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STRUCTURAL FEATURES OF THE *Bordetella pertussis* ENDOTOXIN

Ladislas Szabò

Equipe n°55 du C.N.R.S. Institut de Biochimie, Université de Paris-Sud, Centre d'Orsay, 91405 Orsay, France.

Abstract. Graded acidic hydrolysis sequentially released from the endotoxin of *Bordetella pertussis* (Brucellaceae) two polysaccharides each of which possesses a single molecule of 3-deoxy-2-octulosonic acid as the terminal reducing sugar; in one the octulosonic acid is phosphorylated in position 5 and substituted in position 4, in the other it is not phosphorylated, but is substituted in position 5. Following treatment of the endotoxin with strong base a fragment, containing bound, non phosphorylated 3-deoxy-2-octulosonic acid, glucosamine phosphate and fatty acids, was isolated. This indicated that both polysaccharides were bound to the lipidic region of the endotoxin. The endotoxin structure thus defined is different from that proposed for the lipopolysaccharides of *Enterobacteria*.

As most of you well know the bacterial cell envelope can be considered to consist of three layers. Innermost we find the cytoplasmic membrane which is mainly made up of proteins and phospholipids: the active transport of material going into and flowing out of the cell takes place in this membrane. Covering this soft structure which has no great mechanical resistance, we find the "armour" of the cell : the rigid peptidoglycan layer. This is a tridimensional structure in which glucosaminyl-muramic acid chains are linked to each other by peptide bridges. This rigid peptidoglycan is covered by the so-called "outer membrane": an ill known, ill defined and extremely variable layer whose composition varies according to any parametre in the cell's environment: temperature, composition of the medium, its pH, and so on. In spite of its variability, or perhaps because of it, this is an extremely important part of the cell envelope, as this is the layer that is in permanent contact with the "outer world", the cell's surroundings, to which the cell must constantly adapt for its survival. Inversely, the constituents of this layer are those that are recognised by the cell's environment, the host if the cell is a parasite, as "foreign", "non-self" material, and against which the host will react and direct its defense mechanisms.

It is amongst the macromolecules making up this layer that we find, in gram negative bacteria, the so-called endotoxin: a misnomer obviously because this endo-toxin is, in fact, on the surface of the cell to which it is firmly attached. The name was given to it many years ago when its exact location was not known, in order to distinguish it from the exo-toxins, that is toxins secreted by microorganisms into the medium in which they grow.

Endotoxins can be extracted from whole cells or isolated cell-envelopes by a variety of methods, the most often used being that of Westphal, Lüderitz and Bister which employs a mixture of phenol and water. In most cases the endotoxin appears in the aqueous phase from which it can be recovered by high speed centrifugation.

The most investigated endotoxins are those of Enterobacteria, particularly those of *Salmonellae*. The most often quoted structure for the Salmonella endotoxin is that shown, schematically, on Fig. 1. It can be devided into three regions: region I is the so-called O-specific side chain; it is made up of repeating oligosaccharide units , the degree of polymerisation varying from zero to 35-40. Region II is the core, which is a single sequence of monosaccharides terminated, in the vast majority of cases, by a group or a single molecule of 2-keto-3-deoxy-octulosonic acid, usually referred to as KDO. In two or three cases it has been established that this acid had the D-*manno*-configuration; its systematic

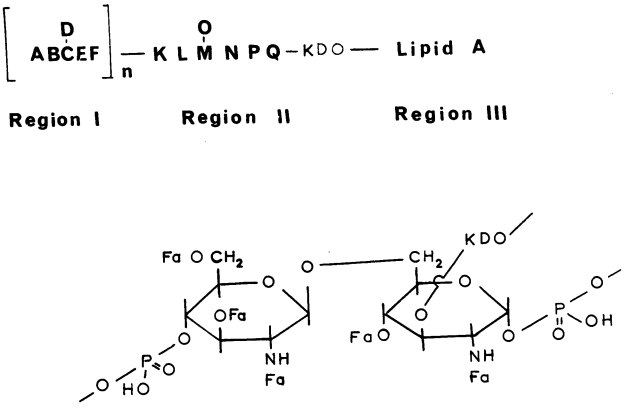


Figure 1.

name is therefore 3-deoxy D-manno-oct-2-ulosonic acid. Region III is the so-called Lipid A, or firmly bound lipid. According to Westphal, Lüderitz and their colleagues, this lipid is a polymer in which β-1, 6-linked glucosaminyl-glucosamine units are joined by pyrophosphate bridges. The hydroxyl and amino groups of the glucosamine molecules are substituted by fatty acids, the characteristic components being tetradecanoic (myristic) and 3-hydroxy-tetradecanoic (β-hydroxy-myristic) acids. To one of the hydroxyl groups is linked, through the glycosidic bond of a KDO molecule, the core. This ketosidic linkage is, of course, very labile to acid: it can be and usually is cleaved by treatment of the endotoxin with acetic acid of pH 3.4 at 100° for one hour: the bond being broken, the lipid part, deprived of its solubilising polysaccharide moiety, appears as a precipitate, while the polysaccharide-core complex remains in solution and can be isolated from the supernatant. This pattern seemed to hold true for most cases investigated hitherto and is often seen to be referred to as "the general structure of the endotoxin of gram negative bacteria", although its Authors never claimed such generalisation.

