PREPARATION AND HOST-REACTIVE PROPERTIES OF ENDOTOXIN WITH LOW CONTENT OF NITROGEN AND LIPID

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Endotoxins are sufficiently complex that, although they have been studied intensively, precise relationships between chemical constitution and the ability to elicit characteristic reactions in mammals are still largely unknown and subject to controversy. The concensus is that these substances, as ordinarily isolated, consist of lipid, polysaccharide, and protein or peptide-like components. However, it has been demonstrated that most of the protein moiety can be removed without altering the biological properties (1–4).

Hydrolysis of endotoxin with dilute acetic acid led to the isolation of a haptenic polysaccharide which no longer retained the ability to stimulate physiological responses characteristic of the starting material (5, 6). The product of similar hydrolysis with dilute acetic or mineral acids, when extracted with common fat solvents, yielded lipids which were originally thought to be inactive. Westphal and Lüderitz (3) showed, however, that chloroform-soluble material, obtained by hydrolysis of bacterial lipopolysaccharides with dilute hydrochloric acid (lipid A), retained some of the endotoxic properties of the original substance, and therefore postulated that "endotoxic" activities of lipopolysaccharides were attributable to the lipid component.

In the Rocky Mountain Laboratory (RML), endotoxins extracted from living cells by aqueous ether at 8–12°C have been shown to contain smaller amounts of bound lipid than endotoxins prepared by other methods, yet they were highly active (4). The low lipid content of such products has been still further reduced by non-hydrolytic means without appreciable loss in potency (4). These toxins were at least as potent as trichloroacetic acid (TCA) or phenol extracts containing 10 to 30 per cent bound lipid. Consequently, it was difficult to visualize lipid as being the toxophore group in endotoxins. Since preparations with the lowest content of bound lipid had not been deproteinized, previous work had thus dealt with either deproteinized endotoxin which had abundant lipid (1, 3)

or endotoxin with little lipid but possibly an appreciable amount of protein (4). An important objective of the present work, therefore, was an investigation of the biologic properties of endotoxins from which essentially all of the protein and most of the bound lipid had been removed.

Materials and Methods

A. Chemical Analyses of Bacterial Fractions.—Contents of nitrogen, phosphorus, and certain organic compounds were determined, as far as possible, by standard methods, as follows:

Nitrogen, by nesslerization following digestion with copper selenite–sulfuric acid according to the procedure of Johnson (7); *phosphorus*, colorimetrically after digestion with sulfuric acid and hydrogen peroxide as recommended by Dryer *et al.* (8); *rhamnose*, by the cysteine–sulfuric acid method described by Dische (9); *hexosamine*, essentially by the procedure described by Rimington (10) based on the Elson-Morgan method using glucosamine hydrochloride as a standard. For this determination the hydrolysis of the sample was carried out in 3 N hydrochloric acid in a boiling water bath for 6 hours, conditions which were found to provide a maximum yield of hexosamine for subsequent colorimetric analysis. Extension of hydrolysis to 24 hours resulted in destruction of 8 per cent of the hexosamine content of the endotoxin and 22 per cent of the glucosamine of a control sample. These observations are in agreement with those of Salton and Pavlik (11).

Hexose was estimated by the anthrone method of Scott and Melvin (12). The use of glucose as a standard for this determination results in low values for the total hexose content. As pointed out by Dische (9), galactose and mannose have lower extinction coefficients than glucose and, furthermore, we find that rhamnose exhibits only 75 per cent of the value given by glucose. It has been shown previously that all 4 of these sugars are present in the polysaccharide portion of the endotoxin from *Salmonella enteritidis* in approximately equal proportions (4). The carbohydrate content was determined by the tryptophan–sulfuric acid method described by Dische (9). Results obtained by this method are somewhat high when calculated as glucose owing to the presence of rhamnose which has an extinction coefficient 200 per cent that of glucose, while the values for galactose and mannose are almost identical to that of glucose. For this reason, a mixture of equal weights of glucose, galactose, mannose, and rhamnose was employed as the standard in this determination.

Esterified fatty acids (FAE) were determined by conversion to hydroxamic acids and colorimetric estimation with ferric ion. The procedure was that of Snyder and Stephens (13) as modified by Tauber (14). This modification compensates for the low solubility of the endotoxins in ethanol. Total fatty acids present in endotoxins are not determined by this method because it does not detect fatty acids bound through amide linkages (FAA). A further modification was therefore devised (15) which involves extending the reaction time to 5 hours and which is believed to give reliable estimates of FAE plus FAA. The latter may then be obtained by difference. The results were calculated as per cent palmitic acid.

