Detoxified Bacterial Endotoxins

II. Preparation and Biological Properties of Chemically Modified Crude Endotoxins from *Salmonella typhimurium*¹

WILLIAM J. MARTIN² AND STANLEY MARCUS

Department of Microbiology, College of Medicine, University of Utah, Salt Lake City, Utah

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ABSTRACT

MARTIN, WILLIAM J. (University of Utah, Salt Lake City), AND STANLEY MARCUS. Detoxified bacterial endotoxins. II. Preparation and biological properties of chemically modified crude endotoxins from *Salmonella typhimurium*. J. Bacteriol. 91: 1750-1758. 1966.—Chemical modification of a crude endotoxin prepared by the Roschka-Edwards (RE) procedure from a strain of *Salmonella typhimurium* yielded products which were nontoxic for mice and had reduced fever effects in rabbits. A reduction in rabbit pyrogenicity of approximately 100 times was noted with a potassium periodate-treated RE preparation when compared with the parent RE preparation. Measured in a similar fashion, pyrogenicity of a potassium methylate-treated RE preparation was reduced by a factor of 10 while pyrogenicity of a boron trifluoride RE preparation was unchanged. All of these endotoxoids, including the parent RE preparation, showed little toxicity for mice. Immunogenicity was determined in mice by comparing Boivin, RE, and endotoxoid preparations with a heat-killed, phenol-preserved (HP) vaccine prepared from the same strain of *S. typhimurium*. Employing a 10 LD₅₀ challenge, the protective immunogenicity of the respective vaccines was determined by active immunized mouse protection tests. Although two 100 µg immunizing doses of the Boivin, RE, and the respective endotoxoid preparations varied in mouse protection (potassium methylate RE > Boivin > RE > boron trifluoride RE > potassium periodate RE), it was evident that, with the exception of the potassium methylate preparation, the HP vaccine yielded greatest protection against the 10 LD₅₀ challenge with *S. typhimurium*. Further mouse protection experiments suggested that the minimal immunogenic dose of the potassium methylate RE vaccine preparation was approximately 50 µg. These data indicated an approximate fivefold difference between the minimal pyrogenic dose (10 µg) and the minimal immunogenic dose (50 µg). These findings further suggest that potassium methylate RE vaccine preparations should be considered in the search for less toxic enteric fever vaccines.

Several O antigen isolation methods have been described since that first reported by Boivin, Mesrobian, and Mesrobian (1). Their trichloroacetic acid extraction procedure enabled these investigators to isolate toxic and immunizing fractions from gram-negative bacteria. This method of isolating endotoxin from entero-

bacteriaceal organisms was followed by many other extraction procedures (11, 17, 19, 20, 22, 23). In addition, the selective reduction of one or more of the toxic properties of endotoxin with retention of antigenic, nontoxic factors of value in immunization of the host has also been the subject of much experimental effort (2, 3, 4, 14, 21). Recently, we described the acetylation of a crude endotoxin material obtained by the Roschka-Edwards (RE) method from a strain of *Salmonella typhimurium* (9). The results indicated that the preparation obtained had reduced pyrogenicity in rabbits and was not lethal in mice unless given in doses exceeding 1 g/kg of body weight. Moreover, experiments in mice with this

¹ This paper is an essential portion of a thesis submitted to the Department of Microbiology, University of Utah, in partial fulfillment of the requirements for the Ph.D. degree.

² Present address: Enteric Bacteriology Unit, Communicable Disease Center, Public Health Service, U.S. Department of Health, Education, and Welfare, Atlanta, Ga.

same acetylated Roschka-Edwards (Acet-RE) preparation indicated a protective capacity comparable to that obtained with heat-killed, phenol-preserved vaccine employed from the same strain of *S. typhimurium*.

In studying the effects of heat and chemicals on erythrocyte-modifying, antigenic, toxic, and pyrogenic properties of enterobacteriaceal lipopolysaccharides, Neter et al. (13) reported that, if lipopolysaccharides are exposed to a periodate solution, they lose their antigenic specificity and toxicity but retain their pyrogenicity. More recently, the use of a variety of other chemical procedures for the purpose of detoxifying endotoxin preparations has been reported by Nowotny (15, 16) and by Johnson and Nowotny (5). Employing endotoxin isolated by the trichloroacetic acid method from *Serratia marcescens*, these investigators found that toxicity could be decreased or completely abolished by chemical methods, such as treatment with boron trifluoride, potassium methylate, or pyridinium formate. In addition, the ability to induce the second fever peak of the biphasic fever response characteristic of endotoxin was diminished; the preparations produced potent antisera except in the case of the boron trifluoride-treated material. These investigators also reported that these endotoxoids enhanced the nonspecific resistance of mice and rabbits against experimental infections.

