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GAS CHROMATOGRAPHIC DETERMINATION OF THE FATTY ACID COMPOSITION OF ENDOTOXINS FROM DIFFERENT BACTERIA

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SUMMARY

Endotoxins from four bacterial species extracted by three different procedures were acid-methanolyzed and the methyl esters of the fatty acids were analyzed by packed-column gas chromatography. There were qualitative and quantitative differences in the fatty acid profiles of the lipopolysaccharides isolated from four Gram-negative bacteria. Our data show considerable lot-to-lot variations in amounts of four methyl esters from the same bacterial serotype extracted by the same procedure and in the same bacterial serotype extracted by different procedures. These results indicate that extraction and perhaps culture conditions, as well as bacterial species, affect the fatty acid composition of endotoxins, hydrolyzed and derivatized by these procedures.

INTRODUCTION

Endotoxins, which are components of the cell walls of Gram-negative bacteria, are considered responsible for many of the symptoms that occur during Gram-negative infections. According to Wolff [1], the human being is the most sensitive animal to bacterial endotoxins. Recently, Rylander and co-workers [2—4] have reported that endotoxins are responsible for many of the symptoms of byssinosis. Structural investigations of endotoxins from *Salmonella, Escherichia coli* and related bacteria have shown them to be composed of three regions: (a) a core polysaccharide; (b) a polysaccharide chain with repeating oligosaccharide units of varying length (the O-specific side-chains); (c) a lipid portion, referred to by some workers as lipid A [5, 6].

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These researchers have reported that the lipid A moiety is the primary agent responsible for the endotoxic activity of Gram-negative bacteria. A number of authors have published on the composition of lipopolysaccharide (LPS) from specific bacteria [7, 8]. Moss [9] and Dees et al. [10] have used gas- liquid chromatographic (GLC) profiles of fatty acids from bacterial cells as a means of identifying and classifying bacterial species.

The *Limulus* amoebocyte lysate (LAL) test is commonly used to determine LPS content. Wachtel and Tsuju [11] have found differences in sensitivity of up to 100-fold among different commercial LAL reagent preparations. Weary et al. [12] reported the relative potencies of four reference endotoxin standards measured by LAL and rabbit pyrogen tests. They found variations in potency of three- to six-fold and also that some of the endotoxin standards exhibited considerable lot-to-lot variation. With the inherent variability of both reagent and standards in mind, we began investigation of a potential chemical approach to the analysis of endotoxins. Quantitation of the lipid A portion of the endotoxin was chosen for our initial work because of its implications as the primary agent responsible for the endotoxin activity of Gram-negative bacteria. The fatty acid profiles of LPS isolated from four bacteria by two extraction procedures were determined to ascertain if these profiles will be useful as a means of identifying endotoxin from specific bacteria or as a quantitative measure of lipopolysaccharide. Morris et al. [13] used the method described in this paper to estimate the amount of endotoxin in extracts of cotton dusts.

EXPERIMENTAL

Lyophylized powders of LPS extracted from the cell wall of four Gram-negative bacteria [*E. coli* (serotype 011:B4), *Serratia marcescens*, *Salmonella typhimurium*, and *Salmonella abortus equi*] were purchased from Sigma*. Two types of extracts were obtained for the four bacteria: phenol–water [14] and trichloroacetic acid (TCA) [15]. An additional butanol [16] extract was obtained for *E. coli*. Six different lots of the phenol–water-extracted LPS from the same serotype of *E. coli* were also analyzed. The LPS extracts were used without further purification. Fatty acid methyl esters of 99.9% purity were obtained as follows: methyl laurate, methyl myristate, methyl palmitate and methyl palmitoleate from Supelco; methyl 3-hydroxymyristate and methyl 2-hydroxymyristate from Applied Sciences Labs.

