THE CHEMICAL SYNTHESIS OF 2-KETO-3-DEOXY-OCTONATE AND ITS CLEAVAGE BY A SPECIFIC ALDOLASE[1]

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We have previously reported (Heath and Ghalambor, 1963) the occurrence of 2-keto-3-deoxy-octonate (KDO) as a glycosidically-bound constituent of cell wall lipopolysaccharide (LPS) of Escherichia coli O111-B_4 and the enzymatic synthesis of CMP-KDO (Ghalambor and Heath, 1963). This report presents evidence for the chemical synthesis of KDO and its enzymatic cleavage (KDO-aldolase) to yield equimolar quantities of pyruvate and D-arabinose.

Materials and Methods--- All compounds and enzymes were obtained from commercial sources except as indicated below. The following materials were prepared as previously described (Heath and Ghalambor, 1963): D-arabinose 5-phosphate; natural KDO isolated from LPS or prepared enzymatically; synthetic barium 3-deoxy-D-arabino-heptulosonate 7-phosphate (Sprinson, 1960), a gift from Dr. David B. Sprinson, Columbia University, New York, was converted to its dephosphorylated derivative (KDH). N-Acetyl-neuraminate, N-acetyl-D-mannosamine, and 2-keto-3-deoxy-gluconate (KDG) were gifts from Dr. Saul Roseman of this Unit. D-Ribulose was prepared chemically from D-arabinose (Barnett and Reichstein, 1937).

The following analytical methods were employed: 2-keto-3-deoxy-onic acids by the thiobarbituric acid (TBA) method of Waravdekar and Saslaw (1959)

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as modified by Weissbach and Hurwitz (1959); pentose by the method of Mejbaum (1939); ketoses by the cysteine-carbazole procedure (Dische and Borenfreund, (1951); and pyruvate by a spectrophotometric method using lactic acid dehydrogenase. Paper chromatography was performed on Whatman No. 1 filter paper.[3]

Chemical Synthesis of KDO--- The conditions employed for the synthesis of KDO are essentially the same as those previously described for the synthesis of N-acetyl-neuraminate (Cornforth, Firth and Gottschalk, 1958; Carroll and Cornforth, 1960). Oxalacetic acid (91 mmoles) in 100 ml of water was adjusted to pH 11 at 10°, treated with 250 mmoles of D-arabinose in 150 ml of water, warmed to 23°, and the pH maintained at 11 with KOH. Assay of aliquots (TBA method) indicated a linear rate of KDO synthesis to the theoretical yield in approximately 2 hours.[4] The mixture was adjusted to pH 6.5 and applied to a column which contained Dowex 1-$CO_3^=$ resin (500 ml; 200-400 mesh), the column washed with water, eluted with 0.5 M ammonium bicarbonate (4 liters), the eluate treated with an excess of Dowex 50-H^+ resin, and concentrated *in vacuo* to a syrup. Paper chromatography (repeated development in solvent system A) of this material indicated that it consisted of a mixture of at least three TBA-reactive components which exhibited the following relative migration rates (R_{KDO}): I, 0.75; II, 1.00; III, 1.15. After elution of each of the components from the chromatogram, analysis by the TBA method indicated that the three compounds occurred in the mixture in the following proportions: I, 12%; II, 70%; III, 18%.

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[3]The following solvent systems were used: A, n-butanol, pyridine, 0.1 N HCl (5:3:2); B, ethyl acetate, acetic acid, water (3:1:3); C, isopropanol, n-butanol, water (7:1:2); D, ethyl acetate, pyridine, water (7:1:2). Methods for the detection of compounds on paper chromatograms were as follows: 2-keto-3-deoxy-onic acids by the method of Warren (1960); aldoses by the method of Trevelyan, Procter, and Harrison (1950); and ketoses by the method of Bevenue and Williams (1951).

[4]Although no attempt was made to identify the products of the reactions, similar rates of reaction and yields were obtained when any of the following compounds were substituted for D-arabinose: D-ribose, D-erythrose, or D-glyceraldehyde.

KDO-Aldolase

Aerobacter cloacae (Roseman, Hayes, and Ghosh, 1960) was grown (24 hours at 37°) in a mineral medium (Heath and Ghalambor, 1962) which contained the chemically synthesized KDO mixture (25 millimoles per liter) as the sole source of carbon. The washed cells were sonicated for 15 minutes, the suspension centrifuged, the supernatant fluid treated with 1/8 volume of 2% protamine sulfate solution, centrifuged, and the supernatant fluid fractionated with ammonium sulfate. The fraction that precipitated between 45% and 65% saturation contained most of the activity (15-20 fold purified) and was used for the remainder of the studies.

Characterization of the Products--- Pyruvate was characterized by its reactivity with lactic acid dehydrogenase in the presence of DPNH. D-Arabinose was characterized as the other product of the cleavage by the following criteria: (1) reactivity with the orcinol reagent; (2) paper chromatography in solvent systems B, C, and D, revealed a single neutral component which migrated with authentic arabinose and was clearly distinguishable from the other known aldopentoses; (3) reactivity of the neutral product with L-fucose isomerase[5] (Green and Cohen, 1956).

Stoichiometry--- For this determination, the following incubation mixture was prepared (μmoles in 0.4 ml): KDO, 1.55; phosphate buffer, pH 7, 25; DPNH, 1.5; 100 μg lactic acid dehydrogenase; and 500 μg of KDO-aldolase. After incubation at 37° for 30 minutes, analysis of the mixture indicated the following stoichiometry (μmoles): KDO, -0.65; pyruvate, +0.59; arabinose, +0.66.

