The Biosynthesis of Cell Wall Lipopolysaccharide in
Escherichia coli

V. PURIFICATION AND PROPERTIES OF 3-DEOXY-D-MANNO-OCTULOSONATE ALDOLASE*

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SUMMARY

3-Deoxy-D-manno-octulosonate aldolase, an inducible enzyme isolated from extracts of 3-deoxy-D-manno-octulosonate-grown Aerobacter cloacae, has been purified approximately 60-fold. The enzyme catalyzes the following reaction: 3-deoxy-D-manno-octulosonate $\Leftrightarrow$ pyruvate + D-arabinose. Pyruvate was characterized chromatographically and with lactic acid dehydrogenase. D-Arabinose was characterized chromatographically and by its reactivity with L-fucose isomerase to form D-ribulose. The purified enzyme exhibited the following properties: pH optimum, 7; Michaelis constant $= 6 \times 10^{-3}$ M; and equilibrium constant $= 0.077$ M. The enzyme provides a convenient method for the preparation of specifically $^{14}$C-labeled 3-deoxy-D-manno-octulosonate.

The two preceding papers in this series reported the occurrence of 3-deoxy-D-manno-octulosonate as a glycosidically bound constituent of the cell wall lipopolysaccharide of Escherichia coli 0111-B4 and other enteric bacteria (1), and the purification and properties of cytidine monophosphate 3-deoxy-D-manno-octulosonate synthetase which catalyzes the following reaction: cytidine monophosphate + 3-deoxy-D-manno-octulosonate $\Leftrightarrow$ CMP-3-deoxy-D-manno-octulosonate + inorganic pyrophosphate (2). In a preliminary communication (3), an inducible enzyme, 3-deoxy-D-manno-octulosonate aldolase, was reported to catalyze the following reaction:

3-deoxy-D-manno-octulosonate $\Leftrightarrow$ pyruvate + D-arabinose

The purified enzyme provides a specific analytical tool for the determination of 3-deoxy-D-manno-octulosonate and also a simple, inexpensive method of preparation of 14C-3-deoxy-manno-octulosonate.

EXPERIMENTAL PROCEDURE

Materials and Methods—All chemicals were obtained from commercial sources except where otherwise indicated. KDO* and KDG** either isolated from cell wall lipopolysaccharide of E. coli 0111 or synthesized enzymatically or chemically (1). Uniformly labeled 14C-KDO was isolated (1) from cells which were grown in a synthetic medium which contained uniformly labeled 14C-glucose as the sole source of carbon; the specific activity of the KDO prepared in this manner was 40,000 cpm per pmol. KDO-8-phosphate and D-arabinose 5-phosphate were prepared previously described (1). D-Ribulose was prepared chemically from D-arabinose (4). N-Acetylneuraminic acid, KDG, N-acetyl-D-mannosamine were gifts from Dr. Saul Roseman.

* The following abbreviations are used: 3-deoxy-D-manno-octulosonate, KDO; N-acetylneuraminic acid, KDG; N-acetyl-D-mannosamine, N-AN.
The reaction was initiated by the addition of KDO; decrease in absorbance of KDO disappearance were as follows: KDO, 5 moles; phosphate buffer, pH 7, 20 μmoles; and an appropriate amount of KDO aldolase in a final volume of 0.5 ml. The reaction was initiated by the addition of enzyme; 0.05-ml aliquots are withdrawn at zero time and at appropriate intervals thereafter, and transferred to clean tubes mounted in a boiling water bath. After heating for 1 min, denatured protein was removed by centrifugation, and aliquots of the supernatant fluid were analyzed for KDO and for pyruvate.

Incubation mixtures utilized with the colorimetric determination of KDO disappearance from incubation mixtures. An alternate assay procedure, which may be used with more purified preparations of the enzyme (after removal of DPNH oxidase), couples the aldolase reaction with an excess of lactic acid dehydrogenase and DPNH; when it was desirable to follow this reaction continuously, determinations were conducted with a Gilford recording spectrophotometer.