These endotoxins possess a bewildering array of biological activities. Some of these are undesirable, such as their general toxicity, their capacity of provoking the febrile state, their sensibilising action and so on. Others, however, would be of great theoretical and practical interest, for these endotoxins can also establish what is called the state of non specific immunity, most of them are adjuvants and they are one the few B-cell mitogens.

The question then arises: is the totality of the biological activities the property of the macromolecule as such, which acts as a single activator and starts a series of essentially indissociable phenomena, or do we have present within the macromolecule a certain number of effector sites which can each trigger a specific receptor more or less independently ? Data from the litterature indicate that it is the second hypothesis which is more likely to be the correct one; the chemists and biochemists have, therefore, to face the challenge of identifying these affector sites and, if possible, of separating them.

We became interested in this problem and this is why, in 1968, we started working on the structure of the endotoxin of Bordetella pertussis, which, as you know, is the agent that causes whooping-cough. We chose to work on this material for several reasons. Firstly, Bordetella pertussis is not an Enterobacterium: it belongs to the family Brucellaceae; thus either we would find that its endotoxin was similar to that of the enteric bacteria and that would have given some support to the generalisation of endotoxin structure; or we would find that the structure was different, which would make for more interesting work. Secondly, previous work by MacLennan, Kasai, Nakase and others had established that the acute toxicity of this endotoxin was very low as compared to endotoxins obtainable from Enterobacteria. And finally, pertussis being produced by the pharmaceutical industry for making vaccines, is a human pathogen that is relatively easily accessible, and I should like to thank our colleagues, Drs. Ayme, Roumiantzeff, Mynard, and Donikian from the Institut Mérieux,

Lyon, France, who grew several hundreds of litres of pertussis culture for us and prepared the extracts from which the endotoxin was isolated.

The endotoxin was extracted from freshly harvested, wet cells with phenol, and purified by repeated high speed centrifugation, until free of nucleic acids. From 160 litres of culture we obtain about 1.6 g of pure endotoxin i.e. 10 mg per litre of culture medium. The endotoxin thus obtained gave constant analytical figures from one batch to another and all batches had the same biological potency in all tests to which we submitted them, calculated on weight basis. Upon ultracentrifugation the material gave a single peak and had a sedimentation coefficient of about 2 S, but this is, unfortunately, not a proof of homogeneity.

The endotoxin's composition, as far as we could estimate it, is shown in Table 1, together with data taken from the litterature and you will notice immediately the discrepancies between the different Authors, including ourselves. We think that these discrepancies

TABLE 1.

	N	P	Heptose	Hexose	Total sugar	Hexosamine	KDO	Fatty acid
MacLennan	3.9	2.5	34	6		20.3		25
Nakase et al.	2.6	1.2-1.5	9-11		13.5	16-16.5	0.3	25-32
Kasai	4.5	2.3	12		22	12		17
Ribi et al.	3.0	2.5	9.9	9.7	15	7.3	1.25	18
Chaby, Le Dur	3.7	2.4	10.0	4.9	13.8	18.0	0.34	30

are mainly due to two factors. The first is that we have observed that the yield and the composition of the endotoxin could be kept constant only if the growth and the harvesting of the cells were very regorously controlled: small deviations, for instance not extracting the cells immediately after the harvest, affected the yield and the composition of the endotoxin considerably. The second factor is the appearance of a glucan that accompanies the endotoxin and from which it is well-nigh impossible to separate it. We know now how to grow pertussis which will give no, or negligible amounts of glucan, but we still do not know which exact parametres of the culture are responsible for its appearance or disappearance. The figures you see were obtained by colorimetric methods and thus are subject to caution. This is particularly true for the figures shown for KDO: these certainly bear no relationship to the real value. We must admit that for the time being we have no reliable method for the estimation of KDO in complex molecules. I shall come back to this point later. It is also clear from these figures that we are very far from having identified all components of the endotoxin. Indeed, if we add up all of the figures shown and those we obtained for components which we have identified, but which are not shown because there was no counterpart for them in the previous litterature, we still do not account for more than about 75 % of the endotoxin's mass. Our situation is thus no better than that of our colleagues working with Enterobacteria.

As I mentioned a minute ago, in the case of endotoxins obtained from Enterobacteria it is customary to separate regions 1 + 2, that is the specific polysaccharide + core fragment, from the Lipid A moiety, by treatment of the endotoxin with acetic acid of pH 3.4 at 100° for 1 hour. It appeared to us that this purely empirical method was not very satisfactory and we tried to find conditions in which the hydrolysis of the deoxy ketosidic bond of KDO would be the only glycosidic bond broken. Please note that I stressed the word "glycosidic", because we know that in the conditions we finally found suitable other bonds, especially phosphate bonds, are broken.

