The Relation of 3-Deoxy-2-oxo-octonate to the Serological and Physical Properties of a Lipopolysaccharide from a Rough Strain of *Escherichia coli*

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1. A water-soluble preparation of lipopolysaccharide was isolated from the extracellular lipopolysaccharide-phospholipid-protein complex formed by *Escherichia coli* A.T.C.C. 12408. 2. Heating at 100° and pH 4.6 caused a rapid decrease in serological activity concomitant with the release of 3-deoxy-2-oxo-octonate; aggregation of the molecules also occurred. 3. Further evidence that the release of 3-deoxy-2-oxo-octonate is related to loss of serological activity was obtained by comparing the effects of heating for 1 hr. at 100° and pH values between 5.0 and 9.0; detectable changes still occurred at pH 7.5. 4. 3-Deoxy-2-oxo-octonate was isolated from the products of hydrolysis of lipopolysaccharide and shown to be an effective inhibitor of the precipitin reaction between lipopolysaccharide and homologous antiserum. 5. The possibility that 3-deoxy-2-oxo-octonate is joined to the lipopolysaccharide through a phosphodiester linkage is discussed.

When a lysine-requiring mutant of *Escherichia coli*, A.T.C.C. 12408, is grown under lysine-limiting conditions a number of products accumulate in the culture fluid. These include a polymer, designated lipoglycopeptide (Bishop & Work, 1965), which contains a lipopolysaccharide component indistinguishable from the lipopolysaccharide of the cell wall. The carbohydrate components of the lipopolysaccharide were glucosamine, glucose, galactose, heptose and KDO†; all except glucosamine are components of the polysaccharide moiety, which is consistent with the organism being a rough strain (Taylor, Knox & Work, 1966).

Studies on the immunological properties of lipopolysaccharides from strains of *Salmonella* and *Escherichia* have led to the conclusion that the polysaccharide moiety is the serologically reactive component, and that the specificity depends on the sequence of, and linkages between, the carbohydrate components that are attached as side chains to the heptose-containing 'backbone' of the polysaccharide (Staub & Westphal, 1964). However, the polysaccharide component obtained from the extracellular lipopolysaccharide of *E. coli* A.T.C.C. 12408 by mild acid hydrolysis did not react with antiserum to the lipopolysaccharide nor did it inhibit the precipitin reaction between

lipopolysaccharide and homologous antiserum (K. W. Knox, unpublished work). Apart from the removal of lipid, the only known difference between preparations of the lipopolysaccharide and its polysaccharide component was the lower content of KDO in the polysaccharide (Taylor *et al.* 1966). Osborn (1963) showed that the conditions employed for hydrolysing the linkage between the polysaccharide and lipid moieties of lipopolysaccharide result in the liberation of free KDO. KDO is also the terminal reducing group of the polysaccharide moiety but the liberation of free KDO proceeds more rapidly than the hydrolysis of the linkage between the polysaccharide and lipid moieties. Thus the conditions required for release of the polysaccharide also cause its partial degradation.

The present paper describes the results of physical and serological studies on the changes associated with the release of free KDO from the lipopolysaccharide from *E. coli* A.T.C.C. 12408. Further, it was shown that KDO isolated from the products of partial hydrolysis is an effective inhibitor of the precipitin reaction between lipopolysaccharide and homologous antiserum.

MATERIALS AND METHODS

Preparation of lipopolysaccharide. *E. coli* A.T.C.C. 12408 was grown under conditions of lysine-limitation for 26 hr. (Bishop & Work, 1965). The extracellular lipopolysaccharide was present in the culture fluid as a complex with phospholipid and protein and was isolated by a

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† Abbreviation: KDO, 3-deoxy-2-oxo-octonate ('2-keto-3-deoxyoctonate').

modification of the method of Bishop & Work (1965). The cell-free culture fluid was shaken with 2.5% chloroform and allowed to stand overnight at room temperature. The opalescent solution was then centrifuged at 14000g for 20 min. and the residue suspended in water. The suspension was transferred to a vacuum rotary evaporator. With the removal of chloroform *in vacuo* the contents of the flask changed from a milky suspension to an opalescent solution.