With these observations in mind, as well as for purposes of comparison, the chemical modification procedures of transesterification with boron trifluoride, deacylation with potassium methylate, and oxidation with potassium periodate were applied to the same crude endotoxin material obtained by the RE method from a strain of *S. typhimurium*. By use of the previously reported biological parameters of pyrogenicity, lethality, and immunogenicity, data were obtained which suggest the feasibility of using these chemical methods as potential procedures for producing a less toxic enteric fever vaccine.

MATERIALS AND METHODS

Experimental animals. Testing for pyrogenicity was done in rabbits that had been properly "screened" by methods previously reported (8). Rabbits which yielded positive fever results, and were used in more than one experiment involving pyrogenicity, were "rested" for 2 or more weeks as outlined by the *United States Pharmacopeia XVI*.

Tests for immunogenicity and lethality were performed in white mice weighing between 20 and 25 g; deaths were recorded for a period of 1 week.

Organism. A mouse-virulent strain of *S. typhimurium* originally obtained from the Utah State Department of Health was employed in all experiments. This organism has been maintained by transfer culture

at monthly intervals on tryptose-phosphate-agar slants. It is incubated at 37 C for 24 hr and thereafter kept at room temperature.

RE procedure. The method and handling of the parent RE preparation was described in a previous publication (9). To summarize, pooled washings of previously heat-killed *S. typhimurium* organisms were taken through three changes of absolute ethyl alcohol and acetone. The remaining sediment was dried at 37 C and then ground to a fine powder. This powder, consisting primarily of the "hulls" of these *Salmonella* organisms, was further disintegrated by the use of an ultrasonic disintegrating device. A 15-ml amount of water was added to 1,500 mg of this crude endotoxin and disintegrated (Sonifier, Branson Instruments, Stamford, Conn.) for 15 min under sterile conditions. The suspension was filtered (Millipore filter), and the resulting precipitate was dried overnight at 37 C. As previously reported, employing this procedure of ultrasonic exposure produced no visible changes in yield or in alteration of this crude endotoxin.

Periodate procedure (13). The periodate-treated RE crude endotoxin was prepared as follows: 200 mg of the parent RE preparation was suspended in 160 ml of distilled water; 20 ml of 1.0 N sodium acetate buffer (pH 5.0) as well as 20 ml of 0.1 N potassium periodate solution were added. This material was kept in the dark for 24 hr at room temperature and then dialyzed in cellophane (Visking) casing against distilled water for 24 hr. Finally, the material was concentrated by pervaporation at room temperature and freeze-dried. The resulting product was a light, white, fluffy powder with a yield of 112 mg (56%). Although Neter and his associates employed 0.1 N sodium periodate solution in their procedure, it is of interest to note that the potassium periodate-treated RE material in identical concentrations reacted in a similar manner; i.e., our freeze-dried oxidation product was also insoluble in distilled water, but was soluble upon the addition of small amounts of 1.0 M NaOH and subsequent neutralization with dilute acetic acid. The soluble stock suspension of 50 mg/10 ml of saline was readily prepared and had a characteristic milky appearance.

Potassium methylate procedure (15). The parent RE preparation was treated by extraction with anhydrous methanol, a procedure which removes small amounts of inert impurities but does not alter the biological properties of endotoxins (15). To 1 g of dry RE preparation, 250 ml of methanol was added, and the resulting suspension was refluxed on a hot-plate with continuous magnet-stirring for 60 min, cooled, and finally centrifuged in the cold at 2,500 rev/min for 10 min. The clear supernatant fluid was drawn off, and the precipitate was dried in a vacuum desiccator for 48 hr. This "purified" RE preparation was subjected to the following chemical procedures. To 200 mg of the above described RE preparation, 40 ml of 0.02 M potassium methylate in anhydrous methanol was added, and the mixture was refluxed for 60 min. The insoluble residue was centrifuged in the cold at 2,500 rev/min for 10 min, washed with methanol, and dried in a vacuum desiccator. This preparation was a very fine, light powder which was ivory in color, and

sparingly soluble in saline in small amounts. The yield was 169.1 mg (84.6%).

