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Comparisons of Five Toxicity Parameters of *Serratia marcescens* Endotoxins* (32854)

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Interrelationships between the numerous endotoxic reactions have not been thoroughly investigated. Dissociation of lethality from Shwartzman reactivity in the rabbit has been demonstrated by the administration of large doses of cortisone prior to injection of endotoxin as reported by Thomas and Smith (1). It is generally believed, however, that different endotoxicity parameters are equivalent and may be used interchangeably as measurements of toxic potency. While this may be true for a few measurements under certain experimental conditions, great differences can be demonstrated in other instances, as observations in this laboratory have indicated.

Experiments aimed at investigating the relationship of structure to endotoxic activity have utilized chemically altered preparations. A variety of different biological properties have been measured and compared to the original potency of the preparations prior to chemical alteration of the structure with boron trifluoride, potassium methylate, or pyridinium formate. Differentiation between the biological properties of such preparations was established by diminishing or abolishing some toxic properties, including lethality in mice and rabbits, while maintaining other biological actions, such as stimulation of nonspecific resistance or the adjuvant effect (2). Using milder chemical procedures such as 0.1 *N* NaOH which resulted in partial loss of toxicity, it was observed that while some of the toxicity assays showed no alteration in degree of potency, other methods indicated complete inactivation. These observations initiated the experiments reported here. In these investigations, quantitative comparisons of the five most commonly used endotoxicity parameters were performed on preparations with different degrees of purity or chemical degradation.

Materials and Methods. Endotoxin preparations. A partially purified Boivin-type, trichloroacetic acid (TCA) extracted *Serratia marcescens* endotoxin (3) served as the original preparation which was further treated by different chemical means. A phenol–water purification of the original starting material was prepared according to the method of Westphal *et al.* (4). A type of chemically detoxified endotoxin (endotoxoid-4) was prepared by treating the Boivin-type TCA extracted endotoxin with 0.1 *N* NaOH at room temperature for varying periods of time, followed by neutralization with 0.1 *N* hydrochloric acid to pH 7.0 (5).

Assay methods. (a) The chick embryo (CE) lethality tests using the chorioallantoic membrane (CAM) route of inoculation were patterned after the method of Smith and Thomas (6). (b) The CE lethality tests employing an intravenous (iv) route of injection followed the procedure of Smith and Thomas (6), modified by Finkelstein (7). (c) Mouse lethality of each preparation was measured by injecting graded doses intraperitoneally into 18–20 gm random bred Swiss albino mice. Each dose level was injected into at least five mice at different dilutions and deaths were recorded 12–72 hours following injection. The calculation of median lethal dose (LD_{50}) was made by the method of Irwin and Cheeseman (8). (d) Shwartzman reactivity was carried out by intradermal injection into the shaved abdomen of New Zealand white rabbits

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TABLE I. Biological Activities of TCA Extracted *S. marcescens* Endotoxin and Its Purified Phenol–Water Derivative.

Treatment of *S. marcescens* endotoxin	Chick embryo LD_{50} (μg/embryo $\pm$ 1 SD)		Mouse LD_{50} (μg/mouse)	FI_{40} (μg)	Shwartzman[a]
	Chorioallantoic inoculation	Intravenous inoculation			
Untreated	10.2 $\pm$ 1.1	.015 $\pm$ .001	580	0.52	2.9
Phenol–water purified	6.2 $\pm$ 1.7	.0023 $\pm$ .0002	310	0.44	8.7

[a] Values represent product of two diameters of hemorrhagic necrosis (measured in cm) taken at right angles.

weighing 2–3 kg. In most cases 10 μg of material to be tested was injected as the preparative dose in 0.2 ml volume of saline, followed by a 20 μg challenge dose of standard endotoxin given iv 24 hours later (9). The spots of hemorrhagic necrosis were measured and the products of two diameters obtained at right angles were reported. (e) Pyrogenicity was measured in rabbits similar to those above which were acclimatized and had constant rectal temperature between 38.5 and 40°C prior to injection. Temperatures were usually recorded in the carefully restrained rabbits at 15-min intervals for 1–3 hours before and 5 hours following injection of varying amounts of endotoxin samples contained in 0.5 ml of pyrogen-free saline (9). Pyrogenicity was expressed as FI_{40}, or that dose in μg which produced an area under the fever curve of 40 cm^2 when 1 hour and 1°C were plotted as 1 inch (10). Each FI_{40} represents a composite figure obtained from the temperature of 3 rabbits at each dose level. When the fever curves did not return to the base line at the end of 5 hours, they were extrapolated to that point.

