. The free KDO liberated by this acid hydrolysis was then determined by the thiobarbituric acid assay as described previously (17). Standards (5 to 50 nmol) were added to tubes after the addition of 20.0 N H_3PO_4 immediately following the 37°C acid hydrolysis. Under the conditions of the assay, 1.0 μmol of KDO gave an absorbance of between 16 and 18 at 549 nm. One unit of enzyme activity equals 1.0 μmol of CMP-KDO formed per min. Specific activity has the dimensions of units per milligram of protein.

Preparation of substrate. KDO was prepared by using *E. coli* alkaline phosphatase to dephosphorylate KDO-8-phosphate prepared as previously described (17) or was a gift of Frank Unger of the Sandoz Forschungsinstitut (Vienna, Austria). The Unger method of synthesis (Adv. Carbohydrate Chem. Biochem., in press) is a modification of that of Hershberger et al. (7).

Enzyme purification. All buffers used during the purification of CMP-KDO synthetase contained 0.5 mM dithiothreitol, were adjusted to the designated pH at 23°C, and were equilibrated to 4°C before use. Of various purification procedures attempted, the following procedure is the method of choice for simplicity and yield of purified enzyme.

Step 1: crude extract. The frozen cell paste (454 g) was suspended in 400 ml of 0.05 M potassium phosphate buffer (pH 7.4) containing 50 mg of lysozyme, 10 mg of RNase, and 10 mg of DNase and was thawed at 37°C. After thawing, the cell suspension was homogenized in an Omnimixer (Sorvall), cooled to 4°C, and divided into two aliquots, and the cells were disrupted by sonication for 30 s (10 times) at maximal output, using a Branson W 350 Sonifier while maintaining the temperature below 5°C. Whole cells and cellular debris were removed by centrifugation at 20,000 × *g* for 60 min. The pellets were discarded and the supernatants were combined.

Step 2: protamine sulfate and acid precipitation. Nucleic acids and some proteins were removed from the combined crude extract by the subsurface addition of 2.2% protamine sulfate (pH 7.0) at a flow rate of 2 ml min^{-1}, to give a final concentration of 0.26%. The suspension was stirred for 15 min and clarified by centrifugation at 48,000 × *g* for 60 min. The pellets were discarded. The enzyme activity was precipitated from the supernatant by the addition of $(NH_4)_2SO_4$ to 65% saturation (438 g $liter^{-1}$ of supernatant). After stirring in an ice bath for 15 min, the protein was precipitated by centrifugation at 16,000 × *g* for 15 min. The supernatant was discarded, and the pellets were suspended in 150 ml of distilled water. The protein solution was dialyzed overnight against 4 liters (twice) of 0.015 M potassium-acetate buffer (pH 5.0). The resulting precipitate was removed by centrifugation, and the supernatant was adjusted to pH 7.6 by the addition of 2.0 M Tris base.

Step 3: DEAE-Sephadex. The supernatant was further dialyzed against 4 liters of potassium phosphate buffer (pH 7.8) for 1.5 h and adsorbed onto a column of DEAE-Sephadex (5 by 30 cm), which had been equilibrated with the same buffer, at a flow rate of 60 ml h^{-1}. After adsorption of the protein solution, the column was washed overnight with 0.1 M potassium phosphate (pH 7.8) at the same flow rate. The protein was eluted from the column with a linear phosphate gradient (0.1 to 0.3 M potassium phosphate, pH 7.8; 4-liter total gradient) at a flow rate of 60 ml h^{-1}. Fractions from the column were assayed for protein (280 nm), inorganic phosphate, and enzyme activity. Those containing CMP-KDO synthetase activity (0.21 to 0.25 M phosphate) were pooled and concentrated by the addition of $(NH_4)_2SO_4$ to 65% saturation. After centrifugation, the supernatants were discarded, and the pellets were suspended in 40 ml of distilled water and dialyzed overnight against 4 liters (twice) of 0.01 M phosphate buffer (pH 7.0).