To establish such conditions we used the endotoxin of Salmonella typhimurium, an Enterobacterium, because this endotoxin contains, besides the "usual" sugars: galactose, mannose, rhamnose, glucose, glucosamine, heptose and of course KDO, a 3,6-bis-deoxy-sugar, called abequose. The rate of acid catalysed hydrolysis of the glycosidic bond of this abequose is considerably faster than that of the "usual" sugars, including rhamnose, but

much slower than that of KDO, and free abequose can be readily detected by the very sensitive thiobarbiturate method. It was, therefore, reasonable to assume that if we could find conditions in which the glycosidic bond of KDO was cleaved and the glycosidic bond of abequose was not cleaved, we could be reasonably certain that the only glycosidic bond cleaved would be that of KDO. A young colleague of mine Dr. S.R. Sarfati has established that hydrolysis with trifluoroacetic acid of pH 2.4 at 50° will satisfy our requirements and, as shown in Figure 2, when the pertussis endotoxin was thus treated and the kinetics of the cleavage followed by the thiobarbiturate reaction, a plateau was attained. As at the same time a precipitate appeared it looked as if the pertussis endotoxin would behave just as those of the Enterobacteria. Indeed, from the supernatant Dr. A. Le Dur could isolate a polysaccharide, which we called Polysaccharide I, that was homogeneous as judged by ultracentrifugation and by gel-filtration, in a yield of about 20 % w/w. The reducing terminal sugar of this polysaccharide was a single molecule of 3-deoxy-octulosonic acid.

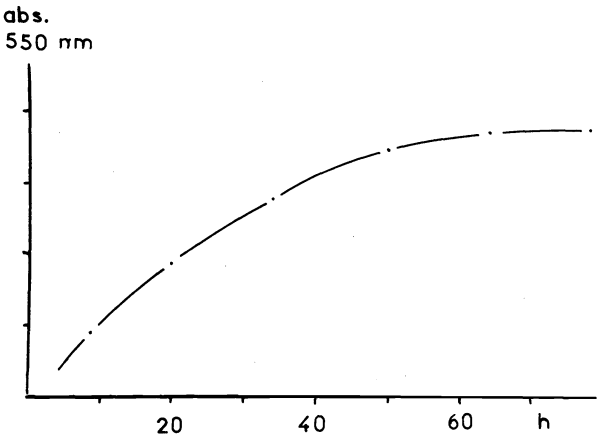
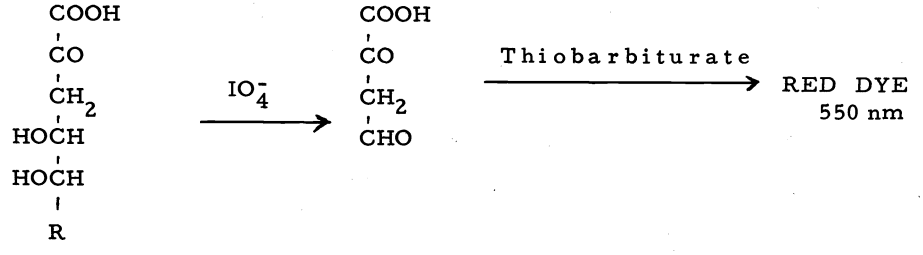


Figure 2.

And now I must open a parenthesis about the estimation of KDO. When we started this work, there were two main methods available for the estimation of KDO. The first one is the so-called semicarbazide method introduced by McGee and Doudoroff; it is based on the fact that any α-keto acid will condense with semicarbazide at pH 3-4 to give a semicarbazone. The absorption band of this semicarbazone centred at 250 nm is proportional to the α-keto-acid concentration. The drawback of this method is that any free carbonyl group will interfere. The second, somewhat more specific method is the so-called thiobarbiturate method, the principle of which is shown in Scheme 1: periodate cleaved KDO to give a fragment called "β-formyl-pyruvate" which, when condensed with thiobarbituric acid gives a red dye. The absorption band of this dye centred at 550 nm is used to estimate the KDO-content of the sample. For obvious reasons it was first thought that 4- or 5-O-substituted KDO would not give a positive reaction in this test. However the molar absorption coefficient



Scheme 1.

of 5-O-substituted octulosonate is about one seventh, and that of 4-O-substituted octulosonate about one thirtieth of that of the parent, unsubstituted compound. It is clear that in these conditions, meaningful estimation of a sample's KDO content by this method is hardly feasible, unless the substitution pattern of the KDO is fully established - which is rarely the case.

To remedy this situation two of my colleagues, Drs. Chaby and Sarfati tried to find another method for estimating KDO. They soon found that KDO, like many other sugars, when heated in acid solution with diphenylalanine, produces a chromophore that can be used to estimate KDO. The absorption maximum given by KDO (Fig. 3) is quite remote from the maxima given by other sugars. The method is reliable even in the presence of large amounts of other sugars and the molar absorption coefficient of KDO is the same for unsubstituted and 5-O-substituted KDO, as long as the substituent is as labile to acid as a glucosyl-residue: N-acetyl-glucosaminyl-, phosphoryl-, or glucuronyl-KDO have much lower absorption coefficients than glucosyl- or rhamnosyl-KDO (Table 2). And this is why I said that we still do not have a reliable method for the estimation of KDO.

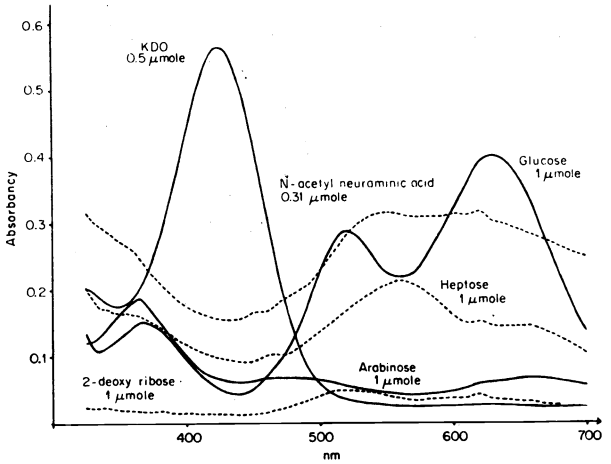


Figure 3.

