SYMPOSIUM ON BACTERIAL ENDOTOXINS[1]

I. Relationship of Chemical Composition to Biological Activity

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Although endotoxins have been studied intensively by competent investigators during the past several decades, exact relationships between chemical constitution and capacity to evoke characteristic reactions in mammals remain largely unknown. As ordinarily isolated, endotoxins are complexes of lipid, polysaccharide, and protein or peptide-like substances; but the proportions of each have been found to vary with the method of isolation. The question arose whether all of these components were essential to the properties ordinarily referred to as "endotoxic." Morgan and Partridge (9) found the protein moiety necessary to endotoxic potency. However, Westphal and Lüderitz (19), Webster et al. (16), and Landy et al. (7) demonstrated that an essentially protein-free lipopolysaccharide retained the ability to stimulate all the characteristic physiological effects, thus rendering an important role for protein unlikely. This conclusion is supported by our own findings (12).

Boivin and Mesrobeanu (1) and Freeman (3) demonstrated that hydrolysis of endotoxin with dilute acetic acid destroyed antigenicity in vivo and most of the endotoxic potency, leaving the serologically specific polysaccharide hapten and a lipid component which was extractable in chloroform. Lipids freed from endotoxins by hydrolysis with acid were originally thought to be physiologically inactive, but Westphal and Lüderitz (19) showed that certain of them retained at least some endotoxic properties. The material which they called *Lipoid A* (lipid A) appeared as chloroform- or pyridine-soluble substance in the product of hydrolysis of endotoxin with 1 N HCl. They found that redispersion of lipid A in aqueous menstruum by detergents or by suspension in low molecular weight dextrans, or by coupling it to an inert soluble protein restored activity to as much as one-tenth, or even one-fifth, the potency of intact endotoxin (17–20). Since the polysaccharides present in such hydrolyzates were physiologically inert, it was concluded that the "toxicity" of endotoxins could be adequately accounted for in the firmly bound lipid moiety. We have employed a variety of experimental approaches to evaluate this important concept. Some of the findings reported here have been abstracted from our detailed publications (5, 11), in which full descriptions of methods are given.

Evidence which appears to demonstrate the importance of lipid for endotoxic activity may be readily obtained. The experiment on nonspecific protection outlined in Table 1 was performed with endotoxins extracted from *Salmonella abortus equi* and *Shigella flexneri* by the phenol-water method. One was a commercially available material (Difco); the other was sent to us by O. Westphal. Mice were prepared by intravenous, graded doses of materials under test and challenged intraperitoneally 24 hr later with 8×10^7 cells of *Salmonella typhosa*, strain Ty_2, in saline. Protective ED_{50} values were obtained graphically from the proportion of survivors at each dose level. The dose range for endotoxins was not well chosen, but it is apparent that doses of 2 μg protected about half the animals in each case. Acid hydrolysis of these endotoxins yielded preparations of lipid A with ED_{50} values of about 30 μg and 19 μg for the lipids from *S. abortus equi* and *S. flexneri*, respectively. Thus the lipids had about one-tenth the activity of the original endotoxins in this particular test.

Additional experience with these materials showed, however, that, although the lipids were of unusually high potency in the protection test, the parent endotoxins, despite their high content of lipid A (*see* Table 6), were rather weak in this

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respect. Table 2 shows how they compare with a typical aqueous-ether-extracted endotoxin (Se 204) prepared from *Salmonella enteritidis* in our laboratories (11). Although the three toxins were of similar lethality for mice, the ether-toxin was at least tenfold more active in protection. It must not be supposed, either, that this was because *S. enteritidis* and the challenge organism have antigens in common: certain endotoxins from bacteria wholly unrelated to *S. typhosa* consistently have been just as active as preparation Se 204 in the same test.

