Reaction of Endotoxin and Surfactants

I. Physical and Biological Properties of Endotoxin Treated with Sodium Deoxycholate

E. RIBI, R. L. ANACKER, R. BROWN, W. T. HASKINS, B. MALMGREN,
K. C. MILNER, AND J. A. RUDBACH

Rocky Mountain Laboratory, National Institute of Allergy and Infectious Diseases, Hamilton, Montana

Received for publication 25 April 1966

ABSTRACT

RIBI, E. (Rocky Mountain Laboratory, Hamilton, Mont.), R. L. ANACKER, R. BROWN, W. T. HASKINS, B. MALMGREN, K. C. MILNER, AND J. A. RUDBACH. Reaction of endotoxin and surfactants. I. Physical and biological properties of endotoxin treated with sodium desoxycholate. *J. Bacteriol.* 92:1493-1509. 1966.—Endotoxins from three species of gram-negative bacteria were shown to be dissociated by the bile salt sodium deoxycholate (NaD) into nontoxic subunits with molecular weights of about 20,000. When the bile salt was removed by dialysis, the subunits reaggregated in an orderly manner to form a relatively uniform population of biologically active endotoxin particles with average molecular weights of 500,000 to 1,000,000. If a small amount of human plasma was added to the dissociated endotoxin before removal of the NaD, reassociation apparently did not occur and the preparation remained nonpyrogenic. However, the plasma protein could subsequently be removed from the endotoxin subunits, and reaggregation to the toxic form would then occur. The studies on the physical nature of endotoxin performed with biophysical solution techniques were supplemented and confirmed by direct examination of the endotoxin polymers by electron microscopy. The results of these studies were consonant with the theory that the biologically active endotoxin elements are composed of micellar aggregates of linear lipopolysaccharide subunits.

In the search for the fundamental toxic unit of endotoxins from gram-negative bacteria, controlled acidic and alkaline hydrolysis (2, 5, 7, 12, 13, 15, 22, 31) and other chemical procedures that alter the primary structure of the particles (4, 8, 16, 17), as well as numerous measures designed to refine and purify the active material (14, 20, 21, 23, 32, 33), have thus far yielded only fragmentary information. Typical endotoxins usually have a minimal particle weight on the order of 1,000,000, and most preparations appear to consist of much larger molecules or aggregates. When the kinetics of mild acid hydrolysis of endotoxin was observed with reference to both particle size and biological activity, the endotoxin was converted to a particle with a molecular weight in the range of 10,000 to 20,000 (3, 19, 22), and biological activity declined in parallel with this conversion. Such experiments were not entirely suitable for determining the minimal size of the toxic particle, because hydrolysis appeared to produce the small acid hapten in one step, without formation of particles of intermediate size. Conversion

to the acid hapten also appeared to be an irreversible reaction; therefore, the hapten's role as a basic structural unit of endotoxin could not be assessed by reforming the toxin (22).

Recently it was reported (18) that treatment of endotoxin from *Serratia marcescens* with a surfactant, the detergent sodium lauryl sulfate (SLS), resulted in dissociation of the particles into subunits which lacked the tumor-damaging potency of the original. Upon removal of the SLS, the subunits appeared to reaggregate into toxic particles. These studies provided an approach for testing the relationship of size to biological activity in the endotoxin polymer. An extension of the method might also permit identification and characterization of the nontoxic subunits of the polymer.

The present investigation was undertaken to establish the conditions under which another surfactant, the bile salt sodium deoxycholate (NaD), depolymerizes endotoxins from three different species of bacteria. The subunits produced were characterized physically, and their occurrence

could be correlated with a reduction in pyrogenicity of the preparations. In addition, it was found that minute quantities of human plasma would stabilize the subunits of dissociated endotoxin and permit dilution of the NaD without reaggregating the subunits into pyrogenic endotoxin.

MATERIALS AND METHODS

Endotoxin preparations. A conventional aqueous ether extract of *Salmonella enteritidis* was prepared from whole cells of strain S-795, as described previously (20, 21). This endotoxin was used without further purification and possessed the biological and chemical properties indicated in Table 1.

Two strains of *Escherichia coli*, O113 (Braude) and O111:B4 (Difco), were grown in M-9 medium (24) containing 0.5% glucose. After repeated washings in cold saline, the cells were disrupted at 20,000 psi in a Sorvall Refrigerated Cell Fractionator and the walls were isolated by differential centrifugation (6). Endotoxin was extracted from these cell walls by the phenol-water method of Westphal, Lüderitz, and Bister (34). The biological and chemical properties of these preparations are given in Table 1.

The mixture of three strains of *Bordetella pertussis* used for routine production of standard vaccine was cultivated by Parke, Davis & Co. Frozen pastes of harvested cells were used as starting material for the extraction of endotoxin by the phenol-water procedure (34). The residual cells were re-extracted with phenol-water five times, and the aqueous phases were subjected to differential ultracentrifugation until ultraviolet spectroscopy showed them to be free from nucleic acid (Table 1).

Physical measurements. To prepare specimens for electron microscopy, the aqueous ether extract of *S. enteritidis* or the phenol-water extract of cell walls from *E. coli* was suspended in distilled water to give a concentration of 1.0 mg/ml. Each of these preparations was negatively stained by adding an equal volume of 3% potassium phosphotungstate (PTA), pH 7.2. A drop of each endotoxin-PTA suspension

was placed on a separate phosphor bronze specimen grid which had been previously coated with Formvar. After 3 to 5 min, the droplets were removed with a fine capillary pipette. Micrographs were made with a Siemens Elmiskop I electron microscope. Shadowed preparations of *B. pertussis*'s endotoxin were prepared by standard procedures.

Sedimentation velocity determinations on the endotoxins were performed in a Spinco model E ultracentrifuge. The observed sedimentation coefficients, reported in Svedberg units (1S is 10^{-13} cm per sec per unit field), were corrected to values corresponding to a solvent with the viscosity and density of water at 20°C.