The resulting solution of the lipopolysaccharide complex was dialysed and freeze-dried. Phospholipid was removed by extraction with methanol-chloroform and the lipopolysaccharide-protein residues were freeze-dried. The lipopolysaccharide and protein components were then separated by heating in aq. 45% (w/v) phenol at 65° for 10 min., followed by cooling to 4° and centrifuging to separate the aqueous and phenolic phases (Westphal, Lüderitz & Bister, 1952). The aqueous phase was dialysed and centrifuged at 100000g for 1 hr.; the supernatant fluid contained approx. 90% of the recovered lipopolysaccharide, the remainder being deposited as a gel. The solution, which contained 9 mg. of lipopolysaccharide/ml., was water-clear and viscous. Because of the known purity of the lipopolysaccharide-phospholipid-protein complex, further purification of the lipopolysaccharide component was not considered necessary, particularly as such procedures decrease its solubility. Freeze-drying also rendered the lipopolysaccharide insoluble in water and accordingly the solution was sterilized by membrane filtration and stored at 2°, the concentration (9 mg./ml.) being determined by freeze-drying a portion of the solution. The freeze-dried material has been analysed and shown to be similar in composition to the lipopolysaccharide isolated by Taylor *et al.* (1966).

For the quantitative recovery of lipopolysaccharide, Osborn, Rosen, Rothfield & Horecker (1962) extracted phenol from the aqueous phase with ether, and then added $MgCl_2$ solution to give a final concentration of 25 mM. The presence of 25 mM- $MgCl_2$ in the dialysed solution of lipopolysaccharide under investigation did not give a precipitate unless ether was added to give a final concentration of about 5% (v/v); under these conditions 90% of the lipopolysaccharide preparation was recovered in the precipitate and 1% in the supernatant liquid.

Polysaccharide component of lipopolysaccharide. Freeze-dried lipopolysaccharide (200 mg.) was heated at 100° for 15 min. in 10 ml. of 0.01 N-acetic acid. The suspension was then cooled and centrifuged at 100000g for 2 hr.; the supernatant liquid was dialysed and freeze-dried (yield, 60 mg.) (Taylor *et al.* 1966).

Isolation of KDO. KDO was isolated from a freeze-dried preparation of lipopolysaccharide. The material (2.1 g.) was suspended in 100 ml. of 25 mM-ammonium acetate buffer, pH 4.6, and heated at 100° for 1 hr. After cooling, the suspension was centrifuged at 100000g for 1 hr. The supernatant solution was concentrated *in vacuo* at 35° to 12 ml., applied to a column (94 cm. $\times$ 2.5 cm.) of Sephadex G-10 and the column eluted with water (5 ml. of eluate/tube). Carbohydrate-containing polymers were eluted in fractions 37-47 and free KDO was eluted in fractions 51-53. An authentic sample of KDO was kindly supplied by Dr E. C. Heath, School of Medicine, The Johns Hopkins University, Baltimore, Md., U.S.A.

The eluates containing KDO were examined for purity by paper chromatography in butan-1-ol-acetic acid-water

(67:10:23, by vol.), followed by detection of carbohydrates with thiobarbituric acid (Warren, 1960) and $AgNO_3$ (Trevelyan, Procter & Harrison, 1950); only one component was present. The fractions were then pooled and the purity confirmed by chromatography in butan-1-ol-pyridine-water (6:4:3, by vol.), and aq. phenol, NH_3 atmosphere. The yield of KDO was 20 mg. The solution was freeze-dried, redissolved in 1 ml. of veronal-sodium acetate-HCl buffer, pH 7.0 (Michaelis, 1931), and diluted with 0.9% NaCl to 20 μ moles/ml.

Analytical methods. Free and polysaccharide-bound KDO were estimated by the procedures described by Osborn (1963).