Step 4: hydroxylapatite chromatography. The dialyzed enzyme solution was passed through a column of hydroxylapatite (2.5 by 5.0 cm) that had been equilibrated with 0.01 M potassium phosphate (pH 7.2) (CMP-KDO synthetase does not adsorb to hydroxylapatite), and the column was washed with 75 ml

of the same buffer. The loading volume and eluant were directly adsorbed onto a DEAE-Sephadex column (2.5 by 25 cm; equilibrated with 0.025 M potassium phosphate buffer, pH 7.2). The column was washed overnight with 0.075 M potassium phosphate buffer, and the protein was eluted with a linear potassium phosphate gradient (0.1 to 0.3 M, pH 7.2; 2-liter total gradient). The fractions containing CMP-KDO synthetase activity were pooled and concentrated by the addition of $(NH_4)_2SO_4$ to 65% saturation. After centrifugation, the pellets were suspended in 10 ml of distilled water and dialyzed overnight against 0.1 M phosphate buffer (pH 7.2).

Step 5: G-200 Sephadex. The enzyme solution, containing 5% glycerol, was loaded onto a G-200 Sephadex column (2.5 by 100 cm), equilibrated with 0.1 M potassium phosphate (pH 7.2), and eluted at a flow rate of 18 ml h^{-1}. The peak tubes (3-ml fractions) of activity (void volume/elution volume $\cong$ 1.8) were concentrated as before. After centrifugation, the pellets were suspended in 5 ml of distilled water and dialyzed overnight against 4 liters of 0.005 M Tris-acetate buffer (pH 7.4).

Step 6: isoelectric focusing. The concentrated enzyme solution was added to a final volume of 50 ml containing 5% sucrose and 2.8% ampholytes (pH 4.0 to 6.5). The enzyme was distributed throughout a linear 5 to 50% sucrose gradient containing 2.8% ampholytes in a 110-ml LKB electrofocusing column. The upper electrode (anode) contained 1% H_3PO_4, and the lower electrode (cathode) contained 1% NaOH in 57% sucrose. The gradient was initially run at 2.0 mA for 1 h, and the proteins were focused at 400 v for 65 h. The gradient was removed from the bottom of the column at a flow rate of 1.0 ml min^{-1}, and 30-drop fractions were collected. Fractions were assayed for protein at 280 nm, pH, and CMP-KDO synthetase activity (0.01 ml in a 0.1-ml total volume for 40 min). The fractions containing CMP-KDO synthetase activity (pI = 4.1 to 4.4) were pooled and dialyzed against 2 liters of 0.1 M potassium phosphate buffer (pH 7.2) to remove the sucrose. After dialysis, the enzyme was concentrated by the addition of $(NH_4)_2SO_4$ to 65% saturation. After centrifugation, the pellets were dissolved in 2 ml of distilled water and dialyzed against 0.1 M potassium phosphate (pH 7.2) for 2 h.

Step 7: G-100 Sephadex. To remove the ampholytes which interfere with protein measurements, the enzyme solution was loaded onto a G-100 Sephadex column (1.5 by 120 cm), which had been equilibrated with the above-mentioned buffer, and eluted at a flow rate of 9 ml h^{-1}. The tubes containing the highest specific activity (elution volume/void volume = 1.31 to 1.42) were retained, concentrated by $(NH_4)_2SO_4$ precipitation, and dialyzed against 0.05 M potassium phosphate buffer (pH 7.2). The enzyme was stored at 4°C or −90°C in the above-mentioned buffer until utilized. CMP-KDO synthetase at this point in purification was 70% pure as determined by electrophoresis on polyacrylamide gels. The enzyme has been purified to homogeneity by preparative gel electrophoresis as described previously for the purification of KDO-8-phosphate synthase (17). Polyacrylamide gels were prepared, stained, and scanned as previously described (17).

RESULTS

CMP-KDO synthetase was isolated and purified 2,300-fold from *E. coli* B cells by using conventional techniques (Table 1). The enzyme could be further purified to homogeneity (with a specific activity of 9.3 to 9.6 U mg of protein^{-1}) by preparative polyacrylamide gel electrophoresis (see Fig. 3A). Binding of the enzyme to various affinity resins including Matrix Screening Affinity Gels (Pharmacia) and CTP-, CDP-, and CMP-agarose gels (Sigma) was not effective under the conditions tested. The assay for CMP-KDO, described in the previous section, is 50% more sensitive than that used previously (5); the main difference in the assay is the destruction of $NaBH_4$ at 0°C by the addition of H_3PO_4 rather than the addition of acetone at room temperature. The enzyme obtained after column chromatography on G-200 Sephadex (step 5) was devoid of KDO-8-phosphate synthase and KDO-8-phosphate phosphatase activities and was stable for up to 3 months when stored at 4°C or −90°C in 0.05 M potassium phosphate buffer containing 0.5 mM dithiothreitol.