Paper chromatography was performed in a descending manner on Whatman No. 1 or Schleicher and Schuell No. 589 blue ribbon papers with the following solvent systems: Solvent A, ethyl acetate-acetic acid-water (14:3:3); B, butan-1-ol-pyridine-water (2:2:1); and C, butan-1-ol-pyridine-water (5:3:2). Sugars were detected on paper with either alcoholic silver nitrate (9) or aniline trichloracetate-acetic acid reagent (11).

**Purification of KDO aldolase**

**Assay Procedure**—KDO aldolase may be most conveniently assayed in crude preparations by the colorimetric determination of the disappearance of KDO from incubation mixtures. An alternate assay procedure, which may be used with more purified preparations of the enzyme (after removal of DPNH oxidase), couples the aldolase reaction with an excess of lactic acid dehydrogenase and DPNH; when it was desirable to follow this reaction continuously, determinations were conducted with a Gilford recording spectrophotometer.

The procedure described in this paper for the cultivation of a variety of other organisms (Escherichia coli, strains 0111, B, and K12, as well as Salmonella typhimurium and Salmonella alcalaide) grown in a medium that consisted of the following (grams per liter): NH₄Cl, 5; NaH₂PO₄, 10; KH₂PO₄, 3; K₂SO₄, 1; NaCl, 1; MgSO₄·7H₂O, 0.2; CaCl₂·6H₂O, 0.02; FeSO₄·7H₂O, 0.001; glucose, 0.25; yeast extract, 1; and crude synthetic potassium KDO, 4.5. The carbohydrate constituents were sterilized separately and aseptically added to sterile media; glucose was autoclaved as a 50% solution, and a 10% solution of KDO was sterilized by filtration. The medium (1 liter contained in a 2-liter flask) was inoculated with 25 ml of an overnight culture of the organism and incubated at 37° in a rotary shaker for 18 to 24 hours. The cells (approximately 6 g) were harvested by centrifugation, washed with cold 0.15 M KCl, resuspended in 20 ml of water, and sonically disrupted for 10 min. The suspension was centrifuged at 25,000 × g for 10 min, and the supernatant fluid was retained. All procedures of the purification were conducted at 0 to 4°.

**Protamine Sulfate Precipitation**—To 24 ml of crude extract were added 33 ml of 0.025 M phosphate buffer, pH 7, and 3 ml of 2% protamine sulfate solution. The suspension was stirred for 5 min, and the precipitate was removed by centrifugation at 25,000 × g for 10 min and discarded.

**Ammonium Sulfate I**—The protamine sulfate supernatant fluid (58 ml) was treated with 13 g of solid ammonium sulfate. The precipitate was discarded, and to the supernatant fluid (66 ml) were added 12 g of solid ammonium sulfate; the suspension was stirred for 5 min, centrifuged at 25,000 × g for 10 min, and the precipitate was dissolved in 0.025 M phosphate buffer, pH 7, to a final volume of 6 ml.

**pH 5 Dialysis**—A flask containing 250 ml of 0.01 M potassium acetate buffer, pH 5.1, was placed in a salt-ice mixture until the temperature of the solution reached 0°. The ammonium sulfate fraction from the preceding step was transferred to dialysis tubing, chilled to 0°, and placed in the acetate buffer solution which was stirred for 4 hours; during this period, the temperature of the system was maintained at 0°. Care was taken to avoid a rise in temperature during this procedure as temperatures significantly above 2° resulted in rapid inactivation of the enzyme. After dialysis, the suspension was centrifuged at 25,000 × g for 10 min, and the precipitate was discarded. The supernatant fluid (7.2 ml) was adjusted to pH 6.5 with 2 M Tris base.

**Calcium Phosphate Gel**—The supernatant solution (7.2 ml) from the preceding step was diluted with 14.4 ml of water and then treated with 14.4 ml of calcium phosphate gel (14 mg per ml). The suspension was stirred for 10 min, centrifuged at 10,000 × g for 5 min, and the calcium phosphate gel was washed successively with 10-ml portions of water, 0.005 M, 0.01 M, and 0.015 M phosphate buffer, pH 7. Most of the KDO aldolase activity was obtained in the fraction cluted with 0.01 M phosphate buffer; this fraction was retained for further purification.

