BIOLOGICAL PROPERTIES OF PARENT ENDOTOXINS AND LIPOID FRACTIONS, WITH A KINETIC STUDY OF ACID-HYDROLYZED ENDOTOXIN

By WILLARD T. HASKINS, Ph.D., MAURICE LANDY, Ph.D., KELSEY C. MILNER, Ph.D., and EDGAR RIBI, Ph.D.

(From the United States Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Montana, and the National Cancer Institute, Bethesda)

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The investigations of Westphal and his associates on the isolated moiety of bacterial endotoxin that they designated *lipoid A* (lipid A) have attracted much interest. These workers emphasized that for lipid A to exert endotoxic effects it must be suitably dispersed in aqueous menstruum, and they speculated that, were it possible to disperse lipid A in water in a manner comparable to its original state as bound to the polysaccharide carrier, it would then display biological activity similar to that of the intact endotoxin. Experimentally, they found that lipid A, dispersed by detergents or in low molecular weight dextrans, or coupled to an inert soluble protein, exerted at most one-fifth the potency of the endotoxin itself (1–4).

However, comparisons of the potency of dispersed lipid A with that of the very endotoxin from which the lipid was derived are not readily identifiable in existing reports. In view of our findings (5, 6), in which correlations of potency with lipid content were not evident, and the continuing scarcity of relevant biologic data, we considered it necessary to obtain, by well established dose-response assays, such comparative data. These assays showed that lipids isolated from endotoxins of high initial potency exerted an insignificantly small fraction of the potency of the parent endotoxins.

When the polysaccharide moiety is liberated by acid hydrolysis (*e.g.*, during the preparation of lipid A), it no longer possesses any endotoxic potency. Comparison of such a totally inactive fraction with active lipids does, of course, highlight the apparently greater importance of the lipid part. But we also found that lipid obtained by a non-hydrolytic procedure, which did not destroy the activity of the remainder of the endotoxin complex, was as active as lipid A. This suggested that the activity of these lipids is separate from, and of a different order from, the activity of the principal toxic moiety of bacterial lipopolysaccharides. The activity of lipids is obviously resistant to acids, but the major activity of endotoxins is not.

Hydrolysis of endotoxin with acid did, in another way, elucidate the function of lipid in endotoxin. When samples were taken for bio-assay at brief intervals following initiation of the hydrolytic process, potency was seen to be rapidly and progressively reduced before measurable amounts of lipid were released. This experiment provided an alternative explanation for the activity, intermediate between that of lipids and that of endotoxins, of so called "artificial lipoproteins" produced by *Umkupplung* (1, 3).

Materials and Methods

A. Chemical Analysis.—The methods used for chemical analysis of the bacterial fractions are described in an accompanying paper (6). Since other investigators (7) have used more vigorous conditions of hydrolysis than we have for determination of hexosamine in lipoid material, parallel determinations of hexosamine on a representative sample of lipid A were made to compare the effects of different conditions of hydrolysis. Values obtained after 6 hours of hydrolysis with 3 N hydrochloric acid were not significantly different from those found when hydrolysis with 6 N hydrochloric acid was prolonged for 12 hours at 100°C.

B. Preparation of Endotoxins and Lipids.—Unless otherwise stated, endotoxins and "hydrolytic" lipid A and lipid W were prepared as described under Materials and Methods in an accompanying paper (6). The methods for non-hydrolytic dissociation of bound lipid from endotoxin are described under Results in the same report. The fraction referred to as non-hydrolytic lipid I represents the chloroform-soluble extractives recovered by evaporation of the filtered chloroform-methanol reaction mixture. The fractions designated non-hydrolytic lipid II a and II b were derived from the extractives recovered by evaporation of the filtered monochlorobenzene-ethanol reaction mixture; lipid II a was the chloroform-soluble portion and lipid II b the water-soluble remainder of the residue after evaporation. Preparations from other sources are acknowledged in the text.

C. Endotoxin Bio-Assays.—

1. General considerations: In addition to the specific requirements for the individual assays, as detailed below, the following were considered to be basic prerequisites for the quantitation of endotoxin. Animals employed for each of the assays were uniform as regards strain, source, and weight. The size of the experimental groups varied in accordance with the requirements of each assay, *i.e.*, the number considered to provide statistically reliable data. These groups ranged in size from as many as 32 (for measurement of resistance to infection) to as few as 2 (for determination of febrile response). Reference endotoxins were included in each test to ascertain the responsiveness of each set of animals. The primary objective in these assays was to administer to groups of animals amounts of the test materials which would evoke the particular response in 15 to 85 per cent of the animals, *i.e.*, bracket the median dose.

2. Lethality for mice: Assays were performed with 21-day-old male white mice of the Rocky Mountain Laboratory (RML) strain weighing 10 to 12 gm. Graded doses of endotoxin were injected intraperitoneally into groups of 5 mice each. Mice were observed for 6 days and any dying more than 72 hours after injection were examined in the gross and by culture for evidence of extraneous cause of death. Median lethal doses (LD_{50}) were calculated by the method of Kärber (8). Analysis of the variation to be expected in this method has been reported earlier (9).