All glassware was made pyrogen-free by rinsing the cleaned glassware with pyrogen-free water (McGaw Labs., Irvine, CA, U.S.A.) and subsequently heating at 250°C for 0.5 h [17]. Endotoxin (5 mg) was weighed in duplicate into 12.9-cm PTFE-lined, screw-capped culture tubes. A 2-ml volume of 5% methanolic sulfuric acid (v/v) was added and the tubes were sonicated for 10 min to disperse the endotoxin. The mixture was maintained at 60°C for 2.5 h in a water bath. Pyrogen-free water (1 ml), which was also equilibrated at 60°C,

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was added to each sample to quench the reaction. The fatty acid methyl esters were extracted with two portions of light petroleum (1 ml, boiling range 35—60°C).

Injections of 5 μl of these extracts were analyzed on a Tracor 560 gas chromatograph equipped with a flame ionization detector using nitrogen as the carrier gas. The column (3.05 m × 2 mm I.D.) was silanized glass packed with GP 3% SP-2100 DOH on 100—120 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). The oven was at 150°C initially and temperature-programmed to 225°C at 4°C/min. Injection port temperature was 250°C, and the detector temperature was 300°C. The chromatographic data were acquired and integrated by the Hewlett-Packard 3354 Laboratory Automation System. The fatty acid methyl esters were quantitated by a linear least-squares regression of a standard curve prepared by plotting the integrated areas of standard solutions of methyl laurate, methyl myristate and methyl palmitate against concentration. The methyl 3-hydroxymyristate was quantitated using a determined response factor and the measured methyl myristate in each extract. Identification of several of the methyl esters was confirmed by analysis of the extracts with a Finnigan 4000 gas chromatograph—mass spectrometer.

RESULTS AND DISCUSSION

A chromatogram of the fatty acid methyl esters of endotoxin extracted from *S. typhimurium* by the TCA procedure is shown in Fig. 1. The identifications of five of the nine peaks have been confirmed by gas chromatography–mass spectrometry (GC—MS). These are methyl laurate ($C_{12:0}$), methyl myristate ($C_{14:0}$), methyl 2-hydroxymyristate (2OH $C_{14:0}$), methyl 3-hydroxymyristate (3OH $C_{14:0}$) and methyl palmitate ($C_{16:0}$). A peak at about 13.5 min has been

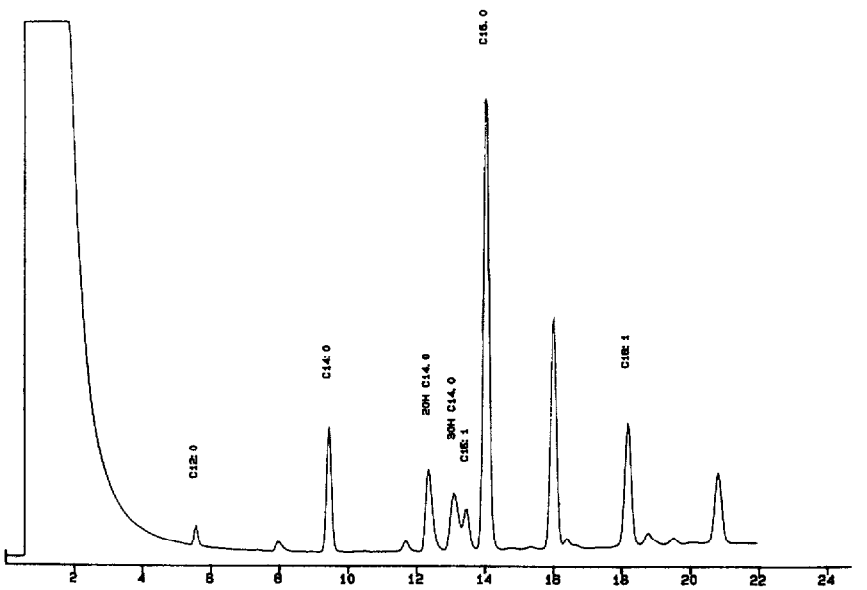

Fig. 1. Chromatogram of fatty acid methyl esters from *Salmonella typhimurium* endotoxin extracted by the TCA procedure.