Biological methods. Antiserum was prepared by repeated injection of rabbits with the lipopolysaccharide complex. The reaction between the soluble lipopolysaccharide preparation and antiserum was determined by the quantitative precipitin method, the amount of protein in the precipitate being estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). A preliminary experiment on the amount of precipitate obtained on adding increasing amounts of lipopolysaccharide (5-50 μ g. in 1 ml. of 0.9% NaCl) to 0.5 ml. of antiserum showed that studies could be carried out on 40 μ g. of material. The amounts were decreased for the investigation of inhibition of the precipitin reaction by component sugars. A solution of the carbohydrate to be tested (in 0.5 ml. of 0.9% NaCl) was mixed with 0.2 ml. of antiserum and kept at 37° for 1 hr., and the lipopolysaccharide solution (15 μ g./0.3 ml.) was then added; the mixtures were left at 37° for 1 hr. and then at 4° for 3 days. The degree of inhibition was determined by comparing the results of triplicate experiments with those obtained in the absence of added carbohydrate.

The primary inflammatory response of rabbits to endotoxin was examined by the procedure described by Taylor *et al.* (1966).

The toxicity of lipopolysaccharide preparations was compared by injecting 0.2 ml. of a sterile solution into the yolk sac of 5-day-old fertile eggs. The doses employed were 25, 12.5, 6.25, 3.12 and 1.66 μ g., each being injected into ten eggs. After 48 hr. at 37° the eggs were examined for viability, and the LD₅₀ was estimated by the method of Reed & Muench (1938). Each result is the mean of three estimations.

Determination of the effect of alkaline phosphatase on polysaccharide and partially degraded lipopolysaccharide. The polysaccharide component of lipopolysaccharide was dissolved in veronal-sodium acetate-HCl buffer, pH 8.0 (Michaelis, 1931). To 0.4 ml. of solution (containing 1.5 mg.) was added *E. coli* alkaline phosphatase (0.025 ml.) (Worthington Biochemical Corp., Freehold, N.J., U.S.A.), 0.1 M- $MgCl_2$ (0.025 ml.) and water (0.05 ml.) (Morton, 1957). The experiment was carried out in duplicate, and controls were included without enzyme. After incubation at 37° for 16 hr., 10% (w/v) trichloroacetic acid (2 ml.) was added and inorganic phosphate estimated (Chen, Toribara & Warner, 1956).

Lipopolysaccharide (1 ml. containing 9 mg.) was mixed with 0.25 ml. of veronal-sodium acetate-HCl buffer, pH 5.0 and heated for 1 hr. at 100° in a stoppered tube. After cooling, the pH was adjusted to 8.0 and the action of alkaline phosphatase investigated as described above. The experiment was carried out in duplicate, with duplicate

controls set up as follows: (a) solution not heated, enzyme not added; (b) solution not heated, enzyme added; (c) solution heated, no enzyme added. The procedure was repeated on lipopolysaccharide that had been heated for 4 hr. in veronal-sodium acetate-HCl buffer, pH 7, pH 8 and pH 9.

RESULTS

Biological and physical changes associated with the release of KDO at pH 4.6. Sealed tubes containing 2 ml. of a solution of lipopolysaccharide (8 mg./ml.) in 25 mM-ammonium acetate buffer, pH 4.6, were heated at 100° for appropriate intervals up to 4 hr., and the rate of decrease in serological reactivity was compared with the rate of release of free KDO (Fig. 1). The serological reactivity had decreased by approx. 90% in 30 min., the decrease occurring concomitantly with the release of free KDO. The maximum amount of free KDO (obtained after 30 min. heating) was 2.3% of the dry wt. of starting material. Release of lipid, revealed by the appearance of a turbidity in the solution, was detected after heating for 90 min.; on more prolonged heating, a gradual increase in the opalescence of the solution indicated a continuing slow release of lipid.

Although samples heated for 30, 45 and 60 min. were clear immediately after heating, a gel settled out when the samples were left overnight at 4°, the amount of gel progressively decreasing with the longer heating times. Heating for less than 30 min. produced no visible gel formation, but evidence for

aggregation of molecules was provided by examination of the solutions in the Spinco model E analytical ultracentrifuge. The unheated lipopolysaccharide solution contained only one detectable component, the sedimentation coefficient (corrected to 20° in water) being 4.1 s. Immediately after heating for 10, 20 and 30 min. the sedimentation coefficients for the major component were 4.9 s, 8.8 s and 9.5 s respectively.