Data for diffusion coefficients were obtained with a synthetic boundary cell in a Spinco ultracentrifuge. The diffusion coefficients were calculated by plotting the squares of the second moments against time, as suggested by Schachman (28), and were corrected to correspond to a temperature of 20°C in a solvent with the viscosity of water ($D_{20,w}$). They are reported in units of 10^{-7} cm²/sec.

Viscosities were measured in an Ostwald capillary-type viscometer and were calculated according to the method described by Schachman (28). To obtain straight-line relationships, the procedure was modified by calculating the slope of the line which resulted from plotting the logarithms of relative viscosity against solute concentration (grams per 100 ml). Values are reported as the intrinsic viscosities.

The dimensions of the molecules were determined from the physical data as follows. With the help of Einstein's viscosity increment, the axial ratio for oblong ellipsoidal molecules was obtained by interpolation from a graph prepared from the table of Mehl, Oncley, and Simha (10). With the axial ratio, the frictional ratio was then determined by Perrin's formula (see 30). In this manner, average values were estimated for molecular weights and for lengths and diameters of the particles in solution.

Pyrogenicity determinations. The assay procedure, which has been described previously (7), consisted of injecting the test material intravenously into rabbits which were restrained in metal stocks. The changes

TABLE 1. Chemical and biological characterization of the endotoxins used in this study

Organism	N ^a	P	Hexose	Total CHO	Hexosamine	FAA + FAE	Hep-tose	DDS	KDO	Mouse LD ₅₀ (ip)	Pyrogenicity (FI ₅₀)	Chick embryo LD ₅₀ (iv)
										mg	μg	μg
<i>Salmonella enteritidis</i>	2.08	1.37	54.1	61.7	3.33	9.41	1.5	13.6	2.2	ND ^b	0.18	0.0081
<i>Escherichia coli</i>												
O111:B4.....	1.71	1.48	26.2	36.8	12.6	12.5	5.1	15.6	6.2	ND	0.27	0.0045
<i>E. coli</i> O113.....	2.50	2.01	16.8	31.1	22.7	33.3	4.1	0	5.2	0.54	0.27	0.0049
<i>Bordetella pertussis</i> ^c	3.0	2.5	9.7	15	7.3	18	9.9	0	1.25	2.1	0.64	0.0072

^a Abbreviations: N, nitrogen; P, phosphorus; CHO, carbohydrate; FAA + FAE, fatty acid amide + fatty acid ester; DDS, dideoxysugar; KDO, 2-keto-3-deoxyoctulosonic acid.

^b ND = not done.

^c Free of nucleic acid.

in the rabbits' rectal temperatures were detected with indwelling thermocouple probes and transcribed on an automatic multipoint recorder. The febrile responses thus obtained were plotted on arithmetic graph paper with a scale such that 1 C and 1 hr equaled 1 inch. The areas under the curves were measured in square centimeters with a compensating polar planimeter. The fever indices (FI) obtained in this manner represent the areas under the fever curves, and are affected by both the height and duration of the febrile responses. It has been shown previously that a dose of endotoxin which gives a fever index of 40 (FI₄₀) falls in the center of the linear portion of the dose-response curve (9). All fever indices and fever curves shown in this paper represent the average responses of three rabbits.

Reaction system for endotoxin and NaD. Endotoxin was worked into solution or into fine suspension in tris(hydroxymethyl)aminomethane (Tris)-Cl buffer (pH 8.0). To samples of the endotoxin solution, necessary amounts of concentrated NaD solution, and other reagents if any, were added so that the desired concentration of NaD would be reached and the Tris-Cl concentration would be 0.1 M. Control solutions were prepared in the same fashion except that one or more of the reagents were excluded. Whenever possible, the samples were examined in the various tests after standing for about 15 min at room temperature. If immediate testing was not possible, i.e., if varying concentrations had to be run in the ultracentrifuge or several types of tests were made on the same preparation, the samples were stored at 4 C.

When human plasma was incorporated into the reaction system, alteration of the endotoxin by plasma alone was prevented by keeping the samples at 4 C and by maintaining a low plasma to endotoxin ratio.

Restoration procedure. The protocol outlined by Rudbach and Johnson (27) was employed with 1.6 mg of Pronase (Calbiochem, B grade, lot no. 109080) added for the initial digestion.

RESULTS

Physical measurements of endotoxin treated with NaD. The endotoxins were dissolved as completely as possible in 0.2 M Tris-Cl (pH 8.0). To these were added an equal volume of aqueous NaD such that the final NaD concentration would be 2% with the *E. coli* endotoxins and 0.5% with the *S. enteritidis* endotoxin. These solutions were allowed to stand overnight at 4 C before any physical studies were undertaken. In those experiments which required several dilutions of endotoxin, the dilutions were all made with the appropriate concentration of NaD in 0.1 M Tris-Cl (pH 8.0).

Physical measurements of the endotoxins are presented in Table 2. The aqueous ether extract of *S. enteritidis* was the most soluble of the endotoxins employed in this study. However, it still presented an opalescent solution in Tris-Cl when viewed with oblique lighting. When the NaD was

added, this opalescence was immediately almost completely lost. Much more dramatic clearing was observed when NaD was added to the phenol-water extracted endotoxins of *E. coli*. In Tris-Cl, the *E. coli* endotoxins formed suspensions rather than solutions; the addition of NaD resulted in complete and instantaneous dissolution of the endotoxin particles. This insolubility and heterogeneity of the particle sizes of the *E. coli* endotoxins in Tris-Cl alone precluded meaningful physical measurements. That the bile salt did in fact cause a dissociation of the endotoxins is shown by the physical measurements undertaken in the presence of NaD (Table 2). The large complex polymers were reduced in size to smaller elements which were relatively monodisperse. These elements displayed diminished length and diameter as compared with the original endotoxin (see Fig. 2-4). In fact, they approached the dimensions of a linear chain of sugars with only a slight degree of branching allowable.