Lipid W in the present work designates the chloroform-soluble material separated from an endotoxin by hydrolysis with acetic acid as employed by Webster *et al.* (1). A 0.1 per cent solution of the endotoxin in 0.1 N acetic acid was refluxed for 6 hours. After cooling, the mixture was extracted 5 to 6 times with redistilled chloroform. The chloroform extracts were dried with anhydrous sodium sulfate, filtered, and concentrated to dryness with a current of air at room temperature. The dry residue was dissolved in a small volume of chloroform, filtered into a tared dish, evaporated, dried *in vacuo* over phosphorus pentoxide, and weighed. In some cases, instead of refluxing the acetic acid solution of the endotoxin, a more concentrated solution was prepared (1 to 3 per cent) and heated in a sealed tube in a boiling water bath for 6 hours. Extraction of the lipid was made in the same way.

The term *lipid A* is employed here to designate the chloroform-soluble material released from endotoxins by hydrolysis with hydrochloric acid according to the instructions of Westphal *et al.* (3, 16, 17). This fraction was determined by two slightly different methods. In the first (3, 17), a 10 to 20 per cent solution of the endotoxin in water was prepared, an equal volume of 2 N hydrochloric acid was added, and the mixture was heated in a sealed tube in a boiling water bath for 45 minutes, by which time a precipitate had formed and coagulated. After the mixture had been cooled it was extracted 4 or 5 times with redistilled chloroform. The chloroform extract was washed with water, dried with anhydrous sodium sulfate, filtered, and evaporated to dryness at room temperature in a current of air. The dry residue was dissolved in a small volume of chloroform, filtered into a tared dish, evaporated, dried *in vacuo* over phosphorus pentoxide, and weighed. The second method (16) varied from the first in that the acid solution, after being heated for 45 minutes, was centrifuged at 1600 *g* for 30 minutes. The clear supernatant fluid was decanted and the sediment washed twice with water by centrifugation. The washed sediment was dried *in vacuo* and then extracted with several small portions of chloroform. The chloroform was filtered to recover any insoluble material, which was dried and weighed separately. The filtered extract was evaporated, dried, and weighed as before. The aqueous supernatant was neutralized and preserved in the cold for subsequent analyses. The two methods, when applied to endotoxins extracted from *S. enteritidis* with aqueous ether, gave approximately the same values.

B. Preparation of Endotoxin from S. enteritidis.—

1. Strain employed and cultivation of the organism: Strain S795 of *S. enteritidis* was used throughout because it has been studied extensively in biological systems and has consistently yielded potent endotoxin when extracted by the aqueous ether method (4, 18). Organisms from lyophilized stock cultures were reconstituted and grown on heart infusion agar slants for 24 hours at 37°C, inoculated into synthetic M-9 medium (19) containing 0.5 per cent glucose (100 ml volumes in 500 ml Erlenmeyer flasks), and incubated for 24 hours at 37°C under continuous shaking. Three additional transfers in liquid medium were made before the experimental batch was inoculated by transferring 0.1 to 0.2 ml per flask. The cells were usually harvested by centrifugation after 18 hours' incubation and washed twice with 0.15 M sodium chloride.

2. Extraction of endotoxin with aqueous ether: Freshly harvested and washed cells were resuspended in saline to an appropriate concentration (3 to 5 mg dried bacteria per ml) on the basis of turbidity (scale reading of 770 in a Klett-Summerson colorimeter, filter No. 520). Two volumes of precooled (6–12°C) diethyl ether were added to the cell suspension at the same temperature and the mixture was shaken gently in a separatory funnel for 6 consecutive 10 second intervals. Pressure was released after each interval of shaking. The suspension was left overnight in the funnel at 6–12°C, after which time the aqueous phase was drawn off and the residual ether removed from it by bubbling air through the suspension. The remaining steps were performed at a temperature of 4–6°C. Centrifugation at 2500 *g* for 70 minutes resulted in a supernatant containing the soluble endotoxin and a sediment containing the residual cells (still intact as revealed by electron microscopy) which was discarded. The supernatant was dialyzed for 5 days in daily changes of glass-distilled water. Sodium chloride was then added to a concentration of 0.15 M and the endotoxin precipitated by slowly adding ethanol, with continuous stirring, to a final concentration of 68 per cent by volume, and the suspension was allowed to stand overnight. The precipitate was collected by centrifugation at 2000 *g* for 45 minutes, dissolved in the same volume of 0.15 M NaCl, reprecipitated, and again collected by centrifugation. The final precipitate was dissolved in water, dialyzed against glass-distilled water, and lyophilized.

This product, referred to as "68 per cent ethanol-precipitated aqueous ether extract" was used as starting material for further fractionation, such as removal of protein by the

method of Webster *et al.* (1), as slightly modified (4), or removal of portions of bound lipid with non-hydrolytic methods which are described in the section on Results.

3. Extraction of endotoxin with trichloroacetic acid (TCA): Boivin type endotoxin was prepared according to the procedure used by Webster *et al.* (1). A wet sediment of saline-washed cells was extracted at 0°C for 3 hours with TCA in a final concentration of 0.25 N. The extract was clarified by centrifugation, dialyzed, poured slowly into two volumes of chilled ethanol, and allowed to stand overnight at −2°C. The precipitate was collected by centrifugation, dissolved in water, and lyophilized.