It has proved possible to fractionate partially degraded lipopolysaccharide, since the release of KDO decreased the precipitability of the material by Mg^{2+} in the presence of ether. A solution of lipopolysaccharide (180 mg./20 ml.) was heated at pH 4.6 and 100° for 10 min. After cooling, 1 ml. of ether was added followed by magnesium chloride to give a final concentration of 25 mM. The precipitate that formed was removed by centrifuging, dissolved in 20 mM-EDTA, dialysed against mM-EDTA followed by water and freeze-dried (fraction 1, 105 mg.). The supernatant solution from the precipitation was also dialysed and freeze-dried (fraction 2, 64 mg.). The amounts of KDO that could be released on further mild acid hydrolysis of fractions 1 and 2 were 0.83% and 0.39% respectively, compared with 2.3% for the starting material. The serological activity, as determined by the quantitative precipitin method, had decreased to 38% for fraction 1 and 2% for fraction 2. However, fraction 2 still retained two of the properties of endotoxin: (1) the LD₅₀ for chick embryos was 12 µg. compared with 7 µg. for the starting material and for fraction 1; (2) it produced inflammatory lesions in the skin of rabbits at the same dose level (0.5 µg.) as untreated lipopolysaccharide, though with less oedema.

Effect of pH on the release of KDO and loss of serological reactivity. The release of free KDO and the associated loss of serological reactivity also took place at pH 7 (25 mM-ammonium acetate); after 1 hr. at 100° the serological reactivity had decreased to 43% of the starting level, and 64% of the total potentially free KDO had been released. Paper chromatography of the hydrolysate confirmed that KDO was present as the monosaccharide. Heating for 4 hr. at pH 7.0 produced changes similar to those obtained after heating at pH 4.6 for 30 min., namely maximal release of KDO, minimal serological activity and formation of a gel on standing at 4°.

To investigate the effect of pH more fully, solutions of lipopolysaccharide (1.0 ml. containing 9 mg.) were heated at 100° for 1 hr. with 0.5 ml. of veronal-sodium acetate-hydrochloric acid buffer at pH 5.0, 6.0, 7.0, 7.5, 8.0 and 9.0 (Fig. 2). These experiments showed that KDO release was not detected at pH 8.0 or above, and it was only at these pH values that serological activity was not

Fig. 1. Comparison of the rate of release of KDO from lipopolysaccharide with the rate of decrease in serological activity at pH 4.6 at 100°. The amount of free KDO (●) is expressed as a percentage of the amount present after heating for 30 min. (2.3% of the dry wt. of the original material). The serological reactivity (○) was determined by the quantitative precipitin method and is expressed as a percentage of the reactivity of original material.

Fig. 2. Effect of pH on the release of KDO and decrease in serological activity of lipopolysaccharide heated for 1 hr. at 100°. The amount of free KDO (●) is expressed as a percentage of the amount present after heating for 1 hr. at pH 5 (2.4% of the dry wt. of the original material). The serological reactivity of heated material (○) was determined by the quantitative precipitin method and is expressed as a percentage of the reactivity of original material.

Table 1. *Inhibition of the precipitin reaction between lipopolysaccharide and homologous antiserum by monosaccharides*

Antiserum (0.2 ml.) was mixed with the monosaccharide (0.5 ml.) and incubated at 37° for 1 hr.; lipopolysaccharide (15 µg./0.3 ml.) was then added, and after 1 hr. at 37° and 3 days at 4° the protein content of the precipitate was determined. The results are expressed as percentage inhibition of the precipitin reaction by the indicated amount of carbohydrate. —, Not estimated.

Amount of carbohydrate (µmoles).....	Percentage inhibition of precipitin reaction			
	25	10	2	0.5
Carbohydrate				
KDO	—	69	17	10
Glucose	17	—	—	—
Galactose	6	—	—	—

altered. The results provide further evidence that the release of KDO is related to a decrease in serological activity.

Inhibition of precipitation reaction by component sugars. More direct evidence for the role of KDO in the serological specificity of the lipopolysaccharide was provided by comparing the ability

of component sugars to inhibit the precipitin reaction. These results, summarized in Table 1, show that KDO is an effective inhibitor. Neuraminic acid is also a 3-deoxy-2-oxo-alonic acid but 5 µmoles did not cause detectable inhibition.