The lack of homogeneity and solubility of preparations of *E. coli* endotoxins isolated by the phenol-water procedure made it impossible to determine the size and shape of endotoxin. This difficulty could be overcome when the endotoxins were treated with bile salt and the surfactant was subsequently removed by dialysis against 0.1 M Tris-Cl (pH 8.0). Reassociation of the endotoxins took place, resulting in soluble and relatively uniform populations of particles which were fully toxic. Figure 1 shows the pattern presented in the ultracentrifuge after a phenol-water extracted endotoxin of *E. coli* had been treated with NaD and then freed from the bile salt by dialysis. Also shown is the ultracentrifugal pattern of a phenol-water extracted endotoxin from *B. pertussis* "solubilized" in this manner. The third pattern shown in Fig. 1 is that obtained from an aqueous ether-extracted endotoxin from *S. enteritidis* after treatment with and removal of the bile salt. It was then possible to compare the physical structures of these toxic "elements" with structural precursors or subunits of the polymer. These results are compiled in Table 2.

Electron microscopy of endotoxin treated with NaD. The size of endotoxin polymers permitted direct observation by electron microscopy. Normally, a phenol-extracted endotoxin appeared as long and short filaments having a uniform width of 50 to 60 A. Figure 2A shows a negatively stained phenol-water extracted endotoxin from cell walls of *E. coli* O111:B4. This picture was taken from a representative field and shows mostly the long string forms along with some shorter rod forms and spherical particles. When this endotoxin was treated with 2% NaD, the

TABLE 2. Effect of treatment with sodium deoxycholate (NaD) on molecular weight and shape of endotoxins from *Escherichia coli* and *Salmonella enteritidis*

Source	Prepn	Solvent	Intrinsic viscosity $[\eta]$	Partial specific volume $\bar{v}$	Sedimentation coefficient $S_0 \times 10^{10a}$	Values from $[\eta]$ and S_0			Values from D_0 and S_0		
						Molecular wt	Shape	Length	Molecular wt	Shape	Length
<i>E. coli</i> O111:B4	Endotoxin extracted with										
	Phenol-water	0.1 M Tris-Cl	—	—	73 ^a	—	A	A	—	A	A
	Phenol-water	0.1 M Tris-Cl + 2% NaD	0.30	0.66	0.94	8,820	9.6	220	20,700	8.2	680
<i>E. coli</i> O113	Phenol-water	0.1 M Tris-Cl + 2% NaD; dialyzed against Tris-Cl	0.60	0.66	17.9	930,000	39	1310	525,000	44	586
	Phenol-water	0.1 M Tris-Cl	—	—	— ^b	—	—	—	—	—	—
	Phenol-water	0.1 M Tris-Cl + 2% NaD	0.36	0.66	1.04	10,750	10.4	259	20,100	8.7	562
	Phenol-water	0.1 M Tris-Cl + 2% NaD; dialyzed against Tris-Cl	0.74	0.66	12.8	607,000	32	1225	270,000	39	371
	Phenol-water, hydrolyzed "acid hapten"	0.2 M NaCl + 0.03 M phosphate buffer	0.18	0.60	1.63	13,600	11	212	—	—	—
<i>S. enteritidis</i>	Aqueous ether	0.1 M Tris-Cl + 0.5% NaD; dialyzed, lyophilized; reconstituted in Tris-Cl	0.88	0.66	18.9	1,175,000	39	1660	752,000	42	886
	Aqueous ether	0.1 M Tris-Cl	0.66	0.66	16.2	788,000	28	1590	—	—	—

^a Slowest moving component.^b Low solubility, not measurable.

FIG. 1. Ultracentrifuge patterns of endotoxin after treatment with, and subsequent removal of, NaD. The solvent was 0.1 M Tris-Cl (pH 8.0). (A) 1% *Escherichia coli* phenol-water extracted endotoxin treated with 2% NaD and dialyzed; (B) 1% *Bordetella pertussis* phenol-water extracted endotoxin treated with 1% NaD and dialyzed; (C) 1% *Salmonella enteritidis* aqueous ether extracted endotoxin treated with 0.5% NaD and dialyzed.

strings dissociated into elements which could not be seen with the electron microscope; i.e., even if the elements were large enough to be resolved, the bile salt which had dried on the supporting membrane would cover them. However, once the bile salt was removed by dialysis against distilled water, the elements reassociated into larger units which could be observed (Fig. 2B). The lengths of the endotoxin polymers were decreased considerably to give a relatively uniform population

of short-chain forms. The greatest number of particles were from 300 to 600 Å long and from 50 to 60 Å wide. From these dimensions, the estimate of the particle weight was from 500,000 to 1,500,000. Physical measurements by two different methods performed on the same sample of endotoxin in solution indicated an average molecular weight of either 525,000 or 930,000 (Table 2). These particles probably were mostly rod-shaped in solution, but during the drying of the sample on the specimen-supporting membrane, some of the elementary fibers changed from a micellar aggregate to a laminar disperse form with a racket-shaped appearance.

Electron microscopy of a heavy metal-shadowed endotoxin which had been extracted from *B. pertussis* with phenol-water revealed both short and long filaments, which again had a rather uniform diameter of 50 to 60 Å (Fig. 3A). The filaments often showed distinct angular bending (see arrows). When the segments between the bends were measured, the most frequently found distances were 300 to 400 Å. This corresponded not only to the length of the most numerous category of single rods observed in this photomicrograph but also to those shown in a shadowed picture of the same endotoxin after treatment with NaD followed by dialysis against distilled water (Fig. 3B). The "unit" particle of this *B. pertussis* endotoxin, which is 300 to 400 Å long and 50 to 60 Å wide, would weigh 500,000 to 1,000,000.

