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Purification and Characterization of the Lipid A Component of the Lipopolysaccharides from *Escherichia coli**

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A crude lipopolysaccharide fraction has been isolated from *Escherichia coli*, strain $O_{111}B_4$, by cold aqueous phenol extraction. Purification of this material gave a lipopolysaccharide preparation (F-III) of high molecular weight in 4–5% yield (based on the dry weight of the cells). In addition, by extending the purification procedures commonly used, another 10–15% yield of lipopolysaccharide was recovered. The lipid content of this second preparation (F-VI) has not been previously observed. Mild aqueous acid degradation of the bacterial lipopolysaccharides released the Lipid A component, which was then purified by acetone fractionation and silicic acid chromatography. A major portion of the lipid was consistently obtained as a well-defined peak from silicic acid columns. The purified lipid derived from F-III and designated as A-III melts at 197–200°, has a molecular weight of 1700, gives the triphenyltetrazolium test for a reducing end group, and shows limited solubility in organic solvents. It is devoid of glycerol, necrosamine, and peptides. The analytical data are consistent with a structure containing two glucosamine molecules, one phosphate, three to four acetyl groups, and five long-chain fatty acids (of which about half are hydroxy acids). Calcium and magnesium are present. On the basis of preliminary results from sodium borohydride reduction and alkaline hydrolysis of the lipid, a structure has been tentatively proposed for Lipid A in which the basic unit consists of two glycosidically linked fully acylated glucosamine molecules. Lipid prepared from F-VI is similar in chemical composition and solubility properties to A-III, but differs in that its molecular weight is approximately twice that of A-III.

From the cells of Gram-negative bacteria it is possible to extract lipopolysaccharide complexes which have marked biological activity. Their antigenic and endotoxic characters are especially important, but these complexes are also pyrogenic, often promote resistance to infection, and will inactivate phages which infect the original bacteria. These bacterial complexes contain a major lipopolysaccharide component associated with protein or peptide and biologically inert lipid in amounts varying with the solvent used in extraction. The chemistry and biological effects of these bacterial complexes are under investigation in a number of laboratories (Westphal, 1960; Osborn *et al.*, 1962; Ribi *et al.*, 1962; Nowotny, 1963).

Westphal and Lüderitz (1954) have shown that mild acid degradation of lipopolysaccharide yields a lipid fraction (designated as Lipid A). Our particular interest in this problem concerns the preparation of Lipid A in a pure form and the characterization of its structure. For this study lipopolysaccharides have been prepared from *Escherichia coli* by cold aqueous phenol extraction. The lipid components of the lipopolysaccharides have been prepared and their composition examined. In this report, we describe the preparation and properties

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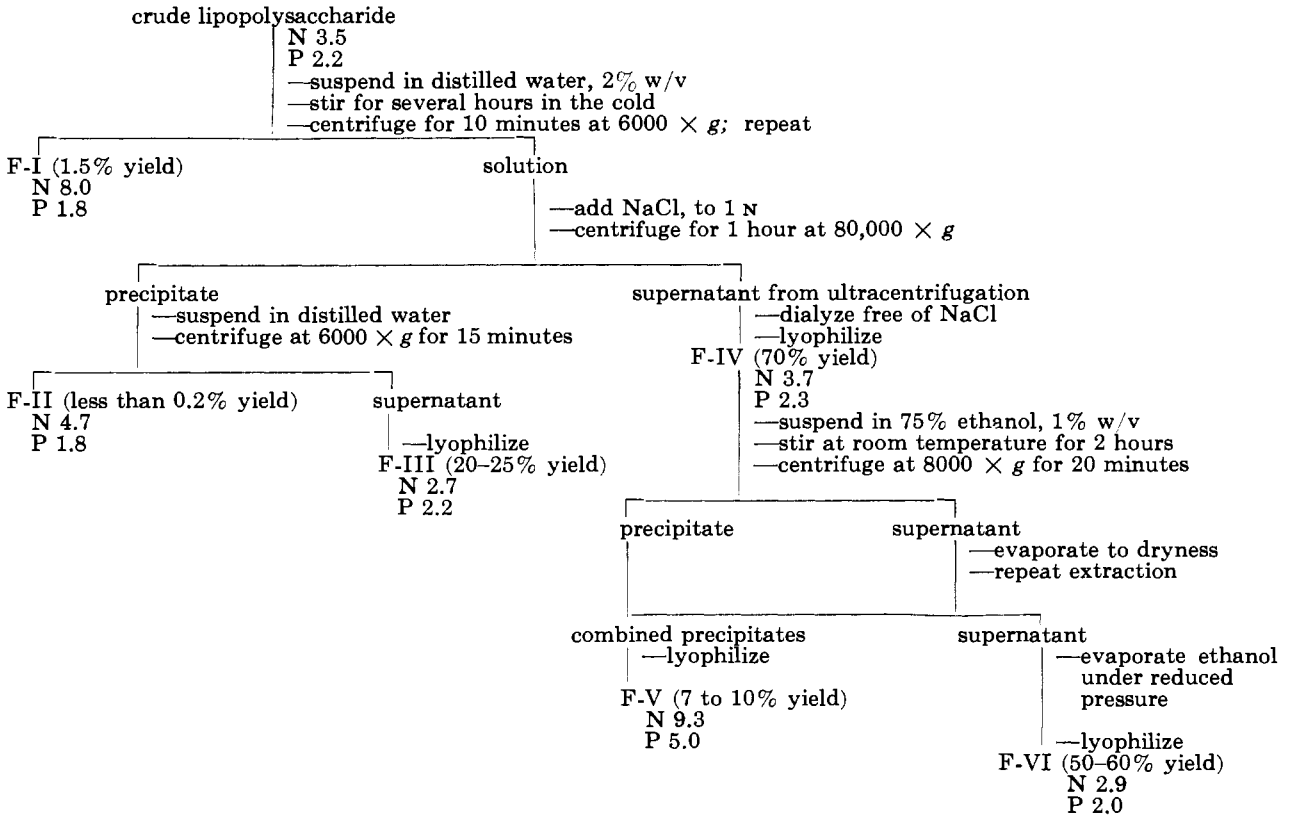