The enzyme appears to be cytoplasmic in that no evidence of membrane association was noted using several methods of cell disruption. The formation of CMP-KDO from CTP and KDO was linear with time for 10 min and linear with protein concentration over a 10-fold range at

TABLE 1. *Purification of CMP-KDO synthetase*

Step and fraction	Volume (ml)	Protein (mg)	U	Sp act (U mg of protein^{-1})	Fold purification	Recovery (%)
1. Crude extract	600	27,660	83	0.003		100
2. Protamine sulfate: acid precipitation	225	10,400	82	0.008	2.6	100
3. DEAE-Sephadex	50	1,800	72	0.040	13.3	86
4. DEAE-Sephadex	13	280	44	0.160	53.3	53
5. G-200 Sephadex	5	60	26	0.430	143.3	31
6. Isoelectric focusing	2.7		16			19
7. G-100 Sephadex	2.0	1.4	9.6	6.90	2,300.0	11

30°C. The enzymatic reaction was dependent upon the addition of CTP, KDO, and Mg^{2+} (Table 2) but did not require a reducing agent such as glutathione, mercaptoethanol, or dithiothreitol as reported previously (5). Even though a reducing agent was not required in the reaction, it was present during the purification procedure. It should be noted that, at 10 mM Hg^{2+} in the presence of 10 mM Mg^{2+}, total inhibition of the enzyme activity occurred. The formation of CMP-KDO was not inhibited by the addition of CDP, CMP, KDO-8-phosphate, or N-acetylneuraminic acid to the complete reaction mixture. Furthermore, in agreement with the data of Ghalambor and Heath (5), neither KDO-8-phosphate nor N-acetylneuraminic acid could substitute for KDO in the reaction (the reaction involving *N*-acetylneuraminic acid was monitored by a radioactive assay utilizing [^{14}C]CTP; data not shown). PP_i, one of the end products, was shown to be a weak inhibitor of the reaction, with an apparent I_{50} of 5.0 mM. A divalent cation, particularly Mg^{2+}, was required for maximal enzymatic activity. Optimal activity occurred at 10.0 mM Mg^{2+} (added as either the chloride or acetate salt) (Fig. 1), with approximately 50% of the rate exhibited at 20 and 5 mM Mg^{2+}. Other divalent cations tested were Cd^{2+}, Mn^{2+}, Zn^{2+}, Ba^{2+}, Ca^{2+}, and Co^{2+}. The percentage of maximal activity in the presence of these cations (10 mM) was 42, 38, 32, 20, 14, and 11%, respectively. Monovalent cations Na^+, Li^+, and K^+ were ineffective in stimulating the enzyme, as were divalent cations Hg^{2+}, Ni^{2+}, and Fe^{2+}.

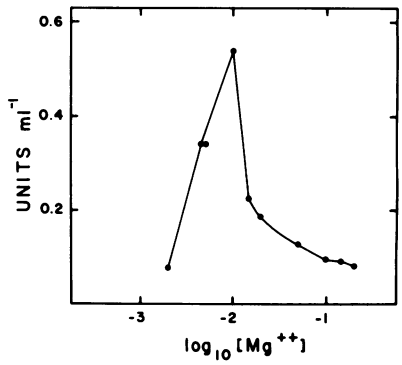


FIG. 1. *Determination of optimum Mg^{2+} concentrations. Reaction mixtures contained in a final volume of 0.1 ml: 20.0 μmol of Tris-acetate (pH 9.8), 1.0 μmol of CTP, 0.2 μmol of KDO, and either magnesium acetate or $MgCl_2$ at final concentrations as indicated. The reaction was initiated at 30°C by the addition of 0.02 ml of enzyme and terminated after 4.0 min by the addition of 0.2 ml of cold ethanol. CMP-KDO formed was assayed as described in Materials and Methods.*

TABLE 2. *Effect of components in the reaction mixture on the activity of CMP-KDO synthetase*[a]