In contrast to the endotoxins extracted with phenol-water, described above, aqueous ether-extracted endotoxins from *S. enteritidis* appeared to consist predominantly of short rodlets 50 to 60 Å in diameter (Fig. 4A). Treatment of this endotoxin with NaD, followed by removal of the bile salt by dialysis, did not appear to alter the size of the particles to any great extent (Fig. 4B and 4C). The shortest clearly discernible rodlets, i.e., not considering the smallest spherulike particles, were about 120 Å long, corresponding to an estimated molecular weight of 200,000 to 300,000; however, the more numerous particles fell within the range of 200 to 250 Å in length and 300,000 to 600,000 in weight. A portion of the rods were longer, with an estimated weight of up to about 1,000,000. The rodlets could be aggregated into long filaments when the NaD-treated endotoxin was extracted in the aqueous phase of a phenol-water mixture (Fig. 4D).

We assume that the long rods or filaments are aggregates of shorter rodlets. The unit particle may not have a definite dimension but may be polydisperse in length, as are the micelles which make up the 50 to 100 Å wide elementary fibrils of natural and synthetic fibers.

FIG. 2. Electron micrographs of negatively stained preparations of a phenol-water extracted endotoxin from *Escherichia coli* (A) and the same endotoxin after treatment with 2% NaD and dialysis (B).

The discrepancy between the dimensions of the molecules as determined by the solution techniques and by electron microscopy is more apparent than real. The assumptions used for calculations of the axial ratio of the molecule from viscosity and ultracentrifugal data are not absolutely valid. These long polysaccharide chains are not perfect rotational ellipsoids, and they are certainly partially solvated. Furthermore, the degree of polydispersity of the preparations contributes to the discrepancies. Nevertheless, the values for the shape of the polymers obtained from the physical data do correlate to a degree with those obtained by direct observation with the electron microscope and are of value in a comparative sense.

Biological activities of endotoxin treated with NaD. The physical measurements have shown that the bile salt dissociated the endotoxin into subunits in the same size range as the hapten produced by acid hydrolysis of the endotoxin (Table 2). If a minimal size of the endotoxin polymer (or a minimal degree of aggregation of basic subunits) is necessary for endotoxic activities, completely disaggregated endotoxin would not be expected to be biologically active. How-

ever, when the NaD-treated endotoxin was tested for pyrogenicity, after dilution in saline, little if any reduction was detected. Figure 5 (A and B) shows that *S. enteritidis* endotoxin treated with NaD had the same or a slightly enhanced pyrogenicity compared with the normal endotoxin when both preparations were diluted in saline. Similar results were obtained with a phenol-extracted endotoxin from *E. coli* cell walls (Fig. 6, A and B). A purified endotoxin from *B. pertussis* failed to show any reduction in pyrogenicity (Fig. 7, A and B). It was, therefore, hypothesized that the dissociated elements of endotoxin were reassociating upon dilution of the bile salt. That this reassociation occurs very rapidly is shown in Fig. 6C, where *E. coli* endotoxin treated with bile salt, diluted rapidly in saline to the proper pyrogenic dose, and injected immediately, had regained full pyrogenicity. Therefore, to test the pyrogenicities of the dissociated elements of the endotoxins, they were diluted with NaD solutions. In all cases, the volumes of materials injected into the rabbits were adjusted so that the amount of NaD never exceeded 5 mg, a nonpyrogenic dose (Fig. 5C). When injected in NaD solutions, doses of *S. enteritidis* endotoxin (Fig. 5D),

FIG. 3A. Electron micrograph of a metal-shadowed preparation of a phenol-water extracted endotoxin from *Bordetella pertussis*.

E. coli endotoxin (Fig. 6D), and *B. pertussis* endotoxin (Fig. 7C) which were usually pyrogenic were all shown to have lost their abilities to produce fever. The simultaneous injection of 5 mg of NaD contralaterally to the intravenous injection of endotoxin did not change the fever response from that elicited by endotoxin alone. When the pyrogenicities of varying doses of *E. coli* endotoxin, with and without bile salt treatment, were used to calculate FI_{40} values, a 100- to 200-fold reduction in pyrogenicity of the bile salt-dissociated endotoxin had occurred. The physical measurements showed that, after treatment with and subsequent removal of the bile

salt, the endotoxins reassociated to form polymers with particle weights of about 0.5 million. That these polymers had regained full pyrogenicity is shown in Fig. 5E, 6E, and 7D for *S. enteritidis*, *E. coli*, and *B. pertussis* endotoxins, respectively.

Correlation of ultracentrifugation pattern with pyrogenicity of endotoxin treated with NaD. In the experiments presented above, the concentrations of NaD were adjusted so that essentially complete dissociation of the endotoxin polymer occurred. This was shown by the single peaks in the ultracentrifuge with sedimentation coefficients of about 1S. In the experiments to be pre-

FIG. 3B. Same endotoxin shown in Fig. 3A after treatment with 1% NaD and dialysis.

sented, endotoxins were prepared in varying concentrations of NaD so that dissociation of the polymers was incomplete. Figure 8 shows the pyrogenic responses and the ultracentrifuge patterns of *S. enteritidis*, *E. coli*, and *B. pertussis* endotoxins in 0.25, 0.125, and 0.062% NaD solutions. Full pyrogenicity appeared to be present in each of the preparations when the endotoxins were in 0.062% NaD. At 0.125% NaD the pyrogenicities were moderately reduced, and at 0.25% NaD almost no fever could be elicited at the doses indicated. Loss of pyrogenicity corresponded to the reduction in rapidly sedimenting components in ultracentrifuge patterns and to the increase in slow sedimenting components.