FIG. 1.—Purification of crude lipopolysaccharide.

of these materials, together with the initial experiments designed to establish the structure of the lipids.

EXPERIMENTAL PROCEDURE AND RESULTS

Preparation of the Lipopolysaccharides.—Generous quantities of *E. coli*, strain $O_{111}B_4$ (virulent form), were provided by the Upjohn Co.[1] The lipopolysaccharides were extracted by the cold aqueous phenol procedure of Westphal *et al.* (1952a). In a typical experiment, 1.25 g crude lipopolysaccharide was obtained from 5 g dry wt of bacteria. In the purification of the crude material, a combination of low-speed centrifugation, ultracentrifugation, and ethanol fractionation gave two lipopolysaccharides, F-III and F-VI. The procedure is outlined in Figure 1. Ultraviolet absorption spectra showed that F-V consisted largely of nucleic acids, while F-III and F-VI were essentially free of nucleic acids. Paper chromatography following acid hydrolysis of F-I and F-II identified these fractions as peptides.

The two lipopolysaccharides, F-III and F-VI, were obtained as fluffy white hygroscopic powders. These materials did not melt, but charred and decomposed at temperatures above 180°. F-III dissolved with difficulty in water to give a highly opalescent solution; F-VI readily yielded an almost clear aqueous solution. A suspension of F-III sedimented too rapidly to allow calculation of its molecular weight which must be greater than 10^7. An estimate of the particle weight of F-VI, using the method of Ehrenburg (1957), is close to 2×10^6. This value represents a change in the material following its preparation in the course of which it remained in solution for an hour during centrifugation at 80,000 × *g*. Analytical data for the two lipopolysaccharides are summarized in Table I. The hexosamine present in the lipopolysaccharides was identified as glucosamine and determined quantitatively as described below.

Since our interest in the lipopolysaccharides was primarily in the lipid moiety, no extensive study of the chemistry or biological properties of these materials was undertaken. However, the lipopolysaccharides did precipitate with antiserum prepared against the intact bacterial cells; tests of toxicity and pyrogenicity, performed for us through the kindness of Dr. Westphal,[2] as well as by Dr. Rhuland of the Upjohn Co., showed that these materials possessed biological, activity qualitatively similar to that of materials prepared by other investigators (Westphal, 1960).

TABLE I
COMPOSITION OF THE LIPOPOLYSACCHARIDES

	F-III	F-VI
N	2.7	2.9
P	2.2	2.0
Acetyl number	5.4	6.2
Nonvolatile fatty acids	20–30	15–25
Carbohydrate	35	48
Glucosamine	18	15
Amino acids	3–5	<1
Lipid A	21	6–8

Preparation of the Lipid A Component.—F-III (5.06 g) was added with stirring to 550 ml 0.1 N hydrochloric acid which had been heated to 85°. The mixture was heated under refluxing conditions for 30 minutes, then cooled in an ice bath. The milky reaction mixture containing suspended white particles was extracted

[1] The authors are grateful to Dr. L. E. Rhuland of the Upjohn Co. for providing the bacteria used in this work.

[2] The authors wish to express their deep appreciation to Dr. Otto Westphal, both for his advice and for his assistance in determination of biological properties of several of the lipopolysaccharide and lipid preparations.

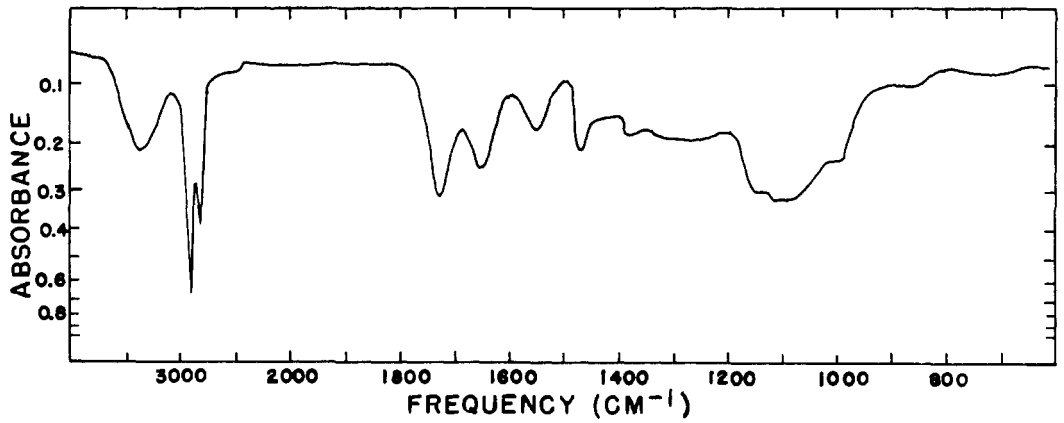


FIG. 2.—The infrared spectrum of Lipid A: a 5% solution of A-III in chloroform.

with five 200-ml portions of chloroform. The combined extracts were washed three times with 200-ml portions of deionized water, then concentrated *in vacuo* to a dry powder weighing 1.066 g (21%).

