THE ENZYMATIC SYNTHESIS OF CYTIDINE MONOPHOSPHO-2-KETO-3-DEOXY-OCTONATE*

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The accompanying paper (Heath and Ghalambor, 1963) presents evidence for the occurrence of 2-keto-3-deoxy-octonate (KDO) as a glycosidically bound constituent in preparations of cell wall lipopolysaccharide (LPS) obtained from Escherichia coli O111-B_4. The purpose of this paper is to report the biosynthesis of cytidine monophospho-KDO (CMP-KDO) by an enzyme purified from this organism.

Materials and Methods--- Unless otherwise noted, all compounds, reagents, and methods used in these studies were the same as those indicated in the preceding paper; abbreviations used here are also the same.

Enzyme purification--- The assay procedure is based on the principle that 2-keto-3-deoxy-onic acids no longer give a positive reaction in the TBA method after the carbonyl group is reduced with $NaBH_4$ (Weissbach and Hurwitz, 1959). Thus, after incubation of CTP, KDO and enzyme, the remaining uncombined KDO is reduced with $NaBH_4$ while the CMP-bound KDO is resistant to reduction. After destruction of the excess borohydride with acetone, followed by mild acid hydrolysis, the KDO released from CMP-KDO was measured by the TBA method. Using this assay procedure, the formation of CMP-KDO was linear for 30 min, over a 5-fold range in protein concentration.

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The organism was grown for 6 hours under forced aeration in Trypticase Soy Broth (Baltimore Biological Labs.), harvested, washed with 0.15 M KCl, and disrupted by sonication. After centrifugation of the broken cell suspension, the crude extract was purified by successive application of the following techniques: (1) protamine sulfate treatment; (2) ammonium sulfate fractionation I; (3) isoelectric precipitation I; (4) calcium phosphate gel adsorption of inactive protein; (5) ammonium sulfate fractionation II; (6) isoelectric precipitation II. These fractionation procedures resulted in an increase in specific activity of the enzyme approximately 160-fold; the final preparation catalyzed the formation of 2 μmoles of CMP-KDO per mg of protein per 10 min at 37°.

Isolation and characterization of CMP-KDO--- For this purpose, an incubation mixture was prepared which contained the following (in μmoles): CTP, 7.5; KDO, 4; Mg^{++}, 15; Tris buffer, pH 8.0, 150; protein, 250 μg; in a final volume of 1.5 ml. After incubation of the mixture at 37° for 30 min, 1.6 μmoles of bound KDO was formed. The incubation mixture was diluted to 10 ml with water and applied to a column containing 20 ml of DEAE-cellulose (Type 40, Brown Co.), previously equilibrated with 0.01 M Tris buffer, pH 7.9. After washing with 20 ml of the same buffer, the column was eluted with a linear gradient of lithium chloride (0 to 0.2 M) contained in 0.01 M Tris buffer, pH 7.9 (Warren and Blacklow, 1962). The entire procedure was conducted at 4°; fractions (4 ml) were collected with an automatic fraction collector. Aliquots were removed from each fraction and analyzed for free and bound KDO, and for UV-absorbing (271 mμ) material. The results of this fractionation are shown in Figure 1. This method provided quantitative recovery and complete resolution of the various components of the incubation mixture: Peak I represents the remaining free KDO; Peak II is composed of bound KDO and cytidine in equimolar quantities (CMP-KDO); Peak III and Peak IV were identified as CDP* and CTP respectively by paper electrophoresis. The material in Peak

* The CTP preparation used was found to be contaminated with CDP.

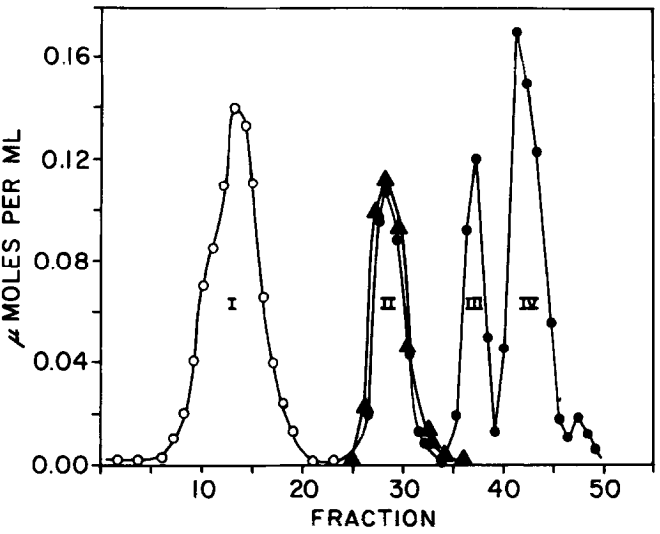


Figure 1. The isolation of CMP-KDO. See text for composition of incubation mixture and details of the fractionation procedure. o——o , free KDO; ▲——▲ , bound KDO; ●——● , UV-absorption.