Reaction mixture	nmol of CMP-KDO formed	% activity
Complete	15.25	100
Complete + reducing agent	15.03	98
− Buffer	3.32	22
$-Mg^{2+}$	<0.1	0
−CTP	<0.1	0
−KDO + KDO-8-phosphate	<0.1	0
− KDO + *N*-acetylneuraminic acid	<0.1	0
Complete + CDP or KDO-8-phosphate	15.5	101
Complete + CMP or *N*-acetylneuraminic acid	15.6	102
Complete + P ~ P_i	3.2	21

[a] The complete reaction mixture contained in a final volume of 0.1 ml: CTP, 1.0 μmol; KDO, 0.2 μmol; Tris-acetate buffer (pH 9.8), 20 μmol; Mg^{2+}, 1.0 μmol; and enzyme, 0.01 ml. The reducing agents tested were glutathione, mercaptoethanol, and dithiothreitol, added to a final concentration of 6.4 mM. CDP, CMP, and PP_i were tested at 10 mM; KDO-8-phosphate and *N*-acetylneuraminic acid were tested at 4 mM. The reaction was initiated at 30°C by the addition of 0.01 ml of enzyme and terminated after 5 min by the addition of 0.2 ml of cold ethanol.

The effect of pH on enzyme activity is depicted in Fig. 2. Maximal enzyme activity was obtained in either 0.2 M glycine (NaOH) buffer at pH 9.3 or 0.2 M Tris-acetate buffer at pH 9.6. A number of other buffers were tested, including phosphate buffer and Tris-chloride buffer, at various concentrations and pH values. For the routine assay of enzyme activity, 0.2 M Tris-acetate buffer (pH 9.8; final pH in the reaction mixture was 9.5) was utilized unless otherwise stated. The enzyme activity was more stable during purification and storage in potassium phosphate buffer than in Tris-acetate or Tris-chloride buffer at pH 7.4 to 8.0.

CMP-KDO synthetase exhibited a requirement for CTP and KDO for the formation of the $NaBH_4$-resistant product (Table 2). KDO could not be replaced by either KDO-8-phosphate or *N*-acetylneuraminic acid. Also, CTP was required for product formation (Table 3). However, CTP could be replaced by dCTP when the reaction was carried out at both pH 8.0 and pH 9.5, and by UTP at pH 9.5 but to a lesser extent at pH 8.0. Neither dUTP nor any of the other nucleotide triphosphates, diphosphates, or monophosphates tested could substitute for CTP in the reaction. The apparent K_m values determined at pH 9.5 for CTP, dCTP, and UTP were 2.0×10^{-4}, 3.4×10^{-4}, and 8.8×10^{-4} M, respectively. The apparent V_{max} values for CTP, dCTP, and UTP were 3.8×10^{-4}, 6.7×10^{-4}, and

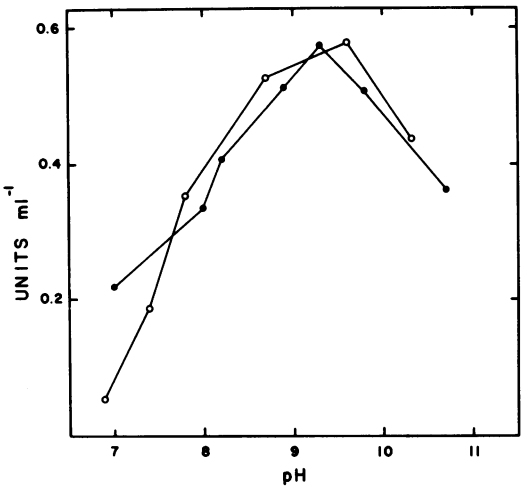


FIG. 2. *Effect of pH on the activity of CMP-KDO synthetase. Reaction mixtures contained in a final volume of 0.1 ml: 1.0 μmol of CTP, 0.2 μmol of KDO, and 20.0 μmol of Tris-acetate or sodium-glycine at the pH values indicated. The reaction was initiated at 30°C with 0.02 ml of enzyme and terminated after 4.0 min by the addition of 0.2 ml of cold ethanol. Fractions were then assayed for CMP-KDO formed as described in Materials and Methods. Control reaction mixtures with no enzyme were included at all pH values to assure complete reduction of unutilized KDO. pH values are represented as the final pH in the reaction mixture. ○, Tris-acetate; ●, sodium-glycine.*

3.3×10^{-4} mol min^{-1} mg of $protein^{-1}$. In the presence of CTP, the apparent K_m value for KDO was determined to be $2.9 \pm 0.2 \times 10^{-4}$ M, with a maximum velocity of $6.2 \pm 0.2 \times 10^{-4}$ mol min^{-1} mg of $protein^{-1}$.