F-VI (5.08 g) was hydrolyzed in a similar manner. The reaction mixture was deep brown in color after hydrolysis and yielded 347 mg (6.8%) of chloroform-soluble material.

The chloroform-soluble extract from F-III (1.066 g) was extracted with three 15-ml portions of hot acetone to remove free fatty acids. The acetone-insoluble lipid was collected on a filter, dissolved in a minimum amount of chloroform, and precipitated with three volumes of acetone. The precipitate was again collected on a filter, dried, and weighed to give 981 mg acetone-insoluble lipid (crude Lipid A). The acetone-soluble lipid, when dried *in vacuo*, weighed 82.3 mg.

The chloroform-soluble fraction from F-VI (347 mg) was treated in the same manner to give 278 mg acetone-insoluble lipid and 69 mg acetone-soluble lipid.

Silicic Acid Chromatography of the Lipids.—The acetone-insoluble lipid from F-III (980 mg) was applied in chloroform (20 ml) to a column prepared from 60 g silicic acid–Celite (4:1) which had been washed previously with chloroform and activated by heating for 12 hours at 110°. After application of the sample the column was eluted with 100 ml of chloroform, then with successive 200-ml volumes of chloroform-methanol in the proportions 9:1, 8:2, and 7:3, respectively, and finally with 200 ml of 95% aqueous methanol. Immediately before each eluent was changed to the next one of increased polarity, 5 ml of the effluent was collected in a separate vessel, dried, and weighed; elution at each polarity was always continued until lipid could not be detected in the effluent. For the chromatography of 1 g of lipid 200-ml volumes of each eluent were sufficient. The results of chromatography are summarized in Table II. Of the lipid applied to the column, 782 mg was eluted by chloroform-methanol (9:1). This fraction was applied to a second silicic acid–Celite column and chromatographed in an identical manner. In this case 735 mg (94%) was eluted as a peak fraction by chloroform-methanol (9:1). This rechromatographed fraction is termed Lipid A-III, the purified Lipid A component of F-III.

The acetone-insoluble lipid from F-VI (280 mg) was chromatographed in a similar manner on a column prepared from 30 g of the silicic acid-Celite mixture. Of the lipid applied to the column, 190 mg was recovered in fraction 2. Rechromatography of this fraction yielded 173 mg (91%) of lipid eluted by chloroform-methanol (9:1), termed Lipid A-VI.

Qualitative Examination of the Fractions from Silicic Acid Chromatography.—The infrared spectrum of fraction 1 from a typical column chromatogram showed marked carboxyl absorption at 1705 cm^{-1} and a series of progression peaks between 1380 cm^{-1} and 1180 cm^{-1}, typical of fatty acid absorption. The spectrum of fraction 2 from the chromatography of the lipid component of F-III is reproduced in Figure 2. The spectra of fractions 3 and 4 were similar to that of fraction 2 though the amount of ester relative to amide absorption was somewhat less for these fractions.

TABLE II
COLUMN CHROMATOGRAPHY OF CRUDE LIPID A FROM F-III

Fraction	Eluent $CHCl_3$-MeOH	Volume of Eluent (ml)	Weight of Fraction (mg)	Yield (%)
F-1	100% $CHCl_3$	100	0.8	1
F-2	9:1	200	782	80
F-3	8:2	200	67	6.8
F-4	7:3	200	37	3.8
F-5	95% aqueous MeOH	200	45	4.6
	Total recovery 96.2%			

The fractions from silicic acid chromatography were characterized by subjecting hydrolysates to paper chromatography. Samples of approximately 10 mg of each of the fractions were hydrolyzed for 6 hours with 1 ml 6 N hydrochloric acid. Suitable dilutions of the samples after hydrolysis were applied to Whatman No. 1 filter paper. The chromatograms were developed in the one-dimensional solvent system, isopropanol–acetic acid–water, 3:1:1 (v/v).

The water-soluble components of the acid hydrolysates were detected, following chromatography, with the ninhydrin spray reagent (Block *et al.*, 1958). Fraction 5 was also chromatographed without prior hydrolysis. Glucosamine, after treatment with the above acidic conditions, and necrosamine[3] (untreated) were also chromatographed. The results of these chromatograms are reproduced in Figure 3. When similar chromatograms were developed with periodate, no glycerol was detected in the hydrolysates of the lipids.

The Hexosamine Determination.—To distinguish qualitatively between the hexosamines, glucosamine, galactosamine, and mannosamine, acid hydrolysates of F-III, F-VI, A-III, and A-VI were acetylated according to the method of Roseman and Ludowieg (1954). The acetylated acid hydrolysates were chromatographed on borate-treated paper (sheets of Whatman

[3] We wish to thank Mr. David Teets, who prepared necrosamine according to the procedures of Ikawa and Niemann (1953).