Experimental. Experiment I was designed simply to compare Boivin-type TCA extracted endotoxin with a derivative of the same preparation further purified by the phenol–water procedure. The five different methods of assay outlined above were used to measure toxicity. Experiment II was specifically designed to compare the TCA extracted endotoxin with samples of the same material treated by exposure to NaOH for different periods of time using the same five methods of assay.

Results. Experiment I demonstrated that the TCA extracted endotoxin and its phenol–water derivative both were active and the toxicity figures for the phenol–water treated material all appeared to show enhancement. These differences were consistent, though they may not be significant at the 95% confidence level in all cases. The findings are summarized in Table I. Figure 1 shows the difference in the shape of the fever curve for equal amounts of phenol–water purified and parent TCA endotoxin preparations. The most striking observation here is the lack of the second fever peak given by 0.1 μg of the TCA endotoxin sample, while there is a typical biphasic curve for an equal amount of the phenol–water purified material.

Experiment II showed that the rate of NaOH inactivation of the five toxic properties was quite different. While chick embryo

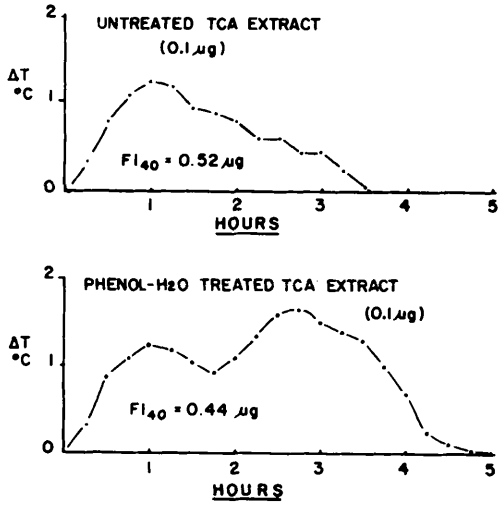

FIG. 1. Pyrogenicity of TCA extracted *S. marcescens* endotoxin and its purified phenol-water derivative.

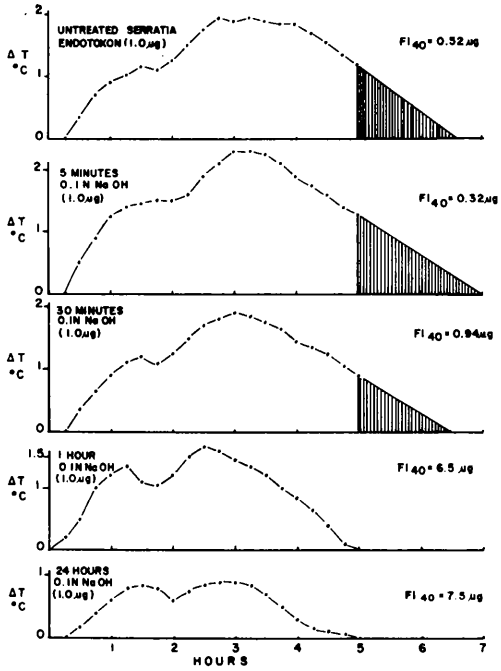

FIG. 2. Pyrogenicity of TCA extracted *S. marcescens* endotoxin and its NaOH treated derivative.

lethality disappeared rapidly, mouse lethality persisted much longer. Complete inactivation of pyrogenic properties could not be achieved even in 24 hours. The same NaOH treatment resulted in a significant transient enhancement in the local Shwartzman reactivity. After 24 hours exposure to 0.1 *N* NaOH, the remaining Shwartzman reactivity of the preparation was still identical to the original starting values. These findings are shown and summarized in Figs. 2, 3, and Table II.