Table 2.

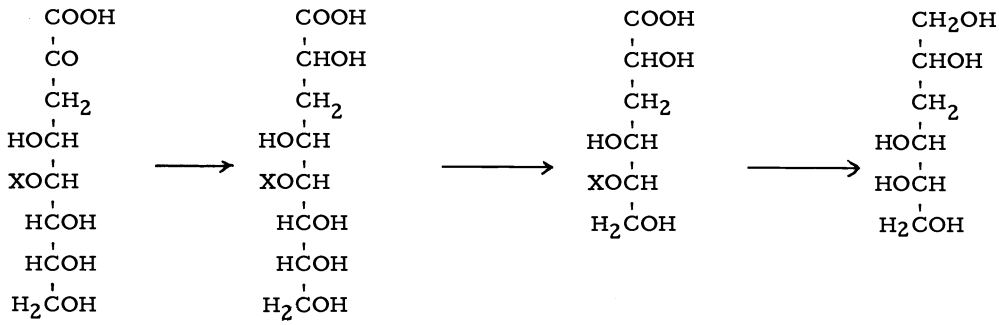
KDO	100 %
Glucosyl 1-5 KDO	100 %
Rhamnosyl 1-5 KDO	95 %
N-acetyl glucosaminyl 1-5 KDO	75 %
D-glucuronyl 1-5 KDO	13 %
KDO 5-phosphate	45 %

Let us now return to our polysaccharide I. We determined its KDO content by three different methods: the values obtained are recorded in Table 3. The diphenylamine and semicarbazide tests give similar figures, while the value given by the thiobarbiturate

Table 3. KDO-content of PS I

Diphenylamine	7,8 %
Semicarbazide	8,8 %
Thiobarbiturate	1,1 %

method was way out. This discrepancy was, however, very useful in this particular case. Indeed you may remember, that I said a few minutes ago that in the thiobarbiturate test a 5-O-substituted KDO has an absorption coefficient which is one seventh of that given by unsubstituted KDO, and you see that by the semicarbazide and diphenylamine tests we measure a KDO content 7 to 8 times higher than by the thiobarbiturate test. We took this as an indication that the KDO of this polysaccharide I could be 5-O-substituted. That this was, in fact, the case could be demonstrated in the following way (Scheme 2). Polysaccharide I was treated with borohydride to reduce the carbonyl group, and then with periodate to cleave all vicinal diol groups. Finally the newly formed aldehyde groups were again reduced with borohydride, and the material obtained was submitted to treatment with mild acid. This led to a pair of epimeric 3-deoxy-hexonic acids, which were identified by gas-liquid chromatography/mass spectrometry following their transformation into alditol acetates. Had the substituent been in position 4, we would have found epimeric 3-deoxy-pentonic acids, whereas substitution in positions 6, 7 or 8 would have given epimeric 3-deoxytetronic acids. As neither of these were detected, we could safely conclude that the substituent was, indeed, in position 5 of the KDO molecule.



Scheme 2.

This then established that mild acid hydrolysis of the pertussis endotoxin released a polysaccharide, just as is the case with the endotoxins of Enterobacteria. We, therefore, expected that the simultaneously formed precipitate would be analogous to the Lipid A. But there we had a surprise, because, as can be seen from Table 4, this precipitate was, in fact, a glycolipid whose composition was not very different from that of the intact endotoxin.

Table 4.

	P	Heptose	Hexose	Total sugars	Glucos-amine	KDO	Fatty acids
Endotoxin	2.6	8.5	5.8	14	18-20	0.32	27.5 %
Glycolipid	2.3	5.4	5.1	9.3	14	0.24	38 %

This glycolipid gave a negative reaction in the thiobarbiturate test, but contained, nevertheless, 3-deoxy-octulosonic acid. To demonstrate the presence of this, we had to treat the glycolipid with 2 N HCl for 90 minutes at 100°: an apparent, maximal, value of 0.24 % of KDO content was then measured. In the same conditions of estimation, the KDO content of the intact endotoxin is 0.35 % ; it is thus of the same order of magnitude.

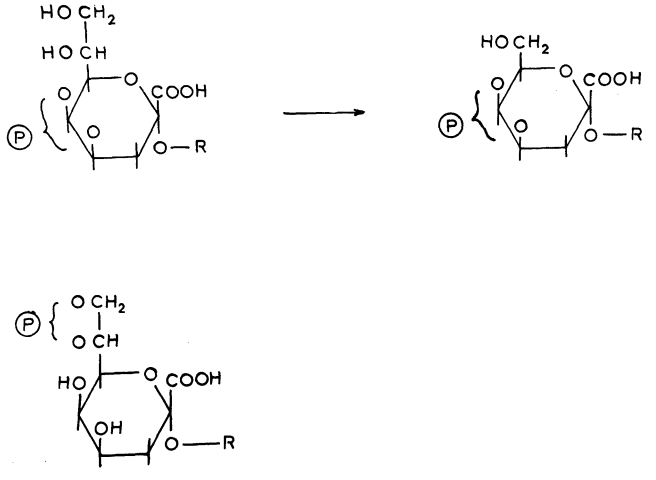
The presence of this KDO strongly suggested, especially in view of the high neutral sugar content of the glycolipid, that there was a second polysaccharide chain terminated by KDO within the pertussis endotoxin. This second polysaccharide was, indeed, found and isolated when the glycolipid was hydrolysed with 0.25 N HCl for 20 minutes at 100°. This polysaccharide II was also found to be homogeneous both by ultracentrifugation and gel permeation chromatography, and it also represented about 20 % of the endotoxin's weight.

