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DETERGENT-ACCELERATED HYDROLYSIS OF BACTERIAL ENDOTOXINS AND DETERMINATION OF THE ANOMERIC CONFIGURATION OF THE GLYCOSYL PHOSPHATE PRESENT IN THE "ISOLATED LIPID A" FRAGMENT OF THE *Bordetella pertussis* ENDOTOXIN*

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ABSTRACT

Due to the formation of micelles, severance of the hydrophilic (poly- or oligosaccharide) and hydrophobic ("Lipid A") domains of bacterial lipopolysaccharides at pH 3.4 or 4.5 and 100° is slow and sometimes does not proceed at all; partially degraded fragments are usually formed. At pH 3.4 (100°) in aqueous 1% sodium dodecylsulphate (SDS), both lipopolysaccharides of the *Bordetella pertussis* endotoxin are cleaved within 20–30 min, but 80% of the glycosidically bound phosphate present in the hydrophobic domain is lost. Other endotoxins behave similarly. At pH 4.5 (100°) and in the absence of detergent, hydrolysis of the glycosidic bonds of 3-deoxy-D-*manno*-2-octulosonic acid residues of the *B. pertussis* endotoxin is negligible but, in aqueous 1% SDS, severance of the two regions of LPS-1 is complete within 1 h (that of LPS-2 requires 3–4 h), and the glycosidically bound phosphate of the isolated hydrophobic region is preserved. Comparison of the rate of acid-catalysed hydrolysis of the glycosidically bound phosphate present in this "isolated Lipid A" preparation with that of 2-deoxy-2-[(3*R*)-3-hydroxytetradecanamido]-α- and -β-D-glucopyranose 1-phosphates established that the former 1-phosphate was the α anomer.

INTRODUCTION

The hydrophobic region (Lipid A) of many endotoxins contains a β-GlcN-(1→6)-GlcN unit that carries fatty acids, typically (3*R*)-3-hydroxytetradecanoic acid, in amide and ester linkages, and phosphate groups at positions 1 and 4′. The anomeric configuration of the GlcN-1-phosphate moiety has been established by polarisation transfer from proton to phosphorus and selective decoupling of the phosphorus n.m.r. spectrum for one endotoxin only, namely, the deep-rough strain *Salmonella minnesota* ·595 (made up of two[1] units of 3-deoxy-D-*manno*-2-

*Dedicated to Professor Rezső Bognár in the year of his 75th birthday.
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octulosonic acid and a hydrophobic region) that was *O*-deacylated with anhydrous hydrazine; the anomeric configuration was found[2] to be α.

O'Brien[3] observed that, in certain conditions of acidity and temperature, the rates of acid-catalysed hydrolysis of 2-acetamido-2-deoxy-α- and -β-D-glucopyranose 1-phosphates are very different and may be used to identify the anomer present. Accordingly, an attempt was made to elaborate a simple general method, applicable to otherwise unmodified "isolated Lipid A" fragments, that could be used to determine the anomeric configuration of the GlcN-1-phosphate moiety.

Because of the availability of well-defined preparations[4], the *B. pertussis* endotoxin was used for the study. This endotoxin is made up of two major lipopolysaccharides (LPS-1 and LPS-2) that account for >90% of the mass. Only LPS-1 is cleaved to yield the hydrophilic domain (PS-1) and "isolated Lipid A-1" by the usual hydrolysis with acetic acid of pH 3.4 for 1 h at 100°, whereas LPS-2 necessitates treatment with mineral acid[5] under conditions where 2-acetamido-2-deoxy-α- and -β-D-glucopyranose 1-phosphates are dephosphorylated completely. Thus, a method was required that would not only sever the polysaccharide and Lipid A regions of the endotoxin, but also preserve the molecular integrity of the hydrophobic regions, and, consequently, their original biological activities. Studies leading to the attainment of this goal are now described.

