The Fatty Acid Content of the *Bordetella pertussis* Endotoxin

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The fatty acid content of *Bordetella pertussis* endotoxin has been estimated by several methods. Expressed as 3-hydroxytetradecanoic acid, it was $0.74 \mu\text{mol}$ (mg lyophilized material)⁻¹, $0.38 \mu\text{mol}$ being ester-bound, and $0.32 \mu\text{mol}$ in amide linkage. Reported molar ratios of ester-bound to amide-bound fatty acids in endotoxins of various bacterial species range from 2.4 to 2 in *B. pertussis*, to 5 to 2 in *Salmonella minnesota*; according to these figures large differences must exist in the degree of substitution, and the substitution pattern of the glucosaminyl- β -1,6-glucosamine unit present in the hydrophobic region of endotoxins. When fatty acids, released by acid and alkaline hydrolyses of the *B. pertussis* endotoxin, were extracted into chloroform, unidentified chromogenic substances appearing in the extract interfered with their colorimetric estimation; no interference was observed when hexane was used instead of chloroform.

INTRODUCTION

Endotoxins of Gram-negative bacteria are lipopolysaccharides built up from a hydrophilic polysaccharide chain, and a hydrophobic region. The latter contains, as major constituent, phosphorylated glucosaminyl- β -1,6-glucosamine, to which various fatty acids are bound by ester and amide linkages (Hase & Rietschel, 1976). The extract structure of the hydrophobic region is not known: when cleaved off by mild acid treatment, it appears as a heterogeneous mixture, insoluble in water, usually referred to as 'lipid A'. Structures derived from the glucosaminyl- β -1,6-glucosamine disaccharide identified (Imoto *et al.*, 1983; Qureshi *et al.*, 1982; Rosner *et al.*, 1979; Strain *et al.*, 1983; Takayama *et al.*, 1983) in 'lipid A' preparations, when produced by chemical synthesis (Charon *et al.*, 1983; Kiso *et al.*, 1981; Kusumoto *et al.*, 1983; Imoto *et al.*, 1984), so far have failed to reproduce the complete set of biological properties of 'lipid A' preparations (Galanos *et al.*, 1984; Kotani *et al.*, 1983; Lüderitz *et al.*, 1983; Matsuura *et al.*, 1983; Yasuda *et al.*, 1982, 1984). It has been suggested that the nature and type of linkage of the fatty acids attached to the phosphorylated glucosamine disaccharide might be critical for the elicitation of the biological properties of the 'lipid A' preparations.

In view of the foregoing, and of our current interest in the chemical structure (Le Dur *et al.*, 1980) and immunological activities (Ayme *et al.*, 1980; Haeffner-Cavaillon *et al.*, 1982, 1983, 1984) of the *Bordetella pertussis* endotoxin, a quantitative study was undertaken of the fatty acids present in this endotoxin, and the conditions of their release; the results are reported in this paper.

METHODS

Cell growth. Culture conditions of *Bordetella pertussis* cells in the liquid medium of Cohen & Wheeler (1946), extraction of the endotoxin by the phenol–water procedure (Westphal *et al.*, 1952), and its purification by sedimentation have been described previously (Ayme *et al.*, 1980).

Fatty acid extraction. Alkaline hydroxylamine at 65 °C (Snyder & Stephens, 1959), 0.05 M-NaOMe (sodium methoxide) in methanol, and methanol saturated with ammonia gas, both at room temperature, were used to

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release ester-bound fatty acids. For estimation of both ester- and amide-bound fatty acids ('total fatty acids'), the endotoxin was treated with 4 M-HCl at 100 °C for 2 h (Haeffner *et al.*, 1977).