3. Tumor damage: Hemorrhagic necrosis was determined with a modification (10) of a previously described method (11). Sarcoma 37 was implanted as a tumor mash into the thigh muscle of 2- to 3-month-old CAF_1 mice. 6 days later mice bearing such implants were randomly distributed into groups of 10 each and the groups were given intraperitoneally graded doses

of endotoxin. Each set of bio-assays included two control groups: the first consisted of 10 untreated mice, to disclose the extent of spontaneous hemorrhage or necrosis in the tumors of the set; the second consisted of 10 mice which had been given 10 μg of *Serratia marcescens* endotoxin to ascertain the responsiveness of the set of tumors to endotoxin. The animals were autopsied the following day. Tumors were cut open and examined in the gross for induced hemorrhage. Values for TD_{50} were read from the slope of dose-response obtained by plotting the percentage of mice with induced tumor damage against log dose of endotoxin.

4. Resistance to challenge with Salmonella typhosa: The test was adapted from procedures commonly employed for assay of typhoid vaccine. Since materials antigenically unrelated to *S. typhosa* were as protective as any homologous preparations, the effect measured is termed "non-specific resistance." Male white mice, 4 to 5 weeks old, housed 2 per jar, were assigned to treatments in groups of 32 mice per dose level by statistical randomization. Graded doses of preparations in physiological saline or in saline containing 0.5 per cent tween 80 were injected intravenously, and 24 hours later the mice were challenged intraperitoneally with 80 (range 50 to 110) million *S. typhosa* (3.6, range 2 to 6, LD_{50}) grown 6 hours on agar and injected without mucin. Survivors were counted 72 hours after challenge, an interval sufficient to include all deaths attributable to *S. typhosa*. Values for ED_{50} were read from the slope of dose-response obtained by plotting percentage (transformed to probit) of surviving mice against log dose of material under test. Equivalent groups of control mice were prepared with injections of diluent. Ordinarily about 90 per cent of these succumbed, but extreme effects ranged from as low as 75 per cent to 100 per cent.

5. Primary inflammation of rabbit skin: These assays were conducted as described by Larson *et al.* (9). Graded doses of materials under test were injected intracutaneously (0.2 ml volume) in bands on the lateral surfaces of at least 4 rabbits each. Primary reactions present 48 hours after injection, consisting of edema and/or erythema extending at least 5×5 mm, were recorded and used for calculation of average threshold dose or SLD_{50} (equivalent to median effective dose except for method of estimating standard errors).

6. Pyrogen response: Data supporting the validity of the procedure have been reported (12). The following summarizes the requirements and the distinctive features of the method. Variability in the pyrogenic response of individual rabbits could be kept to a minimum by observing certain precautions such as: adequate training (in stocks) of the animals to handling and restraint; application of minimal restraint during the test; use of animals from a single source of supply, weighing 2 to 2.5 kg and having a base line temperature of 38.5–39.9°C. For the recording of temperatures, rectal thermocouples, connected to an automatic multipoint recorder (Minneapolis-Honeywell Regulator Co., Minneapolis) were inserted to a depth of 4 inches or more. Temperatures were measured immediately before injection of endotoxin and at 12 minute intervals thereafter until they had returned to normal. The febrile responses were plotted on 1×1 standard graph paper with 1°C and 1 hour equalling 1 inch. The area under the curve was measured in square centimeters with a compensating polar planimeter. The fever index (FI) thus obtained represents a measure of the height and duration of the febrile response. Groups of rabbits were given graded doses of the endotoxin under test so as to obtain data for calculating the dose required to produce a fever index of 40 cm^2 (FI_{40}), a value which was shown to fall on the linear portion of the dose-response curve (12).

RESULTS

Biologic Potencies of Endotoxin (Aqueous Ether Extract) versus Lipid A.—Such biological activity as is possessed by lipoid fractions of endotoxins is most evident when the preparations are well dispersed. By shaking chloroform solutions of the lipoid materials with hot solution of tween 80 in saline, and distilling off

the chloroform in a 70°C water bath, it was possible to obtain stable emulsions or only slightly turbid colloidal solutions. Lipoid fractions thus dispersed were compared in parallel dose-response assays with the parent endotoxins in the same diluent. Specimen data from several such comparisons are presented in Table I. All of the original endotoxins were dialyzed 68 per cent ethanol-precipitated aqueous ether extracts of *Salmonella enteritidis*, of comparable lethality

TABLE I

Endotoxins and Homologous Preparations of Lipid A Compared with Regard to Pyrogenicity for Rabbits, Tumor-Damaging Potency, Stimulation of Non-Specific Resistance to Infection, and Lethality for Mice

Preparations	Pyrogenicity FI_{40}	Tumor damage TD_{50}	Protection ED_{50}	Lethality LD_{60}
	μg	μg	μg	μg
Se 157/161				
Endotoxin	0.18	0.13	0.24	270
Lipid A	>250	550	~50	—
P 1032–1038				
Endotoxin	0.04	0.2	0.25	310
Lipid A	8.8	>100	>50	—
Se 162/168				
Endotoxin	0.39	—	0.20	530
Lipid A	>300	—	>50	—
Se 143/148				
Endotoxin	0.06	0.25	—	270
Lipid A	66	>100	—	—
Se 206/208				
Endotoxin	0.065	0.21	—	220
Lipid A	~210	~390	—	—

for mice, prepared over a period of several months from freshly harvested cells. Lipoid fractions were obtained from these in the manner described earlier for preparation of "lipid A" (6).

