NATURE OF THE ENDOTOXIN-INACTIVATING PRINCIPLE IN GUINEA-PIG LIVER

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ABSTRACT

CORWIN, L. M. (Walter Reed Army Medical Center, Washington, D.C.), AND W. E. FARRAR, JR. Nature of the endotoxin-inactivating principle in guinea-pig liver. *J. Bacteriol.* **87**:832-837. 1964. —Guinea-pig liver preparations inactivate *Serratia marcescens* endotoxin as assayed in chick embryo. The activity is optimal at pH 6.5 to 7.0 and 8.5 to 9.0. Mitochondria and the supernatant fraction containing microsomes possess activity. Mitochondria are only active at the acid pH optimum. The activity of acetone powder extracts of mitochondria is enhanced by adenosine triphosphate and nicotinamide adenine dinucleotide, whereas the mitochondria themselves are also activated by malate. It was concluded that the enzymes which inactivate endotoxin involve fatty acid activation and oxidation. Such a finding suggests that the lipid moiety of endotoxin is required for toxicity.

For the past several years, the nature of the toxic principle in bacterial endotoxins has been the subject of intensive investigation. The problem has focused on the question of whether the lipid or polysaccharide portion of the lipopolysaccharide molecule is responsible for the toxicity. Westphal and Luderitz (1954), Westphal (1957, 1960), and Westphal et al. (1958) found that hydrolysis of endotoxin with dilute HCl yielded a chloroform-soluble lipid (lipid A) which possessed some of the activity of the original lipopolysaccharide. Although the activity was low, they found that it could be increased when the lipid was "solubilized" with detergents or dextrans, or linked with an inert protein. Ribí et al. (1960, 1961) and Haskins et al. (1961) found that endotoxin preparations could be obtained with relatively low quantities of lipid which retained much toxic activity. Furthermore, they demonstrated that acid treatment destroyed endotoxic potency before appreciable lipid was removed. They concluded that lipid is not involved in the

endotoxic activity and, therefore, the acid-labile residue, presumably polysaccharide, is the active principle. If this is true, the chemical nature of the polysaccharide is not specific because an epimeraseless mutant of *Salmonella*, which has an entirely different polysaccharide structure, has endotoxin of the same potency as the parent strain (Nakano, 1962). This problem was also attacked by chemical modification of the lipopolysaccharide. Noll and Braude (1961) treated *Escherichia coli* endotoxin with LiAlH_4 , and thereby reduced the toxicity without affecting immunogenic potency and the serological activity of the original complex. This treatment resulted in the elimination of ester-bound fatty acid. Although only preparations free of ester carbonyl were nonpyrogenic, the crude ester-free reaction product was still highly toxic. They favored the idea that "toxicity is the result of certain physicochemical properties characteristic of the entire molecule." Freedman, Sultzter, and Kleinberg (1961) showed that acetylation of endotoxin reduces pyrogenicity and acute toxicity.

It is the purpose of this report to identify the nature of the enzyme(s) in guinea-pig liver preparations which inactivates endotoxin with respect to its ability to kill 11-day-old chick embryos. Trapani et al. (1962) described rabbit-liver preparations which also inactivate endotoxin. The enzymatic approach to the study of the chemical structures required for the toxicity of the bacterial lipopolysaccharides seems promising. When one is certain of the specificity of the enzyme(s) involved, it is then possible to ascertain what chemical structures are necessary for biological activity.

MATERIALS AND METHODS

The *Serratia marcescens* endotoxin used was prepared by the Shear trypsin method and obtained from Difco. Endotoxin inactivation was

carried out by incubating the endotoxin with the cell fraction under study at 37 C in a water bath for 1 hr. The reaction mixture also contained 2.5 mM phosphate buffer (pH 6.5) and 1.5 mM $MgCl_2$. When used, adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (NAD), and malate were added at a final concentration of 1.5 mM. In all cases, the total volume of the reaction mixture was 2 ml. After 1 hr, 5 volumes of cold saline were added. Further appropriate dilutions (1:5 to 1:20) were made in 0.01 M phosphate buffer (pH 7.4) before injection into eggs for assay.

Residual endotoxin activity was determined by injecting the diluted reaction mixtures intravenously into 11-day-old chick embryos (Hook, Luttrell, and Wagner, 1961). Groups of eggs received either 2 or 4 chick embryo LD_{50} , assuming no inactivation had taken place; mortality was determined after 20 to 24 hr of incubation at 37 C. Control groups of eggs received endotoxin which had been incubated in saline or cell fractions incubated without endotoxin. All components of the reaction mixtures were tested for sterility as part of each experiment.

Fatty acid esters were determined by the method of Snyder and Stephens (1959), as modified by Tauber (1960). The activation of fatty acids in endotoxin by liver preparations was ascertained by the method of Kornberg and Pricer (1953). Liver-cell fractions were separated by differential centrifugation as described previously (Corwin and Schwarz, 1959).