RESULTS AND DISCUSSION

Endotoxins, when treated with acid, are split into a water-soluble polysaccharide and a heterogeneous[6] precipitate (named[7] "isolated Lipid A"), the latter being derived from the hydrophobic region of the macromolecule. Although cleavage of the same covalent bond, *i.e.*, the glycosidic bond of 3-deoxy-D-*manno*-2-octulosonic acid (KDO), is usually necessary to sever the hydrophilic and hydrophobic regions of endotoxins, the acidity required varies from acetate buffer of pH 4.5[8] to 1–2M HCl[9], the conditions used most often being acetic acid of pH 3.4, all at 100° for 1–2 h. The endotoxic activities (pyrogenicity, lethal toxicity, Shwarztman sensitisation, mitogenicity, complement activation, *etc.*) of isolated Lipid A preparations decrease with increasing degree of acidity and duration of the hydrolysis employed[10,11], suggesting that the structure of the hydrophobic part, considered[12] to be responsible for the endotoxic activities, is altered during the acid treatment.

The methyl glycoside of 3-deoxy-α-D-*manno*-2-octulopyranosonic acid has a half-life of <10 min in acetic acid of pH 3.4 at 100°, and 4- or 5-substituents do not change notably the rate of hydrolysis[13]. Therefore, it was concluded that, in the aqueous hydrolysis media, the state of aggregation[14,15] of the endotoxins must be responsible for the discrepancy observed between the rates of hydrolysis of the synthetic glycosides and those present in endotoxins. These aggregates can be disintegrated by detergents, but the concentration of detergent required to produce apparently complete disaggregation varies for different endotoxins. Thus, for

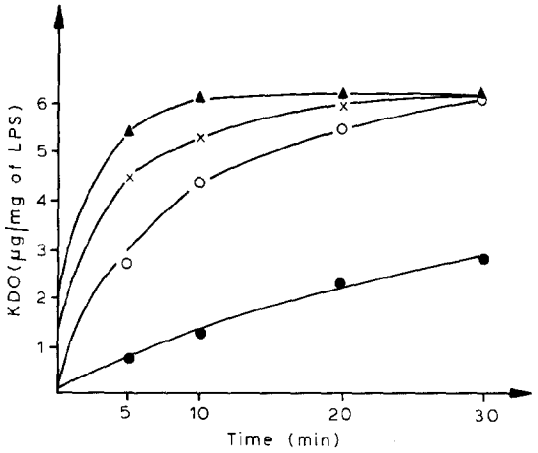

Fig. 1. Influence of the degree of dispersion upon the rate of acid-catalysed release of thiobarbiturate-positive material from the *B. pertussis* endotoxin at pH 3.4 and 100°. Final SDS concentrations: —●—, 0%; —○—, 0.2%; —×—, 0.5%; and —▲—, 1%. Absorbancies, measured in the thiobarbiturate assay, are expressed as µg of KDO/mg of endotoxin; the ammonium salt of KDO was used as reference.

E. coli O113/B4 and O113 endotoxins, at room temperature, treatment with aqueous 2% deoxycholate was required to produce particles having sedimentation coefficients of 0.94 and 1.04 S, respectively[14], whereas, at the same temperature, 3% of this detergent was necessary to disaggregate *Neisseria meningitidis* endotoxin[16]. *B. pertussis* endotoxin required treatment with aqueous 1% SDS for 5 min at 100° to yield particles having 1.01 S; in aqueous 0.2% SDS (100°), much larger particles (16 and 65 S) were observed[17]. Accordingly, the rate of hydrolysis, as measured by the thiobarbiturate assay[18], depended on the concentration of the detergent. In aqueous 1% SDS, cleavage of the glycosidic bonds of the KDO units