RESULTS

Estimation of total fatty acids

By colorimetry. Samples (1–2 mg) of *B. pertussis* endotoxin, dispersed in 0.5 ml 4 M-HCl, were kept at 100 °C for 2 h in sealed tubes. The mixture was brought to dryness by evaporation under vacuum, and dry toluene was evaporated (3–4 times, 1 ml) from the residue to remove any residual mineral acid. Then 2 M-NaOH (0.5 ml) was added to the dry residue; the tube was sealed, and the mixture was kept at 100 °C for 2 h. The cooled mixture was acidified (4 M-HCl) and the fatty acids were extracted with hexane (3 × 1.5 ml). Ethereal diazomethane solution was added; a few minutes later solvents were removed, and the fatty acids were estimated according to the procedure of Snyder & Stephens (1959). The results, expressed as μmol 3-hydroxytetradecanoic acid (mg lyophilized endotoxin) $^{-1}$ (3-hydroxytetradecanoic acid being the most abundant fatty acid present in the endotoxin), gave a value of 0.74 (SD 0.06; $n = 9$). The same value was obtained if the cooled acid hydrolysate was directly extracted with hexane, and the fatty acids estimated after esterification with diazomethane.

Two samples, when similarly treated except that the fatty acids were extracted with chloroform instead of hexane, gave 1.15 and 1.2 μmol 3-hydroxytetradecanoic acid (mg endotoxin) $^{-1}$. Absorption spectra of the coloured solutions obtained with standard methyl 3-hydroxytetradecanoate, with fatty acids of the endotoxin extracted with hexane, and with fatty acids of the endotoxin extracted with chloroform, are reproduced in Fig. 1.

By gas-liquid chromatography (GLC). Methyl stearate (33.84 μg) was added to a sample (0.865 mg) of endotoxin, and the mixture was sequentially treated with 4 M-HCl and 2 M-NaOH as described above. The cooled, acidified mixture was first extracted with hexane (3 × 1.5 ml) and then with chloroform (1 × 1.5 ml): the extracts were then treated separately with ethereal diazomethane solution. The methyl esters remaining after removal of the solvents were dissolved in a known volume (30 μl) of ethyl acetate, and 1 μl samples were injected for analysis by GLC [stainless steel column, 2 m × 3.2 mm, 3% SE30 on Gaschrom Q, 100–120 mesh, 100 °C–200 °C (8 °C min $^{-1}$); or stainless steel column, 2 m × 3.2 mm, 3% OV-1 on Gaschrom Q, 100–120 mesh, 120 °C–260 °C (4 °C min $^{-1}$); carrier gas: He]. Individual peaks were integrated. The amount of 'total fatty acids' (expressed as above) present in the hexane extract was 0.64 μmol (mg endotoxin) $^{-1}$. No fatty acids were found in the chloroform extract.

Sequential release of ester- and amide-bound fatty acids, and colorimetric estimation

Samples (2–4 mg) of *B. pertussis* endotoxin were treated with the alkaline (pH 13–14) hydroxylamine reagent of Snyder & Stephens (1959) at 65 °C for 5 min. The cooled mixture was centrifuged, the supernatant removed, and the sediment washed with ethanol (3 × 1.5 ml). The supernatants and washings were pooled, the solvent evaporated, and the resulting dry residue treated with 4 M-HCl (0.5 ml, 100 °C, 1 h). The cooled mixture was extracted with hexane, ethereal diazomethane solution was added to the extract, and the fatty acids were estimated according to Snyder & Stephens (1959). The mean value of eight determinations was 0.38 μmol (SD 0.04) ester-bound fatty acid (mg endotoxin) $^{-1}$, calculated as 3-hydroxytetradecanoic acid.

The ethanol-washed sediment was dried *in vacuo*, and treated with 4 M-HCl (0.5 ml, 100 °C, 2 h). Fatty acids were extracted from the coloured mixture with hexane (3 × 1.5 ml), ethereal diazomethane solution was added to the extract and, after removal of the solvents, fatty acids were estimated colorimetrically. The mean value of eight determinations was 0.32 (SD 0.043) μmol amide-bound fatty acid (mg endotoxin) $^{-1}$, calculated as 3-hydroxytetradecanoic acid. From these data the calculated fatty acid content of the *B. pertussis* endotoxin was 0.71 (SD 0.064) μmol fatty acid (mg endotoxin) $^{-1}$.