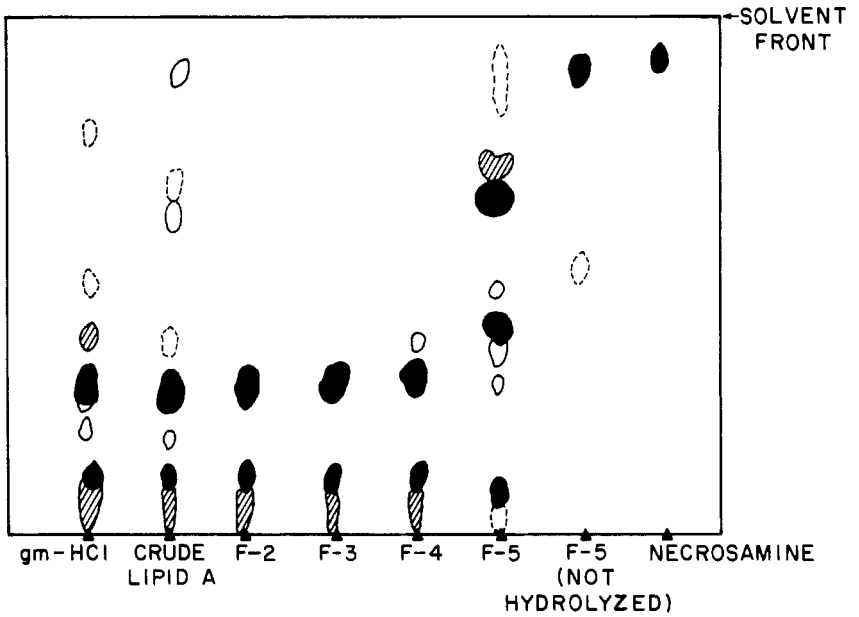


FIG. 3.—Paper chromatography of the acid hydrolysates of silicic acid column fractions.

TABLE III
THE FATTY ACID COMPOSITION OF F-III, F-VI, AND THEIR LIPID A COMPONENTS

Fatty Acid (methyl ester)	Relative Retention Time[a]	A-III (%)[b]	A-VI (%)[b]	F-III (%)[b]	F-VI (%)[b]
Lauric acid	0.10	8	16	13	8
Myristic acid	0.25	37	18	50	24
(β-Hydroxydecanoic acid ?)[c]	0.42	19	12	Trace	Trace
Palmitic acid	0.50	Trace	Trace	17	26
Unsaturated fatty acid (C_{16})	0.58	—	5	—	7
Cyclopropane fatty acid (C_{17})	0.79	—	—	6	7
Cyclopropane fatty acid (C_{19})	1.64	—	—	2	6
β-Hydroxymyristic acid	1.71	27	41	11	11
Unidentified peaks	0.08, 0.17, 0.32, and 0.60	8	6	2	10

[a] The relative retention time is based upon the retention time (1.0) of the methyl ester of stearic acid. [b] This value represents only an approximation of the relative amount of each fatty acid present in the sample: each peak area was measured and its percentage of the total area of the chromatogram was calculated. [c] Characterized by vapor-phase chromatography only and tentatively identified as β-hydroxydecanoic.

No. 1 filter paper which had been dipped in a solution of sodium tetraborate, 12 g/200 ml 0.2 N hydrochloric acid, then air dried) in the descending solvent system *n*-butanol-pyridine-water, 6:4:3 (v/v). It has been reported (Strominger, 1958) that in this solvent *N*-acetyl galactosamine and *N*-acetyl mannosamine move together at approximately half the R_F of *N*-acetyl glucosamine. In this system each of the acetylated acid hydrolysates gave a single Elson-Morgan positive spot (Partridge, 1948) at the same R_F as *N*-acetyl glucosamine, completely separated from *N*-acetyl galactosamine.

The acetylated acid hydrolysate of F-III was also subjected to the fingerprinting technique (Ingram, 1958) as suggested by Salton (1959), who reports a separation of *N*-acetyl muramic acid from *N*-acetyl glucosamine under these conditions. Again a single spot corresponding to the position of *N*-acetyl glucosamine was observed for the acetylated acid hydrolysate of F-III.

The quantitative determination of glucosamine present in the lipopolysaccharides and in the lipids derived from them was carried out according to the method of Elson and Morgan (1933) with later modifications (Rondle and Morgan, 1955; Boas, 1953). The configuration of glucosamine present in these materials was not established.

The Fatty Acid Composition of F-III, F-VI, and Their Lipid A Components.—The methyl esters of the fatty acids obtained from F-III, F-VI, A-III, and A-VI by strong basic hydrolysis were prepared and subjected to vapor-phase chromatography by Dr. N. Z. Stanacev and Dr. John Law. We are indebted to Dr. Law for the assignments of the peaks. Table III summarizes the fatty acid composition observed for these materials.

Total acetyl content was determined quantitatively in the usual way (Clark Microanalytical Laboratories) and was characterized as *O*-acetyl by the hydrazide procedure of Phillips (1963).[4]

Partial Characterization of Lipid A.—A-III is a clear, light tan, crystalline solid, mp 197–200°. Of the common laboratory solvents, only chloroform and pyridine dissolve the lipid; it is insoluble in hot absolute ethanol, ether, methanol, acetone, and benzene. The molecular weight of A-III was determined by Dr. Edwin Flynn of the Eli Lilly Co. The method used

[4] We appreciate the assistance of Mrs. Judy Koob, who performed the *O*-acetyl determination.

was the thermistor technique described by Neumayer (1959) with A-III dissolved in pyridine. A-III has a molecular weight of 1700 ± 40.

To determine the total fatty acid content of the lipid, A-III (18.2 mg) was hydrolyzed with 6 N hydrochloric acid (0.1 ml/2 mg lipid) for 6 hours. The reaction mixture was extracted three times with chloroform. The chloroform extracts were washed, then dried under reduced pressure. The dried extract was completely soluble in acetone. The yield from A-III was 12.1 mg (67%).