RESULTS

Guinea-pig liver has the ability to inactivate endotoxin. Incubation of 20 μg of *S. marcescens* endotoxin with 720 mg of guinea-pig liver homogenate increased the LD_{50} of endotoxin 16-fold (Table 1). When the amount of homogenate was reduced, there was a progressive reduction in the degree of inactivation, but even when the amount of homogenate used was 0.01 of that present in the original 40% suspension, there was still definite evidence of inactivation.

Attempts were made to localize the endotoxin-inactivating activity within a specific cell fraction, but activity was found in all fractions (Table 2). Attempts to purify the enzyme(s) from an acetone powder of guinea-pig liver by means of ammonium sulfate precipitation and

TABLE 1. *Effect of guinea-pig liver homogenate on toxicity of endotoxin**

Homogenate†	Endotoxin dose per egg‡	Mortality (deaths/total eggs)	
		Homogenate	Saline
mg	μg		
720	0.1	6/13	—
720	0.05	1/13	—
720	0.025	0/20	15/16
720	0.0125	0/9	16/18
720	0.0063	0/10	9/19
180	0.0125	5/40	53/60
36	0.0125	13/40	—
7.2	0.0125	23/40	—
1.4	0.0125	31/40	—

* For all assays, 20 μg of endotoxin were used.

† Liver homogenized in 0.01 M phosphate buffer (pH 7.0) as 40% suspension, and supernatant fraction after centrifugation at $6,000 \times g$ for 20 min is termed the homogenate.

‡ Dosages represent dilutions from original experimental flask. Reaction carried out in final volume of 2 ml for 1 hr at 37 C.

TABLE 2. *Effect of cell fractions on inactivation of endotoxin*

Endotoxin	Cell fraction*	Endotoxin dose per egg	Mortality
μg		μg	
20	Nuclei	0.0125	5/10
20	Mitochondria	0.0125	5/9
20	Supernatant	0.0125	3/9
20	Saline	0.0125	13/14
2	Nuclei	0.0188	1/4
2	Mitochondria	0.0188	4/9
2	Supernatant	0.0188	2/9
2	Saline	0.0188	9/9

* Cell fractions were prepared in 0.33 M sucrose by differential centrifugation. They were then re-suspended in sucrose to make a 20% suspension and diluted 1:1 with 0.01 M phosphate buffer (pH 6.5); of this dilution, 1.8 ml was used, equivalent to 180 mg of liver.

calcium phosphate gel absorption were also unsuccessful.

To ascertain the optimal conditions for endotoxin inactivation, a study of the effect of pH on the system was undertaken. There are two distinct pH optima for the reaction, the first at pH 6.5 to 7.0 and the second at 8.5 to 9.0 (Fig. 1).

FIG. 1. Effect of pH on endotoxin inactivation by whole-liver homogenate (●) and by washed mitochondria (△). Homogenate was prepared as a 20% suspension of liver in 0.33 M sucrose after centrifugation at 600 × g. This was diluted 1:1 with 0.01 M phosphate buffer (pH 7.0) and then adjusted to the desired pH with either 0.1 N HCl or 0.1 N NaOH. Reaction mixture contained 1.8 ml of homogenate and 0.2 ml (20 µg) of endotoxin. Mitochondria consisted of a 20% suspension of washed mitochondria in 0.33 M sucrose, diluted 1:1 with 0.1 M buffer of desired pH. Reaction mixture contained 1.5 ml of mitochondria, 1.5 mM ATP, 1.5 mM NAD, 1.5 mM MgCl₂, and 20 µg of endotoxin in a final volume of 2 ml. All eggs received 2 LD₅₀ of endotoxin, which killed 90% of control eggs.

This finding suggested that endotoxin inactivation by liver homogenates may involve more than one enzyme. It was found that mitochondria obtained from the homogenate possess activity only at the more acid pH optimum. Neither the microsomal nor the soluble supernatant fractions had appreciable activity at any pH. However, a combination of the latter fractions had activity at the alkaline pH optimum of the homogenate, as well as at the acid pH optimum.

To ascertain the nature of the enzymatic activity in the mitochondria, an investigation of cofactor requirements was made. Mitochondria were chosen because it is possible to free them for the most part from soluble cofactors. Table 3 indicates a stimulation of "endotoxinase" activity by addition of ATP or malate. However,

it is obvious that the mitochondria alone have substantial activity. It was suspected that even the washed mitochondria have substantial quantities of bound endogenous cofactors. Therefore, an acetone powder was prepared from mitochondria, and a soluble extract in phosphate buffer was made. The activity of the extract was considerably enhanced by ATP and NAD, but malate by itself was almost inactive (Table 4). The effect of the cofactors was considerably reduced at the higher concentrations of endotoxin, but the combination of ATP, NAD, and malate was still quite active.