**Ammonium Sulfate II**—The gel cluate obtained in the preceding step (10 ml) was treated with 4.4 g of solid ammonium sulfate, stirred for 5 min, and centrifuged at 35,000 × g for 15 min. The precipitate was dissolved in 0.025 M phosphate buffer, pH 7, in a final volume of 1.4 ml.

With this fractionation procedure, KDO aldolase was purified followed by the extraction of these cells provided crude preparations that exhibited KDO aldolase activities comparable to those obtained from A. cloacae.

*The procedure described in this paper for the cultivation of a variety of other organisms (Escherichia coli, strains 0111, B, and K12, as well as Salmonella typhimurium and Salmonella alcalade) was used.
approximately 60-fold with recovery of 34% of the activity. These results are summarized in Table I.

KDO aldolase is relatively stable at various stages of purification. Thus, the activity in crude extracts is stable indefinitely when stored at -20°; Ammonium sulfate I and Ammonium sulfate II exhibited only slight (10 to 20%) losses even when the preparations were repeatedly thawed and refrozen over a period of 1 year.

RESULTS

Effect of Time, Protein Concentration, and pH—As illustrated in Fig. 1, under the standard conditions employed for assay of KDO aldolase, the activity was linear with respect to time of incubation and enzyme concentration, regardless of whether the assay was based on KDO disappearance or pyruvate formation. Similarly, the assay system which employed coupling KDO aldolase directly to lactic acid dehydrogenase and recording continuously the oxidation of DPNH, indicated a linear relationship between time and enzyme concentration; these data are illustrated in Fig. 2.

The effect of pH on KDO aldolase activity is shown in Fig. 3. These results indicate that the pH optimum for this activity is approximately 7 with sharp decreases in activity above or below the optimum. Further, it can be seen from these data that KDO aldolase activity is markedly inhibited in the presence of Tris buffer. The effect of phosphate buffer appears to have resulted from an influence on the ionic environment rather than participation of inorganic phosphate in the reaction. Thus, substrates containing inorganic phosphate in the presence of Tris or other buffers at pH 7 exhibited no stimulatory effect on activity.

KDO aldolase activity exhibited no additional requirements for the presence of a variety of divalent cations, and there was no detectable loss of activity when the enzyme was assayed either in the presence of EDTA or assayed after dialysis of the enzyme against EDTA.

Effect of Substrate Concentration—The effect of the concentration of KDO on aldolase activity is illustrated in Fig. 4. Assays were performed as indicated in the text; aliquots of the supernatant fluid were used for the determinations of KDO remaining in the mixture and for the determination of the amount of pyruvate formed.

**Table I**

Summary of purification procedure

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total volume</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>(units/mg X 10²)</td>
<td>%</td>
</tr>
<tr>
<td>1. Crude extract</td>
<td>24</td>
<td>500</td>
<td>9.3</td>
<td></td>
</tr>
<tr>
<td>2. Protamine sulfate</td>
<td>60</td>
<td>250</td>
<td>21</td>
<td>100</td>
</tr>
<tr>
<td>3. Ammonium sulfate I</td>
<td>6</td>
<td>110</td>
<td>43</td>
<td>85</td>
</tr>
<tr>
<td>4. pH 5 dialysis</td>
<td>7.2</td>
<td>60</td>
<td>85</td>
<td>94</td>
</tr>
<tr>
<td>5. Calcium phosphate gel</td>
<td>10</td>
<td>7.6</td>
<td>250</td>
<td>55</td>
</tr>
<tr>
<td>6. Ammonium sulfate II</td>
<td>1.4</td>
<td>3.2</td>
<td>563</td>
<td>34</td>
</tr>
</tbody>
</table>

Assays were performed as described in the text.
substrate saturation curve indicates that a concentration of approximately \(2 \times 10^{-3} \text{ M}\) KDO is required to yield maximal aldolase activity. When these data were plotted according to the method of Lineweaver and Burk (13), the \(K_m\) was estimated to be approximately \(6 \times 10^{-3} \text{ M}\).