4. Extraction of endotoxin with phenol-water: A wet sediment of saline-washed cells was washed 4 times by centrifugation and resuspension in acetone and dried *in vacuo*. Endotoxin was then extracted and purified according to the method described by Westphal and coworkers (20, 21).

C. Other Preparations.—Several endotoxins and a polysaccharide haptene were kindly supplied by other investigators. These sources are identified in the text.

D. Endotoxin Bio-Assays.—The methods used for biological analysis of the bacterial fractions are described in an accompanying paper (22).

RESULTS

Preliminary Experiments with Endotoxins Containing Different Amounts of Bound Lipid (Lipid W).—Water-soluble toxic fractions extracted from freshly harvested *Salmonella enteritidis* and certain other Gram-negative bacteria by treatment with saturated aqueous solutions of diethyl ether possessed all of the biological properties of Boivin type antigens or endotoxins. These extracts had the gross chemical composition of most endotoxins except for an apparently lower content of bound lipid (4). When extraction was carried out at temperatures between 18 and 25°C, the resultant toxins contained up to 14 per cent bound lipid; but extraction at 8 to 12°C yielded products with less than 4 per cent of this component. Removal of about one-half of the bound lipid without measurable reduction in toxicity was achieved by treatment of endotoxins with such non-hydrolytic reagents as boiling mixtures of chloroform-methanol or monochlorobenzene-ethanol. The proportion of bound lipid removed appeared to be about one-half of the total regardless of the amount present in the starting material.

The LD_{50} values for mice of a series of endotoxin preparations containing different amounts of bound lipid appear in Table I. These findings, which represent a more extensive series of data than we have reported previously, make it evident that lipid content does not bear any relationship to toxicity, as measured by lethality for mice. All preparations were potent and the distribution of LD_{50} values was about what would be expected in the same number of replicate determinations on one preparation with an average LD_{50} of 0.25 mg.

The data in Table I and results of other experiments involving tests for various host responses (23), while indicating that the quantity of lipid present in endotoxins did not parallel their host reactivity, were not considered conclusive since the content of bound lipid had been determined by only one method (1).

This method involved hydrolysis of the endotoxin with 0.1 N acetic acid at 100°C for 6 hours, extraction with chloroform, drying, and weighing of the chloroform-soluble material (lipid W). Westphal and Lüderitz (3) removed the lipid by hydrolysis with 1 N HCl for 30 to 45 minutes at 100°C (lipid A). Additional methods have been employed by other investigators (24–28). Since our preparations with the lowest bound lipid (1.2 per cent) had not been deproteinized (2.7 per cent N), the possibility was recognized that the protein might be interfering with the removal of lipid by acetic acid. Therefore, a comparison of methods for the determination of lipid was made on a number of different endotoxins.

The Content of Bound Lipid in Endotoxins as Determined by Different Methods. —Quantities of available materials did not permit us to employ all the methods

TABLE I

Lethality for Mice of 17 Endotoxins (Aqueous Ether Extracts) from Salmonella enteritidis Having Varying Amounts of Bound Lipid (Lipid W)

Lipid	Mouse LD_{50}	Lipid	Mouse LD_{50}
per cent	*mg*	*per cent*	*mg*
1.2	0.53	7.4	0.27
1.3	0.31	8.8	0.19
1.4	0.30	9.4	0.20
2.4	0.35	11.6	0.17
2.8	0.23	11.6	0.18
3.3	0.31	13.8	0.20
3.7	0.23	15.5 (TCA extract)	0.15
4.2	0.27	19.2 (TCA extract)	0.29
6.3	0.12		

which have been used heretofore, but in a number of instances three different methods have been compared on the same preparations; lipid A, lipid W, and total fatty acid ester (FAE), calculated as palmitic acid, were determined. The latter method was chosen because it is less selective and may detect lipoid material present in the endotoxin complex in addition to that soluble in chloroform following hydrolysis with acids; furthermore, very little material is required.

As is revealed in Table II, values for bound lipid depend to a considerable extent on the choice of method. Proportions of lipid A and lipid W may be legitimately compared since both were obtained by gravimetric determination of the chloroform extractives liberated by different conditions of hydrolysis. Determination of FAE, it was hoped, would clarify the quantitative relationship between the fatty acid content of endotoxins and the content of lipids A and W. In general, lipid A was greater than lipid W, and the magnitude of the difference was greatest among the phenol-water extracts. However, in such extracts with

low nitrogen content the differences became negligible or even reversed (see preparations 6 and 5, Table II). Among aqueous ether extracts, preparations with low values for lipid W also gave low values for lipid A, and no relationship was apparent between the nitrogen content of endotoxin and the amount of lipid

