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The Distribution of 2-Keto-3-deoxy-octonic Acid in Bacterial Walls

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SUMMARY

A survey of the walls of over 80 bacterial species has been carried out using chromatographic and colorimetric techniques to demonstrate the presence of 2-keto-3-deoxy-octonic acid (KDO) and or sialic acid. In most Gram-negative bacteria KDO but not sialic acid was found in the wall, whereas the walls of Gram-positive bacteria contained neither compound.

INTRODUCTION

2-Keto-3-deoxy-D-*manno*-octonic acid (KDO) was first recognized as a glycosidic component of the lipopolysaccharide (LPS) of *Escherichia coli* O111B4 (Heath & Ghalambor, 1963; Ghalambor, Levine & Heath, 1966), but subsequently it has been found in the lipopolysaccharides of all the members so far examined of the Enterobacteriaceae (Lüderitz, Staub & Westphal, 1966; Williams & Perry, 1969). Furthermore, some strains of Pasteurella and Brucella contain KDO in the lipopolysaccharide fraction of their cell wall (Ellwood, 1968).

Two other 2-keto-3-deoxy-sugar acids have been reported as structural components of bacteria. 2-Keto-3-deoxy-galactonic acid occurs in an extracellular polysaccharide of *Azotobacter vinelandii*, and sialic acid (*N*-acetylneuraminic acid), an *N*-acetyl amino substituted 2-keto-3-deoxy-sugar acid containing 9 carbon atoms, occurs in a polymeric form in several bacteria (Barry & Goebel, 1957; De Witt & Rowe, 1959). All 2-keto-3-deoxy-sugar acids give a positive reaction with thiobarbituric acid after acid periodate oxidation, and on the basis of this non-specific test it was initially thought that sialic acid was widely distributed in Gram-negative bacteria (Aaronson & Lessie, 1960; Irani & Ganapathi, 1962). Later, on the basis of a similar test, it was suggested that KDO was equally widely distributed (Vincent & Cameron, 1967).

In order to clarify this apparent contradiction a survey has been carried out using both colorimetric and chromatographic analysis for the presence of 2-keto-3-deoxy-sugar acids in the cell walls of a wide selection of bacteria. Since lipopolysaccharides are components of the envelopes of Gram-negative bacteria, KDO is expected to occur in the wall preparations of the Gram-negative bacteria only, nevertheless, both Gram-positive and Gram-negative organisms were examined. A preliminary report has been published (Ellwood, 1966).

METHODS

Organisms. The bacterial species examined are shown in Table 2. They were obtained from the following collections: American Type Culture Collection, Rockville, Md., U.S.A. (ATCC); Microbiological Research Establishment, Salisbury (MRE); National Collection of Industrial Bacteria, Torry Research Station, Aberdeen (NCIB); National Collection of Marine Bacteria, Torry Research Station, Aberdeen (NCMB); National Collection of Type Cultures, Central Public Health Laboratory, London (NCTC); Rothamsted Experimental Station, Harpenden (RES); National Collection of Dairying Organisms, National Institute for Research in Dairying, Reading (NCDO); National Collection of Plant Pathogenic Bacteria, Harpenden, Herts. (NCPPB).

Culture conditions. Bacteria were grown on the surface of a plate count agar (Oxoid) at temperatures facilitating maximum rate of growth. Organisms were harvested by scraping the bacteria from the agar surface with a spatula, washed with saline and disrupted as described below. Three plates (9 cm. diameter) yielded 200 to 300 mg. of bacteria.

Preparation of bacterial walls. Bacteria (*c.* 300 mg. equivalent dry wt in 30 ml. water) were shaken in a vertical shaker (stroke 10 cm.) at 450 strokes/min. in volumes of 10 ml. with Ballotini beads (10 ml. no. 16 grade), for 1 hr. The Ballotini beads were then removed by filtration through a no. 2 glass sinter and the walls were separated by centrifugation (17,000 ***g***; 1 hr), washed 3 times in saline (1 %, w/v) and 3 times in water, lyophilized, and weighed. Yield 30 to 60 mg. initial dry wt of bacteria. The optimum conditions for the release of KDO from wall preparations were established using wall samples of *Escherichia coli* MRE600, and *Klebsiella* (*Aerobacter*) *aerogenes* NCTC418. Samples (50 mg.) of these walls were heated in 0·1 N-H_2SO_4 (5 ml.) at 80 and 100°. The release of KDO was followed by analysing samples by the method described below.