TABLE 3. Effect of cofactors upon activity of mitochondria

Determination*	2 LD ₅₀ (0.02 µg)		4 LD ₅₀ (0.04 µg)	
	Mortal-ity	Per cent protec-tion†	Mortal-ity	Per cent protec-tion†
Mitochondria alone	9/24	45	15/24	35
+ ATP	5/24	69	11/24	52
+ Malate	2/23	87	15/24	35
+ ATP + malate	2/24	88	11/24	52
Saline control	16/24		23/24	

* ATP and malate were added at a concentration of 1.5 mM.

† Per cent protection = $\frac{\% \text{ mortality saline} - \% \text{ mortality sample}}{\% \text{ mortality saline}} \times 100$.

TABLE 4. Effect of cofactors upon activity of mitochondrial acetone powder extract*

Determination	2 LD ₅₀		4 LD ₅₀	
	Mortal-ity	Per cent protec-tion	Mortal-ity	Per cent protec-tion
Extract alone†	20/44	36	33/50	19
+ ATP	8/42	75	24/44	32
+ NAD	12/52	69	30/52	28
+ Malate	21/43	35	24/44	32
+ NAD + malate	14/51	64	26/50	36
+ ATP + NAD + malate	13/50	65	16/51	62
Saline control	40/53		42/52	

* The incubation medium contained 1.4 ml of acetone powder extract in 0.01 M phosphate buffer (pH 6.8) and 20 µg of endotoxin. ATP, NAD, and malate were added at a concentration of 1.5 mM.

† Protein concentration = 1.55 mg/ml.

TABLE 5. *Activation of fatty acid esters by guinea-pig liver homogenates*

Determination	μg of oleic per mg of endotoxin
Fatty acid ester*.....	123
Fatty acids activated†.....	59

* Used 2.66 mg of *Salmonella typhimurium* endotoxin; fatty acid ester was determined with oleic acid as a standard.

† Used 5 mg of endotoxin and 0.2 ml of 20% liver homogenate, centrifuged at $2,000 \times g$ and incubated with tris(hydroxymethyl)aminomethane buffer (pH 7.4), 10 mM ATP, 5 mM MgCl_2 , 0.5 M hydroxylamine (pH 7.4), and 3 mM CoA. The pyrophosphate formed was measured and converted to oleic acid equivalents. Incubation was for 30 min at 37 C.

The enhancing effect of additions of ATP, NAD, and malate is strongly suggestive of fatty acid activation and oxidation. For this reason, the ability of the guinea-pig liver to activate the fatty acids of endotoxin to form the acyl CoA derivatives was tested. Almost half of the fatty acid esters in endotoxin can be activated by the liver homogenate, a reaction stimulated by ATP (Table 5). The activated acid can be oxidized by the fatty acid oxidation system which requires NAD. The function of the citric acid cycle intermediates, such as malate, as a source of ATP for fatty acid oxidation is well known.

Inasmuch as Smith et al. (1963) demonstrated that an endotoxin-inactivating principle from spleen was inhibited by tetraethylpyrophosphate (TEPP), a potent esterase inhibitor, the effect of TEPP on the guinea-pig liver enzyme(s) was tested (Table 6). At a concentration of 0.1 mM TEPP, there was no inhibition of endotoxin inactivation at either acid or alkaline pH optima, despite complete inhibition of liver esterase activity with *p*-nitrophenylbenzoate as substrate.

DISCUSSION

Previous approaches to the problem concerning the chemical identity of the toxic moiety of bacterial lipopolysaccharides led to opposing conclusions. Westphal and his associates (1954, 1958, 1960) tried the direct approach of hydrolyzing the molecule with acid and assaying the toxic effects of the lipid and polysaccharide. The lipid was found to possess some activity and could be activated by detergents, but the potency at best

was far less than the parent endotoxin. Ribi et al. (1961) found the activity of the lipid very low and, furthermore, gave evidence that the toxic moiety is sensitive to concentrations of acid that do not remove the lipid. With their method of preparing endotoxin, they could obtain a compound with as little as 2% lipid, and could not correlate lipid content of other preparations with toxicity. As is the case with most indirect approaches, the conclusions, although reasonable, suffer from the lack of direct evidence to be gained with endotoxin which is truly lipid free. One can still argue, with some precedent, that the residual lipid bound very tightly to the polysaccharide is the active factor or, perhaps, has the same relation to the polysaccharide that a cofactor such as NAD has to a protein; i.e., a synergistic action.

The use of enzymatic inactivation of endotoxin as a means of elucidating the chemical basis of its toxic properties offers some significant advantages over previous approaches to this problem. When drastic chemical procedures, such as acid hydrolysis, are applied to a large and complex molecule of unknown structure, it is impossible to ascertain exactly what changes have been produced in the molecule. Enzymatic degradation represents a gentler and more specific kind of chemical attack. Once the identity of the enzyme(s) is known, then the classification of the substrate (lipid or polysaccharide) becomes obvious.