Its analytical data, shown in Table 5, indicate that it is not very different from polysaccharide I, except for two points. The first is that polysaccharide II did not give a proper reaction in the diphenylamine test and one measures about 25 times more KDO by the semicarbazide test than by the thiobarbiturate reaction. You may remember that in polysaccharide I we found seven to eight times more KDO by the semicarbazide and the diphenylamine tests than by the thiobarbiturate method. The second difference is that polysaccharide II contains phosphate while polysaccharide I does not.

Table 5.

	P	Heptose	Hexose	KDO	Glucosamine	
PS I	nil	20	12.5	1.1 8.8	19	%
PS II	1.8	17.9	10	0.3 6.6	17	%

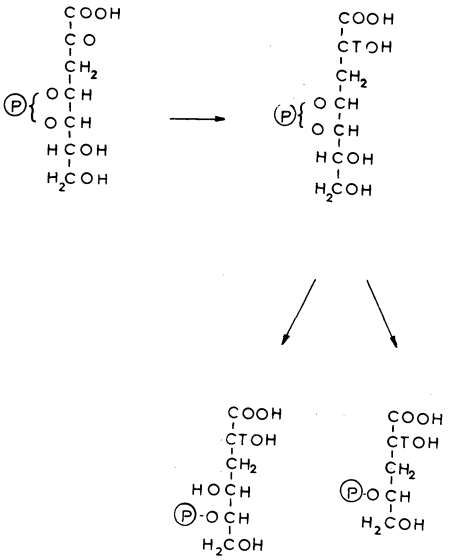
As in polysaccharide I, the KDO molecule is the reducing, terminal sugar in polysaccharide II, but while in polysaccharide I it is glycosylated in position 5, in polysaccharide II the KDO is phosphorylated in that same position. This was demonstrated in the following way. Firstly, when the glycolipid was treated with relatively strong acid (HCl 2 N/2 h/ 100°), upon paper electrophoresis a compound was detected that contained phosphate and gave a positive reaction in the thiobarbiturate test. This substance, when eluted and analysed by paper chromatography and paper electrophoresis in a variety of conditions, was found to be indistinguishable both from KDO-8- and KDO-5-phosphates which have been synthesised in our laboratory by Drs. D. Charon, S.R. Sarfati and Mrs. M. Mondange. Moreover, when treated with acid phosphatase, this compound released inorganic phosphate and a thiobarbiturate positive acid that behaved in all chromatographic conditions as authentic KDO. We therefore concluded that we were dealing with a phosphorylated KDO molecule. To establish the position of the phosphate group we made the hypothesis that in the intact endotoxin the KDO molecule was in the pyranose form. The phosphate group could then be situated (Scheme 3) either on the ring, that is in positions 4 or 5, or on the extracyclic hydroxyl groups 7 or 8



Scheme 3.

In the former case sequential treatment of the intact endotoxin with periodate and borohydride, followed by hydrolysis with acid, should lead to a phosphorylated 3-deoxy-heptulosonic

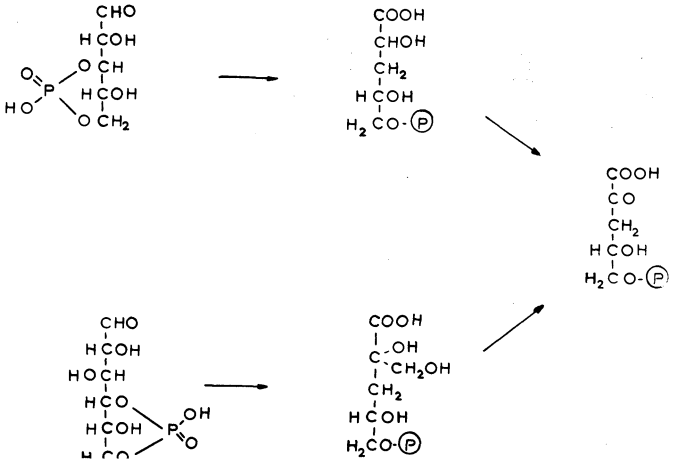
acid; in the latter the same sequence should completely destroy the phosphorylated 3-deoxy-octulosonate. When this experiment was carried out by Dr. Chaby he found that a phosphorylated 3-deoxy heptulosonic acid was formed. It was identified, after isolation, by comparison with a sample of synthetic 3-deoxy-D-arabino-heptulosonic acid 7-phosphate by the usual chromatographic and electrophoretic methods. The phosphate was therefore esterifying hydroxyl groups 4 or 5 of the KDO molecule. To distinguish between these two possibilities the 3-deoxy heptulosonic acid was isolated and treated with tritiated sodium borohydride (Scheme 4):



Scheme 4.