tentatively identified as methyl palmitoleate ($C_{16:1}$) and one at about 18 min as methyl oleate ($C_{18:1}$). The retention times for these latter two esters compare well with known standards, and data from the GC—MS system rank these compounds as 1 or 2. The match with the library spectra is poor, however, even when compared with known standards which have been run on the same instrument and added to the library of mass spectral data. It is possible that these peaks represent more than one component and this would affect the match obtained. The other peaks have not been identified.

In the chromatograms of TCA-extracted LPS, the peaks due to methyl palmitate and higher-molecular-weight fatty acids are usually enhanced. The LPS extracted from the same bacterium by the phenol–water procedure have slightly different fatty acid profiles in that the methyl palmitoleate peak is not found in the phenol—water extracts and the concentrations of the higher-molecular-weight fatty acids are less.

The fatty acid profiles of endotoxins extracted by the same procedure from all of the bacteria in this study were qualitatively very similar. Two representa-

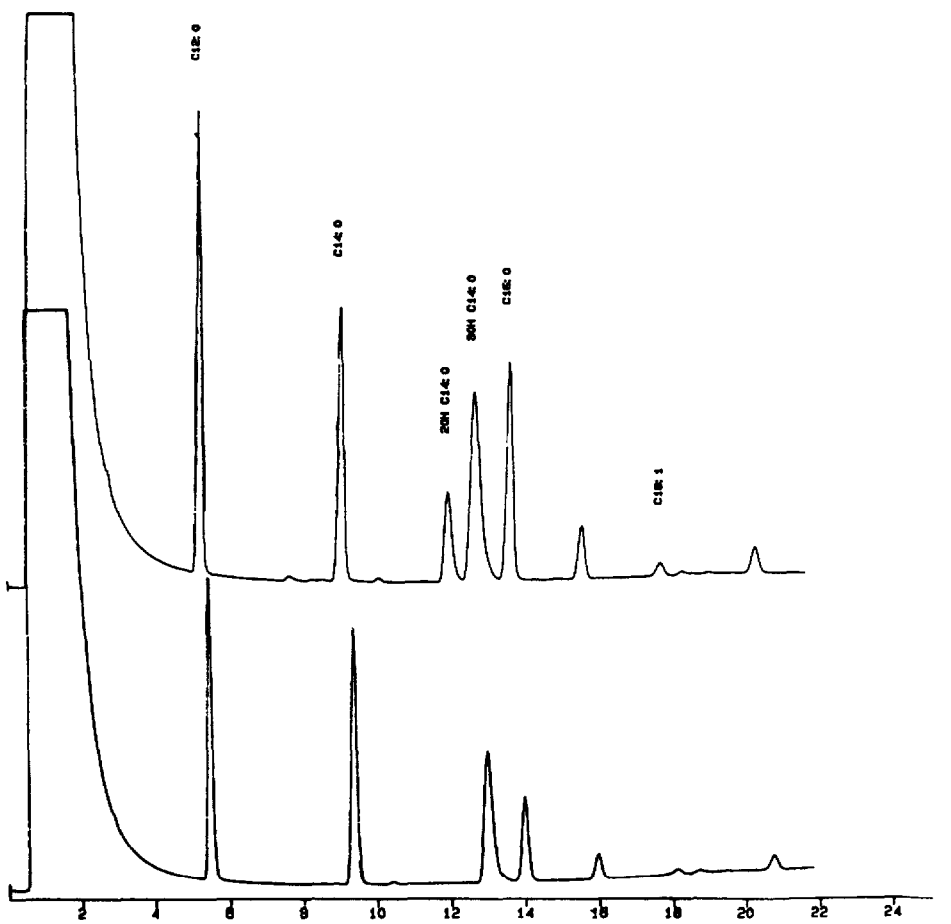

Fig. 2. Chromatograms of fatty acid methyl esters from *Escherichia coli* (bottom) and *Salmonella typhimurium* (top) endotoxins extracted by phenol—water procedures.