TABLE II

Content of Bound Lipid of Endotoxins as Determined by Different Methods

Preparation		Origin	Extractant	Nitrogen	Lipid A	Lipid W	Fatty acid ester
No.	Designation						
				per cent	*per cent*	*per cent*	*per cent*
1	Sf 4103	*Sh. flexneri*	Phenol-water	4.5	11	6.7	18.0
2	Se 902763	*S. enteritidis*	" "	3.6	20	5.0	11.7
3	Se 156 Be	*S. enteritidis*	" "	2.9	14	3.1	
4	Sf 902762	*Sh. flexneri*	" "	2.7	20	13	22.5
5	Co 08-2158 S_5	*E. coli*	" "	1.8	16	24	15.3
6	AE-1859 S_3	*S. abortus equi*	" "	1.2	22	20	13.3
7	Se 153 Bc	*S. enteritidis*	TCA	3.3	14	12	18.0
8	Se 185 A	*S. enteritidis*	Aqueous ether (6°)	5.9	4.0	4.0	7.6
9	Se 185 B	" "	" " (6°)	4.8	4.6	2.0	8.5
10	Se 173/176	" "	" " (6°)	2.8	6.4	2.4	6.9
11	Se 154 A	" "	" " (12°)	2.6	4.9	2.8	6.1
12	Se 155 A	" "	" " (25°)	2.3	18	14	10.8
13	Se 162/168 IIK	*S. enteritidis*	Aqueous ether (6°)	2.9	2.0	2.0	5.7
14	Se 183/188 IE	" "	" " (6°)	1.3	3.4	3.0	3.1

Preparations 1, 2, and 4 were obtained from the Difco laboratories, Detroit. Preparations 5 and 6 were obtained from Drs. Westphal and Lüderitz who reported 17 to 18 per cent lipid A for preparation 5 and 22 per cent for preparation 6. The remaining endotoxins were prepared at the Rocky Mountain Laboratory from *S. enteritidis*. Preparations 8 to 12 were precipitated with 68 per cent ethanol. Preparation 13 is identical with fraction C in Table IV and preparation 14 is identical with fraction B in Tables V and VI. The mouse LD_{50} of these preparations varied from 0.20 to 0.81 mg.

freed by acid hydrolysis. Regardless of the measure chosen, endotoxins extracted at low temperature by the aqueous ether method were found to contain less lipid than phenol-water or TCA extracts.

The Distribution of Fatty Acid Ester in Fractions Resulting from Hydrolysis of Endotoxin.—The FAE determination disclosed higher content of fatty acid in endotoxins than could be accounted for by either the "lipid W" or "lipid A" determinations. Also since lipid A has been reported to contain only about 50 per cent of fatty acids (17), there must obviously be fatty material in fractions

other than the chloroform-soluble portion of acid hydrolysates. We therefore sought to clarify this issue by following the distribution of fatty acids, as FAE, in fractions resulting from hydrolysis with 1 N HCl.

In these experiments, 100 mg of endotoxin was dissolved in 10 ml of 1 N HCl and heated at 100°C for 45 minutes. The suspensions were centrifuged at 1600 *g* for 10 minutes and the sediment dried *in vacuo* over P_2O_5. The dried sediment was extracted with chloroform, the chloroform evaporated, the residue dried *in vacuo*, and the FAE determined (chloroform-soluble fraction). Any portion of the sediment insoluble in chloroform was dried and weighed separately (chloroform-water–insoluble fraction). The supernatants from the HCl hydrolysis were also extracted with chloroform, but in no case was any residue obtained. The water phase was evaporated, dried *in vacuo*, and the FAE determined (water-soluble fraction).

The results obtained with 1 endotoxin and calculations from the data are given below to illustrate the source of figures in Table III. The preparation was a 68 per cent ethanol precipitate of aqueous ether extract from *S. enteritidis* (Se 162/168).

Products of 1 N HCl hydrolysis (100 mg endotoxin)	Total solids recovered	FAE in fraction	FAE in endotoxin	Proportion of total FAE in fractions
	mg or per cent	*per cent*	*mg or per cent*	*per cent*
Chloroform-soluble (lipid A)	6.7	28.50	1.91	25.6
Chloroform- and water-insoluble	13.7	20.55	2.81	37.6
Water-soluble	81.6	3.38	2.76	36.8
Total	102.0		7.48*	100.0

* FAE value of 7.20 per cent determined directly on whole endotoxin.

The data from all endotoxins examined in this manner are listed in Table III. With two exceptions the preparations were selected from those described in Table II. Besides normal phenol-water and aqueous ether extracts, endotoxins were included which had been subjected to salt and alcohol precipitation for removal of protein or treated additionally for non-hydrolytic reduction of lipid. An unexpected finding was that the aqueous ether extracts contained a chloroform- and water-insoluble component whereas the phenol extracts were devoid of this substance. It was apparent that this particular fatty acid ester–containing component was not essential for biological activity and these experiments led us to extract "partially deproteinized" (*e.g.*, 1.3 per cent N) aqueous ether extracts with aqueous phenol as will be described in another section of this report.