Isolation of the 2-keto-3-deoxy-sugar acids. The samples of bacterial walls (50 mg.) were heated with 0·1 N-H_2SO_4 (5 ml.) at 100° for 30 min. The residual walls were sedimented by centrifugation (17,000 ***g***; 15 min.). The supernatant fluid was neutralized with a saturated solution of $Ba(OH)_2$, centrifuged to remove $BaSO_4$, and passed through a column (0·5 cm. long and 1·0 cm. diam.) of Dowex 1 (HCO_3^- form). The column was then washed with water (5·0 ml.) and the eluate and washings discarded. The column was eluted with 0·5 N-ammonium carbonate (5 ml.), the eluate treated with an excess of Dowex 50 (H^+ form) and the resin removed by filtration. The filtrate was lyophilized and then dissolved in water (0·1 ml.) for chromatographic analysis.

Determination of 2-keto-3-deoxy-sugar acids. This was a modification of the methods of Weissbach & Hurwitz (1959) and Aminoff (1961). Samples (0·2 ml.) of the acid hydrolysates of the wall samples were diluted with water (0·1 ml.). Periodic acid (0·025 M in 0·125 N-H_2SO_4; 0·25 ml.) was added and incubated at about 20° for 20 min. Sodium arsenite (2 %, w/v in 0·5 N-HCl; 0·5 ml.) was next added, shaken, and allowed to stand until the yellow colour was discharged (2 to 3 min.). Thiobarbituric acid (0·3 %, w/v, pH 2·0; 2·0 ml.) was then added and, after shaking, the solution heated to 100° for 10 min. The pink solution was cooled to 4° (when it became cloudy) and shaken with 5·0 ml. of conc. HCl + *n*-butanol (5 + 95, v/v). After centrifugation the extinction of the coloured upper layer was measured in a spectrophotometer at 550 mμ. Under these conditions an optical extinction of 0·430 was obtained from 0·025 μmole of KDO.

Paper chromatography. This was done on acid-washed Whatman no. 1 paper with the following solvent systems: (A) butan-2-ol + acetic acid + water (8 + 1 + 1, v/v), and (B) butan-1-ol + pyridine + 0·1 N-HCl (5 + 3 + 2, v/v). Paper chromatograms were dried and developed by the technique of Anderson (1966).

Preparation of 2-keto-3-deoxy-sugar acids (chromatographic standards). These were prepared by aldol condensation between the appropriate aldose and oxaloacetic acid in alkaline solutions, as described by Ghalambor *et al.* (1966). Glyceraldehyde gave a mixture of 2-keto-3-deoxy-gluconic acid (KDG) and 2-keto-3-deoxy-galactonic acid. These acids ran as one component in both solvents and were used as a chromatographic standard for KDG. Erythrose also gave a mixture of two 2-keto-3-deoxy-heptonic acids. The major component was used as a chromatographic standard for 2-keto-3-deoxy-heptonic acid (KDH). Authentic KDO was kindly supplied (as the penta-acetyl methyl ester) by Dr E. C. Heath; *N*-acetylneuraminic acid was purchased from Koch-Light Laboratories (Colnbrook), Bucks., England.

RESULTS

The determination of KDO depends upon oxidation with periodate under acid conditions to give β-formyl pyruvate, which is then reacted with thiobarbituric acid to form a chromagen with an absorption maximum at 550 mμ. The assay of Weissbach & Hurwitz (1959) is based upon these reactions but has been modified by extracting the

Table 1. *Separation of various 2-keto-3-deoxy-sugar acids by paper chromatography*

	R_{KDO}			
Solvent	KDG	KDH	KDO	*N*-acetyl-neuraminic acid
A	1·78	1·41	1·00	0·85
B	1·93	1·37	1·00	0·84

Solvent A: butan-2-ol + acetic acid + water (8 + 1 + 1, v/v).
Solvent B: butan-1-ol–pyridine + 0·1 N-HCl (5 + 3 + 2, v/v).

chromagen into acid butanol (cf. the assay method for sialic acid, Aminoff, 1961). This extraction step prevents precipitation which otherwise tends to occur at room temperature. The results obtained in this assay are largely specific for KDO: equimolar solutions of *N*-acetylneuraminic acid give less than $\frac{1}{200}$ the colour reaction. The R_F values of the series of 2-keto-3-deoxy-sugar acids are shown in Table 1 and it is apparent that the acids may be differentiated by paper chromatography in these solvents.