The analytical data for A-III are summarized in Table IV. Neither halide nor sulfur was detected following sodium fusion, but calcium and magnesium were found to be present using the method of Sommer (1955).

TABLE IV
THE COMPOSITION OF LIPID A-III

Component	Calculated, mw 1680 (%)	Found, mw 1700 (%)
Fatty acids (5)	68	60–70
Acetyl (4)	10.2	9.2
N	1.67	1.62
P	1.85	1.83
Hexosamine	21	20

The lipid was tested for the presence of a reducing end group in the following manner, which is a modification of the method of Mattson and Jensen (1950). A-III was dissolved in pyridine and applied to Whatman No. 1 filter paper. Standard solutions of sucrose, *N*-acetyl glucosamine, and glucosamine were also applied to the paper. A 0.5% solution of triphenyltetrazolium chloride in anhydrous pyridine was used to spray the paper. After drying, the paper was sprayed with 0.5 N potassium hydroxide in 95% ethanol, then heated over steam. No spot was observed for sucrose, *N*-acetyl glucosamine gave a deep purple-red color, and glucosamine gave a cherry red color; the paper surrounding the spots was light pink in color. A-III when tested in this manner gave a deep purple spot.

The corresponding Lipid A component from F-VI is a brown crystalline solid, mp 194–196° (N, 1.73; P, 1.91; acetyl, 9.4; hexosamine, 19; fatty acids, 70). Its solubility properties are identical with those of A-III. The molecular weight of A-VI is 3400 ± 80. It also contains calcium and magnesium and gives a positive reaction to the reducing test.

Preliminary Experiments on the Sodium Borohydride Reduction of Lipid A.—Because of the solubility properties of Lipid A it was necessary to find a suitable solvent for its reduction. *N*-Acetyl glucosamine was chosen as a test compound. An attempt to reduce *N*-acetyl glucosamine in pyridine modifying the procedure of Bragg and Hough (1957) was not successful: after 24 hours at room temperature in the presence of sodium borohydride the product obtained still gave a positive reaction to a reducing test. In absolute ethanol, however, *N*-acetyl glucosamine was reduced completely when the reaction was carried out under refluxing conditions. *N*-Acetyl glucosaminol obtained from this reduction and *N*-acetyl glucosamine were subjected to the quantitative glucosamine determination. A value of 97.5% glucosamine was found for *N*-acetyl glucosamine, while no glucosamine was detected in the preparation of *N*-acetyl glucosaminol.

A-III (409 mg) was suspended in 5 ml absolute ethanol by stirring for 2 hours at 70°. Sodium borohydride (92.5 mg) in 3 ml absolute ethanol was added dropwise with stirring. The reaction mixture was stirred continually under refluxing conditions for 16 hours. An additional 97 mg sodium borohydride was then added and the reaction was continued for another 8 hours. The reaction was stopped after 24 hours, since a clear orange solution of the lipid had been effected. Ethanol was removed from the reaction mixture under reduced pressure. The product was suspended in 10 ml deionized water and chilled, and the *p*H was adjusted to 1. The acidified suspension was extracted repeatedly with chloroform; from the chloroform extracts 169 mg lipid (fraction 1) was obtained. The infrared spectrum of this fraction, obtained in chloroform, showed medium hydroxyl absorption at 3500 cm^{-1} and strong ester absorption at 1730 cm^{-1}; because of the anhydrous reduction condition the fatty acids were released from Lipid A as esters. Fraction 1 contained less than 0.1% nitrogen.

The aqueous layer, which contained a considerable amount of suspended material, was centrifuged at 10,000 × *g*, and the supernatant was removed. The pellet was suspended in a minimum volume of deionized water and centrifuged again at 10,000 × *g*. After removal of the water wash, the pellet (fraction 2) was dried in a vacuum dessicator over P_2O_5 to give 154 mg. The infrared spectrum of fraction 2 showed no ester absorption, and marked amide absorption at 1645 cm^{-1} and 1555 cm^{-1}. This fraction was completely insoluble in water, chloroform, acid, and alkali, dissolving only in pyridine; fraction 2 accounted for 60–65% of the total nitrogen of the lipid.

From the supernatant following centrifugation, two minor fractions were obtained: fraction 3, which was not soluble in water following removal of methyl borate (20.8 mg), and fraction 4, which was water soluble. Fraction 3 contained approximately 10% of the lipid nitrogen; its infrared spectrum showed weak, broad ester absorption between 1700 and 1750 cm^{-1} and amide absorption at 1645 cm^{-1} and 1555 cm^{-1}. Fraction 4 was diluted to 25 ml and aliquots were removed for determination of N and P. Based on these determinations, fraction 4 contained less than 5% of the total nitrogen of the lipid.

Fractions 3 and 4 gave negative reactions to reducing tests, but fraction 2 was still positive. Accordingly, fraction 2 (133 mg) was suspended in 10 ml of 0.1 N potassium hydroxide in absolute ethanol. Sodium borohydride (100 mg) was added and the mixture was stirred for 3 days at room temperature. The reaction mixture remained cloudy. After evaporation to dryness, acidification, and extraction with chloroform, three fractions were obtained. The first (10.9 mg) was chloroform soluble, and exhibited strong hydroxyl and typical fatty acid absorption in the infrared. The other two, fractions 5 (31.4 mg; N, 2.1; P, 2.4) and 6 (17.1 mg; N, 2.5; P, 3.4), differed slightly in water solubility, though both dissolved in alkali to give a clear, light yellow solution, and both were precipitated by acid. When tested for reducing end group with both the triphenyltetrazolium chloride reagent and with alkaline copper sulfate spray reagents, each fraction was negative.

