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Preliminary communication

Detection of 3-deoxy-2-octulosonic acid in thiobarbiturate-negative endotoxins

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Many endotoxins, when exposed to mild acid (pH 3–5) at 100°, are cleaved to yield a water-soluble oligo- or poly-saccharide fragment, and a precipitate, insoluble in water ("free Lipid A"). Inorganic phosphate, ethanolamine phosphate, and ethanolamine diphosphate have been observed to be released simultaneously. Experiments carried out with synthetic models established[1] than while phosphoric esters and glycosyl phosphates are not appreciably hydrolyzed at pH 4.5, 3-deoxy-4-*O*-phospho-2-aldulosonic acids readily eliminate the phosphate group in the pH range 3–7 and form unsaturated derivatives, the reaction being complete within 15 min.

The endotoxin isolated from *Bordetella pertussis* cells by the phenol–water method contains two lipopolysaccharides[2] (LPS-1 and LPS-2) which account for more than 90% of the endotoxin mass. When exposed to pH 3–5 at 100°, LPS-1 readily released its polysaccharide chain (PS-1) which, when isolated, gave a strong positive response in the thiobarbiturate test[3] (Table I), indicating the presence of a 3-deoxy-2-aldulosonic acid that has been shown to be an octulosonic acid[5]. Under

TABLE I

ABSORBANCIES MEASURED FOR *Bordetella pertussis* ENDOTOXIN, ITS COMPONENTS, AND FRAGMENTS DERIVED THEREFROM, IN THE THIOBARBITURATE TEST

Products	*Absorbance*[a]
Endotoxin (LPS-1 + LPS-2)	1.0
Endotoxin treated with HF	2.6
LPS-1	2.2
LPS-2	0.26
LPS-2 treated with HF	2.1
PS-1[b]	2.2
PS-2[b]	0.35

[a]Absorbance, at 549 nm, of the dye formed from 1 mg of endotoxin or LPS, or 0.5 mg of PS treated according to ref. 8, but extracted into 1.8 mL of cyclohexanone. [b]Isolated after hydrolysis at pH 4.5 and 100° in 1% $NaDodSO_4$ solution[4].

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the same conditions, LPS-2 is not cleaved; it requires treatment with mineral acid[2], but could be hydrolyzed at pH 3–5 in aqueous 1% sodium dodecyl sulfate ($NaDodSO_4$) solution[6]; under the latter condition, diphosphorylethanolamine is set free simultaneously[7]. LPS-2, but not LPS-1, contains a phosphorylated 3-deoxy-2-octulosonic acid[5,8]; its presence could, however, be demonstrated only following treatment with 2M mineral acid. PS-2, released from LPS-2 by hydrolysis at pH 4.5 (100°, 1% $NaDodSO_4$), gave but a negligible response in the thiobarbiturate test (Table I), and the characteristic signals at δ 1.9 and 2.2 [H-3ax and H-3eq of 3-deoxy-D-*manno*-2-octulopyranosidonic acid (KDO)] were absent from its ^{1}H-n.m.r. spectrum. A pentasaccharide fragment [prepared by treatment of *B. pertussis* endotoxin first with nitrous acid to shorten the sugar chains, and hydrolysis (pH 4.5, 1% $NaDodSO_4$) to cleave the glycosidic bond of KDO units] contained, beside an acidic sugar of unknown structure, heptose, hexose, and hexuronic acid in the ratio of 2:1:1; it obviously represented the region of the sugar-chain proximal to the hydrophobic domain. This pentasaccharide was found to have a strong absorption band (at 250 nm) that disappeared upon treatment with hydrogen and palladium-on-charcoal catalyst; this suggested the presence, in the unsaturated material, of an olefinic double bond conjugated with a carbonyl group: as a corollary, signals assignable to two methylene groups appeared in the δ 1.5 region of the ^{1}H-n.m.r. spectrum of the hydrogenated material.

Taking into consideration the observations that, in most endotoxins analyzed so far, the terminal units of the polysaccharide chains are KDO molecules, that PS-1 of the *B. pertussis* endotoxin is indubitably terminated by a KDO unit, and that during hydrolysis with weak acid the *B. pertussis* endotoxin invariably released inorganic phosphate and phosphoric esters, it was deduced that the unidentified component in the pentasaccharide was an olefinic derivative of KDO that was formed in an acid-catalyzed elimination reaction, the eliminated substituent having the structure $OP(O)_2HX$.