CMP-KDO synthetase appears to be a monomer with a molecular weight between 35,000 and 45,000. The molecular weight of the native enzyme was estimated by three methods. Measurement of the relative mobility of the enzyme on non-SDS-polyacrylamide gels containing various acrylamide concentrations (7 to 12%) gave a molecular weight by Ferguson analysis (4) of 35,000 to 40,000, with a $-K_r$ value identical to that of pepsin (molecular weight, 34,700). An $s_{20,w}{}^{0.725}$ value of 4.0 to 4.2 S at 4°C, corresponding to a molecular weight of 35,000 to 45,000, was obtained by sucrose density gradient centrifugation by the method of Martin and Ames (12), using lysozyme (molecular weight, 17,200), alkaline phosphatase (molecular weight, 80,000), and catalase (molecular weight, 250,000) as standards. Gel filtration studies using G-100 Sephadex as the matrix gave an elution volume/void volume ratio of 1.4 to 1.44, corresponding to a molecular weight of 40,000 to 46,000; replotting the gel filtration data by the method of Siegel and Monty (21) gave a Stokes radius of 3.0 nm. As shown in Fig. 3B, denaturation of the purified protein (Fig. 3A) by boiling in 2% SDS followed by SDS-polyacrylamide gel electrophoresis revealed a single polypeptide band. Comparison of the relative mobility on 12% SDS-gels of CMP-KDO synthetase to those of the molecular weight standards (Fig. 3C) gave a molecular weight for the denatured enzyme of 36,000 ± 4,000. Thus, it appears that the native enzyme is a monomer with a molecular weight of approximately 36,000. The isoelectric point for CMP-KDO synthetase was determined to be between pH 4.15 and 4.4.

The stoichiometry of the reaction reported by Ghalambor and Heath (5) appears to be correct; however, we have been unable to isolate and stabilize the product CMP-KDO in sufficient quantity for qualitative analysis and for testing as a feedback inhibitor. By radioactive assay (data not shown) we have shown that, using stoichiometric amounts of KDO and [^{14}C]CTP, the reaction can be run to completion, and after mild acid hydrolysis only [^{14}C]CMP is generated.