Fig. 1 shows the rate of release of 2-keto-3-deoxy-sugar acids from walls of *Escherichia coli* and *Klebsiella aerogenes* by acid hydrolysis at 80 and 100°. The maximum release of these acids from both wall samples was obtained after heating for 30 min. at 100° and these conditions were used for all other wall samples investigated. Paper chromatography of hydrolysates prepared by the standard procedure showed the presence of KDO in nearly all the wall samples of Gram-negative bacteria examined. *N*-acetylneuraminic acid was found in only a few samples. In wall preparations of Gram-positive bacteria no trace of either KDO or *N*-acetylneuraminic acid was found. The results are summarized in Table 2.

Table 2. *The 2-keto-3-deoxy-sugar acids found in bacterial walls*

Species	Strain number	KDO*	*N*-acetylneuraminic acid*	KDO in the cell wall (%)
	Gram-negative bacteria			
Athiorhodaceae				
Rhodopseudomonas palustris	NCIB 8252	+	−	0·1
R. spheroides	NCIB 8253	+	−	0·1
Pseudomonadaceae				
Pseudomonas aeruginosa	l.s.	+	−	0·6
P. angulata	NCPPB 263	+	−	0·5
P. chlororaphis	NCIB 9402	+	−	0·5
P. fluorescens	KB I	+	−	0.4
	NCIB 8248	+	−	0·5
P. iodinum	NCDO 613	+	−	0·05
		−		< 0·01
P. primulae	NCPPB 133	+	−	0·1
P. syncyanae	NCDO 759	+	−	0·7
Xanthomonas campestris	NCPPB 528	+	−	0·1
X. hyacinthi	NCPPB 599	+	−	0.1
X. juglandis	NCPPB 362	+	−	0·1
	XJ 107	+	−	0·2
Acetobacter aceti	NCIB 8554	+	−	0·05
Aeromonas hydrophila	NCMB 72	+	−	0·05
A. liquefaciens	NCMB 87	−	−	< 0·01
Protoaminobacter alboflavus	NCIB 8167	−	−	< 0·01
Mycoplana bullata	ATCC 4278	+	−	0·2
Spirillaceae				
Vibrio cuneatus	NCIB 8194	+	−	0·7
V. foetus	ED 148	+	−	0·4
V. percolans	NCIB 8193	+	−	0·6
Rhizobiaceae				
Rhizobium leguminosarum	RES 317	+	−	0·3
Agrobacterium tumefaciens	NCPPB 397	+	−	0·2
Chromobacterium violaceum	NCTC 7150	+	−	0·2
	NCTC 9373	+	−	0·2
Achromobacteraceae				
Alcaligenes faecalis	NCIB 8156	+	−	0·02
A. metaligenes	NCIB 9021	+	−	0·2
Achromobacter lacticum	NCIB 8208	+	+	0·3
Flavobacterium acidificum	NCMB 683	−	−	< 0·01
F. aurantiacum	NCIB 8204	−	−	< 0·01
		+	−	0·02
F. sauveolens	NCIB 8992	−	−	< 0·01
Agarbacterium alginicum	NCMB 886	+	−	0·05
Enterobacteriaceae				
Escherichia coli	MRE 162	+	+	0·4
	NCTC 8164	+	−	0·7
	MRE 600	+	−	0·7
E. freundii	NCTC 8165	+	+	0·5
Klebsiella aerogenes	NCTC 8167	+	−	0·5
Paracolobactrum aerogenoids	NCTC 8105	+	−	0·6
Klebsiella aerogenes	NCTC 418	+	−	0·5
Erwinia amylovora	NCPPB 595	+	−	0·4
E. carnegieana	NCPPB 671	+	−	0·5
Serratia keilensis	NCTC 4619	+	−	0·2
S. marcescens	NCTC 1377	+	−	0·5
Proteus vulgaris	NCIB 8064	+	−	0·4