Only 35 to 62 per cent of the total fatty acid esters in the fractions could be found in the chloroform-soluble or lipid A fraction of the endotoxins prepared by phenol extraction and 38 to 65 per cent FAE was found associated with the water-soluble fraction, *i.e.*, FAE was about equally distributed between lipid A and the water phase. With the aqueous ether extracts, however, 16 to 32 per cent was found in the chloroform-soluble fraction and 16 to 38 per cent in the insoluble fraction. The remainder, comprising 37 to 59 per cent, was found asso-

TABLE III

Distribution of Fatty Acid Ester (FAE) in Fractions Resulting from Hydrolysis of Endotoxin (1 N HCl, 45 minutes, 100°C)

Preparation		Origin	Extraction	Total FAE in untreated endotoxin	Total FAE in 1 N HCl hydrolysate	Proportion of total FAE in fractions of hydrolysate		
No.	Designation					$CHCl_3$-soluble	Insoluble	H_2O-soluble
				*per cent**	*per cent‡*	*per cent*	*per cent*	*per cent*
1	Sf 4103 (Difco)	*Sh. flexneri*	Phenol-water	18.0	9.5	35	0	65
2	Se 902763 (Difco)	*S. enteritidis*	" "	11.7	9.0	46.3	0	53.7
3	Sf 902762 (Difco)	*Sh. flexneri*	" "	22.5	7.8	62	0	38
4	Se 185 B	*S. enteritidis*	Aqueous ether	8.5	9.7	16.2	25.2	58.6
5	Se 173/176	" "	" "	6.9	8.2	27.8	32.1	40.1
6	Se 162/168	" "	" "	7.2	7.5	25.6	37.6	36.8
7	Se 183/188	" "	" "	4.7	7.6	31.6	15.8	52.6
8	Se 183/188 IE	" "	" "	3.1	5.5	22.9	22.9	54.2

* Expressed as per cent of original endotoxin.

‡ Sums of FAE in fractions expressed as per cent of original endotoxin.

ciated with the water-soluble fraction. This analysis demonstrates that the chloroform-soluble (lipid A) portion of an acid hydrolysate of endotoxin does not contain all, and sometimes not even a major portion, of the bound lipid originally present in the complex.

It is noteworthy that the sums of the FAE values determined in hydrolysates of the two phenol extracts prepared from *Shigella flexneri* are only one-third to one-half of the values which were obtained by analysis of the endotoxin prior to hydrolysis with 1 N HCl. This is in contrast to the *S. enteritidis* preparations where FAE values determined prior to and after hydrolysis with 1 N HCl were either the same or slightly greater. It would appear, therefore, that *S. flexneri*

preparations obtained by phenol extraction contain volatile FAE (*e.g.*, acetyl) or that these preparations yield free fatty acids on hydrolysis with HCl.[1]

Biological and Chemical Properties of "Deproteinized" and "Delipified" Aqueous Ether Extracts from S. enteritidis.—Extraction of the original endotoxin (68 per cent ethanol precipitate of dialyzed aqueous ether extract from *S. enteritidis*) with common fat solvents removed about 0.1 to 0.4 per cent of the total material ("free lipid") (4). Attempts to remove protein and bound lipid without destroying toxicity met with varying degrees of success. Two experiments of special interest are described.

Experiment I: A 68 per cent ethanol precipitate of dialyzed aqueous ether extract of *S. enteritidis* (fraction A) was first subjected to a full cycle of salt and ethanol precipitation, essentially by the method of Webster *et al.* (1), for the reduction of protein (fraction B). It was then processed for non-hydrolytic dissociation of lipid in the following manner. Lyophilized fraction B was dried over P_2O_5 and boiled under reflux with a mixture of chloroform-methanol (1:1) for 12 hours; the reaction mixture was filtered and the residue washed with a fresh mixture of chloroform-methanol. The residue was then boiled under reflux with a mixture of monochlorobenzene-ethanol-water (11:8:2) for 12 hours, filtered, washed with a mixture of monochlorobenzene-ethanol (1:1), then with chloroform. The final residue was dissolved in water and lyophilized from the frozen state (fraction C).

The data presented in Table IV show that the final product (fraction C) had its lipid A content reduced from 7.1 to 2.0 per cent and its fatty acid ester content from 7.2 to 5.7 per cent and yet was as effective as the starting material (fraction A) in evoking primary lesions in the skin of rabbits. Pyrogenicity and ability to stimulate resistance to infection in mice (challenged with *Salmonella typhosa*) were also retained. Differences in ED_{50} values of fractions A, B, and C were of questionable significance.

Fraction C, of which the lipid A content had been markedly lowered, still retained 3.0 per cent nitrogen and thus contained appreciable protein which might have exerted an influence in the biological activity of this preparation. For this reason, further studies were conducted as detailed below.