In subsequent experiments it was found that treatment of fraction 2 suspended in aqueous sodium hydroxide with sodium borohydride resulted in complete solution within 1 hour.

Table V summarizes the nitrogen and glucosamine content of the reduction products. These data were obtained on small samples of products which were not completely free of salt, and in most cases were single determinations.

Alkaline Hydrolysis of Completely Reduced Lipid A.—

TABLE V
THE RATIO OF GLUCOSAMINE TO AMINO SUGAR IN THE PRODUCTS OF SODIUM BOROHYDRIDE REDUCTION OF LIPID A

	N (%)	Glucosamine (%)	Glucosamine Found/ Glucosamine Calculated[a]
Fr-2	2.5	18.5	0.58
Fr-3	1.85	11.7	0.50
Fr-5	2.1	13.7	0.51
Fr-6	2.5	13.2	0.41

[a] The calculated value is based on the assumption that all of the nitrogen is present as glucosamine.

Fraction 5 from the reduction of A-III (3.7 mg) was transferred to a 2.5-ml ampoule and dissolved in 0.37 ml 2 N sodium hydroxide. The ampoule was sealed and the solution was heated within a steam cone for 3.5 hours. No change in the initial color of the solution (light yellow) was observed upon heating. Treated in the same manner were chitobiose-diHCl (a gift from Dr. S. Roseman), glucosamine, and glucosaminol. The solution of glucosaminol did not darken upon heating, while the solutions of both glucosamine and chitobiose turned almost black as hydrolysis proceeded. After 3.5 hours the solutions of chitobiose and fraction 5 were concentrated almost to dryness and acidified. In both solutions precipitates formed which were filtered, washed with a minimum amount of water, and dissolved in 0.1-ml volumes of 0.5 N sodium hydroxide. The four samples, in base, were applied to Whatman No. 1 filter paper and chromatographed in the descending solvent system isopropanol–ethyl acetate–water, 7:1:2 (v/v) for 12 hours. The chromatogram was air dried, then sprayed with periodate. While degraded glucosamine moved approximately 9 cm from the origin and glucosaminol moved approximately 8 cm, under these conditions degraded chitobiose gave a diffuse spot at the origin and hydrolyzed fraction 5 gave a single well-defined spot at the origin.

DISCUSSION

For this investigation, lipopolysaccharides have been isolated from *Escherichia coli* by extraction with cold aqueous phenol. A lipopolysaccharide preparation termed F-III, similar to that isolated from Gram-negative organisms by numerous investigators (e.g., Westphal and Lüderitz, 1954) was obtained in 4–5% yield based on the dry weight of the cells. However, in addition to this material another 10–15% yield of lipopolysaccharide was obtained by extending the usual purification procedure (Westphal *et al.*, 1952b). Although previous reports have noted the presence of polysaccharide in supernatant solutions following ultracentrifugation of the crude lipopolysaccharide, the lipid content of this fraction has not been previously observed. Suitable fractionation of the supernatant material gave F-VI, a lipopolysaccharide preparation which is similar in qualitative chemical composition to F-III, but which contained less lipid and more polysaccharide than F-III. Both lipopolysaccharides were shown to be essentially free of nucleic acid but no attempt was made to establish the homogeneity of either preparation. Indeed it seems possible that F-VI was inhomogeneous since any polysaccharide not attached to lipid would be likely to appear in this fraction.

The isolation of the water-soluble lipopolysaccharide fraction (F-VI) with a low lipid content is of interest in connection with the reports of Ribi *et al.* (1960) of the preparation of biologically active polysaccharide fractions of low lipid content.

Our chief interest in the bacterial lipopolysaccharides concerned the lipid component which is obtained by mild acid degradation. This material was designated Lipid A by Westphal and Lüderitz (1954) to distinguish it from biologically inert lipid (Lipid B, a cephalin) sometimes associated with lipopolysaccharide. Westphal, who has studied extensively the biological activity of Lipid A, has reported that purified lipid A contains hexosamine (20%), fatty acids (50%), phosphate (7–8%), and a peptide component (Westphal *et al.*, 1958; Westphal, 1960). Ikawa *et al.* (1953a, 1953b; Ikawa and Niemann, 1953) isolated a possibly similar substance from a tumor-necrosing lipopolysaccharide complex of *E. coli*. This material contained glucosamine, ethanolamine, aspartic acid, a long-chain base (named necrosamine) identified as 4,5-diamino-*n*-eicosane, and fatty acids (lauric, myristic, palmitic, and β-hydroxymyristic).

It should be noted that neither of the above materials was subjected to a rigorous purification step nor were any claims made as to the homogeneity of the preparations. In undertaking structural studies of Lipid A, therefore, it seemed of the utmost importance to devise chromatographic or other purification procedures both as a means of obtaining purified lipid and as a basis for determining whether the lipid contained either a covalently linked peptide component or necrosamine.

To this end a substantial amount of lipopolysaccharide from *E. coli*, strain $O_{111}B_4$, was subjected to mild aqueous acid degradation. The resultant water-insoluble lipid mixture was extracted with acetone to remove free fatty acids. The residue was an amorphous solid which on strong acid hydrolysis yielded glucosamine, inorganic phosphate, fatty acids, amino acids, and a ninhydrin-positive substance which resembled necrosamine on paper chromatography.

