2-KETO-3-DEOXY-OCTONATE, A CONSTITUENT OF CELL WALL LIPOPOLYSACCHARIDE PREPARATIONS OBTAINED FROM ESCHERICHIA COLI*

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The purpose of this paper is to present evidence for the occurrence of a 2-keto-3-deoxy-onic acid (Compound I) as a glycosidically bound constituent in preparations of cell wall lipopolysaccharide (LPS) obtained from Escherichia coli O111-B_4 and from a mutant of this organism, E. coli J-5 (Heath and Elbein, 1962). Based on results obtained by specific colorimetric techniques, chromatography, and chemical degradation studies, Compound I is indistinguishable from 2-keto-3-deoxy-octonate (KDO). Levin and Racker (1959) purified an enzyme (2-keto-3-deoxy-phospho-octonate synthetase) from extracts of Pseudomonas aeruginosa which catalyzed the following reaction: D-arabinose-5-P + PEP → KDO-8-P + Pi. Compound I appears to be identical to the dephosphorylated product of KDO-8-P obtained by Levin and Racker.

Materials and Methods--- All compounds and enzymes were obtained from commercial sources except as indicated below. Crystalline calcium 2-keto-3-deoxy-gluconate (KDG) was a gift from Dr. Saul Roseman of this University. Synthetic barium 3-deoxy-D-arabino-heptulosonate 7-phosphate (Sprinson, 1960) was a gift from Dr. David B. Sprinson, Columbia University, New York. This compound was converted to the potassium salt, treated with potato acid phosphatase, and the dephosphorylated product (KDH) was isolated by ion-exchange

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and charcoal chromatography as described below for the isolation of Compound I. KDO was prepared from D-arabinose-5-P and PEP (Levin and Racker, 1959) with an enzyme preparation from E. coli O111-B_4 obtained as follows: after treatment with protamine sulfate, the crude extract was fractionated with ammonium sulfate; the fraction which precipitated between 50 and 70% saturation contained most of the 2-keto-3-deoxy-phospho-octonate synthetase activity. The preparation was inactive when PEP and either D-ribose-5-P, D-erythrose-4-P, or D-arabinose were used as substrates. After incubation of this protein fraction with PEP and D-arabinose-5-P, 80% of the thiobarbituric acid reactive material formed was isolated (as described below for Compound I) as the phosphate-free compound, presumably due to the presence of phosphatases in this relatively crude enzyme preparation. D-Arabinose-5-P was prepared from crystalline D-glucosamine-6-P (kindly supplied by Dr. G. W. Jourdian, of this University) by degradation with ninhydrin according to the method of Pontis (1955), followed by isolation of the pentose phosphate on ion-exchange resin and precipitation of the barium salt with ethanol. The overall yield of arabinose-5-P obtained in this procedure was approximately 80%. D-Erythrose-4-P was a gift from Dr. C. E. Ballou, University of California, Berkeley.

LPS was prepared by the following methods: phenol extraction (Westphal, et al., 1952); trichloracetic acid extraction (Boivin, et al., 1933); ether extraction (Ribi, et al., 1959); digestion of acetone-dried cells with Pronase (California Corp. for Biochem. Research, Los Angeles), followed by precipitation with Mg^{++} (Osborn, et al., 1962). Alkaline degradation of LPS was performed according to the method of Neter, et al. (1956). The thiobarbituric acid (TBA) method used was that of Waravdekar and Saslaw (1959) as modified by Weissbach and Hurwitz (1959). Paper chromatography was performed on Whatman No. 1 filter paper employing the following solvent systems: A, 2-butanone, acetic acid, water (8:1:1); B, ethyl acetate, acetic acid, water (3:1:3); C, water saturated phenol; D, n-butanol, pyridine, 0.1 N HCl (5:3:2). Methods for the detection of compounds on paper chromatograms were as follows: 2-keto-

3-deoxy-onic acids by the method of Warren (1960); 2-deoxy-aldoses by the method of Gordon, et al. (1956).

Isolation of Compound I from LPS--- The LPS preparations were each dissolved in 0.1 N H_2SO_4 and heated in a boiling water bath for 10 min. The hydrolysate was neutralized by the addition of saturated $Ba(OH)_2$ solution, centrifuged, and the clear supernatant fluid was applied to a column of Dowex-1-formate resin (200-400 mesh). Solutions of increasing concentrations (0.2 to 0.5 N) of formic acid were passed through the column. The TBA-reactive material was eluted in a symmetrical peak with 0.5 N formic acid and the pooled fractions were continuously extracted with ether for 24 hours. The compound was further purified by adsorption on charcoal (Type CAL, 12 x 40 mesh, Pittsburgh Coke and Carbon Co.), followed by elution with methanolic ammonia solution (MeOH, 47; water, 50; conc. NH_4OH, 3). The charcoal eluate was concentrated to dryness, dissolved in water, treated with Dowex-50-H^+ resin, and neutralized with KOH. Analysis of this preparation indicated that it was nitrogen and phosphorus free. (Similarly, the phosphatase-treated KDH and the enzymatically synthesized KDO preparations isolated by this procedure were free of nitrogen and phosphorus).