Discussion. Data presented here tend to show that phenol–water purification of a TCA extracted *S. marcescens* endotoxin produces enhancement of toxicity. This apparent increase in toxicity of the phenol–water treated material might be ascribed merely to a process of purification or to an increase in solubility, i.e., dispersion of the product which is considered to be important in certain assays (11).

The effect of solubility on the biological potency of similar preparations has been emphasized frequently in the past by several authors. Unquestionably this may play a significant role in certain bioassays, but as the results presented here indicate, this does not apply in all instances. The NaOH treatment induces a great decrease in particle size and results in an enhanced solubility, as has been shown by light-scattering photometry (5). The same treatment, however, does not in-

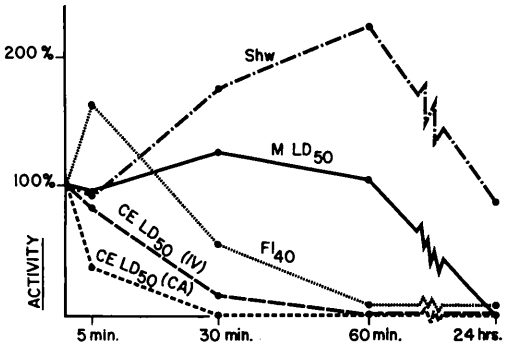

FIG. 3. Changes in biological potency during treatment with 0.1 *N* NaOH.

TABLE II. Biological Activities of TCA Extracted *S. marcescens* Endotoxin and Its NaOH Treated Derivatives.

Treatment of *S. marcescens* endotoxin c̄ 0.1 *N* NaOH	Chick embryo LD_{50} (μg/embryo ± 1 SD)		Mouse LD_{50} (μg/mouse)	FI_{40} (μg)	Shwartzman[a]
	Chorioallantoic inoculation	Intravenous inoculation			
Untreated	10.2 ± 1.1	.015 ± .001	580	0.52	2.9
5 min	27.2 ± 2.2	.018 ± .001	610	0.32	2.7
30 min	>100	.091 ± .015	460	0.94	5.1
1 hour	>160	>100	550	6.5	6.5
24 hours	>160	>100	>2000	7.5	2.6

[a] Values represent product of two diameters of hemorrhagic necrosis (measured in cm) taken at right angles.

crease the biological potency in all assays.

While mouse lethality remains essentially unchanged after at least 1 hour of NaOH treatment and probably persists somewhat longer, previous work by Tripodi and Nowotny showed detoxification has occurred by 6 hours (5). It is of interest to note that this slow decrease in mouse lethality is paralleled by a decrease in the complement fixing ability of endotoxin similarly treated with NaOH, as demonstrated by recent work of Gewurz, Mergenhagen, Nowotny, and Philips (unpublished).

These studies also revealed additional limitations of the quantitative evaluation of some parameters. For example, for the dose levels of endotoxin used to obtain the FI_{40} figures reported here, we were unable to obtain good dose response curves to calculate the minimal pyrogenic dose at 3 hours (MPD-3). We did, indeed, note an increase in MPD-3 after 5 min of NaOH treatment but the erratic results based on measurements made at 3 hours made it impossible to calculate dependable MPD-3 figures for all of the various time periods of NaOH treatment used in our experiments.

We have also observed that FI_{40} figures do not fully reflect the magnitude of the differences in pyrogenicity for different endotoxin preparations. For example, we measured the pyrogenicity of 0.1 μg of endotoxin treated for 1 hour with NaOH and observed a rise in body temperature as measured by 22 cm^2 in area under the curve when plotted as described in "Materials and Methods." To obtain the same 22 cm^2 injecting an endotoxin treated for 24 hours with NaOH, the necessary dose would be 1.6 μg, as calculated from the dose response curves used to obtain the FI_{40} of this preparation. If we compare the FI_{40} values, however, we find no significant difference between these two preparations. It would appear that mere observation of the shape and magnitude of pyrogenic curves frequently enables the experienced observer to judge the comparative potency of different materials, provided the injections have been given in the proper dose range.