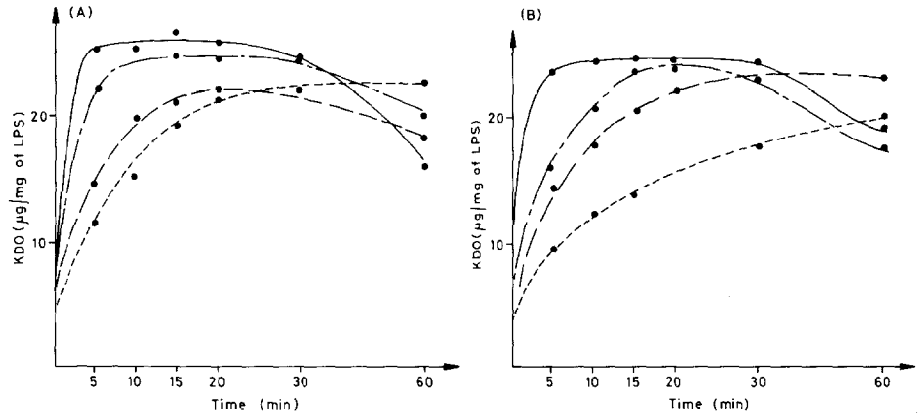

Fig. 2. Influence of the degree of dispersion on the rate of acid-catalysed release of thiobarbiturate-positive material from *S. typhi* (A) and *E. coli* (B) endotoxins. Final SDS concentrations: ----, 0%; — — —, 0.2%, - — -, 0.5%; ——, 1%. Conditions as in Fig. 1.

present in the endotoxins of *B. pertussis* (Fig. 1), *E. coli* (Fig. 2A), and *Salmonella typhimurium* (Fig. 2B) proceeded at rates similar to that of methyl 3-deoxy-α-D-*manno*-2-octulopyranosidonic acid under the same conditions of acidity, but in the absence of detergent. On treatment at pH 3.4 and 100° for 1 h in the absence of detergent, the *B. pertussis* endotoxin released[4] only PS-1 of LPS-1, whereas, in the presence of 1% of SDS, both LPS-1 and LPS-2 were cleaved within 30 min, as shown by the appearance of 90% of the neutral sugars as lipid-free, water-soluble material.

Although these conditions of hydrolysis can be considered as near ideal for the release of the polysaccharide chains of the *B. pertussis* endotoxin, and probably for other endotoxins, particularly those that are cleaved only in relatively harsh conditions, they are not adequate for the isolation of the intact hydrophobic region. Hydrolysis of the *B. pertussis* endotoxin with acetic acid (pH 3.4, 1 h, 100°), even in the absence of detergents, yielded isolated Lipid A preparations that had lost 80% of their acid-labile phosphate (Table I). Acceleration of the acid-catalysed hydrolysis of simple phosphate esters by detergents has been demonstrated[19] and was observed for the *B. pertussis* endotoxin (Fig. 3); its magnitude precluded the use of aqueous 1% SDS at pH 3.4. However, further experiments revealed that

TABLE I

PHOSPHORUS, HEXOSAMINE (GlcN), AND FATTY ACID (FA) CONTENTS (μEQUIV/mg)

	P	*GlcN*	*FA*	*P/GlcN*
Isolated Lipid A-1, pH 3.4	0.77	1.0	2.86	0.77
Isolated Lipid A-1, pH 4.5, SDS	1.14	1.14	2.96	1.0

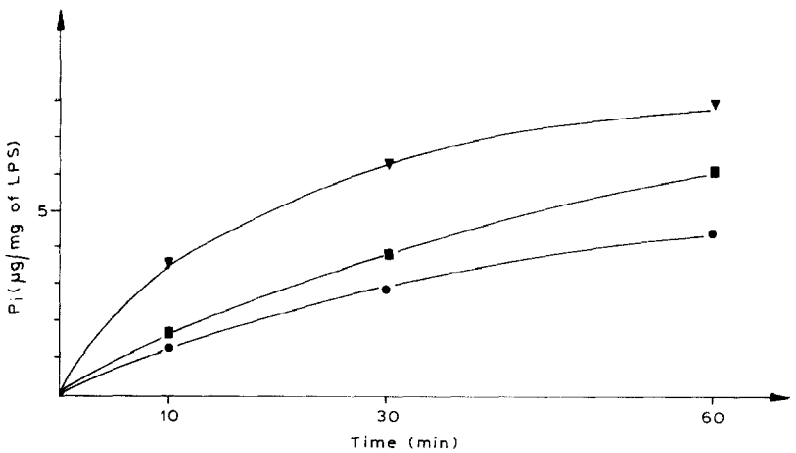

Fig. 3. Influence of the concentration of detergent on the rate of release of inorganic phosphate from *B. pertussis* endotoxin at pH 3.4 and 100°. SDS concentrations: —●—, 0%; —■—, 0.2%; —▲—, 1%.

hydrolysis at pH 4.5 and 100° (ref. 8) in aqueous 1% SDS released only small amounts of inorganic phosphate.