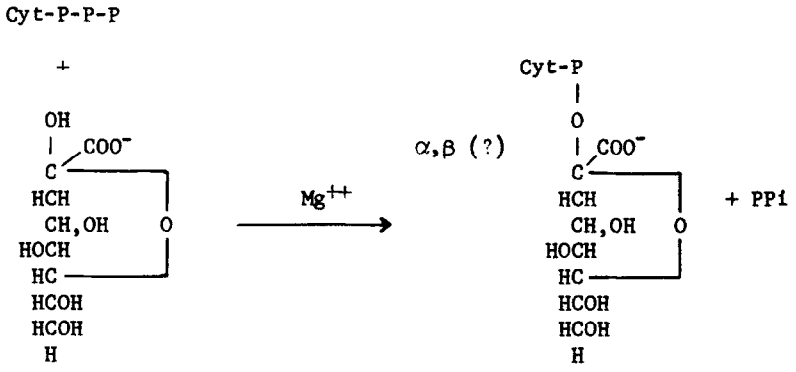
II was further purified by adsorption and elution from charcoal. Analyses of Peak II and of the charcoal eluate gave the following results (molar ratios): DEAE-Peak II, cytidine, 1.00; organic P, 1.05; KDO (resistant to $NaBH_4$), 0.95; charcoal eluate, cytidine, 1.00; organic P, 1.03; KDO (resistant to $NaBH_4$), 0.95. The DEAE eluate contained inorganic P which was absent from the charcoal eluate. Adjustment to pH 2 of a solution containing the nucleotide resulted in its rapid degradation to CMP and free KDO as determined chromatographically, and by the fact that the KDO becomes completely susceptible to reduction by $NaBH_4$. Due to its extreme lability, all attempts to further characterize this nucleotide by paper chromatography or electrophoresis have been unsuccessful. Although some of the nucleotide (10 to 25%) could be recovered intact from papers used in these techniques, most of the material was recovered as free KDO and CMP. In most instances, the UV-absorbing material and the KDO were streaked from the origin suggesting that the nucleotide decomposed on the paper. A variety of brands and grades of papers were employed in these experiments; some were washed with EDTA, acids, and/or buffers before use.

Further work is necessary to determine if conditions can be devised to stabilize this nucleotide during chromatographic or electrophoretic fractionation.

The results of stoichiometry studies were as follows (molar ratios): CMP-KDO, 1.00; PPi, 0.97 (isolated by ion-exchange methods).

Specificity--- The enzyme exhibited a requirement for Mg^{++} and it was specific for KDO and CTP. Thus, ATP, UTP, GTP, ITP, TTP, and CDP were inactive when substituted for CTP. KDO isolated from LPS and enzymatically synthesized KDO exhibited identical rates of activity and yielded identical products. On the other hand, KDH, KDG, and N-acetyl-neuraminic acid (kindly provided by Dr. Saul Roseman of this University) were inactive (less than 2%).

Discussion--- The evidence presented in this paper indicates that the purified enzyme catalyzes the following reaction:



The conclusion that the product is a glycoside of KDO is based primarily on its resistance to reduction by borohydride. Further experiments are in progress to confirm the nature of this new nucleotide.

The enzymatic synthesis of CMP-KDO is the second instance of the occurrence of a cytidine monophospho-2-keto-3-deoxy-onic acid derivative. The first report of the occurrence of a nucleotide of this type was that of Comb, et al. (1959), who isolated CMP-N-acetyl-neuraminic (N-AN) acid from E. coli K-235, an organism that produces a polymer of N-AN (colominic acid). Subsequently, Roseman (1962) reported the enzymatic synthesis of CMP-N-AN by an enzyme isolated from extracts of hog submaxillary gland; a similar enzyme was purified from extracts of Neisseria meningitidis by Warren and Blacklow

(1962). The enzymatic synthesis of CMP-KDO apparently is analogous to the formation of CMP-N-AN, and this may represent a general mechanism for the 'activation' of sugar-acids of this type. It has been demonstrated that CMP-N-AN is, indeed, an activated form of N-AN, since this nucleotide serves as an N-acetyl-neuraminyl donor in the enzymatic synthesis of colominic acid (Aminoff, et al., 1963) and of N-AN-containing oligosaccharides of milk (Jourdian, et al., 1963).

The series of enzymatic reactions outlined below represent an hypothesis for the route of biosynthesis of KDO-containing polymers.

I D-arabinose-5-P + PEP → KDO-8-P + Pi

II (KDO-8-P → KDO + Pi)

III KDO + CTP → CMP-KDO + PPi

IV (CMP-KDO → KDO-Polymer + CMP)

Reaction I was first demonstrated by Levin and Racker (1959). Reaction II is proposed on the basis of the following: (1) non-phosphorylated KDO serves as substrate in the biosynthesis of CMP-KDO (KDO-8-P has not yet been tested); (2) only non-phosphorylated N-AN serves as substrate in the analogous biosynthesis of CMP-N-AN; (3) the protein fraction used in the present studies to prepare KDO apparently contained a potent phosphatase for KDO-8-P; (4) Weissbach and Hurwitz (1959) also observed rapid dephosphorylation of KDH-7-P in crude preparations of the enzyme that synthesized this compound. Whether or not the phosphatase proposed in Reaction II is specific for KDO-8-P remains to be established. Reaction III has been substantiated in the present paper. Reaction IV has not yet been demonstrated and is proposed on the basis of the assumption that CMP-KDO is a precursor of glycosidically bound KDO.

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