Release of ester-bound fatty acids by treatment with ammonia in methanol

A sample (11 mg) of the *B. pertussis* endotoxin, placed in a tube, was dispersed (ultrasonic bath, 20 °C, 5 min) in anhydrous methanol (5 ml); the mixture was cooled to 0 °C, and saturated

Fig. 1. Absorption spectra of the Fe^{3+} -complexes of fatty hydroxamates formed from methyl 3-hydroxytetradecanoate (—) (reference), and from the fatty acid mixture of the *B. pertussis* endotoxin released by consecutive acid and alkaline hydrolyses, esterified, and extracted with hexane (---); the same extracted with chloroform (· · · · ·).

with ammonia gas. The tube was sealed with a teflon-lined cap, and kept for 24 h at room temperature under constant stirring. Ammonia was removed from the mixture by bubbling nitrogen through it, the solvent was evaporated, hexane (1 ml) was added to the residue, the contents were thoroughly mixed (vortex), and then centrifuged. The supernatant was collected and the extraction repeated three times. The pooled extracts were brought to dryness, and treated with 4 M-HCl (0.5 ml, 100 °C, 2 h). The cooled mixture was extracted with hexane (3×1.5 ml), the solvent evaporated, and the free fatty acids converted into their methyl esters (diazomethane) which were estimated colorimetrically. The residue was suspended in methanol, saturated with ammonia as above, and the procedure was repeated daily until the amount of fatty acid released became negligible (7 d). The total amount of ester-bound fatty acids was $0.31 \mu\text{mol}$ (mg endotoxin) $^{-1}$. The amount of amide-bound fatty acids recovered after acid hydrolysis of the sediment was $0.38 \mu\text{mol}$ (mg endotoxin) $^{-1}$.

Release of ester-bound fatty acids with 0.05 M-sodium methoxide in methanol

NaOMe (0.05 M) in methanol (5 ml) was added to a sample (11 mg) of *B. pertussis* endotoxin, and the stirred mixture kept in a sealed vessel for 24 h. The mixture was neutralized with acetic acid (pH paper), and the solvent was evaporated. The dry residue was extracted with hexane (3×2 ml), and the insoluble material sedimented by centrifugation. Pooled extracts were concentrated, treated with a few drops of ethereal diazomethane to ensure that all the fatty acids were present as methyl esters, and the fatty acids estimated colorimetrically. The sediment was treated with 0.05 M-NaOMe as above, and the procedure was repeated until the amount of fatty acids recovered was negligible (7 d). The amount of amide-bound fatty acids remaining in the residue was then determined colorimetrically after acid hydrolysis (4 M-HCl, 100 °C, 2 h), extraction with hexane (3×2 ml) and esterification with diazomethane. The amount of ester-bound fatty acid was $0.35 \mu\text{mol}$ (mg endotoxin) $^{-1}$, that of amide-bound fatty acid was $0.39 \mu\text{mol}$ (mg endotoxin) $^{-1}$, both expressed as 3-hydroxytetradecanoic acid.

DISCUSSION

Both acid and alkaline hydrolyses have been used to release fatty acids from endotoxins, but no generally applied method appears to be accepted, nor have comparative studies been made. Methods used for the estimation of 'total fatty acids' include hydrolysis with 4 M-NaOH or 4 M-HCl at 100 °C for 5 h (Rietschel *et al.*, 1972), 2 M-HCl in anhydrous methanol at 85 °C for 16 h (Bryn & Rietschel, 1978), a mixture of 12 ml conc. HCl (aqueous) and 150 ml MeOH at 85 °C for 18 h (Wollenweber *et al.*, 1983). In the present study 'total fatty acids' were estimated after acid hydrolysis with 4 M-HCl (100 °C, 2 h), followed by treatment with 2 M-NaOH (100 °C, 2 h), as it has been established previously (Haefner *et al.*, 1977) that with *B. pertussis* endotoxin this procedure gives the highest recovery of fatty acids. If the fatty acids were estimated colorimetrically, the alkaline treatment (required to cleave acyloxy derivatives of hydroxy-fatty acids) could be omitted.