Before attempting to sever the two domains of the *B. pertussis* endotoxin under these conditions, the rates of hydrolysis of synthetic[20] 2-deoxy-2-[(3*R*)-3-hydroxytetradecanamido]-α- and -β-D-glucopyranose 1-phosphates were measured. Under both O'Brien's conditions (0.665M H_2SO_4 at 23°) and at pH 4.5 (100°), the rates of release of phosphate from the α and β acetamido and the α and β 3-hydroxytetradecanamido derivatives were identical. The difference in rates of hydrolysis of the α and β anomers of the 3-hydroxytetradecanamido derivatives were as marked as those of their 2-acetamido analogues[3] and, consequently, could serve to identify the anomer present in endotoxins. Thus, at pH 4.5 and 100°, 2-deoxy-2-[(3*R*)-3-hydroxytetradecanamido]-β-D-glucopyranose 1-phosphate was completely dephosphorylated within 30 min, whereas the α anomer retained 94% of its phosphate (Fig. 4).

As judged by the thiobarbiturate assay[18] (which, for the *B. pertussis* endotoxin, monitors cleavage of KDO units of LPS-1 only[4]) under the same conditions of pH and temperature, but in the presence of 1% of SDS, hydrolysis of the glycosidic bond of KDO was complete within 1 h (Fig. 5). Accordingly, an "isolated Lipid A" fragment was prepared by treating the *B. pertussis* endotoxin with aqueous 1% SDS of pH 4.5 for 1 h at 100°. Since, under these conditions, severance of the 2 domains of LPS-2 of the endotoxin requires treatment for ~4 h, the isolated Lipid A recovered was mainly that of LPS-1; it will be referred to as "isolated Lipid A-1, pH 4.5, SDS". This material represented ~40% of the hydrophobic regions present in the endotoxin as judged by its content of 3-hydroxytetradecanoic acid

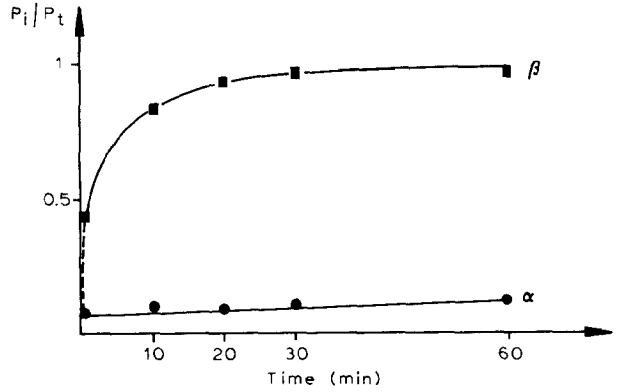

Fig. 4. Kinetics of the release of phosphate from 2-deoxy-2-[(3*R*)-3-hydroxytetradecanamido]-α- (—●—) and -β-D-glucopyranose 1-phosphate (—■—). (*a*) In 0.665M sulphuric acid at 26°. Samples (20 μg) of the anomeric phosphates were treated with 0.665M sulphuric acid (200 μL) at 26° for 10, 20, 30, and 60 min. The mixtures were neutralised (M NaOH), their volumes were brought to 0.5 mL, and inorganic phosphate was determined[27]. (*b*) In 10mM sodium acetate buffer of pH 4.5 at 100°. Samples (20 μg) of the anomers were dissolved in the buffer (200 μL), and the mixtures were kept at 100° for 10, 20, 30, and 60 min. Following adjustment of the volumes to 0.5 mL, the amount of inorganic phosphate was determined[27]. Data points from experiments (*a*) and (*b*) were superimposable.