To prove this hypothesis, the endotoxin on the one hand, and isolated LPS-2 on the other, were dephosphorylated by treatment with 50% aqueous hydrofluoric acid solution[10] (4°, 48 h). The endotoxin (LPS-1 and LPS-2) thus treated, upon subsequent hydrolysis, released the totality of its bound polysaccharide material (PS-1 and PS-2) and gave a response in the thiobarbiturate test that was more than double of that of the endotoxin not treated with hydrofluoric acid (Table I). LPS-2 (which gives but a negligible reaction in this test with a maximum at 532 instead of 549 nm) gave, after treatment with hydrofluoric acid, a strong response similar in intensity to that of LPS-1, with a maximum at 549 nm (Table I). As a corollary, in the ^{1}H-n.m.r. spectrum of the pentasaccharide prepared from hydrofluoric acid-treated endotoxin, signals at δ 1.9 and 2.2 having the required intensity[9] established that the reducing terminal sugar was a mixture of the α and β anomers of KDO in the pyranose form. No olefinic KDO derivative was detectable, the u.v. spectra of both the polysaccharide and the pentasaccharide fragment being monotonous in the 225–260 nm region.

The extreme ease and speed with which the substituent was eliminated, under the very conditions widely employed to sever the hydrophilic and hydrophobic regions of endotoxins, are comparable to those observed for synthetic 3-deoxy-4-*O*-phospho-2-aldulosonic acids, and different from those measured for 4-*O*-glycosyl derivatives[1]. The behavior of the endotoxin and the removal of this substituent by aqueous hydrofluoric acid (which in the conditions employed is a fairly specific *O*-dephosphorylating agent) strongly suggests that the substituent was eliminated from C-4 of the KDO unit and confirmed that it was a phosphoric ester group. In the case of the *B. pertussis* endotoxin, the eliminated phosphoric ester is likely to be diphosphorylethanolamine; independent proof is being sought for confirmation.

One important consequence of this elimination–reaction is the transformation of a KDO unit into a thiobarbiturate-negative derivative which, if this reagent is used to estimate the KDO content of the endotoxin, will unavoidably lead to erroneous results. This could be the case for the endotoxin of *Vibrio cholerae*, in which a phosphorylated KDO-derivative was discovered to be present[11], although the endotoxin was first reported to be free of KDO on the basis of its negative reaction with the thiobarbiturate reagent. The effect of hydrofluoric acid treatment on the response of this endotoxin in the thiobarbiturate reaction is shown in Table II.

It is, therefore, suggested that endotoxins, particularly those unresponsive in the periodate–thiobarbiturate test, should be re-examined after treatment with 50% aqueous, hydrofluoric acid solution, if the presence of KDO is to be assessed on the basis of a positive reaction in this test. It must be born in mind, however, that KDO can be "silent" in this test for quite unrelated reasons, *e.g.*, simultaneous substitution of OH-5 and -7 by (relatively) acid-stable substituents. Finally, it should be recognized that the olefinic KDO derivative was discovered only because, being substituted by the polysaccharide (respectively tetrasaccharide) chain, it was recovered with it. Had it been substituted by another KDO unit and, consequently, released as 2-octulos-3,4-enonic acid into the medium, it would have been rapidly transformed into 5-(trihydroxypropyl)-2-furoic acid[12], and lost.

TABLE II

ABSORBANCIES MEASURED FOR *Vibrio cholerae* (STRAINS INABA AND OGAWA EL TOR) ENDOTOXINS IN THE THIOBARBITURATE TEST BEFORE AND AFTER TREATMENT WITH HYDROFLUORIC ACID

Products	*Absorbance*[a]
Endotoxin *V. cholerae* str. Inaba	0.27
Endotoxin *V. cholerae* str. Inaba treated with HF	2.14
Endotoxin *V. cholerae* str. Ogawa El Tor	0.24
Endotoxin *V. cholerae* str. Ogawa El Tor treated with HF	1.3

[a]Conditions as in Table I.

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