Table 2 (*cont.*)

Species	Strain number	KDO*	*N*-acetylneuraminic acid*	KDO in the cell wall (%)
	Gram-negative bacteria			
P. mirabilis	PR-27	+	−	0·3
Salmonella typhimurium	MRE LT/2	+	−	0·6
Shigella shigae	NCTC4837	−	−	<0·01
S. flexneri	NCTC8192	+	−	0·3
Brucellaceae				
Brucella abortus	544	+	−	0·3
	45/0	+	−	0·2
	45/20	+	−	0·1
B. melitensis	6015	+	−	0·3
B. suis	PS III KG25 MRE43	+	−	0·4
Pasteurella pestis	TS MRE100	+	−	0·5
	EV76 MRE103	+	−	0·4
P. pseudotuberculosis	MRE321V	+	−	0·4
Yersinia enterocolitica	l.s.	+	−	0·3
P. multocida	l.s.	+	−	0·1
P. tularensis	MRE125	−	−	<0·01
P. septica	l.s.	+	−	0·05
Neisseriaceae				
Neisseria catarrhalis	NCTC8554	+	−	0·05
	Gram-positive bacteria			
Micrococcaceae				
Micrococcus lysodeikticus	MRE310	−	−	<0·01
Staphylococcus aureus	NCTC6751	−	−	<0·01
S. epidermidis	l.s.	−	−	<0·01
S. lactis	NCTC7944	−	−	<0·01
Propionibacterium shermanii	l.s.	−	−	
Lactobacillaceae				
Lactobacillus plantarum	l.s.	−	−	<0·01
Brevibacteriaceae				
Kurthia zopfii	NCTC404	−	−	<0·01
Corynebacteriaceae				
Corynebacterium viscosum	NCTC2416	−	−	<0·01
C. xerosis	NCTC9755	−	−	<0·01
Arthrobacter globiformis	NCIB8907	+	−	0·02
Bacillaceae				
Bacillus anthracis	NCTC8234	−	−	<0·01
B. brevis	NCTC7577	−	−	<0·01
B. megaterium	NCTC2607	−	−	<0·01
B. subtilis	ATCC9372	−	−	<0·01
Mycobacteriaceae				
Mycobacterium smegmatus	NCTC8159	−	−	<0·01
Actinomycetaceae				
Actinomycetes bovis	NCTC9430	−	−	<0·01

* KDO and *N*-acetylneuraminic acid were characterized and detected by paper chromatograph in solvents A and B while KDO was estimated by a modification of the method of Weissbach & Hurwitz (1959). Laboratory strains are identified as l.s. Strains whose designations are unspecified in the text were gifts from Dr H. E. Wade and Dr J. Keppie of this establishment.

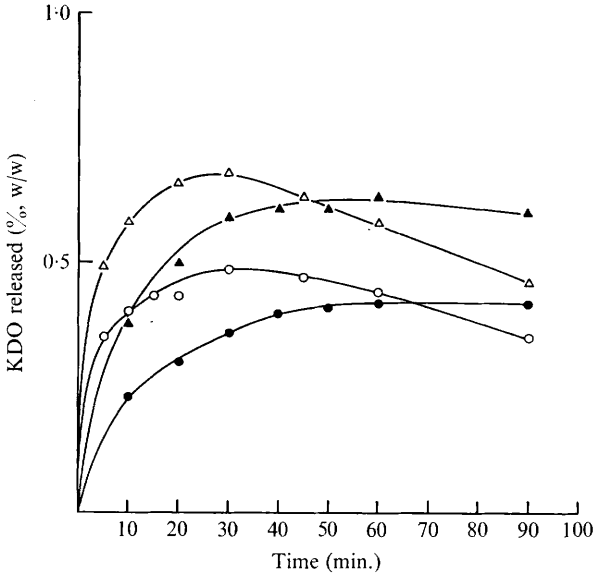

Fig. 1. Release of KDO from walls of *Escherichia coli* MRE600 (50 mg.), at 100° (△) and 80° (▲), and from *Klebsiella aerogenes* NCTC418 (50 mg.), at 100° (○) and at 80° (●), by heating samples with 0·1 N-H_2SO_4 (5 ml.). KDO was estimated by a modification of the method of Weissbach & Hurwitz (1959).