One idea which emerges from our experience is that the potency of a lipid prepared from endotoxin is independent of the potency of the starting material; that is, relatively potent lipids may be derived from weak endotoxins, and vice versa. Tables 3 and 4 show that lipid A is not very active in comparison to potent endotoxins assayed in the same test. Table 3 contains data on pyrogenicity, tumor damage, and protection obtained with specimens of crude and purified lipid A prepared from *Escherichia coli* by a modified Westphal procedure recently outlined in a German patent description (2). Another lipid A from *E. coli* was prepared by Merck, Sharp and Dohme. An aqueous ether extract from *S. enteritidis* and lipid A prepared from it in the manner originally described by Westphal and Lüderitz (19) are included as reference materials. Protection was assayed as before. Median effective doses (TD_{50}) for producing hemorrhagic necrosis in sarcoma 37 implants in mice are listed under tumor damage. Pyrogenicity for rabbits is expressed as the quantity required to produce a fever index of 40 (FI_{40}, meaning an area of 40 cm² under the fever curve, when plotted on a scale of one degree centigrade and one hour per inch). It is a more meaningful and more reproducible measure than the minimal pyrogenic dose (MPD) because it falls on the straight-line portion of the dose-response curve (6). The reference endotoxin gave the customary linear responses to graded doses. The lipids, as usual, provoked erratic responses to graded doses, thus rendering quantitative measurements difficult and leading to much greater variability in repeated tests. The quantities of lipid fractions required ranged from about 100- to several 1,000-fold those for endotoxin.

In Table 4, several potent aqueous-ether endotoxins from *S. enteritidis* are compared with lipid fractions prepared from them by extracting 1 N HCl hydrolyzates (30 to 45 min, 100 C) with

TABLE 1. *Nonspecific protection versus Salmonella typhosa by phenol-extracted endotoxins and lipids derived from them*

Preparation	Dose	Survivors /total	Per cent survival	ED_{50}
	μg			μg
Endotoxin from *Salmonella abortus equi* 1859 S_3 (Westphal)	0.02	10/32	31	(~2)
	0.2	10/32	31	
	2.0	16/32	50	
Lipid A from *S. abortus equi* 1859 S_3	4	14/32	44	(~30)
	20	14/32	44	
	100	19/32	59	
Endotoxin from *Shigella flexneri* 902762 (Difco)	0.02	7/32	22	(~2)
	0.2	7/32	22	
	2.0	17/32	53	
Lipid A from *S. flexneri* 902762	4	4/20	20	19
	20	11/20	55	
	100	15/20	75	
Tween 80*-saline diluent	—	13/68	19	

* Polyoxyethylene sorbitan monooleate.

TABLE 2. *Nonspecific protection versus Salmonella typhosa by phenol-water and aqueous-ether endotoxins*

Endotoxin	Dose	Survivors /total	Per cent survival	ED_{50}
	μg			μg
Salmonella abortus equi 1859 S_3 (Westphal)	0.16	8/32	25	3.0
	0.8	14/32	44	
	4.0	13/32	41	
	20.0	23/32	72	
Shigella flexneri 902762 (Difco)	0.8	10/32	31	3.2*
	4.0	16/32	50	
	20.0	26/32	81	
Salmonella enteritidis Se 204	0.16	10/32	31	0.27†
	0.8	25/32	78	
	4.0	29/32	91	
Saline diluent	—	6/32	19	

* Three separate determinations of ED_{50} gave values of 1.8, 3.2, and 6.8 for an average of 3.9.

† Three separate determinations of ED_{50} gave values of 0.08, 0.15, and 0.27 for an average of 0.17.

chloroform (19). In all comparisons, the febrile responses to endotoxins were greater than those of the corresponding lipids by factors of several 100-fold or more. Variation in the pyrogenicity of the original endotoxins was less pronounced than that of the lipids. Similar comments apply to the results of tumor-damage assays where, it will be noted, TD_{50} values of original endotoxins varied only between 0.1 and 0.3 μg. These may be compared with recent reports of similar effects achieved by doses of phenol-water-extracted endotoxin in the neighborhood of 20 μg (10). Lipid A from the latter material had about the same activity as the lipids described in this and previous tables.