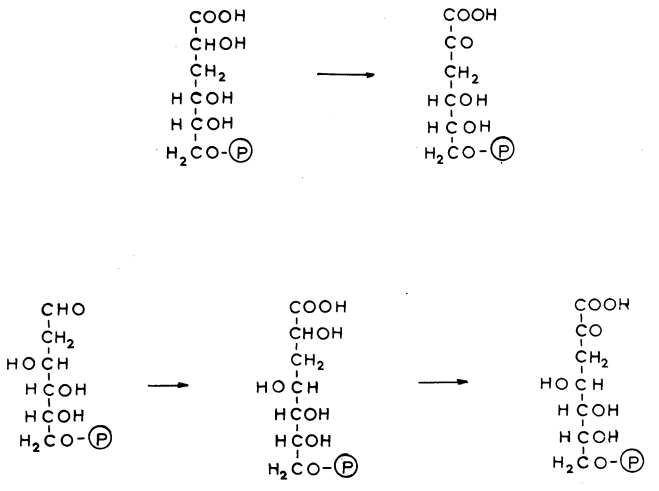
this gave a pair of radioactive 3-deoxy-heptonic acids phosphorylated in positions 4 or 5. This material was again oxidised with periodate and reduced with normal borohydride and the radioactive compounds analysed by paper chromatography and paper electrophoresis. It was found that the only radioactive material present co-migrated with a sample of authentic, phosphorylated 3-deoxy hexonic acid, and was separated from an authentic sample of 3-deoxy pentonic acid phosphate. This then proved that the phosphate group esterified the hydroxyl group in position 5 and not in position 4. It also indicated that our hypothesis was correct: in the intact endotoxin the phosphorylated KDO of polysaccharide II was in the pyranose form, and as it was unsubstituted in positions 7 and 8 the polysaccharide chain was necessarily attached to position 4.

The phosphorylated 3-deoxy aldulosonic acids we needed for these studies were synthesised as follows. The pentulosonic acid 5-phosphate was made either from xylose, via the 3,5-cyclic phosphate, whose alkaline degradation gave "xylometasaccharinic acid" 5-phosphate and this was then oxidised with chlorate and vanadium oxide to the α-keto acid; or it could be made from glucose, via the 4,6-phosphate whose alkaline degradation gave isosaccharinic acid 6-phosphate. When this was treated with one molar equivalent of periodate it gave the phosphorylated pentulosonate (Scheme 5).



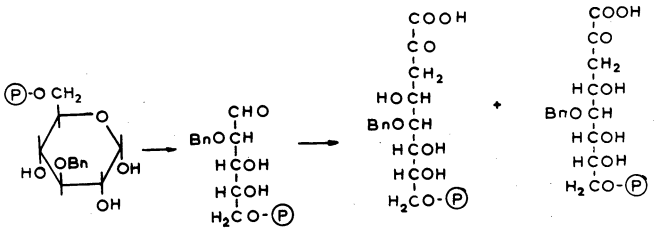
Scheme 5.

The phosphorylated 3-deoxy-hexulosonic acid (Scheme 6) was made from glucometasaccharinic acid 6-phosphate, easily obtained from either 3-O-methyl-glucose 6-phosphate or from glucose 3,6-cyclic phosphate by alkaline degradation, by oxidation with chlorate/vanadium oxide, while the seven carbon analogue was obtained from 2-deoxy-glucose 6-phosphate to which HCN was added and the mixture of epimeric 3-deoxy heptonic acids formed oxidised by the same technique. In the last synthesis it is possible to use $H^{14}CN$ to label the compound. All these syntheses have been published in the Journal of the Chemical Society.



Scheme 6.

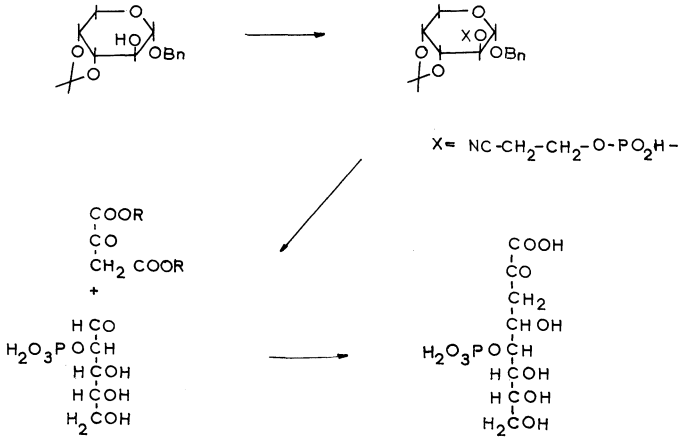
The 3-deoxy-D-manno-octulosonic acid 5- and 8-phosphates were synthesised by a different method, the so-called Cornforth-reaction previously used to obtain unsubstituted 3-deoxy-D-manno-octulosonic acid by several Authors. The 8-phosphate was obtained (Scheme 7) from 2-O-benzyl-D-arabinose 5-phosphate, which can be obtained from 3-O-benzyl-glucose 6-phosphate by cleavage with periodate between C1 and C2. This 2-O-benzyl-arabinose 5-phosphate was condensed with oxalacetic acid to give a mixture of D-manno- and D-gluco-3-deoxy 5-O-benzyl-octulosonic acid 8-phosphates. The ratio of the two isomers



Scheme 7.

is about 7:1 in favour of the D-manno compound. The benzyl groups were next removed by catalytic hydrogenation and the isomers separated by ion exchange chromatography. Like the other phosphate esters they were separated as Li-salts.