Boron trifluoride (BF₃) procedure (15). In this procedure 500 mg of previously "purified" (methanol refluxed) RE preparation was refluxed for 1 hr with 50 ml of 2% boron trifluoride in anhydrous methanol (ca. 80 C). The reaction mixture was diluted with 4 volumes of water and dialyzed against distilled water for 96 hr. The dialyzed material was then centrifuged in the cold at 2,500 rev/min for 10 min, and the resulting residue was dried in a vacuum desiccator. This preparation was tan in color, granular in appearance with a consistency of fine sand, and was mildly lyophobic. The yield was 233.3 mg (46.7%).

Stock solutions (50 mg/10 ml) of the potassium methylate and BF₃ preparations were readily prepared by adding 0.1 ml of saline and triturating with mortar and pestle to thoroughly wet and disperse the solids. Saline was added in small portions as the solids were worked into suspension. It should be noted that, as with the previously described Acet-RE and RE preparations (9), these stock solutions should be thoroughly mixed before testing since both suspensions are dense and separate readily.

In all procedures, precautions to avoid contamination by extraneous pyrogens were rigorously observed. All glassware and other apparatus were heated at 250 C for 2 hr before use, and saline and distilled water were proved to be pyrogen-free (PF).

Boivin preparation. Lipopolysaccharide extracted by trichloroacetic acid from *S. typhimurium* was used as the source of "pure" endotoxin (Difco).

Heat-killed, phenol-preserved (HP) vaccine. HP vaccine of *S. typhimurium* was prepared in the standard manner (6). Dilutions of freshly prepared vaccine were made in saline to give a final concentration of 1.8×10^8 organisms per milliliter (McFarland no. 6) of saline containing 0.5% phenol. The vaccine was then put up in sterile vials, fitted with the appropriate sterile rubber stoppers, and stored in a refrigerator until ready for use.

Active-immunized mouse protection test. The potency of the vaccines was, for the most part, determined by active-immunized mouse protection tests as outlined by the Division of Biologics Standards (12). In each test, groups of 20 mice were immunized with 2 weekly subcutaneous (sc) injections in a volume of 0.5 ml. The number of bacteria in 0.5 ml of the HP vaccine was approximately 900×10^6 organisms. A group of 20 mice given 0.5 ml of saline served as a control for each challenge. All mice were challenged with the same *S. typhimurium* strain 7 days after immunization with approximately 10 LD₅₀ (LD₈₄) by the intraperitoneal (ip) route. This challenge dose was sufficient to kill 80 to 90% of the mice in the saline control group within 24 to 48 hr, and was prepared from the harvest of an 18-hr tryptose-phosphate-agar slant suspended in PF water. This harvest was standardized turbidimetrically by employing the turbidity reference standard (Division of Biologics Standards, National Institutes of Health), and confirming the challenge numbers by subsequent viability counts. The mouse virulence of each challenge culture was ascertained by ip inoculation of 0.5 ml of the cul-

ture suspended in PF water. The LD₅₀ values of approximately 2.66×10^6 bacilli, as calculated by both the methods of Reed and Muench (18) and Miller and Tainter (10), were obtained.

RESULTS

Summary of the pyrogen test responses in rabbits with the use of the endotoxoid preparations. A summary of pyrogen test responses in rabbits with the respective endotoxoid preparations is shown in Table 1. The data indicate a $> 100 < 200$ -fold reduction in rabbit pyrogenicity for the potassium periodate RE preparation compared on a weight basis with the parent RE and Boivin preparations. Although these experiments approximate the upper and lower limits of decrease in pyrogenicity for the preparations described in this report only, it is interesting to note that the pyrogen test results with both the potassium periodate RE and the Acet-RE preparations (9) are identical in terms of reduction of pyrogenicity in the rabbit. On the other hand, the results obtained with the former preparation indicate an approximate 25,000-fold reduction in pyrogenicity when compared with the periodate-treated *Escherichia coli* lipopolysaccharide (minimal pyrogenic dose, 0.0013 μ g/kg) as reported by Neter et al. (13). Table 1 also shows the rabbit pyrogen test results with the potassium methylate and boron trifluoride preparations which yielded a 10-fold loss and no loss of pyrogenicity, respectively, when compared with the parent RE preparation. These results are comparable with those reported by Johnson and Nowotny (5).