Lipids and parent endotoxins could not be quantitatively compared with regard to lethality for mice because the lipids we tested did not kill a significant proportion of mice even in extremely high doses. Amounts of lipid A ranging up to 10,000 μg were injected intravenously into rabbits without producing shock or death, although substantially smaller amounts produced fever. Some original aqueous-ether extracts were lethal for this species in doses of 1 to 20 μg; the one phenol-water extract tested had an LD_{50} of 690 μg for rabbits.

In comparisons based on dry weight, we have not found lipids which exerted more than $\frac{1}{100}$ the activity of the parent endotoxins except in cases where the endotoxin was of markedly inferior potency in the particular assay chosen. To implicate lipoid material as solely responsible for endotoxic effects, one must account for a greater than 100-fold disparity in activity since lipids are a minor constituent of endotoxins and, as will be shown later, some potent "deproteinized" endotoxins have been isolated containing about 3% or less of lipid A.

When endotoxin is subjected to acid hydrolysis for the isolation of lipid, the remaining polysaccharide moiety is so degraded that it no longer possesses any endotoxic properties. Comparison with such an inactive fraction does, of course, highlight the apparently greater importance of the lipid part obtained under these conditions. However, some of the foregoing results suggest that the activity of lipids, which resisted acid hydrolysis, might be separate from and incidental to the major activity of whole endotoxin, which was destroyed by acid hydrolysis. This and other considerations led us to a quantitative examination of the relationship between release of lipid from endotoxin and alteration of biological properties during the course of hydrolysis by acid.

The product selected for this purpose was an aqueous ether extract of *S. enteritidis* which had not been deproteinized (2.8% N) and which contained 6.4% lipid A; that is, it was a protein-

TABLE 3. *Biological properties of "crude" and "purified" samples of lipid A from Escherichia coli*

Preparation	Protection ED_{50}	Pyrogenicity FI_{40}	Tumor damage TD_{50}
	μg	μg	μg
Salmonella enteritidis:			
Aqueous-ether extract endotoxin*	0.25	0.18	0.13
Lipid A*	~60	>250	550
E. coli strain 0111:			
Phenol-water extract "crude" lipid A†	>50	16	~60
"Purified" lipid A†	>50	58	>100
E coli: "Purified" lipid A (Merck, Sharp and Dohme)	~60	200	700

* Control materials prepared at the Rocky Mountain Laboratory.

† Prepared according to patent description of the Dr. A. Wander Forschungsinstitut (2).

TABLE 4. *Biological properties of endotoxins (aqueous-ether extracts from Salmonella enteritidis) and homologous preparations of lipid A*

Preparation	Pyrogenicity FI_{40}	Tumor damage TD_{50}	Protection ED_{50}
	μg	μg	μg
I:			
Endotoxin	0.18	0.13	0.24
Lipid A	>250	550	~50
II:			
Endotoxin	0.04	0.2	0.25
Lipid A	8.8	~100	>50
III:			
Endotoxin	0.39	—	0.20
Lipid A	>300	—	>50
IV:			
Endotoxin	0.06	0.25	—
Lipid A	66	>100	—
V:			
Endotoxin	0.065	0.21	—
Lipid A	210	390	—

TABLE 5. *Changes in biological activities of endotoxin during hydrolysis with 0.1 N acetic acid*

Duration of treatment with acid	Free lipid	Relative potency				
		Resistance to infection	Tumor damage	Mouse lethality	Dermal inflammation	Pyrogenicity
min	%					
0	0.0	1.0	1.0	1.0	1.0	1.0
5	—	1.41	0.5	0.49	0.41	0.27
15	0.0	0.83	0.12	0.21	0.41	0.39
30	—	0.27	0.025	0.14	0.18	0.18
45	0.2	0.066	0.0055	0.054	0.061	0.14
60	—	0.062	0.0025	<0.05	0.033	0.093
90	0.6	<0.009	<0.003	<0.03	0.038	0.013
180	—	<0.002	<0.003	<0.03	0.015	0.0033
360	2.0	<0.006	<0.003	<0.02	0.0077	0.0007

polysaccharide-lipid complex. The material was subjected to hydrolysis with 0.1 N acetic acid at 100 C, and at intervals portions were removed, quickly cooled to room temperature, and immediately neutralized. A summary of the results of chemical and biological tests on these aliquots is given in Table 5.