For reasons elaborated by Keene *et al.* (12), the FI_{40} is considered to provide a more accurate measure of febrile responses in rabbits than the minimum pyrogenic dose (MPD), which has also been determined for several preparations (see Table II). In all comparisons, the FI_{40}'s of lipid preparations were greater than those of the corresponding endotoxins by a factor of several 100-fold, or more. Variation in the pyrogenicity of the original endotoxins was less pronounced than that displayed by the lipids.

In assays for capacity to induce hemorrhagic necrosis in Sarcoma 37 implants in mice, original endotoxins were uniformly effective in small doses (TD_{50}'s, < 0.3 μg); end points were not reached with lipid A unless doses considerably in excess of 100 μg were employed.

Non-specific stimulation of resistance to infection is now regarded as an established property of endotoxins. Lipid A has also been shown to be active in this respect (4), but quantitative measurements are rendered difficult, not only by

TABLE II

Pyrogenicity, Induction of Tumor Damage, and Stimulation of Non-Specific Resistance in Mice by Lipids and by Parent Endotoxins from Salmonella enteritidis

Preparations	Pyrogenicity		Tumor damage TD_{50}	Protection ED_{50}
	MPD*	FI_{40}		
	μg	μg	μg	μg
Aqueous ether extract				
Endotoxin	0.0005	0.18	0.13	0.24
Lipid W	3.1	>250	>1000	>50
Lipid A	0.64	>250	550	~50
Phenol-water extract				
Endotoxin	0.0003	0.14	0.3	<0.1
Lipid W	—	115	—	26
Lipid A	2.6	39	550	13
TCA extract				
Endotoxin	—	0.13	0.3	<0.1
Lipid W	—	32	300	50
Lipid A	—	30	—	7

* MPD (minimum pyrogenic dose) = μg of product per rabbit required to elevate temperature 0.6°C.

the usual variability in mouse protection tests and quantal response data in general, but also by the fact that the period of maximum resistance is dependent on factors peculiar to individual preparations and on the amount of material injected (13). Furthermore, we have found a good deal of seasonal variation in the capacity of mice to respond in this manner. Nevertheless, a test modeled on one employed for assay of typhoid prophylactics, with appropriate selection of dose, route, and interval between injection and challenge of animals, yielded data which could be interpreted numerically and used for comparisons. Complete endotoxins almost invariably gave linear responses to dose in this test. Responses to lipid fractions showed much greater variability in repeated tests. In most instances, amounts of such preparations sufficient to bracket the 50 per

cent response level were not available. In all comparisons, the endotoxins were more effective by margins ranging from at least 100- to several 1000-fold.

One of the most reproducible measures of toxicity, lethality for mice, could not be employed for comparison of lipids and parent endotoxins because the lipid preparations did not kill mice. In limited trials with rabbits, a more susceptible species, intravenous injection of lipid A in amounts ranging from 500 to 10,000 μg (trichloroacetic acid (TCA) extract) (Table II) did not produce shock or death, whereas the original endotoxins employed were lethal in amounts of 2 to 10 μg.

Biologic Potencies of Endotoxins Prepared from S. enteritidis by Various Methods versus Lipids Derived from Them.—In another series of experiments we examined lipids A and W derived from endotoxins extracted by different procedures: a Boivin type TCA extract, a purified phenol-water extract (after Westphal), and a typical aqueous ether extract. These materials were compared for pyrogenicity, induction of tumor damage, and stimulation of non-specific resistance (Table II). The aqueous ether endotoxin yielded a lipid W which was devoid of hexosamine (<1 per cent). Lipid A prepared from the same material had 7.3 per cent hexosamine, which is considerably less than the 18 to 20 per cent reported for lipid A derived from *Escherichia coli* and *Salmonella abortus equi* (1, 7, 14). Both lipids were found to contain about 1 per cent hexose by the anthrone method. Pyrogenic activity, first measured in terms of the MPD, was in the range reported by Westphal for lipid A dispersed in tween (1, 4); values of 0.64 and 3.1 μg for lipids A and W, respectively, were obtained. Although initially impressive, these values represent less than 1/1000 of the activity of the parent endotoxin (MPD = 0.0005 μg).

The phenol extract yielded lipid W containing 2.5 per cent hexosamine and lipid A with 5.6 per cent hexosamine. Hexose contents were 1.4 and 1.8 per cent, respectively. The MPD of this lipid A was about 10,000 times that of the endotoxin. Subsequent comparisons, based on determinations of fever index, revealed that FI_{40}'s for all lipid fractions, including those prepared from the TCA extract, were at least several 100-fold greater than those of the corresponding endotoxins. In tumor damage assays, none of the lipids displayed more than 1/1000 of the activity of the endotoxins. Median effective doses for protecting mice against challenge with *S. typhosa* showed differences in the same direction. In general, the data in Table II indicate that lipids separated from endotoxins by two different conditions of hydrolysis have roughly the same low order of activity in the host reactions studied. Additionally, results of these tests on lipid fractions and parent endotoxins extracted by three standard procedures agree well with results in Table I, where attention is restricted to aqueous ether extracts.