Chromatography of the crude lipid on silicic acid in chloroform-methanol gave substantial purification. Results from a typical column are recorded in Table II. The main peak, which accounted for 70–80% of the crude lipid, was eluted by chloroform-methanol, 9:1 (fraction 2). With increasing concentrations of methanol smaller amounts of more polar fractions (3 and 4) were obtained, and finally a fifth fraction was eluted with 95% aqueous methanol.

Fraction 5, that eluted from the silicic acid columns by 95% aqueous methanol, contained one ninhydrin-positive material which had the same distinctively high R_F on paper chromatograms as that of necrosamine. The material differed from necrosamine, however, in that it reacted more slowly with periodate than does necrosamine, and it reacted with ninhydrin to give a deep purple color while necrosamine gives a red-brown color with ninhydrin. When fraction 5 was subjected to drastic acid hydrolysis the fast-moving component was destroyed almost completely and a spectrum of ninhydrin-positive spots was observed. The fast-moving material is possibly a peptide which is relatively resistant to acid hydrolysis, since it is observed in materials which have been treated for 6 hours with 6 N hydrochloric acid under refluxing conditions. Prolonged hydrolysis (24–48 hours) was necessary to effect complete hydrolysis. Fraction 5 contained essentially all the ninhydrin-positive components, other than glucosamine, present in the crude lipid.

The main lipid fraction (fraction 2), when subjected to rechromatography, behaved as a homogeneous substance, eluted predominantly by chloroform-methanol, 9:1. Closely similar results were obtained in a

number of other preparations. These highly purified lipid preparations (designated as A-III) were used in the characterization and structural studies.

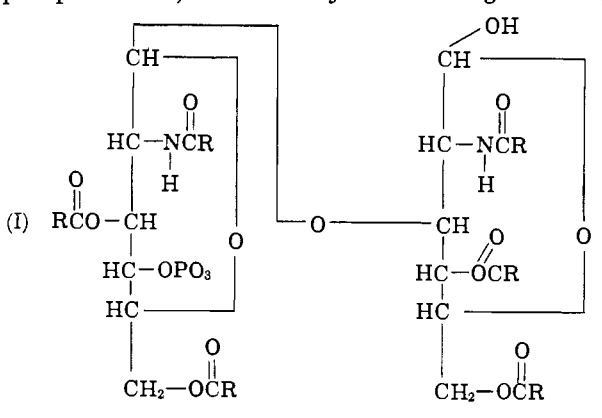
Lipid A-III contained glucosamine, long-chain fatty acids, *O*-acetyl substituents, phosphate, calcium, and magnesium. All of the nitrogen was accounted for by glucosamine and only traces of amino acid could be detected in acid hydrolysates. No necrosamine could be detected and glycerol was also absent. Gas chromatography of the methyl esters of the fatty acids showed four predominant peaks with retention times corresponding, respectively, to the methyl esters of lauric, myristic, and β-hydroxymyristic acids, and of an acid behaving like β-hydroxydecanoic on vapor-phase chromatography. Two to four minor peaks were also observed. An estimation of the relative amounts of the fatty acids present showed that about 45% of the fatty acids of A-III are hydroxy fatty acids and approximately 45% are saturated fatty acids, while the unidentified peaks amount to between 5 and 10% as summarized in Table III. The fatty acid composition of Lipid A-VI, as well as that of each of the lipopolysaccharides, is included for comparison.

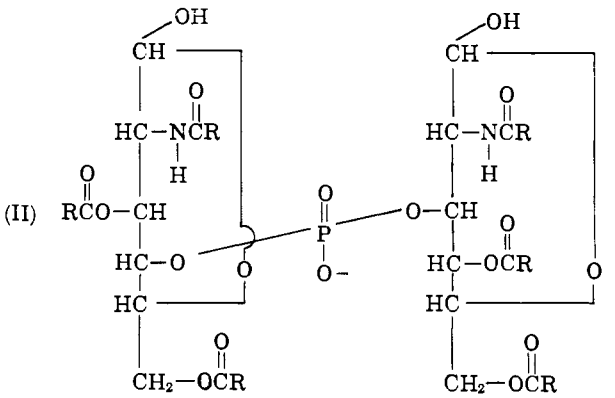
The analytical data for Lipid A are summarized in Table IV. A calculation based on these data shows a nitrogen-phosphorus ratio of 2. The analytical data and molecular weight of A-III are consistent with a unit structure including two glucosamine molecules, one phosphate, three to four acetyl groups, and five long-chain fatty acids. About half the long-chain acids are hydroxy acids, thus providing the extra hydroxyl groups needed to accommodate 8–9 acyl groups in a structure such as (I) or (II), illustrated below. In Table IV calculated data based on such a unit are compared with data found for A-III.

This discussion has concerned the lipid derived from F-III. Lipid has also been prepared in a similar manner from F-VI to give the corresponding lipid, A-VI. The analytical data for this lipid are similar to the data for A-III and fit closely with the two-unit structure described above except that the molecular weight of A-VI was found to be 3400, or twice that of A-III. Further study is needed to determine the basis for this difference in molecular weight.

The minor lipid fractions (fractions 3 and 4) obtained during silicic acid chromatography of the crude lipids are qualitatively similar to fraction 2. A possible explanation for the increased polarity of these fractions is that they are less fully acetylated than fraction 2; comparison of the infrared spectra showed less ester absorption relative to amide for these fractions than for fraction 2.