No significant amount of lipid was released during the early phases of hydrolysis. It was only after 90 min that an appreciable quantity of lipid, amounting to 0.6%, could be extracted from the hydrolyzate with chloroform. At the time the experiment was terminated (6 hr), chloroform-extractable material totaling 2.0% of the endotoxin had been released. Since hydrolysis of this endotoxin with 1 N HCl for 45 min yielded 6.4% chloroform-soluble lipid A, no more than one-tenth of this fraction could have been released by 90 min of hydrolysis with acetic acid. Five standardized procedures were selected to assess changes in the biological properties of the neutralized reaction mixtures. These included the assays, already mentioned, for nonspecific resistance to infection, tumor damage, mouse lethality, and pyrogenicity. In addition, the test for ability to produce primary inflammation in rabbit skin described by Larson et al. (8) was performed. The data from the individual protocols led to potency estimates which are here expressed as potencies relative to those of the unhydrolyzed (zero-time) sample. Inspection of these data shows that as little as 5 min of hydrolysis was sufficient to reduce endotoxic potency 50% or more in 4 out of 5 of the activities measured. Thereafter, at 15, 30, and 45 min, endotoxic activity, as measured in each of the five different tests, continued to deteriorate. There appeared to be some indication that the capacity to induce tumor damage declined more rapidly than the other properties, whereas pyrogenicity was somewhat more resistant to destruction. It was especially noteworthy that up to 60 min, when the loss of biological potency had become pronounced, very little lipid had been released and the turbidity of the reaction mixture did not differ from that of the zero-time sample. At this stage the tumor damaging potency was only $\frac{1}{200}$ that of the zero-time sample. After 90 min, a slight increase in turbidity of the hydrolyzate was observed. At this stage, the decline in pyrogenic potency was 70-fold or about that expected from results of studies with the artificial lipoprotein in which we exposed a casein-lipopolysaccharide complex to the same conditions of hydrolysis. The MPD dose was about 0.01 μg, which compared favorably with the value reported in the literature for so-called artificial lipoproteins (17, 19). With continuing hydrolysis, further deterioration occurred, but available quantities of material precluded attainment of endpoints in all but the most sensitive tests.

The results revealed that progressive loss of biological potency during the course of the hydrolysis occurred before any measurable chloroform-soluble lipid was released. Obviously, separation of the lipid moiety and consequent reduced solubility of this portion were not responsible for loss of potency. It may be recalled that Tal and Goebel (13) have reported that hydrolysis of a purified toxic material, "TM," from *Shigella paradysenteriae* with 1% acetic acid for 1 hr destroyed toxicity without liberating any chloroform-extractable substance. As has been reported (17, 19) and as we have confirmed, the pyrogenic potency of lipoproteins is greater than that of lipid A, a finding explained earlier by assuming a greater dispersion of lipid when bound to an appropriate protein carrier. This kinetic study revealed, however, that the difference in potency is more likely a reflection of the less vigorous hydrolysis to which the protein-lipopolysaccharide complexes were subjected. If solubility effects are involved, they might be adequately explained on the basis that the conditions of hydrolysis employed would not

have rendered the lipids insoluble in water. If coupling to casein actually occurs, it probably does not contribute to the biological potency of the material.