Effect of human plasma upon mixtures of endotoxin and NaD. It has been hypothesized that inactivation of endotoxin by human plasma is the result of plasma proteins binding the endotoxin (27). Since the bile salt-dissociated endotoxin subunits appeared to reaggregate upon removal or dilution of the bile salt, it was thought that plasma proteins which bind endotoxin might prevent reaggregation of the subunits. Therefore, to endotoxins which were dissociated with NaD, human plasma was added before dilution of the preparations in saline for pyrogenicity determinations. The conditions of the tests were adjusted so that the plasma alone would not alter the endotoxin. Figure 9 shows that very small volumes

FIG. 4A and B. Electron micrographs of negatively stained preparations of an aqueous ether-extracted endotoxin from *Salmonella enteritidis* before (A) and after (B) treatment with 0.5% NaD and dialysis.

of fresh human plasma prevented *S. enteritidis* endotoxin, dissociated with NaD, from regaining pyrogenicity after dilution in saline. This figure also demonstrates that the minimal concentration of NaD which effected diminution of the pyro-

genicity of the endotoxin in this system, 0.125%, was the same as was shown previously to diminish the pyrogenicity of endotoxins diluted in NaD. Human plasma also prevented endotoxins from *E. coli* and *B. pertussis*, which had been treated

FIG. 4C and D. Another endotoxin from *Salmonella enteritidis* after treatment with 0.5% NaD and dialysis, resulting in a very uniform population of molecules (C); the NaD-treated endotoxin after extraction with phenol-water (D).

with NaD and diluted in saline for the pyrogenicity test, from regaining pyrogenicity (Fig. 10 and 11).

The effect of the plasma upon the bile salt-dissociated endotoxins was not an irreversible reac-

tion, inasmuch as treatment of these preparations with a proteolytic enzyme, followed by precipitation with ethyl alcohol (27), restored them to apparently normal pyrogenicities (Fig. 9-11).

The above results suggested that, when the

FIG. 5. Pyrogenic responses, including fever indices (FI), of rabbits injected with $0.1 \mu\text{g}$ of endotoxin from *Salmonella enteritidis* treated with NaD.

FIG. 6. Pyrogenic responses, including fever indices (FI), of rabbits injected with $0.25 \mu\text{g}$ of endotoxin from *Escherichia coli* treated with NaD.

FIG. 7. Pyrogenic responses, including fever indices (FI), of rabbits injected with $0.5 \mu\text{g}$ of endotoxin from *Bordetella pertussis* treated with NaD.

endotoxin and NaD mixture was injected into rabbits for the pyrogenicity determination, plasma proteins were bound to the dissociated endotoxin subunits in vivo and prevented their reaggregation to the toxic form. If such were the case, then binding of the plasma proteins to the nontoxic subunits would have to occur very rapidly, since the bile salt would be quickly diluted in the blood stream. When samples of a mixture of *S. enteritidis* endotoxin and NaD, to which human plasma had been added, were removed, diluted in saline, and injected for pyrogenicity determinations either immediately after the plasma was added or several minutes later, little pyrogenicity could be detected in any of the samples (Fig. 12). This in vitro test indicated that the plasma proteins could bind very rapidly with the dissociated endotoxin subunits; probably the binding would be rapid enough in vivo to occur before the endotoxin subunits could reassociate to the toxic form.

Immunodiffusion patterns on endotoxin treated with NaD and human plasma. The experiments described below were all performed with phenol-

FIG. 8. Pyrogenic responses, including fever indices (FI), of rabbits injected with $0.1 \mu\text{g}$ of *Salmonella enteritidis* endotoxin, $0.25 \mu\text{g}$ of *Escherichia coli* endotoxin, or $0.5 \mu\text{g}$ of *Bordetella pertussis* endotoxin contained in varying concentrations of NaD; the ultracentrifuge patterns of the corresponding samples of 0.5% endotoxin concentration (S values given over the peaks).

FIG. 9. Pyrogenic responses, including fever indices (FI), of rabbits injected with 0.1 μ g of *Salmonella enteritidis* endotoxin after treatment with NaD and human plasma. The samples were diluted in saline before injection.

FIG. 10. Pyrogenic responses, including fever indices (FI), of rabbits injected with 0.25 μ g of *Escherichia coli* endotoxin after treatment with NaD and human plasma. The samples were diluted in saline before injection.

FIG. 11. Pyrogenic responses, including fever indices (FI), of rabbits injected with 0.5 μ g of *Bordetella pertussis* endotoxin after treatment with NaD and human plasma. The samples were diluted in saline before injection.

water extracted endotoxin from *E. coli* O111:B4; however, the results are similar to those obtained when an aqueous ether-extracted endotoxin from *S. enteritidis* was tested in the same manner (25). Samples (2-ml) of original mixtures were prepared containing 1 mg of endotoxin, 1 ml of

human plasma, and 2% NaD in 0.1 M Tris-Cl buffer (pH 8.0); 1 mg of endotoxin in 2% NaD in Tris-Cl; 1 mg of endotoxin in Tris-Cl; or 1 mg of endotoxin and 1 ml of human plasma in Tris-Cl. "Restored" samples were treated with Pronase and ethyl alcohol as described in Materials

and Methods. The samples were diluted to contain 100 μ g of endotoxin per ml before their immunodiffusion patterns were determined.