tive chromatograms are shown in Fig. 2. The exception was the absence of the peak assigned to the 2-hydroxymyristate in the chromatograms of endotoxin extracted from *E. coli* by the phenol—water procedure. One lot of endotoxin from *E. coli* extracted by the butanol procedure was analyzed. The peaks assigned to 2- and 3-hydroxymyristate appear in this chromatogram as in those from the TCA-extracted endotoxins. The concentration of all esters beyond methyl laurate was much lower for the endotoxin extracted by this procedure.

The commercially prepared endotoxins were analyzed for free lipids by extracting portions of the endotoxin several times with light petroleum, filtering out the endotoxin and subjecting the extract to the same methanolysis and analysis procedure as before. Very small amounts of methyl myristate and methyl palmitate were observed at the most sensitive GC setting. If methyl laurate is present, it is eluted with the solvent front at this sensitivity setting. At an attenuation high enough to reduce the solvent front prior to the time at which methyl laurate should elute, no laurate is apparent. It was concluded that the fatty acids observed in the chromatograms of the methanolyzed endotoxins were not present as contaminants, but came from the endotoxin itself.

Four methyl esters (methyl laurate, methyl myristate, methyl 3-hydroxymyristate and methyl palmitate) were quantitated for each of the endotoxins. These four esters occurred in the hydrolyzates of all LPS studied. The calibration was linear (typical equation $y = 3.9 \cdot 10^{-6}x + 7.5 \cdot 10^{-3}$) for all of the methyl esters over the concentration range 0.04–1.0 mg/ml with correlation coefficients of 0.9995–0.9999 and a standard error of estimate of 0.6—1.32%. The coefficients of variation for triplicate extractions of all four esters were less than 4% in the concentration range 0.1—1.0 mg/ml and less than 9.5% at a concentration of 0.04 mg/ml. All values reported are the means of duplicate GC analyses of at least two hydrolysis—methanolysis reactions with each endotoxin. The 3-hydroxymyristic acid ester was not included in the standard solution because its presence affected the stability of the standard solution on storage. The concentration of this ester was calculated based on its response factor and the measured concentration of methyl myristate. The recoveries of the methyl esters from the hydrolysis–methanolysis mixture were determined to be 100–114% using the calibration curve at the higher concentration range. Similar results were obtained in the lower range using a bracketing technique. The conversion efficiency of fatty acids to methyl esters was checked by weighing known amounts of the three fatty acids used as standards and subjecting them to the hydrolysis–methanolysis procedure. Conversion efficiencies were calculated by comparing the peak areas of chromatograms of the hydrolyzed, methanolyzed fatty acids with those of weighed samples of the purchased methyl esters. The conversion efficiency was 91.6% for lauric acid, 97.0% for myristic acid and 98.3% for palmitic acid.

To determine the reproducibility of the hydrolysis procedure and the GC analysis, hydrolyses of phenol–water-extracted endotoxin from *E. coli* were performed three weeks apart (Table I). The standard deviation of the determinations for methyl laurate, methyl myristate and methyl palmitate ranged from 0.02 to 0.04%. The ratios of the methyl esters based on methyl myristate show no significant differences for any ester other than the 3-hydroxymyristate, and coefficients of variation for these determinations were

TABLE I

PERCENTAGE METHYL ESTERS FROM SAME LOT OF *ESCHERICHIA COLI* EXTRACTED BY PHENOL—WATER PROCEDURE

The ratio for laurate:myristate:3-hydroxymyristate:palmitate for run 1 = 0.92:1.00:1.03:0.34 and for run 2 = 0.93:1.00:0.83:0.33.