Additional studies of hydrolyzed endotoxin in the optical ultracentrifuge revealed that, on the average, the dissociation of the endotoxin into particles of the size of the Freeman-type hapten (sedimentation constant, 1.3 to 1.6 S) proceeded at the same rate as the destruction of endotoxic potency by acid (Table 5). This was evident from measurements of areas under Schlieren patterns: areas produced by the small particles reached a maximum (essentially complete dissociation of endotoxin) in samples exposed to about 90 min of hydrolysis, i.e., in samples in which the major biological activity had been destroyed.

Up to this point we have used the word lipid a number of times, mostly in the sense conveyed by Westphal's term "*Lipoid A*." The word also has a more general meaning, subject to individual interpretations, but implying a substance insoluble in water, soluble in common fat solvents, and frequently containing esterified fatty acids. Table 6 gives values for three different determinations of lipid on each of several endotoxins. Lipid A and lipid W were determined by weighing the chloroform-extractable material released by acid hydrolysis (11). In the former case, endotoxins were boiled with 1 N HCl for 30 to 45 min. Lipid W was released by boiling with 0.1 N acetic acid for 6 hr. Total fatty acid ester was determined by the hydroxamic acid method of Snyder and Stephens (15) as modified by Tauber [(14) and *personal communication*]. This method, which had the advantage of requiring very little material, was included in the hope of clarifying the relationship between fatty acid content of endotoxins and the content of lipid A.

As is revealed in Table 6, values for bound lipid depend to a considerable extent on the method by which they are determined. In general, values for lipid A were greater than for 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 4 and 5). 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 freed by acid hydrolysis. Regardless of the analytic method used, endotoxins extracted at low temperature by the aqueous ether method (11) were found to contain less lipid than phenol-water or trichloroacetic acid extracts.

The determination of fatty acid ester (FAE) disclosed a higher content of fatty acid in endotoxins than could be accounted for by either the "lipid W" or "lipid A" determinations. Also, since fatty acids account for only about 50% of the composition of lipid A (O. Westphal, *personal communication*) obviously there must be lipoid material in fractions other than the chloroform-soluble portion of acid hydrolyzates. We therefore followed the distribution of fatty acids, as FAE,

TABLE 6. *Content of bound lipid of endotoxins as determined by different methods*

No.	Origin	Extractant	Nitrogen	Lipid A	Lipid W	Fatty acid ester
			%	%	%	%
1	*Shigella flexneri*	Phenol-water	4.5	11.0	6.7	18.0
2	*Salmonella enteritidis*	Phenol-water	3.6	20.0	5.0	11.7
3	*Shigella flexneri**	Phenol-water	2.7	20.0	13.0	22.5
4	*Escherichia coli*	Phenol-water	1.8	16.0	24.0	15.3
5	*Salmonella abortus equi**	Phenol-water	1.2	22.0	20.0	13.3
6	*S enteritidis*	Trichloroacetic acid	3.3	14.0	12.0	18.0
7	*S. enteritidis*	Aqueous ether (6 C)	2.8	6.4	2.4	6.9
8	*S. enteritidis*	Aqueous ether (12 C)	2.6	4.9	2.8	6.1
9	*S. enteritidis*	Aqueous ether (25 C)	2.3	18.0	14.0	10.8
10	*S. enteritidis*	Aqueous ether (6 C)	2.9	2.0	2.0	5.7
11	*S. enteritidis*†	Aqueous ether (6 C)	1.3	3.4	3.0	3.1

* Protection data for these preparations given in Tables 1 and 2.

† For biological properties, see fraction B in Table 9.

in fractions resulting from hydrolysis with boiling 1 N HCl for 45 min with results which appear in Table 7. These fractions were (i) the chloroform-soluble portion, i.e., lipid A, (ii) the chloroform- and water-insoluble portion, and (iii) the remaining water-soluble portion. The data resulting from chemical analysis were recalculated to obtain an expression of the proportions, as percentage, of total FAE in the fractions of the hydrolyzates.