Thus far only tests with the chloroform-soluble lipids present in acid hydrolysates of endotoxin have been considered. As was demonstrated earlier (6),

approximately one-half of the lipoid substance in aqueous ether- or phenol-extracted toxins, determined as fatty acid ester (FAE), was found in the water-soluble fraction which also contained the polysaccharide haptene. Such fractions were tested in doses adjusted to contain FAE equivalent to that of the chloroform-soluble lipids with which they were compared. No measurable activity was observed. Another portion of the FAE in aqueous ether (but not in phenol) extracts, along with a major portion of amide-bound fatty acids (FAA) appeared in a water- and chloroform-insoluble fraction (6). One sample of such a fraction gave the following analysis: 6.7 per cent N, 30.5 per cent FAE, 6.2 per cent FAA, 10.8 per cent hexosamine, 2.2 per cent hexose. For testing, it was evenly dispersed in tween-saline with the aid of ultrasonic vibration. This fraction also was without measurable activity in tests for pyrogenicity and non-specific protection. It therefore appears that the active lipoid materials in an acid hydrolysate of endotoxin reside in the chloroform-soluble portion.

Tests with Lipid A Prepared from E. coli.—A specimen of lipid A from *E. coli*, prepared at the Merck Institute for Therapeutic Research, Rahway, New Jersey was furnished by Dr. H. J. Robinson. Other samples of "crude" and "purified" lipid A were prepared from the same organism by a modified procedure recently described by Nowotny (15) and others (14). It has been reported that lipid purified in this manner is a derivative of D-glucosamine phosphate (4, 16) containing about 20 per cent hexosamine (14). Since endotoxins from which these lipids has been obtained were not available, an aqueous ether extract from *S. enteritidis* and lipid A derived from it (Se 157/161, Table I) were included as reference materials for the protection test. The protection data in Table III were obtained in two separate tests. Characteristically, results with the endotoxin were uniform and similar to those obtained earlier (Table I). The lipids which were retested, however, gave disparate results in the two tests, although the range of doses employed in the second test was better suited to the potency of the materials. The findings in test I support the conclusion that lipid A prepared from an aqueous ether extract of *S. enteritidis* was not less potent than the lipid A from a phenol-water extract of *E. coli*. In neither test was there any significant difference in potency between the "crude" and "purified" preparations of lipid A.

The lipids from *E. coli* were also tested for pyrogenicity and tumor-damaging potency. In these tests they exhibited a range of values similar to that observed for RML preparations (Table I).

Biologic Properties of Lipids Obtained from Endotoxins by Non-Hydrolytic Procedures.—An additional experiment was designed to compare the properties of parent endotoxin, lipoid materials prepared by non-hydrolytic methods (see Materials and Methods), and the residual endotoxin.

The data in Table IV show that the original and final endotoxins did not differ in their capacity to stimulate non-specific resistance of mice to infection with *S.*

typhosa, to induce primary inflammation of rabbit skin, and to produce fever in rabbits. The LD_{50}'s for mice of original and final endotoxin were 0.53 mg and 0.71 mg, respectively. Of the lipoid fractions, the chloroform- and water-soluble portions of the monochlorobenzene-ethanol extract (non-hydrolytic lipids II a and II b) had discernible activity. As has often been observed in tests of "hydrolytic" lipids, the non-hydrolytic lipids likewise gave erratic responses to graded doses in the protection test. Although mice receiving these products fared better

TABLE III

"Crude" and "Purified" Samples of Lipid A from Escherichia coli Compared with Endotoxin and Lipid A from Salmonella enteritidis with Respect to Pyrogenicity, Tumor Damage, and Stimulation of Non-Specific Resistance

Preparations	Protection ED_{50}		Pyrogenicity FI_{40}	Tumor damage TD_{50}
	Test 1	Test 2		
	μg	μg	μg	μg
Phenol-water extract of *E. coli*—0111				
"Crude" lipid A*	>50	32	16	~60
"Purified" lipid A*	>50	27	58	>100
E. coli, "purified" lipid A‡	—	~60	200	700
Aqueous ether extract of *S. enteritidis*				
Endotoxin§	0.3	0.2	0.18‖	0.13‖
Lipid A§	~60	—	>250‖	550‖

* Prepared according to patent description of Dr. A. Wander, Forschungsinstitut, Freiburg (Breisgau)-Zähringen (14).

‡ Prepared by Merck, Sharp and Dohme, Research Laboratories, Rahway, New Jersey, lot No. L670077-0-6.

§ Control materials prepared at the Rocky Mountain Laboratory (6).

‖ Data from Table I.

than the controls, the range of doses tested did not attain protection of 50 per cent of the animals. Non-hydrolytic lipid II a was found consistently to possess activity of at least the same order of magnitude as that of lipid A. Its FAE content (*e.g.*, 26.4 per cent) was similar to that of preparations of lipid A from aqueous ether extracts (6). Attention is directed to the high FAE content (*e.g.*, 58.5 per cent) of the water-soluble non-hydrolytic lipid II b. The chloroform-methanol extracts (non-hydrolytic lipid I) which also were rich in FAE (*e.g.*, 51.2 per cent) displayed no measurable activity in the bio-assays, although at high dose levels they occasionally produced atypical dermal lesions in rabbits. In four different non-hydrolytic lipid fractions examined (including those in Table IV),

the content of hexosamine was low (1.5 to 3.3 per cent) as was that of hexose (0.9 to 1.2 per cent, anthrone method). Thus, among lipids derived from endotoxins by non-hydrolytic, as well as by hydrolytic, procedures, no correlation was evident between content of hexosamine, or of hexose, and biologic potency.