Characterization of Products of KDO Cleavage—Pyruvate was characterized as one of the products of the KDO aldolase catalyzed cleavage of KDO by (a) its reactivity with lactic acid dehydrogenase in the presence of DPNH, (b) its co-chromatography on ion exchange resins with authentic pyruvate, (c) its correspondence to authentic pyruvate when subjected to paper electrophoresis, and (d) the requirement of pyruvate as a substrate for KDO aldolase in the direction of condensation.

Tentative characterization of arabinose as the second product of KDO cleavage was based on co-chromatography of the enzymatically formed pentose with authentic arabinose in a variety of paper chromatographic solvent systems, the reactivity of the neutral enzymatic product with the orcinol reagent (7) to yield a chromogen with a spectrum typical of aldopentoses, and the specificity (Table II) of KDO aldolase for D-arabinose when the condensation reaction was studied. Final characterization of the enzymatically formed arabinose as the \(\alpha\) isomer was obtained as a result of its reactivity with \(L\)-fucose isomerase. Green and Cohen (14) established that \(L\)-fucose isomerase catalyzed the isomerization of \(\alpha\)-arabinose to \(\alpha\)-ribulose nearly as efficiently as its reaction with \(L\)-fucose, but that the enzyme was inactive with \(L\)-arabinose. These authors explained these results by noting that the configuration of hydroxy groups at C-2, C-3, and C-4 is identical in \(L\)-fucose and in \(\alpha\)-arabinose, and that this partial structural identity renders either sugar an appropriate substrate for the isomerase.

As illustrated in Table II, the neutral product of KDO cleavage serves as substrate for \(L\)-fucose isomerase in a manner essentially identical with that of authentic \(\alpha\)-arabinose. As previously observed by Green and Cohen (14), the preparation of \(L\)-fucose isomerase used in the present studies was inactive with authentic \(L\)-arabinose, \(\beta\)-ribose, \(\beta\)-xylose, and \(\beta\)-lyxose.

To corroborate the identity of the pentose as \(\alpha\)-arabinose, uniformly labeled \(^{14}\)C-KDO was incubated with KDO aldolase and the \(^{14}\)C-labeled, neutral product was isolated. As shown in Fig. 5, upon paper chromatography the radioactive product exhibited a mobility identical with authentic \(\alpha\)-arabinose. Further, after treatment of the radioactive pentose with the \(L\)-fucose isomerase preparation, a second radioactive component was detected, the mobility of which corresponded to authentic \(\beta\)-ribulose and gave a typical ketose reaction with the orcinol-trichloracetate spray reagent (11).

On the basis of these studies, the products of the KDO aldolase reaction were concluded to be pyruvate and \(\alpha\)-arabinose.

Substrate Specificity and Stoichiometry—KDO aldolase exhibited a high degree of specificity for KDO in the direction of cleavage, and for pyruvate and \(\alpha\)-arabinose in the direction of condensation. Thus, as summarized in Table III, 3-deoxy-\(\alpha\)-arabinohexitolose, 3-deoxy-\(\beta\)-ribo-hexulosonate, \(N\)-AN, and KDO-8-phosphate were not cleaved to a significant extent by KDO aldolase. Similarly, while incubation of pyruvate and \(\alpha\)-arabinose with the aldolase resulted in the formation of KDO,

### Table II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ketose formed</th>
</tr>
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<tbody>
<tr>
<td>(L)-Fucose</td>
<td>84 mmoles</td>
</tr>
<tr>
<td>(\alpha)-Arabinose</td>
<td>68 mmoles</td>
</tr>
<tr>
<td>Enzymatic product*</td>
<td>74 mmoles</td>
</tr>
<tr>
<td>(L)-Arabinose, (\beta)-ribose, (\beta)-xylose, or (\beta)-lyxose</td>
<td>&lt;2 mmoles</td>
</tr>
</tbody>
</table>

* Incubation mixtures contained the following: substrate, 1 \(\mu\)mole; Tris buffer, pH 8, 25 \(\mu\)moles; and 0.025 ml of crude extract of \(L\)-fucose-grown \(E\). coli 6111-B1, (15) in a final volume of 0.1 ml. The mixture was incubated at 37° for 15 min; the reaction stopped by the addition of 0.1 ml of 20% trichloracetic acid, and ketose was determined by the cysteine-carbazole method (6).