Lethality of potassium periodate RE, potassium methylate RE, boron trifluoride RE, RE, and

TABLE 1. Summary of the pyrogen test responses in rabbits with the use of the endotoxoid preparations of *Salmonella typhimurium*

Amt tested*	Fever responses† with				
	RE	Boivin	Boron trifluoride RE	Potassium periodate RE	Potassium methylate RE
0.1	—	—			
1.0	+	+	+	—	—
10	+		+	—	+
50				—	
100			+	—	+
200				+	
500			+		
1000					

* Micrograms per 33 ml of PF saline.

† Minus and plus indicate negative and positive rabbit pyrogen tests as interpreted by USP procedure.

Boivin preparations injected intraperitoneally into mice. Table 2 presents the data from experiments comparing the lethality in mice of ip injections of five individual potassium periodate RE concentrations with that of the parent RE and Boivin preparations, respectively. Both the periodate-treated RE and the parent RE preparations, under similar conditions, were nonlethal in mice at the concentrations shown (2,500 μg). These data suggest that the oxidation of the parent RE preparations with potassium periodate had little effect on mouse lethality. Moreover, these results appear to correlate well with the findings of Neter et al. (13) in that the periodate procedure yielded a compound of low toxicity. The results shown for the Boivin (trichloroacetic acid) preparation indicate, under similar conditions, that a "pure" endotoxin is much more lethal at higher concentrations than either the potassium periodate RE or parent RE preparations.

Table 3 shows the data from experiments comparing the lethality in mice by ip injection of five individual potassium methylate and BF_3 preparations with that of the parent RE preparation. A comparison of the parent RE preparation with similar amounts of potassium methylate and BF_3

TABLE 3. *Lethality of potassium methylate RE, boron trifluoride RE, and RE preparations of Salmonella typhimurium injected intraperitoneally into mice*

Prepn	Dose*	Cumulative deaths			
		1 day	2 days	3 days	7 days
Potassium methylate RE	μg				
	2.5	0	0	0	0
	25	0	0	0	0
	250	0	0	0	0
	500	0	0	0	0
Boron trifluoride RE	2,500	0	0	0	0
	2.5	0	0	0	0
	25	0	0	0	0
	250	0	0	0	0
	500	1	1	1	1
RE	2,500	0	0	0	0
	2.5	0	0	0	0
	25	0	0	0	0
	250	1	1	1	1
	500	1	1	1	1
Saline controls	2,500	0	0	0	0
	—	0	0	0	0

* All preparations were given in 0.5 ml of PF saline. Each dosage was administered to eight mice.

TABLE 2. *Lethality of potassium periodate RE, RE, and Boivin preparations of Salmonella typhimurium injected intraperitoneally into mice*

Prepn	Dose*	Cumulative deaths			
		1 day	2 days	3 days	7 days
Potassium periodate RE	μg				
	2.5	0	0	0	0
	25	0	0	0	0
	250	0	0	0	0
	500	0	0	0	0
RE	2,500	0	0	0	0
	2.5	0	0	0	0
	25	0	0	0	0
	250	0	0	0	0
	500	0	0	0	1
Boivin	2,500	0	0	1	1
	2.5	0	0	0	0
	25	0	0	0	0
	250	0	0	0	1
	500	2	2	3	3
Saline controls	2,500	5	7	7	8
	—	0	0	0	0

* All preparations were given in 0.5 ml of PF saline. Each dosage was administered to eight mice.

preparations suggests that neither of these procedures altered the lethality of the parent endotoxin molecule. That is, the mouse lethality data shown for the potassium methylate and BF_3 preparations in Table 3 agree favorably with those of Johnson and Nowotny (5), who reported similar findings with their preparations.