Action of alkaline phosphatase on partially degraded lipopolysaccharide and the polysaccharide component. The results of the action of alkaline phosphatase on lipopolysaccharide and its polysaccharide component are summarized in Table 2. The enzyme did not act on unheated lipopolysaccharide but did release inorganic phosphate (0.3% of the dry wt. as phosphorus) from the polysaccharide component; the total phosphorus content of the polysaccharide was 3.8% compared with 4.0% for the lipopolysaccharide. Heating a solution of the lipopolysaccharide at pH values between 5.0 and 9.0 resulted in the release of some phosphate as inorganic phosphorus (0.7–1.0% of the dry wt.) in the absence of added enzyme. The subsequent addition of enzyme released an additional 0.2–0.3% of the dry wt. as inorganic phosphate. Since both types of phosphate release occurred at alkaline reactions, they were not dependent on the prior release of detectable KDO (cf. Fig. 2).

DISCUSSION

The presence of KDO as a component of lipopolysaccharide from Gram-negative bacteria was first reported by Heath & Ghalambor (1963). Subsequently Osborn (1963) presented evidence that the major portion of the KDO was released as free sugar from an insoluble preparation of *Salmonella typhimurium* lipopolysaccharide under very mild conditions of acid hydrolysis, 50% being released in 35 min. at 100° and pH 4.5. Under the same conditions, 50% hydrolysis of the linkage between the polysaccharide and lipid components occurred in 135 min., yielding a polysaccharide with a terminal reducing KDO residue. In the present investigation with a soluble extracellular lipopolysaccharide preparation from a strain of *E. coli*, KDO was released even more rapidly, more than 50% of the maximum being free after 5 min. at 100° and pH 4.6. This difference in rates of release from the two types of lipopolysaccharide probably reflects differences in aggregation and solubility rather than in sources of the lipopolysaccharides.

An investigation of the effect of pH on the rate of release of KDO showed detectable hydrolysis in 1 hr. at pH 7.5; only at pH 8 or above was no hydrolysis detected. Heath & Ghalambor (1963) concluded that KDO was linked glycosidically in their lipopolysaccharide, rather than through an ester linkage, and the stability of the extracellular

Table 2. Release of inorganic phosphate from polysaccharide and partially degraded lipopolysaccharide by alkaline phosphatase

The polysaccharide component isolated from lipopolysaccharide after heating in 0.01 N-acetic acid at 100° for 15 min. was incubated with alkaline phosphatase. Lipopolysaccharide previously heated at 100° and pH 5.0 for 1 hr. or at pH 7.0, 8.0 and 9.0 for 4 hr. was adjusted to pH 8 and the mixture incubated with alkaline phosphatase. The amount of phosphorus present as inorganic phosphate after 16 hr. at 37° was then estimated (% of dry wt. of original material); the results are the means of duplicate experiments. —, Not estimated.

Pretreatment	Enzyme	Polysaccharide	Inorganic phosphate (% of dry wt. as phosphorus)			
			Lipopolysaccharide			
			pH 5	pH 7	pH 8	pH 9
Not heated	Absent		0.23	0.24	—	—
Not heated	Present		0.22	0.23	—	—
Heated	Absent	0.30	0.70	1.01	1.03	1.01
Heated	Present	0.60	0.98	1.25	1.23	1.21

lipopolysaccharide at pH 8 and 9 is in agreement with their conclusions. However, hydrolysis at pH 7 indicates that the linkage is extremely labile. KDO is very vulnerable to acid hydrolysis since it is a ketose with a deoxy grouping on the carbon atom next to the keto group. This structure is very similar to that of neuraminic acid, and the rate of release of neuraminic acid from mucoid polymers (Gottschalk, 1960a) is comparable with the rate of hydrolysis of the linkage between the polysaccharide and lipid components of lipopolysaccharide (Osborn, 1963). The more rapid release of free KDO indicates a linkage even more labile than that of KDO joined glycosidically to another sugar, and suggests the presence of a phosphodiester. The effect of such a linkage on the acid-lability of cell-wall polymers has been observed by Knox & Hall (1965). Both KDO (Ghalambor & Heath, 1963) and neuraminic acid (Comb, Shimizu & Roseman, 1959) are known to occur as their cytidine monophosphate derivatives, which contain such a phosphodiester linkage, and both mononucleotides are hydrolysed very rapidly at room temperature and pH 2.