Run No.	Percentage methyl esters			
	Laurate	Myristate	3-Hydroxymyristate	Palmitate
1	1.20	1.31	1.35	0.45
	1.24	1.34	1.38	0.46
2	1.19	1.28	1.07	0.41
	1.17	1.26	1.04	0.44

TABLE II

HYDROLYSIS—METHANOLYSIS OF LPS FROM *SALMONELLA TYPHIMURIUM*

Time of hydrolysis—methanolysis (h)	Percentage methyl esters			
	Laurate	Myristate	3-Hydroxymyristate	Palmitate
2.50	1.58	1.23	1.92	1.07
3.75	1.64	1.28	2.59	1.10

TABLE III

PERCENTAGE METHYL ESTERS FROM ENDOTOXIN FROM FOUR DIFFERENT BACTERIA EXTRACTED BY PHENOL—WATER

Bacterium	Percentage methyl esters			
	Laurate	Myristate	3-Hydroxymyristate	Palmitate
Escherichia coli	1.20	1.30	1.21	0.44
Serratia marcescens	0.14	2.18	1.56	0.74
Salmonella typhimurium	1.61	1.29	1.92	1.14
Salmonella abortus equi	0.97	0.68	1.09	0.72

2.36--4.82% which indicates the constancy of the procedure for these esters. The hydrolysis conditions used apparently do not complete cleavage of the 3-hydroxymyristate in the time used for hydrolysis. For example, *S. typhimurium*, comparing the 2.5-h hydrolysis products with those from a 3.75-h reaction time experiment, showed an increase in the 3-hydroxymyristate from 1.92 to 2.59% while the other fatty acid esters remained almost constant (Table II).

The percentage methyl esters from phenol-extracted endotoxin from each of the bacteria is given in Table III. Similar data are given in Table IV for the same bacteria extracted by the TCA procedure. The TCA-extracted endotoxin

generally has a higher concentration of palmitic acid and a lower concentration of lauric acid than the phenol—water-extracted LPS. This can be clearly seen by comparing the ratios of the fatty acid esters based on methyl myristate, summarized in Table V. In one case, that of *S. abortus equi*, the only ester that differed significantly in concentration between the phenol- water- and TCA-extracted endotoxins was methyl palmitate. This was the only bacterial endotoxin tested which showed only small differences for even a portion of the fatty acids from endotoxin from the same bacteria obtained by different extraction procedures.

To check the constancy of the fatty acid contents of the LPS isolated from the same bacteria using the same extraction procedure, profiles of different

TABLE IV

PERCENTAGE METHYL ESTERS FROM ENDOTOXIN FROM FOUR DIFFERENT BACTERIA EXTRACTED BY TCA

Bacterium	Percentage methyl esters			
	Laurate	Myristate	3-Hydroxymyristate	Palmitate
Escherichia coli	0.36	1.38	1.20	2.52
Serratia marcescens	0.03	0.69	0.64	2.88
Salmonella typhimurium	0.77	0.94	0.91	2.91
Salmonella abortus equi	0.92	0.60	1.08	4.35

TABLE V

RATIOS OF METHYL ESTERS FROM LPS LAURATE:MYRISTATE:3-HYDROXY-MYRISTATE: PALMITATE

Bacterium	Ratio of methyl esters	
	Phenol extraction	TCA extraction
Escherichia coli	0.92:1.00:1.04:0.35	0.26:1.00:0.87:1.83
Serratia marcescens	0.06:1.00:0.72:0.34	0.12:1.00:0.93:4.17
Salmonella typhimurium	1.25:1.00:1.49:0.88	0.82:1.00:0.97:3.09
Salmonella abortus equi	1.43:1.00:1.61:1.06	1.53:1.00:1.80:7.25