For the estimation of ester-bound fatty acids, transesterification with 0.25 M-NaOMe at 37 °C for 15 h (Broady *et al.*, 1981) or brief (2–3 min) hydroxylaminolysis (Rietschel *et al.*, 1972) have been applied. In the present case the latter method was used, but the time of hydroxylaminolysis at 65 °C was extended to 5 min. Lengthening the treatment with hydroxylamine was necessary to achieve complete removal of ester-bound fatty acids. The original method was elaborated for the estimation of fatty acids in triglycerides and lecithin, and these substances are more soluble, or at least more easily dispersed in the medium than the very insoluble endotoxin. Further extension of the reaction-time is, however, not advisable, as in the strongly alkaline medium (pH 13–14) incipient release of amide-bound fatty acids also occurs.

For quantitative estimation both the colorimetric method of Snyder & Stephens (1959), and integration of peaks observed upon GLC of fatty acid methyl esters were used; as the latter gave somewhat lower values, the colorimetric method was used preferentially. When chloroform was used to extract the fatty acids released from the endotoxin by successive acid and alkaline hydrolyses, much higher values were measured by the colorimetric method than in those instances when hexane was used. That this was not due to incomplete extraction could be established by extracting the aqueous solution first with hexane, and then with chloroform: no fatty acids could be detected by GLC in such chloroform extracts. Furthermore, the internal standard (octadecanoic acid) was quantitatively recovered in the hexane extract. Examination of the spectra of the coloured complex formed upon addition of ferric chloride to solutions of the fatty acid hydroxamates revealed that while samples extracted with hexane gave rise to spectra identical to that obtained with pure, authentic fatty acids, samples extracted with chloroform gave spectra unsuitable for quantitative estimations (Fig. 1).

The total fatty acid content of the *B. pertussis* endotoxin, calculated as 3-hydroxytetradecanoic acid, was 0.74 µmol (mg lyophilized endotoxin)⁻¹, comparable to the figure of 0.75 µmol (mg endotoxin)⁻¹ published for *Chromobacterium violaceum* NCTC 9694 (Hase & Rietschel, 1977), of 1.1 µmol (mg endotoxin)⁻¹ for *Vibrio cholerae* (Broady *et al.*, 1981), but much lower than the 2.45 µmol (mg endotoxin)⁻¹ measured for *Salmonella minnesota* R595 endotoxin (Wollenweber *et al.*, 1983), which is only composed of two KDO (2-keto-3-deoxyoctonic acid) units and the hydrophobic region. It is somewhat higher than the value of 0.54 µmol fatty acids (mg endotoxin)⁻¹ found in *Bacteroides fragilis* NCTC 9342 (Wollenweber *et al.*, 1980).

According to the data obtained by hydroxylaminolysis for ester-bound fatty acids [0.38 µmol (mg endotoxin)⁻¹], and by acid hydrolysis of the de-esterified insoluble residue for the amide-bound fatty acids [0.32 µmol (mg endotoxin)⁻¹], in *B. pertussis* endotoxin 2.4 ester-bound fatty acids appear to be present for every 2 amide-bound fatty acids. Almost identical figures were obtained when ester-bound fatty acids were released by treatment with ammonia in methanol [0.31 µmol (mg endotoxin)⁻¹], or with 0.05 M-NaOMe in methanol [0.35 µmol (mg endotoxin)⁻¹]; in all cases the sum of ester- and amide-bound fatty acids came close to the value found for the total amount of fatty acids [0.74 µmol (mg endotoxin)⁻¹].

Reported ratios of ester-to-amide-bound fatty acids are 3:2 for *Chromobacterium violaceum* NCTC 9694 (Hase & Rietschel, 1977), 4:2 for *Vibrio cholerae* (Broady *et al.*, 1981) and 5:2 for *Salmonella minnesota* R595 (Wollenweber *et al.*, 1982) endotoxins; reported ratios for 'lipid A' preparations are 3:2 for *Escherichia coli* K12, strain D31m4 (Strain *et al.*, 1983), and 4:2 for *E.*

coli 08 K27, strain F515 (Imoto *et al.*, 1983) and for *Salmonella typhimurium*, strain G30/C21 (Qureshi *et al.*, 1982; Takayama *et al.*, 1983). These data suggest that rather large differences are likely to exist in the fine structures of the hydrophobic regions isolated from various Gram-negative bacteria.

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