The neutral product of KDO aldolase was isolated as described in the text; the pentose concentration of this material was determined by the orcinol method (7) with an arabinose standard.

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** The effect of substrate concentration on KDO aldolase. Assays were performed as described in the text except that the concentration of KDO was varied. The formation of pyruvate was followed continuously for a period of 10 min.

![Fig. 5](http://www.jbc.org/)

**Fig. 5.** Identification of \(\alpha\)-arabinose. The neutral product resulting from the cleavage of uniformly labeled \(^{14}\)C-KDO by the aldolase was chromatographed on Whatman No. 1 paper in Solvent A. Duplicate chromatograms were sprayed with aniline-trichloroacetic acid (10) for aldoses or with orcinol-trichloroacetic acid (11) for ketoses. Paper strips were scanned for radioactivity with a commercial \(4\pi\) scanner.
The results of equilibrium studies on KDO aldolase are illustrated in Fig. 6 and indicate that regardless of the direction in which the reaction was studied (KDO or pyruvate and D-arabinose), all 2-keto-3-deoxy-onic acids were formed in this reaction was shown to be KDO by its identity with authentic KDO in several paper chromatographic solvent systems as well as by paper electrophoresis and its reactivity with CMP-KDO synthetase (2).

As illustrated in Table IV, the results of stoichiometry studies on the KDO aldolase reaction indicated that 1 mole each of pyruvate and pentose (D-arabinose standard) were formed for each mole of KDO that disappeared.

Reversibility and Equilibrium Studies—As already shown in the substrate specificity studies (Table II), KDO aldolase appeared to be reversible. Thus, incubation of D-arabinose and pyruvate with the aldolase resulted in the formation of thiobarbituric acid-reactive material; the 2-keto-3-deoxy-onic acid formed in this reaction was shown to be KDO by its identity with authentic KDO in several paper chromatographic solvent systems as well as by paper electrophoresis and its reactivity with CMP-KDO synthetase (2).

The results of equilibrium studies on KDO aldolase are illustrated in Fig. 6 and indicate that regardless of the direction in which the reaction was studied (KDO or pyruvate and D-arabinose as substrates), an equilibrium mixture of the various constituents was attained which consisted of approximately 12% of KDO and 88% of pyruvate and D-arabinose. Further additions of enzyme after this equilibrium had been reached caused no change. From the data obtained in these experiments, the equilibrium constant \( K_{eq} = \frac{[\text{pyruvate}][\text{D-arabinose}]}{[\text{KDO}]} \) was calculated and found to be 0.077 M.

Enzymatic Synthesis of 1-14C-KDO—A variety of studies related to the metabolism of KDO (16) are greatly facilitated by the availability of radioactive KDO of reasonably high specific activity. Preparation of uniformly labeled 14C-KDO (for "Materials and Methods") by isolation from bacterial cell grown in a medium containing a radioactive carbon source.

**Table III**

<table>
<thead>
<tr>
<th>Component</th>
<th>Used or formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>KDO ......</td>
<td>- 0.62</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>+ 0.54</td>
</tr>
<tr>
<td>Arabinose</td>
<td>+ 0.58</td>
</tr>
</tbody>
</table>

* a Incubation mixtures were prepared as described in the text except that the substrate was varied.

* b Incubation mixtures contained the following: sodium pyruvate, 20 μmoles; pentose (or hexosamine), 10 μmoles; phosphate buffer, pH 7, 15 μmoles; and 27 μg of KDO aldolase. The mixtures were incubated for 15 min at 37°, the reaction stopped with trichloracetic acid, and 2-keto-3-deoxy-onic acid was determined as described in the text.