DISCUSSION

Sialic acid occurs either as a polyneuraminic acid (called colominic acid) in several bacterial species or as part of other heteropolysaccharides, such as the K antigen, in other bacterial species (for review see Lüderitz, Jann & Wheat, 1968). A sensitive test for sialic acid is based on the formation of β-formyl pyruvic acid upon periodate oxidation and the subsequent formation of a chromagen formed on heating the β-formyl pyruvate with thiobarbituric acid. This procedure provides the basis of quantitative estimation for sialic acid (Warren, 1969; Aminoff, 1961). The periodate oxidation is carried out in 9 M-phosphoric acid in the Warren procedure and Aminoff used 37° for the oxidation. The high acid concentration or increased temperature is necessary to get a full release of β-formyl pyruvate. However, KDO will also yield β-formyl pyruvate on periodate oxidation and thus give a chromagen with thiobarbituric acid identical to that given by sialic acid.

The Warren procedure was used by Aaronson & Lessie (1960) and Irani & Ganapathi (1962), who considered the test to be sufficiently specific to demonstrate the wide distribution of sialic acid in bacteria. Vincent & Cameron (1967), using the assay system of Weissbach & Hurwitz (1959) in which periodate oxidation is carried out at room temperature, showed that KDO occurred in a similar range of bacteria to those thought to contain sialic acid by Aaronson & Lessie (1960). Our results using room temperature for the periodate oxidation confirm in general the presence of KDO and absence of *N*-acetylneuraminic acid. Recently Hackenthal (1969) showed by ion exchange and paper chromatography that the group-specific polysaccharide of type C meningococci contained both KDO and sialic acid.

A further complication in comparing the present work with that of Aaronson & Lessie (1960) and of Vincent & Cameron (1967) is that they measured material reactive to thiobarbituric acid in hydrolysates of whole bacteria. These preparations would also contain 2-keto-3-deoxy-sugar acids involved in the metabolic pathways of bacteria. We have used wall preparations because this is the location of LPS and capsular fractions of the organisms. The walls were hydrolysed with acid under conditions known to produce maximum yields of thiobarbituric-acid-reactive material in *Escherichia coli* and *Klebsiella aerogenes* and there was no evidence for the presence of 2-keto-3-deoxy-sugar acids with less than 8-carbon atoms.

Nearly all wall samples of Gram-negative bacteria contained KDO. The wide variation in amounts of KDO found may reflect differences in the composition of the walls of different bacteria. However, it is known that the KDO content of the wall of *Klebsiella aerogenes* depends on the way the organisms are grown. The faster they are grown under Mg^{2+} limitation the more KDO is found in the wall, whereas under carbon-limiting conditions the KDO content of the walls falls with increasing growth rate (Ellwood & Tempest, 1967).

Sialic acid was found with KDO in only three organisms, *Achromobacter lacticum* NCIB 8208, *Escherichia coli* MRE 162 and *E. freundii* NCTC 8165, but sialic acid was never found without KDO. Several Gram-negative bacteria appear to have no KDO in their wall, indicating that the LPS may be different in its structure. Kasai (1966) has shown that the LPS of Bordetella species do not contain KDO. None of the wall samples or Gram-positive bacteria examined contained either KDO or sialic acid except for *Arthrobacter globiformis* NCIB 8907, which contained a small amount of KDO. This organism is not unequivocally Gram-positive.

These results indicate that in contrast to the work of Aaronson & Lessie (1960) and Irani & Ganapathi (1962), and in agreement with the work of Vincent & Cameron (1967), KDO is present in most Gram-negative bacteria. The latter authors also found thiobarbituric-acid-reactive material in Gram-positive bacteria. However, KDG and KDH occur as normal metabolites in bacteria and would give a thiobarbituric acid reaction in hydrolysates of whole organisms.

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