Among phenol-water extracts, it is evident that, on the average, esterified fatty acids were distributed about equally between the chloroform-soluble lipid A and the water-soluble portion. Among aqueous ether extracts, about one-fourth of the total FAE appeared in a water- and chloroform-insoluble fraction. About one-half was found in the water-soluble fraction, and only one-fourth in the fraction corresponding to lipid A. The determination of FAE, therefore, does not estimate the content of lipid A, nor could one expect any measure of the total fatty acids to do so.

In hydrolyzates of both types of endotoxin, active lipoid material was found to reside only in the chloroform-soluble portion. The inactive water- and chloroform-insoluble fractions, found only in aqueous ether extracts, were, curiously, the richest in hexosamine as well as in nitrogen of all the lipoid fractions. This observation prompted us to extract aqueous ether endotoxins with aqueous phenol in the hope of further reducing nitrogen and lipid.

Before continuing with this topic, it is necessary to describe some additional procedures which we have employed for the refinement of endotoxin. As was reported earlier (12), the initially low lipid content of aqueous-ether extracts could be further reduced by nonhydrolytic means without significant loss of endotoxic potency. A typical experiment of this nature is outlined in Table 8. Endotoxin was first refluxed with a boiling mixture of chloroform and methanol from which was recovered an inactive chloroform-soluble material designated "nonhydrolytic lipid I." This contained about 50% FAE and the yield was 3.9% of total solids. The residual endotoxin was then refluxed with a mixture of monochlorobenzene-ethanol. The extractives, totaling 2.4% of total solids, were three-quarters chloroform soluble and one-quarter water soluble. The chloroform-soluble lipid IIa, which contained about 30% FAE, was a material with appreciable biological activity, as may be seen from the results of tests for pyrogenicity and inflammation of rabbit skin. The proportion of FAE in the water-soluble fraction was, surprisingly, nearly 60%. The proportion of lipid A in the residual endotoxin (Table 8, last line) had been reduced by approximately 50%, but the biological activity was scarcely different from that of the starting material (Table 8, first line). Thus, when hydrolytic conditions were avoided, lipoid material of activity comparable to that of lipid A could be removed without significantly decreasing the activity of the endotoxin proper. This is the result that had been predicted from the relative potencies of intact endotoxins and lipids derived from them.

The data in Table 9 were obtained when

TABLE 7. *Distribution of fatty acid ester (FAE) in fractions resulting from hydrolysis of endotoxin (1 N HCl, 45 min, 100 C)*

No.	Origin	Extractant	Proportion of total FAE in fractions of hydrolyzate					
			$CHCl_3$ soluble		Insoluble		H_2O soluble	
			%	Avg	%	Avg	%	Avg
1	*Shigella flexneri*	Phenol-water	35		0		65	
2	*Salmonella enteritidis*	Phenol-water	46.3	48	0	0	53.7	52
3	*S. flexneri*	Phenol-water	62		0		38	
4	*S. enteritidis*	Aqueous ether	16.2		25.2		58.6	
5	*S. enteritidis*	Aqueous ether	27.8		32.1		40.1	
6	*S. enteritidis*	Aqueous ether	25.6	25	37.6	27	36.8	48
7	*S. enteritidis*	Aqueous ether	31.6		15.8		52.6	
8	*S. enteritidis*	Aqueous ether	22.9		22.9		54.2	

application of these procedures was followed by a phenol-water extraction as suggested earlier. Fraction A was a conventional aqueous-ether extract which had been subjected to salt and ethanol precipitation, essentially by the method of Webster et al. (16), for reduction of protein. This fraction contained 1.35% nitrogen, 6.8% lipid A, and 4.7% FAE. It exhibited biological reactivity typical of such products except that its lethality for mice was somewhat reduced. When this starting material was partially freed of lipid by the nonhydrolytic procedures just outlined, the resulting fraction B contained 3.4% lipid A and 3.1% FAE. After treatment of fraction B with phenol-water (1:1) at 65 C, fraction C was recovered, which had an FAE content of 1.7% and nitrogen content of only 0.5%. Similar treatment of another starting material led to recovery of fraction C′ (0.4% N) and other similar products have since been obtained. By and large, fractions A, B, C, and C′ have biological potencies which do not differ significantly, and all are potent endotoxins by any criteria.