Specificity and Reversibility--- To determine the specificity of KDO-aldolase, the following incubation mixture was prepared (final volume = 0.125

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[5]When 600 mμmoles of the pentose were incubated for 15 minutes with 0.025 ml of crude extract of L-fucose-grown E. coli, 80 mμmoles of ketose were formed; chromatography (solvent systems B and C) of the products of this reaction indicated the presence only of arabinose and a ketose which migrated identically with authentic D-ribulose. While authentic D-arabinose and L-fucose were active in the isomerase system, no detectable amounts of ketose were formed when L-arabinose, D-ribose, D-lyxose, or D-xylose were used as substrates.

ml): 300 mμmoles substrate; 10 μmoles phosphate buffer, pH 7; 200 μg protein. After incubation for 15 minutes at 37°, the mixture was deproteinized and analyzed by the TBA method; substrate was added to controls after deproteinization. Results were as follows (mμmoles substrate disappearance): KDO (prepared enzymatically), 140; KDO (isolated from LPS), 140; KDH, KDG, and N-acetyl-neuraminate all were inactive (less than 8).

Reversibility of the enzymatic reaction was demonstrated when the aldolase was incubated with pyruvate and D-arabinose; KDO was detected with the TBA reaction. Since the enzyme is relatively specific in the direction of cleavage, its specificity was determined in the reverse direction using incubation mixtures containing (μmoles in 0.25 ml): aldose, 10; sodium pyruvate, 20; phosphate buffer, pH 7.0, 10; and 200 μg of protein. After 15 min at 37°, the reaction was stopped, aldoses added to the control tubes, and the mixtures were analyzed by the TBA method with the following results (mμmoles of TBA-reactive material): D-arabinose, 125; D-ribose, 36; D-arabinose-5-P, L-arabinose, D-xylose, D-lyxose, and N-acetyl-D-mannosamine were inactive (less than 3).

Enzymatic Identification of the Components of Chemically Synthesized KDO– As indicated previously in this report, the chemically synthesized KDO mixture was resolved into three components by paper chromatography; Compounds I, II, and III. The synthetic compounds were analyzed with two enzymes specific for KDO (KDO-aldolase and CMP-KDO synthetase). The results of these analyses, given in Table 1, show that both enzymes exhibited essentially identical activities with Compound II as they did with natural KDO.

Discussion--- The chemical procedure originally employed in the synthesis of N-acetyl-neuraminate (Cornforth, Firth, and Gottschalk, 1958) has now been demonstrated to be generally applicable to the synthesis of 2-keto-3-deoxy-onic acids.[4] In contrast to the acetylhexosamines, the aldoses examined in the present experiments react at a very rapid rate and give essentially quantitative yields of products, although it is apparent that mixtures of

Table 1

Enzymatic Identification of the Components of Chemically Synthesized KDO

SUBSTRATE	ACTIVITY WITH	
	KDO-ALDOLASE[6]	CMP-KDO SYNTHETASE[7]
Natural KDO	92	54
Compound I	3	2
Compound II	84	54
Compound III	9	3

[6]Incubation mixtures contained the following (final volume 0.105 ml): 140 mμmoles substrate; phosphate buffer, pH 7, 10; 250 μg protein. After incubation for 15 minutes at 37°, the mixture was deproteinized and an aliquot was analyzed by the TBA method. Activity is expressed as mμmoles substrate disappearance.

[7]Incubation mixtures contained the following (final volume 0.125 ml): 150 mμmoles substrate; 1 μmole CTP; 0.5 μmole Mg^{++}; 12 μmoles Tris buffer, pH 8; 200 μg protein. After incubation for 15 minutes at 37°, the mixtures were analyzed for CMP-KDO as previously described (Ghalambor and Heath, 1963). Activity is expressed as mμmoles of CMP-KDO formed.

isomers are formed from a single aldose. Further study of this reaction is necessary to determine whether the conditions can be improved to yield only the desired products.

Evidence has been presented to indicate that KDO-aldolase isolated from extracts of A. cloacae catalyzes an aldol cleavage of KDO as follows:

2-Keto-3-Deoxy-Octonate ⇄ Pyruvate + D-Arabinose

As previously discussed (Ghalambor and Heath, 1963) the biosynthesis and activation of KDO and the sialic acids are analogous. This analogy can now be extended to another enzymatic reaction, KDO-aldolase and N-acyl-neuraminic acid aldolase; the latter enzyme catalyzes the reversible cleavage of the sialic acids to pyruvate and N-acyl-D-mannosamine (Comb and Roseman, 1960). Similarly, it is anticipated that KDO-aldolase will be as useful a tool in

the study of the metabolism of KDO (as a means of specific analysis of KDO and the preparation of specifically-labeled KDO) as NAN-aldolase has been in studies on the metabolism of the sialic acids (Brunetti, Jourdian, and Roseman, 1962). A similar aldolytic cleavage of 2-keto-3-deoxy-D-glucarate has been reported (Fish and Blumenthal, 1963).

The availability of KDO-aldolase provides an opportunity for more definitive characterization of KDO isolated from natural sources (Heath and Ghalambor, 1963), although the configuration of the hydroxyl group at carbon atom 4 of natural KDO remains to be established.

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