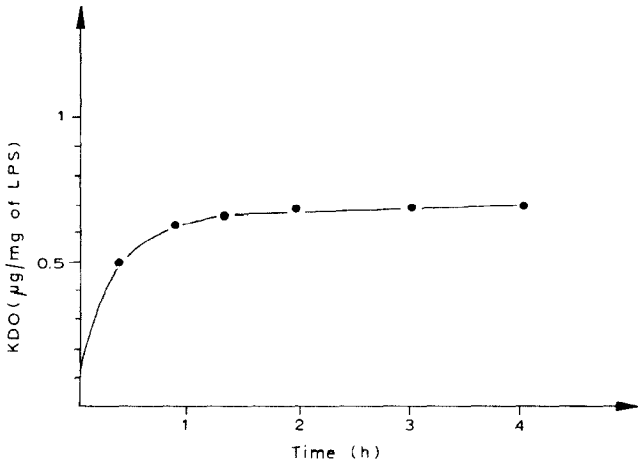

Fig. 5. Kinetics of the release of PS-1 of the *B. pertussis* endotoxin at pH 4.5 and 100° in aqueous 1% SDS as measured by the thiobarbiturate assay; the ammonium salt of KDO was used as reference.

and its composition was that of a GlcN–GlcN bisphosphate substituted by fatty acids (Table I). The presence of a β-GlcN-(1→6)-GlcN unit in the hydrophobic region of the *B. pertussis* endotoxin has been established[21].

When the "isolated Lipid A-1, pH 4.5, SDS" preparation was hydrolysed either under O'Brien's conditions[3], or at pH 4.5 and 100°, both in the presence and in the absence of SDS, negligible amounts of inorganic phosphate were formed and reducing *N*-acylhexosamine units were not detected either before or after hydrolysis.

In order to demonstrate that glycosidically bound phosphate was present, the "isolated Lipid A-1, pH 4.5, SDS" was hydrolysed with 0.25M HCl at 100° which released, within 30 min, 43% of the phosphate as inorganic phosphate, 0.7 mol of "reducing" *N*-acylhexosamine units being formed concomitantly (Fig. 6). This apparent discrepancy was accounted for by the fact that, under similar conditions of hydrolysis, 2-deoxy-2-[(3*R*)-3-hydroxytetradecanamido]-α-D-glucopyranose 1-phosphate quantitatively released its phosphate content within 30 min and produced, concomitantly, 0.7 mol of "reducing" *N*-acyl-GlcN units and 0.3 mol of NH_2 groups. Thus, the 2-deoxy-2-[(3*R*)-3-hydroxytetradecanamido]-D-glucopyranose 1-phosphate present in the hydrophobic region of the *B. pertussis* endotoxin was the α anomer.

"Isolated Lipid A-1, pH 4.5, SDS" was considerably more pyrogenic[22] than Lipid A preparations obtained[23] by hydrolysis in the absence of SDS and its activity was similar or superior to that of the intact endotoxin. This finding suggests that those molecular structures that are required to trigger appearance of fever have been conserved during the hydrolysis. Other biological properties of "isolated Lipid A-1, pH 4.5" are under investigation.

EXPERIMENTAL

Culture conditions of *B. pertussis* (strain 1414, phase I, vaccinal strain, Institut Mérieux, Lyon, France), extraction, and purification of the endotoxin have been described[5]; endotoxins of *Salmonella typhi* and *Escherichia coli* were prepared by the same procedure.

Analytical methods. — Hexosamines were determined[24] after hydrolysis with 4M HCl for 6 h at 100° using a solution of acetylacetone in 0.5M sodium carbonate. Reducing, *N*-acylated GlcN units of "Lipid A" preparations were determined[25] using an incubation period of 25 min at 100°; 2-deoxy-2-[(3*R*)-3-hydroxytetradecanamido]-D-glucose was used as reference compound. Phosphorus was determined by the methods of Chen *et al.*[26] or Parvin and Smith[27], as specified. The modified thiobarbiturate reaction[18] was employed to follow the kinetics of cleavage of the glycosidic bond of 3-deoxy-2-octulosonic acid (KDO). Fatty acids were determined[28] after hydrolysis with 4M HCl for 2 h at 100° (ref. 29), 3-hydroxytetradecanoic acid being used as reference. Primary amines were determined by the method of Moore and Stein[30], and neutral sugars by the method of Dubois *et al.*[31]. T.l.c. of Lipid A preparations was performed on Kieselgel 60 F_{254} (Merck), using solvents that were distilled immediately before use. G.l.c. was performed as described[32]. Sodium dodecyl sulphate (SDS), reagent grade, was purchased from Serva (Heidelberg, FRG), ^{14}C-SDS was from the Commissariat à l'Energie Atomique (Saclay, France).