Biologic Properties of Lipid-Casein Complexes.—Those who suggest that toxic and other host-reactive properties of endotoxins reside chiefly in the lipid moiety account for the feeble activity of isolated lipids by their hydrophobic nature (1, 3). This view is supported by the fact that activity is increased when lipoid fractions are redispersed in aqueous menstruum by detergents. Therefore, providing a new hydrophilic carrier by *Umkupplung* (transference) of lipid from the

TABLE IV

Some Biological Properties of "Non-Hydrolytic Lipids" versus Parent Endotoxin (Aqueous Ether Extract from Salmonella enteritidis)

Procedure	Preparation	Recovery	Protection ED_{50}	Toxicity SLD_{60}	Pyrogenicity FI_{40}	Hexosamine
		per cent	*μg*	*μg*	*μg*	*per cent*
Endotoxin	Original	100	1.1	0.20	0.39	2.2
$CHCl_3$-MeOH-extracted →	$CHCl_3$-soluble (non-hydrolytic lipid I)	3.9	>50*	≥125	>1000	1.5 (0.9)‡
	$CHCl_3$-soluble (non-hydrolytic lipid II a)	1.8	>50§	10.9	28	2.4 (1.2)‡
C_6H_5Cl-ETOH-extracted →	Water-soluble (non-hydrolytic lipid II b)	1.6	>50§	—	160	—
Endotoxin	Final	85	2.0	0.24	0.43	2.3

* No evidence of protection by doses up to 50 μg.

‡ Per cent hexose as determined by the anthrone method.

§ Significant protection, but less than 50 per cent survival with dose of 50 μg.

original polysaccharide carrier to an inert protein such as casein would be expected to permit the lipid to react in a nearly optimal degree of dispersion and thus increase its biological activity. Such artificial lipoproteins have been reported to be toxic and to have as much as one-fifth the pyrogenic activity of the original endotoxin (3).

In the present work, the artificial lipoproteins were prepared (1, 3) from a phenol-water extract of *S. enteritidis*, isolated and purified by the method of Westphal *et al.* (17), and from aqueous ether extracts whose nitrogen content had been reduced by ethanol-salt fractionation (18). A 0.3 per cent solution of endotoxin was mixed with an equal volume of a 0.055 per cent solution of casein and the pH adjusted to 8 by addition of 0.1 N sodium hydroxide. After the mixture had stood for 15 minutes, the pH was adjusted to 6 with 0.1 N hydrochloric acid. Glacial acetic acid was then added to make a concentration of 1 per cent

(*i.e.*, about one-sixth N) and the mixture was heated in a boiling water bath for 1.5 hours and then neutralized. It is believed that, under the influence of alkali, endotoxin couples with casein; then, during acid hydrolysis, degraded polysaccharide is split off, leaving only lipid from the original endotoxin coupled to casein (1, 3). The complex is rendered soluble when the reaction mixture is neutralized.

Pertinent data from biological tests of these neutralized solutions appear in Table V. The LD_{50}'s for mice of the starting endotoxins were of the order of 250 μg, whereas the homologous lipoproteins, compared on the basis of their original content of endotoxin, were not lethal for mice in doses as high as 4 mg. The

TABLE V

Biological Properties of Endotoxins from Salmonella enteritidis and Artificial "Lipoproteins" Prepared from Them

	Nitrogen	Mouse LD_{50}	Rabbit SLD_{50}	Pyrogenicity MPD
	per cent	μg	μg	μg
Aqueous ether				
Endotoxin	1.3	230	0.4	0.0002
"Lipocasein"		>1500	19	0.012
Aqueous ether				
Endotoxin	1.6	270	0.39	0.00001
"Lipocasein"		>2000	38	0.018
Phenol-water				
Endotoxin	2.5	230	1.2	0.0003
"Lipocasein"		>4000	>200	>0.1; <1.0

original endotoxin produced typical raised primary lesions in rabbit skin in amounts varying between 0.4 and 1.2 μg; however, the "lipocaseins" produced atypical dermal reactions in rabbit skin and consequently the values obtained are difficult to interpret. Nevertheless, lipoproteins had, at best, about $\frac{1}{50}$ the activity of the original endotoxin. The regression of LD_{50}'s (mice) on SLD_{50}'s (rabbits) (9) indicates that the highest doses of "lipocasein" tested in mice did not approach the LD_{50}. The final products, while appreciably pyrogenic for rabbits (MPD, 0.01 to >0.1 μg), had no more than $\frac{1}{60}$ the potency of the starting materials (MPD, 0.00001 to 0.0003 μg). This loss of potency was evidently brought about during the limited acid hydrolysis since, prior to that step, the endotoxin-casein mixture was found to possess full activity. Furthermore, the pyrogenic potency of "lipocaseins" appeared to be greater than that of lipid A (see Table II). The question arose as to whether this might simply reflect the less vigorous hydrolysis to which the lipopolysaccharide-protein complexes were

subjected. Accordingly, a kinetic study of the hydrolysis of endotoxin was designed in order to determine the rate at which changes were effected in chemical composition and in biological activity.