Summary. Phenol–water purification of TCA extracted *Serratia marcescens* endotoxin results in an apparent enhancement of toxicity as measured by two methods for testing biological activity, whereas three other methods indicate only an insignificant increase in toxicity. Partial degradation of endotoxin by NaOH shows a rapid loss of chick embryo lethality both by the iv and CAM routes of inoculation. Mouse lethality persists longer, almost complete detoxification occurring between 1 and 6 hours of NaOH treatment. The FI_{40} figures in rabbits tend to show an increase in pyrogenicity after 5 min of treatment and a progressive decrease between 30 min and 24 hours, although a low level of pyrogenicity is retained at the latter time. Shwartzman reactivity increases up to 1 hour of NaOH treatment, followed by a decrease to approximately the initial level after 24 hours of treatment and thus would appear to be a less reliable indicator of detoxification when compared to the other testing procedures. It is thus seen that one method of testing endotoxin may indicate an active preparation whereas another parameter may indicate otherwise to a greater or lesser extent. As a result, it is important to recognize the inherent danger involved in using a single assay technique for describing the toxic potency of an endotoxin preparation. The observed discrepancies in biological assays also indicate that the mode of endotoxic action is obviously triggered and channeled by a number of different pathways in the various host systems.

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Bile Acids and Lipid Metabolism. I. Stimulation of Bile Lipid Excretion by Various Bile Acids (32855)

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Although considerable information is available on the role of bile acids in the intestinal absorption of lipids, very little is known concerning the function of these compounds in the transport of lipids by other tissues. Rat hepatic bile contains, in addition to bile salts, such lipids as cholesterol, phospholipids and glycerides, as well as bile pigments, and steroids (1). A relationship between bile acids and bile lipids can be inferred from the finding of macromolecular complexes in bile containing these compounds (2). In addition, Kay and Entenman (1), showed a progressive decrease in bile acid and certain bile lipids using bile fistula rats as well as with the isolated, perfused rat liver. With the bile fistula rats, biliary taurocholic acid, phospholipids and free cholesterol decreased whereas the biliary levels of glycerides and esterified cholesterol remained unchanged. With the isolated perfused rat liver the levels of biliary phospholipid and free cholesterol fell to essentially zero 90 min after the start of perfusion. Upon addition of cholic acid to the perfusate, biliary taurocholic acid increased as much as 30-fold, free cholesterol by 2-fold, and phospholipids as much as 50-fold.

In the present paper, data on the initial studies on the role of the bile acids in the synthesis, metabolism, and excretion of bile lipids by the isolated perfused rat liver is reported. It is shown that both primary and secondary bile acids normally present in the enterohepatic circulation of the rat (3) are effective in stimulating biliary lipid excretion by the isolated perfused rat liver.

Methods and Materials. Bile salt preparations. The following bile salts were used: sodium taurocholate (Miles, Ames, Research Division); sodium deoxycholate, special enzyme grade (Mann Research Laboratories); hyodeoxycholic acid (K and K Laboratories); chenodeoxycholic acid, Grade A (Cal-Biochem); sodium lithocholate (Steraloids, Inc.); sodium dehydrocholate (Ames Company); and sodium glycocholate (Mann Research Laboratories). Each preparation used was shown to give a single spot on a thin-layer chromatogram using the techniques of Kritchevsky (4) and Gregg (5). The compounds were also treated by the alkaline hydrolysis technique of Kottke *et al.* (6) and the extracts applied to a thin-layer plate. The free acids again gave only a single spot on the chromatogram.

Solutions for injection into the perfusates were prepared as follows: taurocholate, deoxycholate, and glycocholate were dissolved in isotonic saline; hyodeoxycholate was dissolved in isotonic saline that had been adjusted to pH 8.0 with NaOH; and chenodeoxycholate was dissolved in 20 m*M* sodium bicarbonate. Lithocholate (40 mg) was dissolved in 3 ml of methyl alcohol, combined with 0.25 ml of propylene glycol, and the methanol was evaporated under a stream of nitrogen at 80°C. The propylene glycol–lithocholate solution was mixed with 12 ml of rat plasma that had been cleared of the vasoconstrictor factor (i.e., prepared from blood that had been perfused through a liver for 90 min).

Liver perfusion technique. The apparatus and general techniques used for rat liver perfusions were those described by Brauer *et al.* (7). Livers were taken from ether-anesthetized donor, fed, male Sprague-Dawley rats