The nitrogen values for fractions C and C′ approach those reported for polysaccharide haptens which are devoid of endotoxic host-reactivity. As Table 10 shows, there is indeed a remarkable similarity in chemical composition between these refined endotoxins and Freeman-type haptens. A nitrogen content of 0.4 to 0.5%

TABLE 8. *Some biological properties of "nonhydrolytic lipids" versus parent endotoxin (aqueous-ether extract from Salmonella enteritidis)*

Procedure	Preparation	Recovery	Toxicity* SLD_{50}	Pyrogenicity FI_{40}
		%	μg	μg
Endotoxin	Original	100	0.20	0.39
↓				
$CHCl_3$—MeOH extracted →	$CHCl_3$ soluble (nonhydrolytic lipid I)	3.9	≥125	>1,000
↓				
C_6H_5Cl—ETOH extracted →	$CHCl_3$ soluble (nonhydrolytic lipid IIa)	1.8	10.9	28
	Water soluble (nonhydrolytic lipid IIb)	0.6	—	160
↓				
Endotoxin	Final	85	0.24	0.43

* SLD_{50} = mean threshold dose for producing inflammatory lesion in skin of rabbit (8), also used in Table 9.

TABLE 9. *Some chemical and biological properties of an endotoxin (aqueous-ether extract) from Salmonella enteritidis at various stages of fractionation*

Analysis	Fractions			
	A: partially deproteinized	B: partially defatted	C: "deproteinized" and "defatted"	C′:* "deproteinized" and "defatted"
Nitrogen (%)	1.35	1.3	0.53	0.40
Lipid A (%)	6.8	3.4	—	—
Fatty acid ester (FAE, %)	4.7	3.1	1.7	2.5
Protection of mice against *Salmonella 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.

is, we believe, the lowest reported for an active endotoxin. About half of this value is attributable to hexosamine. Attention is directed to the finding that nontoxic haptens contained up to 4% FAE, although they have been assumed to be lipid-free. By an extension of the hydroxamic acid method, we were able to determine fatty acid amides (FAA) as well as FAE (4). Together they are believed to give a reasonable estimate of total fatty acids. It is noteworthy that FAA values are in the neighborhood of 2% in haptens and endotoxins alike, whereas FAE content may be over 20% in some endotoxins. Aside from nitrogen and fatty acid, the other analytical values show that active fractions C and C′ did not differ appreciably from haptens in their content of phosphorus, hexosamine, hexose, or total carbohydrate. As expected, hydrolysis of a preparation similar to C and C′ with 0.1 N acetic acid for 90 min liberated no trace of extractable lipid, and the nondialyzable portion of the hydrolysis product ("90 min hapten") did not differ from the starting material in chemical composition and had approximately the same sedimentation constant (1.4 S) as the highly purified Freeman hapten (1.3 S). A small amount of fast moving component in the neutralized 90 min hydrolyzate, separable from the haptenic component by differential centrifugation, was found to be responsible for most of the residual endotoxic activity in the original reaction mixture.

TABLE 10. *Chemical analysis of an endotoxin (aqueous-ether extract) from Salmonella enteritidis at various stages of fractionation and of haptens from S. enteritidis and Salmonella typhosa*

Analysis	Endotoxins		Haptens	
	C: "deproteinized" and "defatted"	C′: "deproteinized" and "defatted"	*S. enteritidis*	*S. typhosa**
	%	%	%	%
Nitrogen	0.53	0.40	0.14	0.3(0.4)†
Fatty acid ester (FAE)	1.7	2.5	1.6	4.2
Fatty acid amide (FAA)	2.1	2.2	—	1.7
Phosphorus	0.71	0.78	0.57	0.69
Hexosamine	2.1	3.0	1.1	1.6(2.8)†
Hexose	47	56	57	53
Carbohydrate	76	80	—	74
Rhamnose	20.7	26.0	27.0	20.0(19.3)†

* Prepared by Anne-Marie Staub.