Influence of the concentration of the detergent on the hydrolysis of the B. pertussis *endotoxin at pH 3.4 and 100°. — (a) On the rate of appearance of thiobarbiturate-positive material.* Three samples (5 mg each) of the endotoxin were each dispersed (vortex) in aqueous 1% SDS (0.25 mL) and the dispersions were immersed in boiling water for 5 min. To the cooled resulting clear "solutions" were added water (1, 0.625, and 0 mL, respectively) and aqueous 1% SDS (0, 0.375, and 1 mL, respectively) to obtain endotoxin concentrations of 4 mg/mL and 0.2, 0.5, and

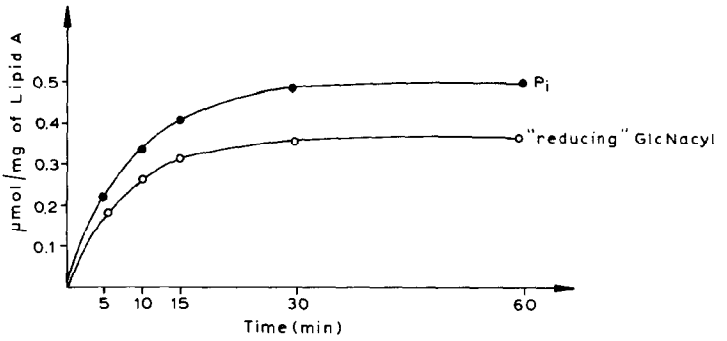

Fig. 6. Kinetics of the simultaneous formation of inorganic phosphate and "reducing" *N*-acyl-GlcN upon treatment of "isolated Lipid A-1, SDS, pH 4.5" of *B. pertussis* endotoxin with 0.25M HCl at 100°. For details, see Experimental.

1% SDS in a final volume of 1.250 mL. A sample (4 mg/mL, 1.25 mL) of endotoxin, dispersed in water, served as "reference". The pH of the mixtures was brought to 3.4 by the addition of glacial acetic acid, and the mixtures were then immersed in boiling water. KDO was determined with thiobarbiturate in samples (0.125 mL) withdrawn at 0, 5, 10, 20, and 30 min. The results are shown in Fig. 1.

(b) On the rate of release of inorganic phosphate. Samples (5 mg) were treated as in (*a*) and phosphate was determined[26] on aliquots (0.015 mL) withdrawn at intervals. The results are shown in Fig. 3.

Preparative hydrolysis of endotoxins at pH 3.4 and 100° in aqueous 1% SDS: preparation of polysaccharide chains. — B. pertussis endotoxin (100 mg) was dispersed (Bransonic ultrasonic bath, 1 min) in aqueous 1% SDS (25 mL) at 20°. The mixture was immersed in boiling water for 5 min and then cooled, the pH was brought to 3.4 by the addition of acetic acid, and the mixture was again immersed in boiling water for 30 min. The cooled mixture was lyophilised. In order to remove the detergent, ethanol (15 mL) was added to the residue, the suspension was thoroughly mixed (vortex), and centrifuged (2000*g*, 10 min). The supernatant solution was discarded and the extraction was repeated twice. Water (25 mL) was then added to the residue and the mixture was centrifuged (150,000*g*, 90 min). Of the neutral sugars present in the sample, 90% (11 mg) were recovered from the aqueous supernatant solution.

When *Salmonella typhimurium* endotoxin was treated similarly, 97% of the neutral sugars were found in the supernatant solution.