A Kinetic Study of the Acid Hydrolysis of Endotoxin.—

The product employed for this investigation was an aqueous ether extract of *S. enteritidis* which contained 2.8 per cent nitrogen and 6.4 per cent lipid A. Samples of 125 mg were placed in each of eight screw-capped tubes; each sample was dissolved in 25 ml of distilled water and the tubes were heated in a boiling water bath. To each tube was added an equal volume (25 ml) of boiling 0.2 N acetic acid. The tubes were

TABLE VI

Alteration of Endotoxin During Acid Hydrolysis: Stimulation of Resistance to Infection

Test No.*	Duration of treatment with acid	Surviving mice, dose per animal, μg				ED_{50}
		0.05	0.5	5.0	50	
	min.					μg
I	0	6/32‡	13/30	29/32	—	0.45
II	0	10/32	15/32	29/32	—	0.31
III	0	15/32	20/32	29/32	—	0.10
II	5	11/32	18/32	29/32	—	0.22
I	15	11/32	15/32	19/32	—	0.54
III	30	—	17/32	18/32	21/32	0.37
I	45	—	7/32	16/32	21/32	6.8
II	60	—	6/32	15/32	25/32	5.0
I	90	—	4/32	11/32	12/30	>50
III	180	—	9/32	9/32	16/32	>50
II	360	—	8/32	5/32	10/32	>50

* Considerations of time and numbers of animals involved made it necessary to conduct these tests on 3 separate occasions.

‡ Survivors/number of mice challenged with 80 million *S. typhosa* in saline.

sealed, while in the boiling water bath, and were removed individually at intervals ranging from 5 to 360 minutes. A control (zero time sample) was prepared by dissolving 125 mg of the same preparation in 25 ml of water at room temperature, adding 25 ml of 0.2 N acetic acid, and immediately neutralizing the solution with 3 N NaOH. Upon removal from the bath the tubes were quickly cooled to room temperature and 20 ml samples (50 mg endotoxin) were withdrawn for lipid determination; the remaining 30 ml, in each case, was neutralized with 3 N NaOH. The latter portions were reduced in volume *in vacuo* to provide appropriate concentrations for bio-assays, and stored in the frozen state (−20°C) until used. To determine the amount of lipid released at the various stages, hydrolysates were extracted 4 times with 5 ml portions of purified chloroform; the chloroform solutions were dried with Na_2SO_4, filtered, evaporated to dryness in tared dishes, and weighed. In each case, the remainder

(water phase) was immediately neutralized with 3 N NaOH; a 2 ml sample was removed, dialyzed against distilled water, and lyophilized for chemical analysis.

TABLE VII

Alteration of Endotoxin During Acid Hydrolysis: Induction of Tumor Damage in Mice

Duration of treatment with acid	Induction of hemorrhagic necrosis in Sarcoma 37, dose per mouse, μg								Dose required to produce tumor damage in 50 per cent of the mice (TD_{50})
	100	30	10	3	1	0.3	0.1	0.03	
min.									μg
0				6/6*	11/11	10/10	5/10	0/10	0.1
5				6/6	9/10	7/10	0/10	0/10	0.2
15			11/11	10/10	6/10	0/10	0/10		0.8
30		10/10	9/10	5/10	0/10	0/10			4.0
45	10/10	7/10	3/10	0/10	0/10				18
60	10/10	2/10	0/10	0/10	0/6				40
90		0/5	0/5	0/5					>30
180		0/6	0/5						>30
360		0/6	0/5						>30

* Number damaged tumors/Number mice tested.

TABLE VIII

Alteration of Endotoxin During Acid Hydrolysis: Lethality for Mice

Duration of treatment with acid	Lethality for mice, dose per animal, *mg*										LD_{50}
	0.125	0.25	0.5	1.0	2.0	3.0	4.0	6.0	8.0	10.0	
min.											μg
0	1/5*	4/5	4/5	5/5							200
5	0/5	0/5	4/5	5/5							410
15		1/5	1/5	2/5	5/5						930
30			0/5	1/5	4/5		5/5				1,400
45				0/5	0/5		3/5				3,700
60					0/5		1/5				>4,000
90						0/5		1/5			>6,000
180							0/5		0/5		>8,000
360										0/5	>10,000

* Deaths/No. of mice tested.

In order to assess the biologic implications of the physicochemical changes wrought by acid hydrolysis, samples of the reaction mixtures for the various intervals were submitted to bio-assay. In seeking to determine which assay was most suitable for this purpose we were mindful of the fact that endotoxins elicit a diverse array of reactions in the mammalian host and that there is no general agreement as to which of these is the most reliable or objective measure of the potency of these complexes.

TABLE IX

Alteration of Endotoxin During Acid Hydrolysis: Capacity to Produce Inflammation in Rabbit Skin

Duration of treatment with acid	Rabbit No.	Dermal reaction, dose per site injected, μg										
		100	50	25	12.5	6.25	3.12	1.56	0.78	0.39	0.20	0.10
min.												
0	1					[SLD_{50} = 0.24 μg]			+	0	0	0
	2								+	+	0	0
	3								+	+	+	0
	4								+	+	0	0
5	1				[SLD_{50} = 0.58 μg]			+	+	+	0	0
	2							+	+	0	0	0
	3							+	0	0	0	0
15	1				[SLD_{50} = 0.58 μg]			+	+	0	0	
	2							+	+	0	0	
	3							+	+	+	0	
	4							+	0	0	0	
30	1				[SLD_{50} = 1.36 μg]	+	+	+	0	0		
	2					+	+	+	+	0		
	3					+	+	0	0	0		
	4					+	0	0	0	0		
45	1				[SLD_{50} = 3.91 μg]	+	+	+	0			
	2					+	+	+	0			
	3					+	+	0	0			
	4					+	0	0	0			
60	1				[SLD_{50} = 7.31 μg]	+	+	0				
	2					+	0	0				
	3					+	0	0				
90	1			[SLD_{50} = 6.25 μg]	+	+	+	+	0			
	2				+	0	0	0	0			
	3				+	+	+	0	0			
	4				+	0	0	0	0			
180	1	[SLD_{50} = 15.6 μg]	+	0	0	0	0					
	2		+	+	0	0	0					
	3		+	+	+	+	0					
	4		+	+	0	0	0					
360	1	[SLD_{50} = 31.2 μg]	+	+	0	0						
	2		+	+	0	0						
	3		+	+	+	0						
	4		+	+	0	0						