The clue that aided in the characterization of the enzyme(s) in guinea-pig liver responsible for the detoxification of the endotoxin was the inability to obtain activities in the various cell

TABLE 6. *Effect of tetraethylpyrophosphate on endotoxin inactivation by liver homogenate**

Determination	pH	Addition	Mortality
Homogenate.....	6.5	None	1/8
Homogenate.....	6.5	TEPP	1/8
Homogenate.....	9.0	None	3/8
Homogenate.....	9.0	TEPP	3/8
Saline.....	6.5	None	8/8

* Incubation medium contained 20 μg of endotoxin, 180 mg of liver homogenate (supernatant fraction after $600 \times g$ centrifugation), 1.5 mM ATP, and 1.5 mM NAD with 0.04 M phosphate buffer. Incubation was for 1 hr at 37 C; 0.1 mM of tetraethylpyrophosphate was added as shown.

fractions of the same order of magnitude as the homogenate. The activation of mitochondrial activity by ATP and malate, and the enhancement of acetone powder extracts of mitochondria by ATP and NAD provided evidence of the nature of the enzyme. ATP can enhance the activity of a variety of enzymes, but most can be eliminated as possibilities. ATP can activate amino acids, but endotoxin of high toxicity can be prepared with little or no nitrogen; thus, amino acids or proteins are probably not involved. The activation must be concerned with a polysaccharide or a lipid. ATP in the presence of hexokinase can phosphorylate a variety of monosaccharides, but in endotoxin a polysaccharide is involved. Furthermore, incubation of endotoxin with hexokinase, ATP, and Mg^{++} had no effect on toxicity. In general, no enzymes hydrolyzing glycosidic links require ATP for their action, nor are they found in guinea-pig liver mitochondria where much of the ATP-activated "endotoxinase" is to be found.

Kornberg and Pricer (1953) found that fatty acid activation enzymes are to be found in mitochondria and supernatant fractions and are activated by ATP. Endotoxin-inactivating properties were observed in both mitochondria and the supernatant fluid (including microsomes). The difficulty in purifying this system is strongly reminiscent of the experience of Kornberg and Pricer (1953). The effects of NAD and malate fit into this hypothesis very well. NAD can, of course, exert its activation by the oxidation of the activated fatty acids. Malate, in common with other Krebs cycle acids, can spark fatty acid oxidation by the formation of ATP for the activation of the fatty acids, as well as by removing the acetyl CoA molecules to form citrate and continue the cycle. The lack of effect of malate in the acetone powder extracts of mitochondria can be explained by the lack of oxidative phosphorylation to produce ATP. Thus, the activity of liver mitochondrial preparations appears to involve the activation and subsequent oxidations of fatty acids. One is then left with the strong implication that the lipid moiety of endotoxin is a necessary requirement for the toxicity of the lipopolysaccharide.

Evidence for the enzymatic inactivation of endotoxin by tissue homogenates was provided by Trapani et al. (1962) for rabbit liver and Smith et al. (1963) for dog spleen. Both of these groups prepared their cell fractions by homogeniz-

ing tissue in saline with a Waring Blender. However, this procedure drastically disrupts subcellular particles. Therefore, enzymes originally present only in mitochondria, for example, are found in the supernatant fraction after this treatment. Thus, their conclusion that detoxification of endotoxin takes place in the supernatant fluid may be unwarranted.

Whereas Smith et al. (1963) found full activity over a pH range of 5.5 to 8.5, Trapani et al. (1962) reported a pH optimum of 9.5 for their material. With the use of the whole homogenate, we found two distinct pH optima, at 6.5 to 7.0 and 8.5 to 9.0. Mitochondria, as well as the acetone powder extract from them, were active at the lower pH, with almost no activity at the alkaline pH. The enhancement of endotoxin-inactivating activity by NAD suggests fatty acid oxidation. This oxidation is limited to the mitochondria because cytochrome oxidase occurs only in this fraction. Thus, the 6.5 to 7.0 pH optimum of the homogenate is probably due to fatty acid oxidation. Prior to oxidation, the fatty acid must be activated to the acyl CoA derivative by an enzyme of guinea-pig liver not restricted to any one fraction, according to Kornberg and Pricer (1953). Because the liver enzymes are not affected by tetraethylpyrophosphate at either acid or alkaline pH range, it is unlikely that an esterase is one of the enzymes involved in the inactivation of endotoxin by liver. Because the supernatant fraction, after the removal of mitochondria, is active at the alkaline pH optimum, and because this activity is enhanced by ATP, it is possible that fatty acid activation may account for this activity.

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