For the KDO-5-phosphate the starting material was (Scheme 8) D-arabinose which was transformed into the isopropylidene ketal of the benzyl glycoside, a known compound. This was then phosphorylated with cyanoethyl phosphate and dicyclohexyl carbodiimide; the protecting groups were removed by alkaline treatment followed by hydrogenation in mildly acidic medium and the 2-phosphate of D-arabinose was thus obtained. This was then condensed with oxalacetate to yield a mixture of D-gluco- and D-manno-3-deoxyoctulosonic acid 5-phosphates which were cleanly separated by ion exchange chromatography. The ratio of the two isomers is again 6-7 to 1 in favour of the D-manno isomer.



Scheme 8.

Reduction of all of these acids with tritiated sodium borohydride provided us with the phosphorylated, radioactive, 3-deoxy aldonic acids we used in the experiments I described.

It must, however, be remembered that the isolation of a 3-deoxy-octulosonic acid 5-phosphate from the endotoxin does not prove, per se, the presence of this compound in the intact endotoxin. Indeed, it is conceivable that in the intact macromolecule the phosphate occupied position 4 of the octulosonate and the polysaccharide chain position 5. This is so, because, as you will remember, the octulosonate 5-phosphate was set free by strong acidic hydrolysis: it is therefore conceivable that the substituent in position 5 being removed first, the phosphate group migrated from position 4 to position 5. We think however, that this hypothesis can be dismissed for several reasons. Firstly: we have shown that authentic 3-deoxy-D-arabino-heptulosonic acid 4-phosphate when treated in the same conditions as were used for the release of the octulosonate 5-phosphate, liberated 84 % of its phosphate content as inorganic phosphate and no phosphorylated 3-deoxy aldulosonic acid could be detected in the hydrolysate. Secondly: no phosphate migration was observed by Foster, Overend and Stacey when they treated methyl 4, 6-O-benzylidene-2-deoxy-α-D-lyxo-hexopyranoside 3-phosphate with acid. The steric arrangement of the phosphorylated and free hydroxyl groups are the same in this compound as in the postulated 3-deoxy-D-manno-octulopyranosidic acid 4-phosphate (Fig. 4): it is highly unlikely that phosphate migration should occur in the latter but not in the former compound. Thirdly: phosphate migrations lead to equilibrium mixtures; it follows that if phosphate migration had taken place in the reaction sequence we used to establish the position of the phosphate ester grouping, we should have found, besides the phosphorylated 3-deoxy-hexonic acid we identified also some phosphorylated 3-deoxy pentonic acid: however, in spite of the use of radioactive material which would have made easy the detection of such a compound, it was never detected. And finally the fact that only aldulosonic acid 5-phosphates were found whether the hydrolysis was done with strong acid (2 N)

or weak acid (0.25 N) also makes it highly unlikely that the 5-phosphate resulted from phosphate migration.

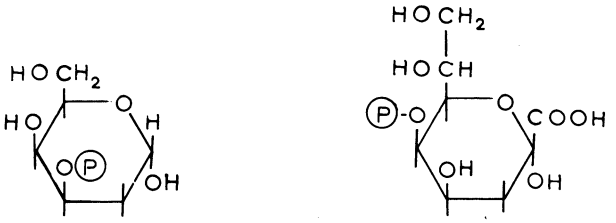
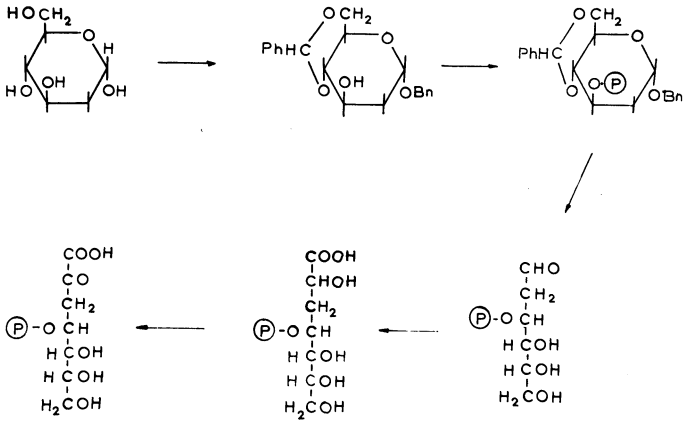


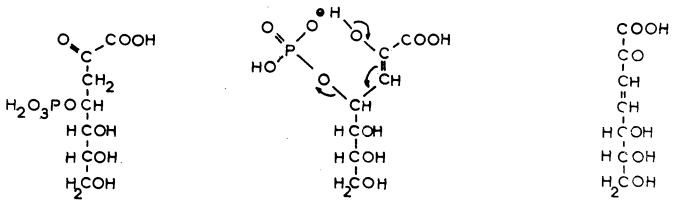
Figure 4.

I just mentioned that 3-deoxy-D-arabino-heptulosonic acid 4-phosphate was used as a model compound. This ester was made (Scheme 9) from 2-deoxy-D-glucose. The benzyl glycoside of this was benzylidinated and then phosphorylated with cyanoethyl phosphate and dicyclohexylcarbodiimide. After removal of the protecting groups the free hexose was transformed into an epimeric mixture of phosphorylated 3-deoxy heptonic acids and then oxidised with chlorate and vanadium oxide to the α-keto acid. The behaviour of this compound in acid medium is worth mentioning. While in N HCl at 100° this 4-phosphate releases its phosphate content about twice as fast as the 5-phosphate of KDO, at pH 3.6 the ratio is several hundred fold. The rate of phosphate release is such that we believe that this is not a hydrolysis but an acid catalysed elimination reaction and we think that the mechanism shown (Scheme 10) accounts for it. At pH 3 to 5 the phosphate is present as a monoanion and it is known that these aldulosonic acids easily undergo enolisation in acid solution.