Experiment II: A 68 per cent ethanol fraction of dialyzed aqueous ether extract was deproteinized more effectively than the corresponding fraction of Experiment I by processing the endotoxin through two cycles of salt and ethanol precipitation instead of one (fraction A). The sample was then processed with boiling solvents to remove bound lipid in a manner similar to Experiment I

[1] Fromme *et al.* (29) reported the presence of free fatty acids in the 1 N HCl hydrolysate of *Salmonella abortus equi* phenol extract. Quantitative data for the amount of these fatty acids were not given, and the report did not indicate that they had been separated from the unsaponified lipid A constituents. The content of lipid A recovered from *S. abortus equi* was referred to as being 26 per cent. This was the same value listed in earlier reports (3).

(fraction B). The protein and lipid contents of this material were then further reduced by extracting with aqueous phenol as described by Westphal *et al.* (3). An equal volume of liquefied phenol was added to a 2 per cent solution of endotoxin in water and the mixture was stirred for 30 minutes at 68°C. After cooling in ice water, the phenol and water phases were separated by centrifugation at 1600 *g* for 10 minutes. The water phase was dialyzed against distilled water and dried from the frozen state. The phenol phase was mixed with an equal volume of water, stirred for 20 minutes at 68°C, and again separated by centrifugation. The second water phase was combined with the first water phase and designated

TABLE IV

Some Chemical and Biological Properties of an Endotoxin (Aqueous Ether Extract) from Salmonella enteritidis at Various Stages of Fractionation

Analysis	Fractions (Experiment I)		
	A Original (Se 162/168)	B Partially deproteinized (Se 162/168 II G)	C Partially delipified (Se 162/168 II K)
Nitrogen, *per cent*	4.3	3.0	2.9
Lipid A, *per cent*	7.1	—	2.0*
Fatty acid ester (FAE), *per cent*	7.2	—	5.7
Protection of mice against *S. typhosa* challenge (ED_{50}), μg	0.2	0.3	1.4
Primary inflammation of rabbit skin (SLD_{50}), μg	0.20	0.15	0.20
Pyrogenicity in rabbits (FI_{40}), μg	0.39	0.35	0.32

* Same value for both lipid A and lipid W.

fraction C. The residual phenol phase contained a brownish precipitate which was discarded.

Results of biological tests and of some chemical analyses are given in Table V. It is shown that processing of the original aqueous ether extract by repeated cycles of ethanol and salt precipitation (1) led to reduction of the nitrogen content to 1.35 per cent (fraction A) and that dissociation of bound lipid with chloroform-methanol and monochlorobenzene-ethanol had reduced the FAE content from 4.7 per cent (fraction A) to 3.1 per cent (fraction B). At the same time lipid A was lowered from 6.8 to 3.4 per cent. The chloroform-methanol extract amounted to 3.2 per cent of the total solids and contained 51.2 per cent FAE. The monochlorobenzene-ethanol extract contained 2.4 per cent of the solids, two-thirds of which was chloroform-soluble having an FAE content of 26.4 per cent and the remaining one-third was water-soluble and had an FAE content of 58.5 per cent.

Following extraction of the partially delipified endotoxin (fraction B) with

aqueous phenol, the FAE content of the recovered endotoxin (fraction C) was reduced from 3.1 to 1.7 per cent; nitrogen was likewise reduced from 1.3 to 0.5 per cent. Fraction C displayed biological potency not significantly different from the starting fraction A and the intermediate fraction B, with the exception of a reduction in its pyrogenic activity. However, when Experiment II was repeated with a starting material similar to fraction A, the final toxic product, fraction *C'*, (Table V) still had pyrogenic potency comparable to that found in the most

TABLE V

Some Chemical and Biological Properties of an Endotoxin (Aqueous Ether Extract) from Salmonella enteritidis at Various Stages of Fractionation

Analysis	Fractions (Experiment II)			
	A Partially deproteinized (Se 183/188)	B Partially delipified (Se 183/188 IE)	C "Deproteinized" and "delipified" (Se 183/188 IL)	C'* "Deproteinized" and "delipified" (Se 179/181 E)
Nitrogen, *per cent*	1.35	1.3	0.53	0.40
Lipid A, *per cent*	6.8	3.4‡	—	—
Fatty acid ester (FAE), *per cent*	4.7	3.1	1.7	2.5
Protection of mice against *S. typhosa* challenge (ED_{50}), μg	0.1	0.4	0.3	2.0
Lethality for mice (LD_{50}), *mg*	0.60	0.81	0.93	0.81
Tumor damage in mice (TD_{50}), μg	0.1	0.25	0.3	0.5
Primary inflammation of rabbit skin (SLD_{50}), μg	0.24	0.31	0.68	—
Pyrogenicity in rabbits (FI_{40}), μg	0.04	0.14	0.44	0.07

* Final fraction containing least amount of nitrogen, obtained from another starting material.

‡ 3 per cent lipid W.

active endotoxin preparations. By and large, fractions A, B, C, and C' all have potencies which compare favorably with those of endotoxins prepared elsewhere.