**Table IV**

<table>
<thead>
<tr>
<th>Component Used or formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>KDO ........................... -0.62</td>
</tr>
<tr>
<td>Pyruvate plus D-arabinose . 83</td>
</tr>
<tr>
<td>Pyruvate plus D-ribose, D-xylene, D-lyxose, L-arabinose, D-arabinose 5-phosphate, or N-acetyl-D-mannosamine .................... &lt;5</td>
</tr>
</tbody>
</table>
 inefficient, expensive, and yields a product of low specific activity. KDO aldolase may be utilized for the efficient preparation of 14C-KDO with readily available and reasonably inexpensive starting materials. As the equilibrium of KDO aldolase at physiological substrate concentrations is favorable to KDO degradation, efficient formation of KDO requires high concentration of pyruvate and D-arabinose. In a typical experiment (see legend to Fig. 7), incubation of an excess of KDO aldolase with high concentrations of 14C-pyruvate and D-arabinose (final concentrations, 0.16 M and 0.32 M, respectively) resulted in the formation of 14C-KDO in approximately 41% yield (based on pyruvate). As shown in Fig. 7, radioactive KDO may be readily separated from the unused pyruvate by ion exchange chromatography. The 14C-KDO solution may then be deionized and concentrated; the unused 14C-pyruvate may be isolated in a similar manner and recycled in the procedure repeatedly until essentially all of it has been converted to 14C-KDO.

Radioactive KDO prepared in this manner was shown to contain all of its radioactivity in C-1 by results obtained in ceric sulfate degradation. Thus, as previously indicated, (1) reduction of C-2 of 2-keto-3-deoxy-onic acids followed by treatment with ceric sulfate results in their oxidative decarboxylation to form the corresponding 2-deoxyaldoses possessing 1 carbon atom less (C-1). Treatment of radioactive KDO, isolated from the KDO aldolase incubation mixture, followed by paper chromatography of the resulting 2-deoxy-aldose (2) indicated that the latter contained no detectable radioactivity. These results substantiated the fact that radioactive KDO prepared from 14C-pyruvate and D-arabinose was, indeed, 14C-KDO.

DISCUSSION

It appears likely that the primary physiological function of KDO aldolase is to provide a means for the enzymatic degradation of KDO to common metabolic intermediates. While the aldolase reaction is reversible, there is no evidence to suggest that this reaction contributes to the biosynthesis of KDO. In fact, the equilibrium constant of the KDO aldolase reaction (Keq = 0.077 M) significantly favors the degradation of KDO and efficient synthesis of KDO can be attained only under abnormal concentrations of pyruvate and D-arabinose. Further, the aldolase appears to be strictly an inducible enzyme, at least among the various species of bacteria tested (see Footnote 5). Thus, unless organisms were grown in the presence of KDO, aldolase activity was not detectable in cell-free extracts. The major route of biosynthesis of KDO apparently is via KDO-8-phosphate synthetase, first reported by Levin and Racker (17). This enzyme, constitutively present in a variety of bacterial species, catalyzes the irreversible condensation of phosphopyruvate and D-arabinose 5-phosphate to form KDO-8-phosphate.

The high degree of specificity of KDO aldolase contributes to the utility of this enzyme for use in studies related to the metabolism of KDO. Thus, the identity of unknown 2-keto-3-deoxy-onic acids may be confirmed as KDO by analysis with KDO aldolase. Secondly, the enzyme provides a convenient and inexpensive method for the preparation of specifically 14C-labeled KDO.

A variety of aldolases that cleave 2-keto-3-deoxy-onic acids (or their phosphorylated derivatives) have been reported. Comb and Roseman (18) characterized N-acetylneuraminate aldolase isolated from E. coli K-235 and later, Brunetti, Jourdian, and Roseman (19) studied an enzyme from mammalian tissues that catalyzes an identical reaction. In many respects, the properties (pH optimum, Keq, Keq) of KDO aldolase and N-acetylneuraminate aldolase are quite similar. In addition, it has been suggested (18) that the primary physiological function of N-acetylneuraminate aldolase is to provide a means for the enzymatic degradation of N-acetylneuraminate.

A variety of aldolases have been reported that act specifically on phosphorylated derivatives of 2-keto-3-deoxy-onic acids. Thus, the aldol cleavage of KDG-6-phosphate (20, 21) and of 3-deoxy-D-threo-hexulosonate 6-phosphate (22) results in the formation of equimolar quantities of pyruvate and D-glyceraldehyde 3-phosphate.

REFERENCES