Having established the composition of a homogeneous preparation of Lipid A, we became interested in the question of the linkage between the two glucosamine molecules. Equally possible are a glycosidic bond and a phosphodiester, illustrated by the following structures:





In both these structures R represents the possible location of long-chain fatty acids. Changes in the position of the glycosidic bond or of the phosphodiester, respectively, allow a number of variations of the two structures.

In evaluating the possible validity of the glycosidic or phosphodiester bond the stability of Lipid A to the mild acid conditions employed in its preparation must be taken into consideration. The unusual stability of glycosides of glucosamine toward acid hydrolysis has long been recognized (Moggridge and Neuberger, 1938). Less is known about the stability of *N*-acyl derivatives and nothing is known about the possible effects of a phosphate substituent on stability. Primary and secondary phosphate esters containing no adjacent free hydroxyl groups are not easily hydrolyzed by acid (Maley and Lardy, 1956) except for a phosphate group in the 1-position. The known acid-lability of *N*-acetyl glucosamine-1-phosphate (Leloir and Cardini, 1956) would exclude this type of structure. One factor bearing on this question is the extreme insolubility of Lipid A preparations in aqueous solvents. It seems probable in view of available data on analogous structures that either structure (I) or (II) would escape extensive degradation in the acid hydrolysis and especially so since the lipid separates from solution as soon as it is formed.

The type of structural unit represented by (II) is inherent in a structure for lipid A proposed by Nowotny (1961):

$$\begin{array}{c} \text{F} \quad \text{F} \quad \text{F} \\ \text{peptide-GA-P-GA-P-GA} \\ \text{FF} \quad \text{FF} \quad \text{FF} \end{array}$$

GA, D-glucosamine; F, fatty acid; P, phosphoric acid. This proposal is based on the analytical data of the lipid studied, on the observation that acid and alkaline phosphatases failed to split free phosphoric acid from the lipids, and on results with venom diesterase which were not presented.

In this study a chemical approach, the sodium borohydride reduction of the lipids, was initiated to distinguish between the two basic possibilities of linkage. From Lipid A a completely reduced fraction was obtained which was not soluble in water or acid but dissolved in alkaline solution. The nitrogen-phosphorus ratio in the reduced lipid was approximately 2:1. A glucosamine determination on this material showed that glucosamine still accounts for nearly half the nitrogen of the lipid (51%). In a structure of the type represented by (II), glucosamine should have been completely reduced to glucosaminol.

When the reduced lipid from A-III was subjected to vigorous alkaline hydrolysis, the solution did not darken upon heating, though solutions of glucosamine and chitobiose turned almost black under the same conditions and glucosaminol remained clear, further evidence

that the lipid was in fact completely reduced under the experimental conditions. When the solutions were chromatographed, glucosamine and glucosaminol moved several cm in the solvent chosen, while chitobiose and the reduced fraction remained at the origin, indicating that the reduced fraction does not contain single units of amino sugar. This stable disaccharide must be isolated and characterized to confirm these observations. Other questions about the reduction experiments were raised by side fractions, one accounting for about 5% of the nitrogen originally present in the lipid, in which the nitrogen-phosphorus ratio is considerably higher than 2, and one accounting for 10% of the lipid nitrogen, in which the same ratio is less than 2. We feel that these fractions represent some degradation of the two-unit structure under the conditions used to reduce the lipid and to remove excess sodium borohydride following the reaction.

The results of the sodium borohydride reduction and alkaline degradation indicate, however, that the two glucosamine molecules are linked glycosidically; after complete reduction a fraction is obtained in which approximately half the amino sugar present is still glucosamine. The apparent stability of the reduced unit to vigorous alkaline hydrolysis indicates that the glycoside joins two glucosamine molecules without involving an hydroxy fatty acid: either an amide or an ester of the latter with a second glucosamine molecule should cleave under the conditions of vigorous alkaline hydrolysis. A phosphodiester linkage does not seem likely; two glucosamine molecules so joined should be completely reduced to glucosaminol. Such a linkage should also cleave under vigorous alkaline conditions. A unit in which a glycosidic bond joins two molecules of glucosamine is therefore tentatively proposed as the basic structural unit of Lipid A. Confirmation of this structure will depend on the outcome of similar experiments conducted on a larger scale.

In conclusion it seems worthwhile to consider how Lipid A is attached to the lipopolysaccharide. Several linkages are possible, including again the glycosidic and phosphodiester bonds, as well as that provided by a di-functional amino acid. A glycosidic bond in which the Lipid A moiety represents a terminal unit attached to a polysaccharide chain is an attractive hypothesis. Studies on the borohydride reduction of intact lipopolysaccharide might well provide decisive evidence on this point.

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Purification of Phytochrome from Oat Seedlings

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Phytochrome, the photoreceptor controlling many aspects of growth and development of higher plants, was extracted from dark-grown Clintland oats. A 60-fold purification was achieved by methods of protein separation: ultrafiltration, calcium phosphate chromatography, gel filtration on Sephadex G-200, and DEAE-cellulose chromatography. The chromoprotein has a molecular weight of between 90,000 and 150,000 and the purity of highest specific activity fractions is estimated to exceed 30%.

Many aspects of plant growth and development are controlled by light quality and the daily duration of light and darkness. Action spectra for the effects of light on flowering, etiolation, germination of light-responsive seeds, plastid development, and many other plant responses (Borthwick and Hendricks, 1960) indicate uniquely that the same reversible photochromic pigment is the effective photoreceptor in all these