Experimental evidence for the presence of a phosphodiester was sought by studying the effect of alkaline phosphatase on the products of hydrolysis. The enzyme did not release inorganic phosphate from lipopolysaccharide but did act on the polysaccharide isolated from the products of mild acid hydrolysis. The molar ratio of KDO released (2.3%) on heating lipopolysaccharide at pH 4.6 to inorganic phosphate released (0.30%) by the enzyme acting on the polysaccharide component was 1:1. This is consistent with a structure in which the reducing end group of each KDO residue is attached to the lipopolysaccharide through a phosphodiester linkage. However, attempts to

verify this conclusion by studying the products of partial hydrolysis at pH values from 5 to 9 gave equivocal results, owing to the large amounts of inorganic phosphate released during the preliminary heating, and to the subsequent enzymic release of more inorganic phosphate under alkaline conditions where prior release of KDO may not have occurred. Smith (1949) observed that, when a purified preparation of O antigen from *Shigella shigae* was heated for 24 hr. at 100° and pH values between 3.3 and 5.6, nearly 50% of the organic phosphate was converted into inorganic phosphate.

Lipopolysaccharides contain two phosphorylated sugars, namely glucosamine phosphate, which is a component of lipid A (Westphal & Lüderitz, 1954), and heptose phosphate, a component of the polysaccharide moiety (Slein & Schnell, 1953). The polysaccharide component of lipopolysaccharides consists of a 'backbone' of heptose phosphate residues with side chains of various structures depending on the serological grouping of the organism (Staub & Westphal, 1964). Each heptose unit is generally assumed to be phosphorylated, though the linkage between the units is not known. The present studies show that only one in 12 of the phosphate groupings in the polysaccharide is hydrolysed by phosphomonoesterase. However, knowledge of the structure of the polysaccharide is not sufficient to enable further conclusions to be drawn.

The release of free KDO at pH 5 was associated with marked changes in the properties of the remaining polymer. Physical changes, evident from increases in sedimentation coefficient, and the appearance of a gel, indicated that aggregation had occurred. KDO, being an α -oxo acid, would be expected to be a relatively strong acid, comparable with neuraminic acid (pK 2.6) (Gottschalk, 1960b).

Ionized neuraminic acid groupings in submaxillary-gland mucin are believed to determine the shape of the molecule (Gottschalk, 1960b). By analogy it is possible that KDO plays a comparable role in determining the shape of the lipopolysaccharide molecule and the interaction between molecules.

The release of KDO from the lipopolysaccharide was also associated with a marked decrease in serological activity. This was shown by comparing the effects of heating for various times at pH 4.6, and also by comparing products heated at pH values between 5.0 and 9.0. The extreme thermal lability of the lipopolysaccharide accounts for the previous observation that the isolated polysaccharide component was not serologically active. Serologically inactive lipopolysaccharide still retains two of the biological properties of lipopolysaccharides, namely toxicity and ability to cause inflammatory lesions in rabbit skin.

It is possible that the loss of serological activity on removal of KDO results from changes in the shape of the molecule and degree of aggregation. However, it is more likely that KDO is the 'immunodominant' sugar (Lüderitz, Staub & Westphal, 1966), as it is a very effective inhibitor of the precipitin reaction. Glucose is a much less effective inhibitor and may be a minor antigenic determinant. However, glucose is not released under the conditions employed for obtaining the serologically inactive polysaccharide (Taylor *et al.* 1966).

By comparison with studies on lipopolysaccharides from other rough strains of *E. coli* and *Salmonella*, it was concluded that the extracellular lipopolysaccharide from *E. coli* A.T.C.C. 12408 contained a heptose 'backbone' to which are attached side chains composed of a glucose and a galactose residue (Taylor *et al.* 1966). Various rough mutants of *E. coli* and *Salmonella* have been described that differ considerably in the structure of their polysaccharide moieties; evidence, where available, indicates that neutral sugars present in the side chains determine serological specificity as in the smooth strains (Staub & Westphal, 1964; Lüderitz *et al.* 1966). KDO is a component of all bacterial lipopolysaccharides so far examined, but

its role as a serological determinant has not previously been reported.

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