TABLE VI

PERCENTAGE METHYL ESTERS FROM SIX DIFFERENT LOTS OF *E. COLI* ENDOTOXIN EXTRACTED BY THE PHENOL—WATER PROCEDURE

Lot No.	Percentage methyl esters			
	Laurate	Myristate	3-Hydroxymyristate	Palmitate
1	1.24	1.34	1.27	0.46
2	0.11	0.52	0.66	0.72
3	0.60	0.70	0.72	0.40
4	0.19	0.76	0.90	0.22
5	0.14	0.74	0.63	1.02
6	1.42	1.61	1.77	0.07

TABLE VII

RATIO OF METHYL ESTERS FROM SIX DIFFERENT LOTS OF *E. COLI* ENDOTOXIN EXTRACTED BY THE PHENOL—WATER PROCEDURE

Lot No.	Ratio of methyl esters (laurate:myristate:3-hydroxymyristate:palmitate)
1	0.92:1.00:0.93:0.34
2	0.21:1.00:1.27:0.50
3	0.86:1.00:1.03:0.57
4	0.25:1.00:1.18:0.30
5	0.19:1.00:0.85:1.37
6	0.88:1.00:1.03:0.05

lots of endotoxin extracted from *E. coli* by the phenol—water procedure were compared. The results are shown in Table VI. The different lots of *E. coli* contained widely varying amounts of fatty acids. The ratios of the fatty acid esters based on methyl myristate, which are given in Table VII, summarize these differences.

Analysis of variance for the laurate, myristate, 3-hydroxymyristate and palmitate were run with respect to injection, replicate, lot, extraction procedure and bacteria. Statistically the results show no differences among the four bacteria for three of the four esters. According to Duncan's multiple range test, only the level of palmitate in *E. coli* endotoxin was significantly different from that in the other three bacterial endotoxins analyzed. Although these results show no differences among the four bacteria in most cases, the statistical test is probably not sensitive enough to distinguish differences among the bacteria. For example, as shown in Table III, the percentage laurate in the phenol—water-extracted endotoxin from the four bacteria ranges from 0.14 to 1.61, that of myristate from 0.68 to 2.18, that of 3-hydroxymyristate from 1.09 to 1.92, and that of palmitate from 0.44 to 1.14. Similar ranges occur for the endotoxin extracted by TCA as shown in Table IV. Similar variations were observed from lot-to-lot for the four esters in the six different lots of *E. coli*. This lot-to-lot variation obscures real differences among the bacteria and indicates that extraction conditions and perhaps culture conditions, as well as bacterial species, affect the fatty acid composition of the endotoxins. The growth and harvesting conditions for the commercial endotoxins are not known. Wartenberg et al. [18] reported changes in fatty acid and sugar composition of LPS from *Yersinia enterocolitica* from cultures grown at different temperatures. Ditter et al. [19] reported differences in the protein patterns determined by isoelectric focusing and in the LAL activity of endotoxins extracted at different times after bacterial growth on solid medium. These endotoxins did not differ significantly in other less sensitive tests, such as acute toxicity in mice, serum colony stimulating factor and pyrogenicity tested in rabbits.

CONCLUSIONS

The hydrolysis—methanolysis and subsequent extraction procedure gives highly reproducible quantitative results for fatty acids from the same lot of

endotoxin taken from a specific lot of bacteria. There appears to be minor qualitative and major quantitative differences in the fatty acid profiles from endotoxin isolated by the phenol–water and TCA extraction procedures. However, differences in both absolute quantity and in ratio of fatty acids based on methyl myristate occur for *E. coli* endotoxin when obtained from different lots of endotoxin isolated from the same bacteria by the same procedure. These results indicate that extraction conditions, as well as bacterial species, affect the fatty acid composition of endotoxins. Care should be exercised in using GC data from fatty acid profiles of the isolated LPS to identify bacterial species and quantitate endotoxin levels. All growth and extraction conditions must be kept the same in order to reliably use the data obtained.

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