Figure 13 shows the precipitation patterns ob-

FIG. 12. Pyrogenic responses, including fever indices (FI), of rabbits to 0.1 μ g of *Salmonella enteritidis* endotoxin after treatment with NaD and human plasma for varying times. The samples were diluted in saline before injection.

tained when the various endotoxin preparations were reacted with homologous antiserum. The endotoxin alone (wells A, D and G) gave several lines of precipitate, which would be expected from the polydispersity of the phenol-water extracted preparation. However, after this endotoxin had been treated with NaD and then dialyzed (well K), a single precipitation band was seen. This latter pattern was similar to that obtained when endotoxin in NaD was tested (well B). In this case, the NaD probably diffused rapidly into the agar, which allowed the subunits of endotoxin to reassociate. When endotoxin was treated with NaD and human plasma (wells C and H), three bands became visible in the plate. The fastest migrating band appeared to be present in the greatest concentration and indicated the presence of dissociated endotoxin units. The dispersion of the endotoxin and the stabilization of the dispersed subunits required both plasma and NaD as shown by the lack of dispersion of endotoxin by plasma alone (well E), or of endotoxin by NaD alone (well B). Note that the endotoxin and plasma, mixed under the same conditions as the other samples, without incubation, did not give the dispersed pattern normally seen after incubation (26). The stabilized dispersion or disaggregation seen with the endotoxin in the presence of NaD and plasma (wells C and H) was a reversible phenomenon. This sample was restored with Pronase and ethyl alcohol treatment (wells I and L) to give a

FIG. 13. Immunodiffusion plates containing anti-*Escherichia coli* serum in the center wells and 100 μ g/ml of the following *E. coli* endotoxin preparations in the peripheral wells: endotoxin alone, wells A, D, and G; endotoxin treated with NaD and dialyzed, well K; endotoxin in NaD solution, well B; endotoxin treated with NaD and human plasma, wells C and H; endotoxin and plasma, well E; endotoxin treated with NaD and human plasma and then restored with Pronase and ethyl alcohol, wells I and L; endotoxin treated with Pronase and ethyl alcohol, wells F and J.

precipitation pattern similar to that of endotoxin treated with Pronase and ethyl alcohol (wells F and J), or of endotoxin treated with and dialyzed free from NaD (wells B and K). These results are consonant with those obtained when the various forms of endotoxin treated and untreated with NaD were subjected to physical analyses and electron microscopy. The basic toxic element of endotoxin is probably represented by the dense precipitation line diffusing from all of the wells except C and H. The slower diffusing compounds from these latter wells appear to be some of the undissociated endotoxin responsible for the residual biological activity of the preparations which was observed in the previous sections of the report.

DISCUSSION

During the course of studies attempting to correlate biological activity of endotoxins with their structure, attention has been focused on the importance of size or degree of aggregation of the polymer (11). It was thought that the basic elements of endotoxin were necessarily aggregated into particles of colloidal dimensions before antigenicity and toxic properties could be manifested. The basic subunits of endotoxin were visualized as chainlike molecules which associated in parallel arrangements to form either fringe or continuous micelles. The present data support this hypothesis and suggest that the basic chainlike subunits associate in a fringe micellar relationship. These elements appear to have dimensions with an average size of 10 Å by 250 to 600 Å. The diameter of these chains is about the same as chains of glucose molecules, i.e., 8 to 9 Å. There is little space left in the 10 Å diameter for chain branching; however, some can occur because the 10 Å reflects an average dimension.

The nonpyrogenic basic subunits were formed by treatment of the endotoxin with bile salt under conditions which should not cause the breaking of covalent or primary chemical bonds. Therefore, secondary or tertiary forces must be responsible for binding the elements together in the toxic unit. This points out a further similarity of the proposed endotoxin structure to a micellar system. The dimensions and particle weight of the endotoxin unit obtained after removal of bile salt are close to those of the smallest toxic units of endotoxin extracted by the conventional aqueous ether method. We, therefore, are not proposing that a new type of polymer is formed after removal of bile salt from an endotoxin, but rather that the bile salt appears to dissociate the endotoxin into its basic subunits which reaggregate, when the bile salt is removed, to form a

relatively monodisperse solution of endotoxin elements possessing the biological activities of the original extract. Thus, a practical application of the reaction of endotoxin with bile salt would be the preparation of a soluble, monodisperse endotoxin from an insoluble, polydisperse endotoxin. These toxic elements, with particle weights of about 500,000, can be aggregated by phenol to form the sparingly soluble long fibers commonly observed in endotoxin extracts.

Much of the impetus for the above work came from reports that a detergent, SLS, dissociated endotoxin from *Serratia marcescens* with concomitant loss of tumor-damaging ability (18). The results obtained in that study were similar to our findings. However, a recent report by other workers, using SLS, suggested that the dissociated subunits of endotoxin provoked the Shwartzman reaction, produced fever and leukopenia in rabbits, and were lethal for rabbits (1). These data were interpreted as indicating that the toxicity of endotoxin is not a function of particle size. Our data are in conflict with this interpretation; however, we used a bile salt, not a detergent, to dissociate endotoxin, a fact which may account for the discrepancies. We also have shown that the endotoxin subunits reassociate readily to form the toxic elements unless special precautions are taken to keep them disaggregated. The procedure which we used to stabilize the disaggregated subunits was treatment of the endotoxin in the presence of bile salt with small amounts of human plasma. Indeed, it was shown that the endotoxin subunits plus plasma did not regain biological activity when the bile salt concentration was reduced by dilution. That the plasma proteins would bind to the dissociated subunits of endotoxin was not surprising, since previous studies had indicated that the alteration of endotoxin by human plasma was occurring through such a binding (27). Also, the observation that such small amounts of plasma proteins were so effective in binding the endotoxin subunits, which had been dissociated by bile salt, raised a further question on the mechanism of the alteration of endotoxin during incubation in fresh human plasma; i.e., did the alteration occur solely by binding of the endotoxin by plasma proteins, or was the endotoxin first dissociated by bile salts present in the plasma and then bound by plasma proteins? The altered immunodiffusion pattern of endotoxin incubated in plasma (26) suggested the formation of a haptenlike component. Furthermore, other evidence has been presented which suggests that plasma alters the endotoxin in a two-step reaction (29). The first step might be dissociation of the endotoxin polymer by the bile salt, followed by binding of

the endotoxin by plasma proteins. A preliminary discussion of the bile salt-protein binding mechanism for the alteration of endotoxin by plasma has been already presented (25). A schematic outline of the proposed mechanism of reversible disaggregation of endotoxin and the subsequent binding of the elements by plasma proteins is presented in Fig. 14. Starting with the long complex string of endotoxin elements observed in an endotoxin obtained by phenol extraction, the bile salt appears not only to dissociate the toxic elements from one another, but also to disaggregate these elements into nontoxic subunits. These elements can then be bound by plasma proteins and kept in the disaggregated nontoxic form. All of these reactions are reversible, such that the elements recombine to form the toxic elements when the protein is removed from the subunits and the bile salt is either dialyzed or diluted. These can then be reaggregated by phenol to form again the long threadlike particles. With such a mechanism, the rationale for the protease digestion and the ethyl alcohol precipitation steps in the restoration procedure for plasma-altered endotoxin would become obvious.