† Data from Webster et al. (16).

In another case, a 90-min hapten was prepared from an aqueous ether endotoxin containing 8% FAE + FAA. This endotoxin had been largely deproteinized (0.7% N) by treatment with phenol-water, but had not been processed for reduction of lipid. After refinement by extraction with chloroform, dialysis, and differential centrifugation, the hapten was completely soluble in water and it sedimented as a single component (1.4 S) in the ultracentrifuge. Its biological activity, less than $1/1000$ that of the endotoxin, was too low to measure with the quantities available. When this hapten, which still contained 8% bound fatty acid, was hydrolyzed further with 1 N hydrochloric acid (45 min at 100 C), it yielded 5% of a chloroform-soluble substance indistinguishable from lipid A.[2]

SUMMARY

These studies have been concerned with the relationship between chemical structure and biological properties of endotoxin. They have been carried out principally with endotoxin prepared from *Salmonella enteritidis* and the conclusions drawn are restricted by this fact. We recognize that endotoxins are usually potent antigens as well, but for the present have not concerned ourselves with that aspect of their dual nature, but have sought instead to determine the minimal chemical structure necessary for elicitation of the host-reactive properties commonly referred to as "endotoxic." To date, the

[2] One of our highly potent preparations (Se 154A) containing 2.8% lipid W, 4.9% lipid A, and 8.3% FAE + FAA calculated as palmitic acid was examined by Dr. Westphal who found 8% total fatty acid also calculated as palmitic acid. On the assumption that lipid A contains about 50% total fatty acids, he estimated its lipid A content to be 15% (Kabat and Mayer's *Experimental Immunochemistry*, 1961, p. 833, Charles C Thomas, Springfield, Ill., Publisher). (If this assumption were valid, then the inactive hapten described above would contain about 16% lipid A.) However, as has been described in this presentation and elsewhere (11), only about one-fourth of the total fatty acids of an aqueous ether endotoxin can be found in the chloroform-soluble portion (lipid A).

positive findings are not numerous. We have succeeded in stripping away much extraneous material and, in the process, have all but eliminated protein and lipid, components in which other investigators have believed the ultimate toxic principle to reside. Our most highly refined products are still large polysaccharide complexes which defy exact structural analysis and which contain, in addition, small amounts of fatty acids, nitrogenous material, and phosphorus.

If the major toxic activity is to be attributed to a lipid, one must postulate a special lipid, present in minute quantity and quickly inactivated by acids. The most active lipids which have been liberated by acids or by nonhydrolytic means are biologically feeble when compared directly with the most potent endotoxins, yet such activity as they possess is undeniable and requires explanation. One possibility already mentioned is that lipids may have a separate distinctive activity, resistant to hydrolysis and measurable in the isolated lipid, but not of sufficient potency to contribute appreciably to the hydrolysis-sensitive activity of the major component. Alternatively, the true toxin may be some relatively small, acid-sensitive component distributed throughout the endotoxin complex in lipid and nonlipid alike which, because of encapsulation or some other restriction of availability, is both resistant to acids and limited in activity in lipid fractions. Still another interpretation may involve properties of macromolecules. It might well be that, in addition to a certain chemical composition and unit structure, a complex of a critically large size is necessary for toxicity, and that the destructive effect of acid is simple depolymerization due to splitting of bonds by which particles of the size of the hapten are held together. Or, the major toxic properties may reside in some small component which is active only when attached to an appropriate macromolecular carrier. At the moment there is no reason to endorse specifically any of these interpretations.

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