Scheme 9.

The elimination reaction could then be initiated by a simple proton transfer from the undissociated enol (or even from the non enolised compound) to the phosphomonoanion. Supporting evidence for this mechanism is that a derivative of the postulated olefinic keto-acid was isolated and identified.



Scheme 10.

Let us now return to our endotoxin. We have established that mild acidic treatment gave polysaccharide I plus a glycolipid; treatment of this glycolipid with somewhat stronger acid gave polysaccharide II and material that contained elements characteristic for the lipid. A moiety of the endotoxin of enterobacteria: glucosamine, glucosamine-phosphate and fatty acids, but no neutral sugars. The question then arose how were these fragments joined to each other ? It was clear that polysaccharide II was joined to the lipid, but as regards polysaccharide I it could be linked either to polysaccharide II or to the lipid.

To distinguish between these two structures the endotoxin was submitted to strong alkaline hydrolysis: 2 N KOH was used at 100° for 8 hours. We have ascertained that in these conditions no destruction of neutral sugars took place and that no thiobarbiturate positive material was lost. It is of interest to note that only 48 % of the total fatty acid content of the samples was saponified; it is likely that the amide bound fatty acids resisted even in these harsh conditions. Fragmentation of the lipid part occurred and these fragments could be separated (Fig. 5). The fragments were then analysed by Dr. M. Laporte for KDO and KDO-phosphate, the underlying idea being that if we could find a fragment containing KDO, glucosamine, phosphate and fatty acids, that is components characteristic for polysaccharide I and the lipid, and if this fragment did not contain any phosphorylated KDO, that would be strong evidence that polysaccharide I, like polysaccharide II was joined to the lipid moiety of the macromolecule. We found two fragments which satisfied our requirements:

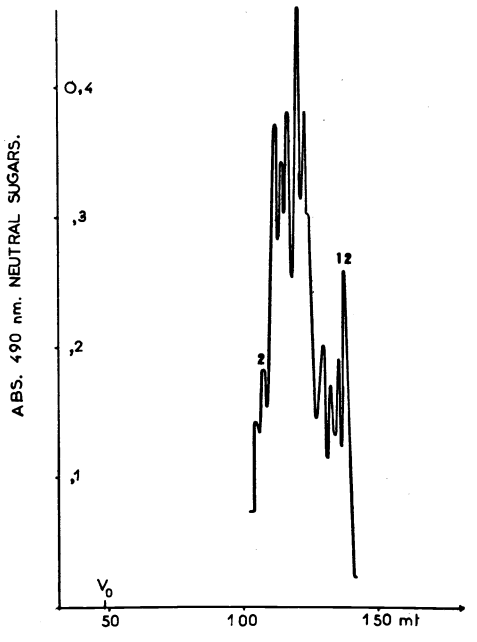


Figure 5.

peaks 2 and 12. In peak 2 negligible amounts of KDO phosphate were found, in peak 12 there was no detectable amount of phosphorylated KDO. All peaks, including peaks 2 and 12, contained fatty acids, glucosamine, phosphorylated glucosamine and neutral sugars. Upon paper electrophoresis material contained in peaks 2 and 12 did not migrate, but when the material was first submitted to hydrolysis with acetic acid of pH 3.4, that is conditions in which the ketosidic bond of KDO is cleaved, two and only two spots could be detected upon paper electrophoresis. One migrated as a positively charged entity and stained with alkaline silver nitrate and it did not contain any phosphate. The other fragment was negatively charged and it contained the totality of the sample's phosphate content. In other words these two peaks appeared to represent fragments in which a non-phosphorylated KDO molecule and the polysaccharide chain attached to it were bound to material that possessed the elements characteristic for the lipid moiety of endotoxins. As the non-phosphorylated KDO is the characteristic element of polysaccharide I, we concluded that polysaccharide I, like polysaccharide II was bound to the lipid moiety of the endotoxin.

The objection can be raised that it is conceivable that the endotoxin preparation we used was, in fact, a mixture of two macromolecules, one containing the phosphorylated, the other the non-phosphorylated KDO and their respective polysaccharide chains. We consider this possibility unlikely, because the alkaline fragmentation gave mostly fragments containing both phosphorylated and non-phosphorylated KDO in comparable amounts. If we were dealing with a mixture, we should have found two series of peaks: one containing only phosphorylated and the other only non-phosphorylated KDO. As this is not the case we believe that our conclusion is valid.

We were able to detect and separate the two polysaccharide chains present in the pertussis endotoxin only because the two KDO molecules have very different rates of hydrolysis. One can imagine, but this is pure speculation, that similar structural features are also present in the endotoxins of other gram negative bacteria, but remained, until now, undetected, because of the similar or identical rates of hydrolysis of the KDO molecules they contain. If this hypothesis is correct, this could be the basis of the so-called microheterogeneity of endotoxins observed and described by several Authors.