Hydrolysis of the B. pertussis *endotoxin at pH 4.5 and 100° in aqueous 1% SDS: preparation of "isolated Lipid A-1, pH 4.5, SDS".* — The endotoxin (100 mg) in sodium acetate buffer (10mM, pH 4.5, containing 1% of SDS; 5 mL) was first sonicated (5 min, Bransonic bath), then immersed in boiling water (5 min), and finally diluted with the same buffer (20 mL; final endotoxin concentration, 4 mg/mL). The mixture was kept for 1 h at 100°, then cooled, and lyophilised. The dry residue was dispersed in water (2 mL) by sonication, and the suspension was poured into acidified (100 μL of 4M HCl) ethanol (20 mL), again sonicated, and centrifuged (2000*g*, 5 min). The supernatant solution was removed and the sediment was washed with ethanol (3 × 20 mL). In order to ascertain that removal of the detergent was complete, the experiment was also performed with labelled SDS (^{14}C, specific activity, 54.5 mCi; 50 μCi): no radioactivity was detected in the pellet. The lyophilised material (60 mg) contained (colorimetry) 2.7 μmol/mg of neutral sugars; 3 spots (R_F 0, 0.19, and 0.32) were detected (charring with sulphuric acid) by t.l.c. (chloroform–methanol–water–triethylamine, 30:14:2.5:0.1). In order to remove the material containing neutral sugars (R_F 0), the material (60 mg) was suspended in chloroform–methanol–water (30:14:2.5, 1 mL), and the thick suspension was carefully layered on a glass filter-paper disc (60 mm ∅) supported by a sintered glass filter (Sovirel No. 4) while being continuously dried by a stream of warm air, so as to form a uniform layer. Lipid material, free of neutral sugars, was then eluted by dropwise addition of the same solvent, the elution being

monitored by t.l.c.: no material having R_F 0 should be present in the eluate. If all of the lipid material is not eluted by 50 ml of the solvent, the same mixture containing triethylamine (0.1, v/v) may be used. Organic solvents were removed from the filtrate at ~12 mmHg at room temperature and the aqueous residue was lyophilised. The residue (12 mg) ("isolated Lipid A-1, pH 4.5, SDS") contained P, 1.14 μequiv/mg; hexosamine, 1.13 μequiv/mg; and fatty acids (as 3-hydroxytetradecanoic acid), 2.96 μequiv/mg. In t.l.c., two spots (R_F 0.19 and 0.32) were detectable (charring with sulphuric acid).

Absence of measurable amounts of polysaccharide material in the lipid was ascertained by determination of its heptose content as follows. A sample (1 mg) of the lipid was hydrolysed with HCl (0.5 mL, 2M; 2 h, 100°). Fatty acids were extracted with chloroform (3 × 0.5 mL), the aqueous phase was repeatedly concentrated with toluene in order to remove all the acid, and the residue was dissolved in water (0.25 mL) and treated with aqueous $NaBH_4$ (0.25 mL; 5 mg/mL) for 4 h. Solvents were removed, and methanol containing some acetic acid was repeatedly evaporated from the residue, which was then acetylated and analysed[32] by g.l.c. No detectable amounts of heptitol were found upon injection of aliquots corresponding to 200 μg of "isolated Lipid A-1, pH 4.5, SDS".

Kinetics of release of inorganic phosphate, and formation of reducing N-*acylhexosamine units from 2-deoxy-2-[(3*R*)-3-hydroxytetradecanamido]-α-*D*-glucopyranose 1-phosphate upon treatment with 0.25*M *HCl at 100°.* — Samples (20 μg) of synthetic 2-deoxy-2-[(3*R*)-3-hydroxytetradecanamido]-α-D-glucopyranose 1-phosphate were treated with 0.25M HCl (300 μL) for 5, 10, 15, and 30 min at 100°, and the amount of inorganic phosphate was determined[26]; the release of phosphate was complete within 15 min. Under similar conditions (60-μg samples, 300 μL of 0.25M HCl), the release of "reducing" *N*-acylhexosamine units[24] was concomitant, but only 0.7 mol of *N*-acylhexosamine was found per mol of inorganic phosphate. Free NH_2 groups were estimated on aliquots of the 15-min hydrolysate: 0.3 mol was found per mol of inorganic phosphate present.

Release of inorganic phosphate and formation of reducing 2-acylamino-2-deoxyglucose upon treatment of "isolated Lipid A-1, pH 4.5, SDS" of the B. pertussis *endotoxin with 0.25*M *HCl at 100°.* — Samples of the lipid were treated as above and the amount of inorganic phosphate produced was determined[26] after 5, 10, 15, 30, and 60 min of hydrolysis. When phosphate release (Fig. 6) appeared to be complete (30 min), 43% of the total phosphorus-content of the sample had been released as inorganic phosphate. The kinetics of the formation of reducing *N*-acylhexosamine units were established by treating samples (300 μg) with 0.25M HCl as described above, and the reducing GlcN was determined in the dried samples. The results are shown in Fig. 6.

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