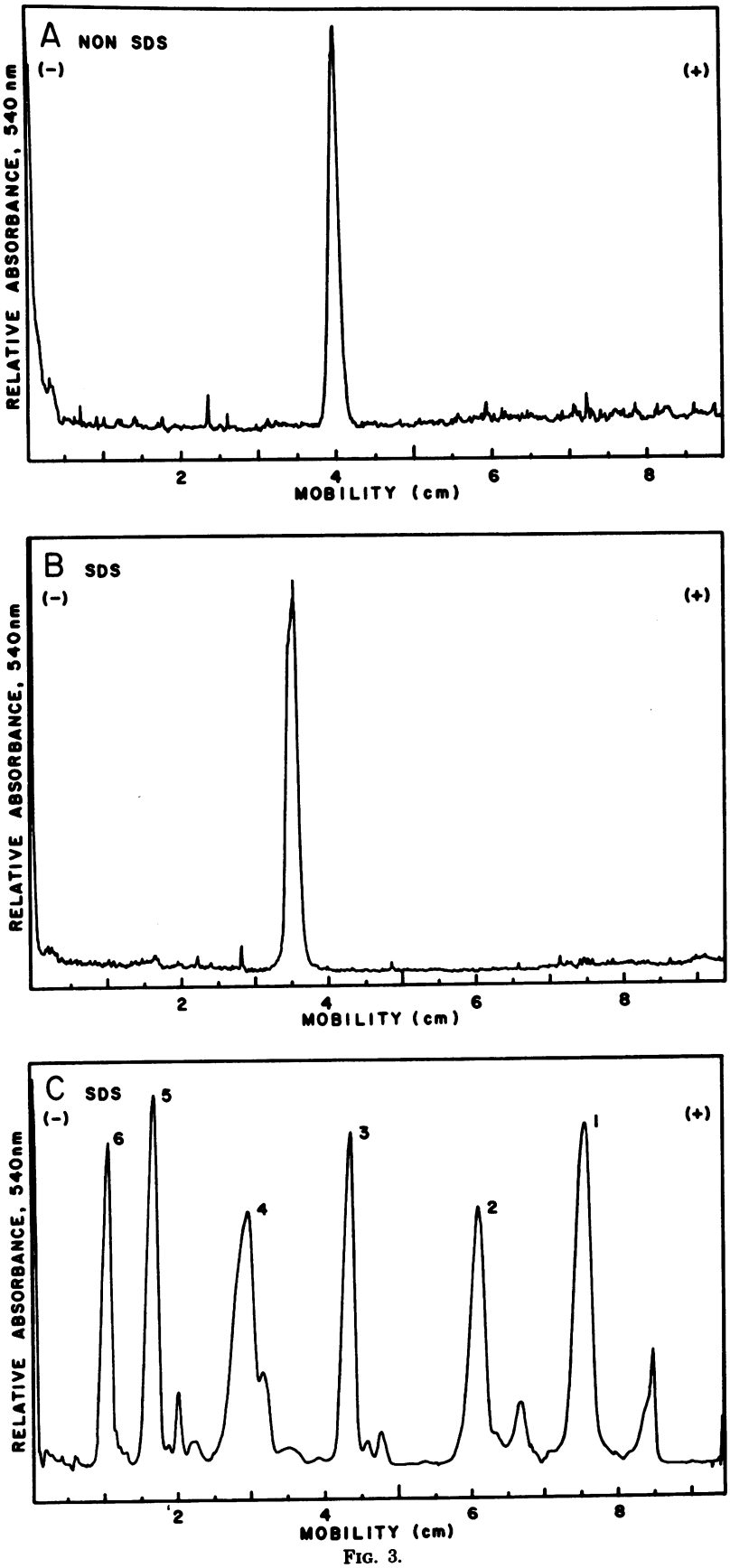
DISCUSSION

KDO is a site-specific constituent of the lipopolysaccharide of most gram-negative bacteria, providing the link between lipid A and the growing polysaccharide chain. There are at least four sequential enzymes involved in the synthesis and utilization of KDO after the formation of D-arabinose-5-phosphate. Preceding papers (17,

TABLE 3. *Nucleotide specificity studies on CMP-KDO synthetase*[a]

Nucleotide	nmol of $NaBH_4$-resistant KDO formed at:		K_m (M)
	pH 8.0	pH 9.5	
CTP	24.3	30.0	2.0×10^{-4}
dCTP	15.0	29.0	3.4×10^{-4}
UTP	1.7	9.4	8.8×10^{-4}
dUTP	0.7	0.5	N.D.
ATP, dATP, GTP dGTP, ITP, dITP TTP, CDP, UDP, CMP	0.1	0.1	

[a] Reaction mixtures contained in a final volume of 0.1 ml: Tris-acetate (pH 8.0 or pH 9.8), 20 μmol; magnesium acetate, 1.0 μmol; KDO, 0.2 μmol; nucleotide, 1.0 μmol each; and enzyme (to initiate the reaction), 0.01 ml. The reaction was terminated after 10 min at 30°C by the addition of 0.2 ml of cold ethanol, and 0.1-ml portions were assayed for CMP-KDO as described in Materials and Methods. K_m values were determined in Tris-acetate (pH 9.5).


A NON SDS
(-)
(+)
RELATIVE ABSORBANCE, 540 nm
2
4
6
8
MOBILITY (cm)
B SDS
(-)
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RELATIVE ABSORBANCE, 540nm
2
4
6
8
MOBILITY (cm)
C SDS
(-)
(+)
6
5
4
3
2
1
RELATIVE ABSORBANCE, 540nm
2
4
6
8
MOBILITY (cm)

FIG. 3.