All of the LPS samples examined, regardless of their source (parent or mutant) or the method of preparation, contained Compound I, in amounts ranging from 1 to 4% of the dry weight. Treatment of these preparations with alkali or with a proteolytic enzyme (Pronase) did not significantly alter their composition. Compound I contained in the preparations (including those treated with alkali or Pronase) could not be separated from LPS by high voltage paper electrophoresis (100 volts/cm, for 2 hours). When LPS preparations were partially precipitated by treatment with limiting quantities of specific antiserum, Compound I (bound to polymer) was precipitated to the same degree as other carbohydrate constituents of LPS, such as colitose. Preliminary studies indicated that Compound I is terminally located in the polymer; thus, hydrolysis of LPS preparations with soluble cation exchange resin (Painter, 1960) resulted in rapid and complete removal of Compound I before the liberation of any of

the other sugar components was detected. The nature of the bond between Compound I and polymer was concluded to be glycosidic on the basis of the following: acid lability; alkaline stability; and resistance of the carbonyl group to reduction with $NaBH_4$ prior to acid hydrolysis.

Characterization of Compound I--- Analysis of Compound I by the TBA method produced a chromogen typical of those obtained with 2-keto-3-deoxy-onic acids (Weissbach and Hurwitz, 1959; Waravdekar and Saslaw, 1959); no TBA reaction was obtained when Compound I was reduced with $NaBH_4$. Compound I reacted with semicarbazide to give a typical α-keto acid semicarbazone derivative (MacGee and Doudoroff, 1954) with an absorption maximum at 250 mμ.

Chromatography in a variety of solvent systems that distinguish between KDO, KDH, and KDG indicated that Compound I migrated identically to enzymatically synthesized KDO (Table 1).

Table 1

Paper Chromatography of Compound I

Compound	R_{KDG} in Solvent Systems			
	A	B	C	D
I	.56	.70	.59	.51
KDO	.56	.70	.58	.52
KDH	.79	.85	.81	.71
KDG	1.00	1.00	1.00	1.00

Additional evidence was sought to establish the identity of Compound I by oxidative decarboxylation to the corresponding 2-deoxy-aldose. The procedure involved reduction of Compound I and the reference compounds with $NaBH_4$, followed by oxidation with ceric sulfate. The yields of 2-deoxy-aldoses ranged from 30 to 40% as determined by the TBA method (the chromogen obtained with 2-deoxy-aldoses absorbs at 532 mμ and is alkali stable). Two of

the products were further characterized as follows: (1) 2-deoxyribose (from KDG) colorimetrically (Dische, 1930); (2) 2-deoxy-D-glucose (from KDH) with glucose oxidase (Sols and Fuente, 1957). Paper chromatography of the oxidation products obtained from Compound I and the reference compounds gave the results shown in Table 2, and indicate that Compound I is, indeed, KDO.

Table 2

Paper Chromatography of Degradation Products*

Solvent System	2-Deoxy Ribose	2-Deoxy Glucose	Degradation Product of			
			Comp. I	KDO	KDH	KDG
A	1.00	.65	.37	.36	.64	.97
B	1.00	.75	.54	.54	.75	.98

* Mobilities expressed as $R_{2\text{-deoxyribose}}$

All of the evidence listed above, and that presented in the accompanying paper, are consistent with the conclusion that Compound I is identical to the KDO originally obtained by Levin and Racker (1959). We should note, however, that stereoisomers of KDO were not available for examination, and it is therefore conceivable that Compound I is such an isomer.

Discussion--- The evidence presented in this paper clearly establishes the occurrence of 2-keto-3-deoxy-octonate as a glycosidically bound constituent of cell wall lipopolysaccharide preparations obtained from E. coli O111-B_4 and of E. coli J-5. This compound may be similar to the TBA-reactive material observed by Sundararajan, et al. (1962) in a variety of strains of E. coli K-12. We have also observed (by colorimetric analyses) the presence of a nitrogen-free, KDO-like substance(s) in a variety of strains of E. coli, Salmonella typhi-murium, S. typhosa, and S. adelaide. It is of interest to

note that Osborn* has observed the presence of a KDO-like compound in oligosaccharides obtained by mild acid hydrolysis of LPS from S. typhi-murium.

The previously described constituents of LPS from E. coli 0111-B_4 included lipid, phosphorus, hexosamine, heptose, glucose, galactose, and colitose (Westphal and Luderitz, 1960). While all of the data presented above suggest that KDO is indeed, an integral component of LPS of these organisms, further proof is necessary to definitively establish this point. For instance, none of the criteria presented above rule out the possibility that a KDO-containing substance, other than LPS, may be present in these preparations.

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