A comparison of the chemical properties of fractions A, B, C, and C' with haptenic polysaccharides from *S. enteritidis* and *S. typhosa* is presented in Table VI. Attention is directed to the finding that these haptenic polysaccharides contained 1.6 and 4.2 per cent FAE, respectively. The FAE value for fraction C (1.7 per cent) was, therefore, not different from that of the corresponding haptene. The nitrogen content of fraction C (0.53 per cent) was somewhat greater than that of the *S. enteritidis* haptene (0.14 per cent), while the rhamnose and total hexose were somewhat less. Fraction C', on the other hand, had almost the same content of rhamnose and total hexose as the haptene. The nitrogen content of 0.4 per cent represents, to the best of our knowledge, the lowest value thus far

reported for any potent endotoxin. Of the nitrogen content in fractions C and C′, 30 and 60 per cent, respectively, are attributable to hexosamine.

The disclosure of fatty acids in the haptenes prepared by acid hydrolysis requires some elaboration. It has been stated that these haptenes do not contain lipid (3, 16, 21). However, it appears that this depends upon the definition of lipid and the method of determining it. Thus, Freeman (6) and Pon and Staub

TABLE VI

Chemical Analysis of an Endotoxin (Aqueous Ether Extract) from Salmonella enteritidis at Various Stages of Fractionation and of Haptenes from S. enteritidis and Salmonella typhosa

Analysis	Fractions (Experiment II)				Haptenes	
	A Partially deproteinized (Se 183/188)	B Partially delipified (Se 183/188 IE)	C "Deproteinized" and "delipified" (Se 183/188 IL)	C'* "Deproteinized" and "delipified" (Se 179/181 E)	*S. enteritidis*‡	*S. typhosa*§
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Nitrogen	1.35	1.3	0.53	0.40	0.14	0.3 (0.4)‖
Fatty acid ester (FAE)	4.7	3.1	1.7	2.5	1.6	4.2
Fatty acid amide (FAA)	3.2	4.6	2.1	2.2	—¶	1.7
Phosphorus	0.73	0.74	0.71	0.78	0.57	0.69
Hexosamine	2.7	2.7	2.1	3.0	1.1	1.6 (2.8)‖
Hexose	43	50	47	56	57	53
Carbohydrate	76	74	76	80	—¶	74
Rhamnose	19.9	19.3	20.7	26.0	27.0	20 (19.3)‖

* Final fraction containing least amount of nitrogen obtained from another starting material.

‡ Prepared by the method of Freeman (5) as modified by Webster *et al.* (1).

§ Prepared by Dr. Anne-Marie Staub by the method of Freeman (5).

‖ Data from Webster *et al.* (1) on similar preparation.

¶ Preparation exhausted.

(30) have reported the presence of 2.2 to 3 per cent of volatile fatty acid (calculated as acetic acid) in these substances. The hydroxamic acid method of Snyder and Stephens, as modified by Tauber, gives definite evidence of fatty acid esters in the products we have examined. Furthermore, acting upon information from Dr. A. Nowotny to the effect that some of the fatty acids in endotoxins prepared by the phenol method were attached through amide linkages (31), efforts were made to extend the use of the method to determination of total fatty acids, *i.e.*, fatty acid esters plus fatty acid amides (FAE + FAA). This was accomplished by increasing the time of reaction with the alkaline hydroxylamine reagent. In this manner, additional fatty material has been revealed in all endo-

toxins and haptenic polysaccharides so far tested. The amounts have been similar in all such products, without regard to values for hydrolytic lipid or FAE. Thus, for example, a phenol-water extract from *Sh. flexneri* (preparation 4 in Table II) had 20 per cent lipid A and 22.5 per cent FAE. The value for FAA was found to be 4.1 per cent. The data for endotoxins from *S. enteritidis* at various stages of fractionation (fractions A, B, and C) and for the *S. typhosa* haptene are listed in Table VI. The FAA value of the final fraction C (2.1 per cent) was similar to that found for the *S. typhosa* haptene (1.7 per cent).

DISCUSSION

This investigation has shown that potent endotoxins may contain less lipid and nitrogenous material than heretofore reported. Three different methods of analysis, for total fatty acid ester (FAE), for lipid A, and for lipid W, were in accord in demonstrating that powerful endotoxins extracted with aqueous ether contained less bound lipid than preparations of similar potency extracted by other methods. Application of these methods to study of the distribution of lipids in endotoxins revealed that only about one-fourth of the total FAE in toxins extracted with aqueous ether was present in the chloroform-soluble portion after hydrolysis with 1 N hydrochloric acid. Even in toxins extracted with phenol-water, the chloroform-soluble lipid in similar hydrolysates contained only 35 to 62 per cent of the total FAE. The determination of FAE, therefore, does not estimate the content of lipid A, nor would one expect any measure of total fatty acids to do so.