18) have described the purification and characterization of KDO-8-phosphate synthase and the highly specific KDO-8-phosphate phosphatase. In this paper, we describe the purification and partial characterization of the last soluble enzyme involved in the synthesis and utilization of KDO, CMP-KDO synthetase. The product of this reaction, CMP-KDO, is very labile and is required for the transfer of KDO to the lipid A precursor (5, 13, 15). KDO transferase is tightly membrane bound and has been studied by Osborn and co-workers (13, 16).

Ghalambor and Heath (5) first reported the formation of CMP-KDO and briefly described some of the properties of CMP-KDO synthetase. Our results differ from those previously published in the following manner: (i) the initial specific activity in crude extracts; (ii) in the determination of the optimum pH; (iii) in the stimulation of the reaction by a reducing agent; and (iv) in the specificity of the reaction for CTP. In our initial studies with CMP-KDO synthetase, the mechanics of the thiobarbituric acid assay for borohydride-resistant KDO (the C-2 carbonyl group of KDO linked ketosidically to CMP is resistant to borohydride reduction) were studied in detail. It was found that when the product of the reaction (CMP-KDO) was maintained at 0°C until the acid hydrolysis of the CMP-KDO bond, and when the borohydride was destroyed at 0°C by the addition of acid rather than by the addition of acetone, the amount of borohydride-resistant KDO detected was 50% greater than previously described (5). This modification of the assay could explain some of the differences noted above. In addition, the activity of CMP-KDO synthetase with UTP is significantly less at pH 8.0 (Table 3), the optimum pH noted by Ghalambor and Heath (5), than at pH 9.5 and could have gone undetected at the lower pH.

The specific activity of CMP-KDO synthetase in crude extracts of *E. coli* B grown on glucose minimal medium is in the range required for lipopolysaccharide synthesis in vivo (2 nmol min^{-1} mg of $protein^{-1}$ [data not shown]), and would suggest that the formation of CMP-KDO may be the rate-limiting step in lipopolysaccharide biosynthesis. The specific activities in crude extracts of *E. coli* B of D-arabinose-5-phosphate isomerase, KDO-8-phosphate synthase, and KDO-8-phosphate phosphatase are at least 15-fold greater than that of CMP-KDO synthetase (17, 18). Also of interest is the substrate specificity of CMP-KDO synthetase. Osborn and coworkers have shown that the product of the CMP-KDO transferase reaction in *S. typhimurium* is a lipid A precursor with 2 mol of KDO attached rather than the expected 3 mol and that the nonreducing KDO in this disaccharide corresponds to the branch KDO rather than to the unit within the main chain (13, 15). The third KDO residue, which is necessary for the elongation of the polysaccharide chain was not detected.

We have shown that KDO can be activated not only by CTP but also with dCTP and UTP as well (Table 3). Considering that the maximum velocities obtained with all three nucleotides are similar (3.3×10^{-4} to 7×10^{-4} mol min^{-1} mg of $protein^{-1}$) and the affinity of the enzyme for CTP is only three- to fourfold greater than for UTP, one cannot exclude the possibility that the enzyme catalyzing the transfer of the third KDO residue might utilize UMP-KDO or possibly dCMP-KDO, rather than CMP-KDO. Further investigation into the nature of KDO activation and utilization is being conducted, and the possible physiological implications of this reaction may prove important in the overall control of lipopolysaccharide biosynthesis.

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FIG. 3. *Densitometer tracings of CMP-KDO synthetase electrophoresed on non-SDS- and SDS-acrylamide gels: estimation of subunit molecular weight. CMP-KDO synthetase was prepared as described in Table 1, and a portion was subjected to preparative 11% acrylamide slab gel electrophoresis utilizing a single well (3.5 cm wide and 3 mm thick) containing approximately 300 μg of protein. The enzyme was electrophoresed as described previously (17). 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-acetate buffer (pH 7.4) containing 0.5 mM dithiothreitol. (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 10% acrylamide slab gel. After staining, the gel was scanned as described previously (17). (B) SDS-gel: Another portion of the enzyme, prepared as described above, was denatured by boiling in SDS and mercaptoethanol and was electrophoresed as described previously (17), using a 12% polyacrylamide gel containing 0.1% SDS in all buffers. (C) SDS-gel: The densitometer tracing labeled "C" is a scan of an SDS-gel containing the Low Molecular Weight Calibration Kit protein supplied by Pharmacia Fine Chemicals. 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 CMP-KDO synthetase.*

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