During the development of our structural

model of endotoxin, we hypothesized that a minimal degree of aggregation or molecular size was necessary for endotoxin to display biological properties. However, we would not imply that a certain macromolecular size, in itself, is a sufficient attribute for a preparation to display endotoxicity. Indeed, we have detoxified endotoxin without reducing its size below that of the molecules in a toxic preparation (*to be published*). Similarly, although our data indicate that reduction in the size of endotoxin is sufficient to alter the biological activity, we are wary of committing the fallacy of confusing conditions and cause. Actually what we have done is to establish a set of conditions whereby the endotoxin is reduced in size and, concomitantly, loses biological activity. We would therefore do no more than hypothesize that a causal relationship exists between the reduction in size and loss of toxicity.

The model to which we have been led by the physical and biological data presented may, of course, be difficult to harmonize with models constructed on the basis of rapidly developing studies of the biosynthesis of cell wall antigens. If errors have been made, these will inevitably come to light. It seems much more probable,

FIG. 14. Schematic diagram of the working hypothesis to explain the reaction of endotoxin with NaD and plasma.

however, that matters of interpretation are chiefly responsible for the contrasting models. Significant differences between cultures and extracted products no doubt occur in different laboratories and account for some of the disparate findings; but, despite these, the biosynthetic approach has led, with a good deal of consistency, to construction of one type of model which is scarcely compatible with that demanded by physical measurements and electron micrographs.

It is probably important to explore carefully the likelihood that extracted lipopolysaccharides may be very different in form, and even in composition, from the original substances as they occur *in situ* in the bacterial cell. The protean quality of endotoxin is sufficiently demonstrated by the variety of forms seen in electron micrographs. And, indeed, the structure of the cell wall, as revealed by electron microscopy, has no room to accommodate a network of fibers of the dimensions found in a phenol-water extracted endotoxin, for example. A fundamental requirement for a good model, therefore, is that it account for all the observed variations in form.

The greatest difficulty is encountered in attempts to reconcile the concept of micellar structure with the "core" theory that has been so vigorously supported elsewhere. Perhaps the haptenic subunits identified in surfactant-treated preparations are simply the specific side chains that have become detached from the carrier structure. In the preparations we have studied, then, the core must be exceedingly small, since we find no component to be identified with it. Alternatively, each haptenic chain may carry its own portion of core substance. Unfortunately, no method has yet been found whereby the "surfactant hapten" may be isolated in a pure state for chemical study.

Almost certainly the "native hapten" we have described does represent a pure preparation of the specific side chains, although its abundance in particular cultures may result from surplus or aberrant production. It is lacking in long-chain fatty acids, phosphorus, and heptose, which are regarded as core substances; and, in contrast to the "surfactant hapten," it has no ability (even with our most artful encouragement) to aggregate into complete endotoxic O antigen.

LITERATURE CITED

1. BEER, H., T. STAHELIN, H. DOUGLAS, AND A. I. BRAUDE. 1965. Relationship between particle size and biological activity of *E. coli* Boivin endotoxin. *J. Clin. Invest.* **44**:592-602.
2. BOIVIN, A., AND L. MESROBEANU. 1935. Recherches sur les antigènes somatiques et sur les endotoxines des bactéries. I. Considérations générales et exposé des techniques utilisées. *Rev. Immunol.* **1**:553-569.
3. DAVIES, D. A. L., AND W. T. J. MORGAN. 1953. The undegraded specific polysaccharide of *Shigella dysenteriae* (Shiga). *Intern. Congr. Microbiol.*, 6th, Rome, p. 457-458.
4. FREEDMAN, H. H., B. M. SULTZER, AND W. KLEINBERG. 1961. Detoxification of bacterial endotoxin with retention of ability to stimulate nonspecific resistance to infection. *Proc. Soc. Exptl. Biol. Med.* **107**:819-821.
5. FREEMAN, G. G. 1942. The preparation and properties of a specific polysaccharide from *Bact. typhosum* Ty2. *Biochem. J.* **36**:340-356.
6. FUKUSHI, K., R. L. ANACKER, W. T. HASKINS, M. LANDY, K. C. MILNER, AND E. RIBI. 1964. Extraction and purification of endotoxin from Enterobacteriaceae: a comparison of selected methods and sources. *J. Bacteriol.* **87**:391-400.
7. HASKINS, W. T., M. LANDY, K. C. MILNER, AND E. RIBI. 1961. Biological properties of parent endotoxins and lipid fractions, with a kinetic study of acid-hydrolyzed endotoxin. *J. Exptl. Med.* **114**:665-684.
8. JOHNSON, A. G., AND A. NOWOTNY. 1964. Relationship of structure to function in bacterial O antigens. III. Biological properties of endotoxoids. *J. Bacteriol.* **87**:809-814.
9. KEENE, W. R., H. R. SILBERMAN, AND M. LANDY. 1961. Observations on the pyrogenic response and its application to the bioassay of endotoxin. *J. Clin. Invest.* **40**:295-301.
10. MEHL, J. W., J. L. ONCLEY, AND R. SIMHA. 1940. Viscosity and the shape of protein molecules. *Science* **92**:132-133.
11. MILNER, K. C., R. L. ANACKER, K. FUKUSHI, W. T. HASKINS, M. LANDY, B. MALMGREN, AND E. RIBI. 1963. Symposium on relationship of structure of microorganisms to their immunological properties. III. Structure and biological properties of surface antigens from gram-negative bacteria. *Bacteriol. Rev.* **27**:352-368.
12. MORGAN, W. T. J. 1937. Studies in immunochemistry. II. The isolation and properties of a specific antigenic substance from *B. dysenteriae* (Shiga). *Biochem. J.* **31**:2003-2021.
13. MORGAN, W. T. J., AND S. M. PARTRIDGE. 1941. Studies in immunochemistry. 6. The use of phenol and of alkali in the degradation of antigenic material isolated from *Bact. dysenteriae* (Shiga). *Biochem. J.* **35**:1140-1163.
14. MORGAN, W. T. J., AND S. M. PARTRIDGE. 1942. An examination of the O antigenic complex of *Bact. typhosum*. *Brit. J. Exptl. Pathol.* **23**:151-165.
15. NETER, E., O. WESTPHAL, O. LÜDERITZ, E. A. GORZYNSKI, AND E. EICHENBERGER. 1956. Studies of enterobacterial lipopolysaccharides. Effects of heat and chemicals on erythrocyte-modifying, antigenic, toxic and pyrogenic properties. *J. Immunol.* **76**:377-385.
16. NOLL, H., AND A. I. BRAUDE. 1961. Preparation and biological properties of a chemically