Values for total fatty acids (*i.e.*, both ester- and amide-linked fatty acids) obtained by the hydroxamic acid method agree well with other estimates. For example, an aqueous ether extract which had FAE and FAA values of 6.5 and 1.8, respectively, was also analyzed by Drs. Westphal and Lüderitz. The content of free fatty acids, determined titrimetrically in the ether extractives released by "total hydrolysis" (6 N hydrochloric acid for 12 hours) and calculated as palmitic acid, was 8 per cent (17). It was possible that amidic-bound fatty acid could be a typical constituent of lipid A. However, in a recent experiment to determine its distribution in a 1 N HCl hydrolysate, the chloroform-soluble lipid A fraction was found to be free of FAA. This component was present in the water-soluble and the insoluble portions of the hydrolysate. Also, the FAA content of 12 different endotoxins varied only between 1.0 and 4.6 per cent without relation to the FAE content, which varied between 1.7 and 22.5 per cent.

At the present time it is not entirely clear why the lipid content of endotoxins derived from phenol-water and from aqueous ether extracts should differ so markedly. Products prepared by the aqueous ether procedure also differ from those obtained by phenol-water in that even the crude extracts contain little if any nucleic acid. Since nucleic acid is a cytoplasmic constituent, a procedure such as the phenol-water method, which extracts this material from whole bac-

teria, may also extract other cytoplasmic constituents, including fatty acid-containing material. There is no direct evidence for such an occurrence, however, and endotoxins extracted at higher temperatures with aqueous ether may contain as much lipid as phenol-water or TCA extracts. The additional finding that non-hydrolytic procedures will remove substantial amounts of lipid from purified endotoxins without loss of potency indicates that some of the so called "bound" lipids may be a non-integral part of the active complex. This is also evident from the finding that different methods extract endotoxins of comparable potency having strikingly different content of lipid.

In like manner, aqueous ether appears to extract some lipoid material which is not present in phenol-water extracts. This is found in the chloroform- and water-insoluble portions of acid hydrolysates and is thought to consist of fatty acids bound to long peptide chains. To eliminate this inactive (22) material, a phenol extraction was performed as the final stage in purification of the endotoxin initially isolated by the aqueous ether method, and it proved to be effective, at this stage, in reducing both lipid and nitrogen.

Thus, potent endotoxins were prepared whose content of nitrogen (0.40 to 0.53 per cent) and of lipoid material (1.7 to 2.5 per cent FAE; 2.1 to 2.2 per cent FAA) closely resembled that of non-toxic Freeman type haptenes (0.14 to 0.4 per cent N; 1.6 to 4.2 per cent FAE; 1.7 per cent FAA). A completely non-toxic, chiefly polysaccharide material, prepared from an aqueous ether extract by dialysis and lyophilization of the chloroform-extracted water phase of an acetic acid hydrolysate (0.1 N acetic acid for 6 hours at 100°C), also was found to contain over 6 per cent FAE + FAA. These crude and purified haptenes from *S. enteritidis* and *S. typhosa* were not lethal for mice and did not produce inflammation in the skin of rabbits in the highest doses tested (300 μg), whereas final fraction C, with similar content of lipid, was toxic to mice and highly active in rabbit skin (SLD_{50} = 0.7 μg). There thus appears to be no basis for correlating lipid content with the toxic properties of these two types of materials. It remains for future work to determine whether or not the small amounts of lipid they contain are made up of the same fatty acids and whether they exist in the same relationship to the polysaccharide moiety.

The presence of fatty acids in Freeman type haptenes from *Salmonella* of group D was anticipated in view of the finding (Table III) of water-soluble FAE-containing material in 1 N hydrochloric acid hydrolysates of endotoxins. These fatty acids must be attached to the degraded polysaccharide moiety, and it is unlikely that they would be removed during the usual process of refining the haptene.

An ensuing portion of this study (22) deals with the biological activity of isolated lipids, and attempts to evaluate the contribution which these substances make to the total activity of endotoxic polysaccharides.

SUMMARY

Endotoxins of low lipid content prepared from *S. enteritidis* by the aqueous ether method have been further treated to remove bound lipid by non-hydrolytic procedures. Such endotoxins, containing as little as 2 per cent lipid A, were as potent in stimulating a variety of physiological responses as those prepared by the well known phenol-water or Boivin procedures which yield products containing as much as 30 per cent lipid A.

To verify the difference in lipid content between the aqueous ether preparations and other types of endotoxins, three different methods of lipid analysis were employed: determination of chloroform-soluble material released by hydrolysis with hydrochloric acid (lipid A) or with acetic acid (lipid W), and estimation of total bound fatty acids. These methods were in accord in showing the magnitude of the difference. No more than one-half of the fatty acids present in endotoxin were associated with the fraction designated lipid A.

Methods are described for the preparation of potent endotoxins with analytical values for nitrogen, phosphorus, hexosamine, carbohydrate, and fatty acid which do not differ appreciably from those of the classical, non-toxic, haptenic polysaccharides.

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