TABLE X

Alteration of Endotoxin During Acid Hydrolysis: Pyrogenic Response

Duration of treatment with acid	Fever indices of individual rabbits, dose per animal, μg							Dose required to produce a fever index of 40 cm² (FI_{40})
	0.04	0.2	1.0	5.0	25.0	125.0	250.0	
min.								μg
0	30, 34, 34 → 33	47, 51, 51, 55 → 51	59, 61, 63, 63 → 62					0.07
5	10, 11, 16, 19 → 14	35, 38, 38, 39 → 38	50, 51, 60, 61 → 56					0.26
15	20, 23 → 22	36, 39, 46 → 40	60, 63 → 62					0.18
30	23, 24, 25 → 24	27, 28, 38 → 31	49, 50 → 50					0.38
45		25, 26 → 26	47, 55 → 51	60, 65 → 63				0.50
60		24, 29, 30, 32 → 29	36, 39 → 38	61, 65 → 63				0.75
90			28, 29, 29 → 29	34, 40 → 37	51, 55 → 53			5.2
180				19, 24 → 22	42, 46 → 44	55, 60 → 58		21
360					19, 22, 28 → 23	41, 41 → 41	51, 56 → 54	100

Furthermore, it was possible that changes produced in the endotoxin might be selective and not apply equally for the different properties. It was therefore deemed essential to utilize a number of the established dose-related bio-assays based on characteristic host reactions to endotoxin.

Five standardized procedures were selected. They included assays for pyrogenicity,

tumor damage, lethality, protection, and dermal inflammation. In addition to their obvious pertinence to the present objective, the data obtained may have additional value since they provide a quantitative comparison of the different host reactions to a single endotoxin. Data from the individual protocols (Tables VI to X) were recalculated to obtain an expression of the reduced potencies as related to the original endotoxin, *i.e.*, relative potencies, and these are given in Table XI. It is apparent that as little as 5 minutes of hydrolysis was sufficient to reduce endotoxin potency 50 per cent or more in four out of five of the activities measured. Thereafter, at 15, 30, and 45 minutes, endotoxic activity, as measured in each of the five different tests, continued to deteriorate. There appeared to be some indication that the capacity to

TABLE XI

Changes in Biological Activities of Endotoxin During Hydrolysis with 0.1 N Acetic Acid

Duration of treatment with acid	Free lipid*	Resistance to infection	Tumor damage	Mouse lethality	Dermal inflammation	Pyrogenicity
	Relative potency					
min.	*per cent*					
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

* Chloroform-soluble material extracted from hydrolysates.

induce tumor damage declined more rapidly than the other properties, whereas pyrogenic capability was somewhat more resistant to destruction, but in general as hydrolysis proceeded the diverse properties declined at rates which were remarkably similar. It is especially noteworthy that up to 60 minutes, when the loss of biologic 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 (Table XI). After 90 minutes, a slight increase in turbidity of the hydrolysate was observed, and a measurable quantity (0.6 per cent) of bound lipid which had been set free was recovered. At this stage, the decline in pyrogenic potency was 77-fold which was about that expected from results of studies with the artificial lipoprotein in which the endotoxin complex had been exposed to the same conditions of hydrolysis. Other activities measured had declined similarly. With continuing hydrolysis, further deterioration occurred, but available quantities of material precluded attainment of end points in all but the most sensitive tests. At the time the experiment was terminated (6 hours), chloroform-extractable material totaling 2.0 per cent of the endo-

toxin had been released. Since hydrolysis of this endotoxin with 1 N hydrochloric acid for 45 minutes yielded 6.4 per cent chloroform-soluble lipid A, no more than one-tenth of this fraction could have been released by 90 minutes of hydrolysis with acetic acid.

Chemical analyses carried out on samples of the endotoxin at the various stages of hydrolysis (non-dialyzable residues) revealed that the content of nitrogen, hexose, total carbohydrate, and rhamnose did not undergo significant change during hydrolysis.

DISCUSSION

In this study we have utilized a number of dose-related assays in parallel tests to determine the relative potencies of endotoxins and lipoid fractions derived from them. The results of these bio-assays consistently demonstrated that endotoxins were, to an unexpected degree, far more potent than their corresponding lipids. When the lipid was separated following protracted acid hydrolysis, smaller polysaccharide units remained which were devoid of endotoxic properties. Employment of non-hydrolytic methods for separation of lipid left a residual material of full endotoxic potency. It was not surprising, therefore, that the lipids separated in this manner did not contain a significant part of the initial potency. Extension of such methods eventually resulted in preparations of active endotoxin with total fatty acid content no greater than that found in the haptenic polysaccharides produced by acid hydrolysis (6).