- modified *Escherichia coli* endotoxin of high immunogenic potency and low toxicity. *J. Clin. Invest.* **40**:1935-1951.
17. NOWOTNY, A. 1963. Endotoxoid preparations. *Nature* **197**:721-722.
18. OROSZLAN, S. I., AND P. T. MORA. 1963. Dissociation and reconstitution of an endotoxin. *Biochem. Biophys. Res. Commun.* **12**:345-349.
19. PHILPOT, J. St. L. Examination in the ultracentrifuge. Addendum to G. G. Freeman. 1942. The preparation and properties of a specific polysaccharide from *Bact. typhosum* Ty2. *Biochem. J.* **36**:340-356.
20. RIBI, E., R. L. ANACKER, K. FUKUSHI, W. T. HASKINS, M. LANDY, AND K. C. MILNER. 1964. Relationship of chemical composition to biologic activity, p. 16-28. In M. Landy and W. Braun [ed.], *Bacterial endotoxins*. Rutgers Univ. Press, New Brunswick, N.J.
21. RIBI, E., W. T. HASKINS, M. LANDY, AND K. C. MILNER. 1961. Preparation and host-reactive properties of endotoxin with low content of nitrogen and lipid. *J. Exptl. Med.* **114**:647-663.
22. RIBI, E., W. T. HASKINS, K. C. MILNER, R. L. ANACKER, D. B. RITTER, G. GOODE, R.-J. TRAPANI, AND M. LANDY. 1962. Physicochemical changes in endotoxin associated with loss of biological potency. *J. Bacteriol.* **84**:803-814.
23. RIBI, E., B. H. HOYER, K. C. MILNER, T. D. PERRINE, C. L. LARSON, AND G. GOODE. 1960. Physical and chemical analysis of endotoxin from *Salmonella enteritidis*. *J. Immunol.* **84**:32-47.
24. ROBERTS, R. B., P. H. ABELSON, D. B. COWIE, E. T. BOLTON, AND R. J. BRITTEN. 1955. Studies of biosynthesis in *Escherichia coli*. Carnegie Inst. Wash. Publ. 607, p. 5.
25. RUDBACH, J. A., R. L. ANACKER, W. T. HASKINS, A. G. JOHNSON, K. C. MILNER, AND E. RIBI. 1966. Physical aspects of reversible inactivation of endotoxin. *Ann. N.Y. Acad. Sci.* **133**:629-643.
26. RUDBACH, J. A., AND A. G. JOHNSON. 1962. Changes in serologic reactivity of endotoxin induced by Fraction IV₁ (Cohn) of normal human serum. *Proc. Soc. Exptl. Biol. Med.* **111**:651-655.
27. RUDBACH, J. A., AND A. G. JOHNSON. 1964. Restoration of endotoxin activity following alteration by plasma. *Nature* **202**:811-812.
28. SCHACHMAN, H. K. 1957. Ultracentrifugation, diffusion, and viscometry. In S. P. Colowick and N. O. Kaplan [ed.], *Methods in enzymology*, vol. 4, p. 32-103. Academic Press, Inc., New York.
29. SKARNES, R. C., AND L. C. CHEDID. 1964. Biological degradation and inactivation of endotoxin (chromate-labeled), p. 575-587. In M. Landy and W. Braun [ed.], *Bacterial endotoxins*. Rutgers Univ. Press, New Brunswick, N.J.
30. SVEDBERG, T., AND K. O. PEDERSEN. 1940. The ultracentrifuge, p. 40-41. Oxford Univ. Press, New York.
31. TAUBER, H., H. RUSSELL, AND W. J. GUEST. 1961. Nature of polysaccharides obtained from endotoxins by hydroxylaminolysis. *Proc. Soc. Exptl. Biol. Med.* **107**:964-965.
32. WEBSTER, M. E., J. F. SAGIN, M. LANDY, AND A. G. JOHNSON. 1955. Studies on the O antigen of *Salmonella typhosa*. I. Purification of the antigen. *J. Immunol.* **74**:455-465.
33. WESTPHAL, O., AND O. LÜDERITZ. 1954. Chemische Erforschung von Lipopolysacchariden gramnegativer Bakterien. *Angew. Chem.* **66**:407-417.
34. WESTPHAL, O., O. LÜDERITZ, AND F. BISTER. 1952. Über die Extraktion von Bakterien mit Phenol/Wasser. *Z. Naturforsch.* **7b**:148-155.