Examination of the various lipoid fractions themselves did indeed show that, whether obtained by hydrolytic or by non-hydrolytic methods, some of them displayed activity, but of a low order. The fact that the potencies were of similar magnitude may have a bearing on whether or not the low potency of lipid A is to be attributed to the damage inflicted by acid hydrolysis. Nowotny has found the "firmly bound" lipid (purified lipid A) to be a mixture of at least 16 different components (personal communication) and has reported the structure of 1 of these components, a phosphomucolipid (15). This new lipid, containing a poly-D-glucosamine phosphate chain in which glucosamine is esterified with fatty acids, was reported by Westphal (16) to be responsible for many of the pharmacological effects characteristic of endotoxins. We have not tested any of the pure component but, as reported here, there was no correlation between hexosamine content and biological activity in any of the lipid fractions tested.

In general, our experiments provided no support for the concept that the lipid moiety is solely, or even chiefly, responsible for the biologic activity of endotoxin. When the biologic data, expressed in terms of the weight of lipid employed, are calculated in terms of lipid content in the starting material, the disparity is even greater than appears at first sight. If lipid were solely responsible for the endotoxic activity of lipopolysaccharides, and if it constituted 10 per cent of the complex, then it should have, on an absolute weight basis, 10

times the activity of the whole endotoxin, provided it were suitably dispersed and not altered in the process of isolation. By the same reasoning, the lipid from an active preparation containing only 2 per cent of this constituent should have a correspondingly greater relative potency. We have, in fact, not yet obtained a lipid fraction from 1 of our endotoxins, which, on an absolute weight basis, exhibited more than $1/100$ of the activity of the parent endotoxin. The implication of our findings, then, is that the contribution of this moiety to the characteristic activities of endotoxin is of a minor nature and, because of its resistance to acid, entirely separate from the principal activity.

In the study of the rate at which the biologic activity of endotoxin diminished during the early stages of acid hydrolysis, the bio-assays were paralleled by determination of released lipid. Progressive loss of biologic potency became evident in the course of the hydrolysis before any measurable chloroform-soluble lipid was released. Obviously, separation and decreased solubility of the lipid moiety were not responsible for loss of potency. The intermediate activity of artificial lipoproteins could, however, be accounted for by the fact that their activity was just about what might be expected from the conditions of hydrolysis to which the original endotoxin used in their preparation had been subjected.

This kinetic study was carried out with endotoxin prepared from *S. enteritidis*, and the conclusions drawn are restricted to this preparation. Analogous examinations of endotoxin, prepared by various methods from various bacterial species, will, of course, be required before any generalization on this subject is made. Since Boivin and Mesrobeanu showed in 1935 (19) that dilute acetic acid was capable of splitting endotoxin with attendant loss of biological activity, except for the *in vitro* specificity manifested by the degraded haptenic polysaccharide, there has been ample confirmation (18, 20–22). Tal and Goebel (23) reported that, when a purified toxic material (TM) from *Shigella paradysenteriae* was heated for 1 hour with 1 per cent acetic acid, toxicity was destroyed but the solution remained clear and chloroform-extractable material was not liberated. They also showed the progressive destruction of lethality for mice by brief acid hydrolysis of a crude antigen from the same organism. Superficially, at least, these findings are in agreement with ours.

Previously, removal of protein and reduction of nitrogen content to 0.6 per cent without loss of endotoxic potency was accomplished (18). Now that it is found that lipid also can be reduced to the same low level found in the haptenic polysaccharide, the features of structure required for the biologic effects remain to be elucidated. With these newer preparations, high potency is present, in sharp contrast to the absence of activity in the haptene, although chemical data thus far have revealed no important difference, at least with regard to nitrogen, phosphorus, hexosamine, carbohydrate, and fatty acid.

An important function for the small amount of lipid and nitrogenous substance in the most highly refined endotoxin cannot be excluded by the present

evidence, although it is conceivable that these may yet be removed completely by suitable non-hydrolytic methods without damage to the residual endotoxin. If major toxic properties are to be attributed to either of these materials, it may be supposed that they are sensitive to acid and that means are not available for isolating them in an active form. They could also be important if the unique effects of endotoxins on the mammalian host are a reflection of macromolecular organization: either or both could be active only if attached to a complex of a critical size, or could contribute to activity by furnishing bonds to give cohesion or form to a large molecule. Questions such as these, and the role of the macromolecular properties of the polymeric sugar components themselves are now being approached experimentally.

SUMMARY

The biological potencies of a number of lipid fractions separated from endotoxins by acid hydrolysis, including the material known as lipid A, were determined in parallel with those of their parent endotoxins, employing bio-assays based on the following dose-related host responses: fever, resistance to infection, tumor damage, primary inflammation of skin, and lethality. Without exception, lipid fractions dispersed by detergents exerted less than 1 per cent of the biological activity of the potent endotoxins from which they were derived.

A study was made of the rate at which biologic activities diminished in relation to the release of bound lipid during progressive hydrolysis of *Salmonella enteritidis* endotoxin with dilute acid. Each of the five assays for endotoxin revealed that biological activity had been reduced to negligible proportions prior to any significant liberation from the endotoxin of water-insoluble firmly bound lipid. The major pharmacological activity of endotoxins, therefore, is acid-labile and cannot be accounted for in isolated lipids. This conclusion is also supported by the finding that lipids with activity similar to that of lipid A could be obtained by non-hydrolytic methods without diminishing the potency of the parent endotoxins.

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