Immunogenic effect of potassium periodate RE, RE, and HP vaccine preparations in mice against a 10 LD₅₀ challenge of S. typhimurium. Since it has been shown previously (9) that a 10 LD₅₀ challenge dose gives a reproducible end point within the 3-day observation period, it was decided to employ this challenge for all experiments involving the various vaccine preparations. Table 4 shows results obtained with three groups of mice immunized with potassium periodate RE, RE, and HP vaccine preparations, respectively, and compared with a saline-treated control group. Each animal received two sc injections, 7 days apart, containing an arbitrarily arrived at dose of 100 $\mu\text{g}/0.5$ ml since < 200 μg of the potassium periodate RE preparation had been found to be the approximate minimal pyrogenic dose in rabbits (see Table 1). All groups of mice, including the saline control group, were challenged 1 week after the last immunizing injection with an ap-

TABLE 4. *Effect of immunization of potassium periodate RE, RE, and HP vaccine preparations in mice against a 10 LD₅₀ challenge of Salmonella typhimurium**

Treatment, 0.5 ml, sc†	Immunizing dose	No. of mice	Cumulative deaths						
			1 day	2 days	3 days	4 days	5 days	6 days	7 days
Potassium periodate RE	100 μ g	20	12	14	17 (S: > .05) ‡ (HP: < .01)	19	20	20	20 (S: > .05) (HP: < .01)
RE	100	20	6	6	11 (S: .01)	14	15	17	18 (S: > .05)
HP	—	19	0	0	0 (S: < .01)	5	6	9	11 (S: < .01)
Saline	—	20	16	19	19	20	20	20	20

* Challenged ip 7 days from last injection with approximately 3.32×10^7 organisms per 0.5 ml.

† Two injections, 7 days apart.

‡ *P* value.

proximate 10 LD₅₀ of *S. typhimurium*. By employing the Division of Biologics Standards criteria of recording lethality over a 3-day period, it can be seen in Table 4 that the HP vaccine-immunized mice were the best protected, followed by mice immunized with the parent RE and potassium periodate RE preparations, respectively. Statistical treatment of these data, as well as all subsequent challenge data, by chi-square analysis (7) suggests with confidence that the potassium periodate (oxidation) procedure destroys immunogenicity. The difference in protective capacity between the periodate vaccine preparation and the saline (S:) control mice at both the 3rd and 7th day of the observation period, as shown in Table 4, indicates no level of protection. However, when the periodate preparation was compared with the HP vaccine (HP:) preparation on the same days of observation, the results were significant at the 1% level. Further evidence that the periodate procedure destroys vaccine immunogenicity is seen in the comparison with the parent RE preparation in Table 4.

Immunogenic effect of potassium methylate RE, boron trifluoride RE, RE, and HP vaccine preparations in mice against a 10 LD₅₀ challenge of S. typhimurium. In this experiment, five groups of mice were immunized with potassium methylate RE, boron trifluoride RE, saline, HP vaccine, and RE preparations, respectively. Each animal received two sc injections, 7 days apart, containing an arbitrary dose of 100 μ g/0.5 ml. All groups of mice, including the saline control group, were challenged ip 1 week after the last immunizing injection with a dose of approximately 10 LD₅₀. It can be seen in Table 5 that, with the exception of the HP vaccine-protected mice, the potassium methylate RE-immunized mice showed the most

mouse protection over a 72-hr observation period, followed by the RE and BF₃ preparations respectively. It is interesting to note that the 100- μ g immunizing dose of the potassium methylate RE preparation gave approximately twice the protection in mice obtained with similar amounts of the BF₃, the RE, and, as elaborated in the Discussion, the Acet-RE immunizing preparations (9). Thus, the cumulative deaths for the potassium methylate RE and the HP vaccine preparations were very similar in their protective capacity in mice throughout the observation period, whereas the boron trifluoride RE and the RE preparations showed a definite breakdown in mouse immunity after 48 hr. The significance of these observations is seen from the respective chi-square analyses shown also in Table 5 for both the 3rd and 7th day after ip challenge. These *P* values again suggest that there is no significant difference (HP: > 0.05) in mouse protection between the potassium methylate RE and the HP vaccine preparation during the entire observation period, whereas the boron trifluoride RE preparation, under similar conditions, shows very little mouse protection.

Immunogenic effect with various concentrations of the potassium methylate RE vaccine preparation in mice against a 10 LD₅₀ challenge of S. typhimurium. Since it had been found previously (Table 1) that the minimal pyrogenic dose of the potassium methylate RE vaccine preparation is approximately 10 μ g ($> 1 < 10$), an attempt was made to determine whether the immunogenic effect in mice is retained by decreasing the immunizing dose of this preparation to two sc injections containing 10 and 50 μ g/0.5 ml, respectively. Table 6 shows the results of this experiment. Compared with the saline-immunized mice

TABLE 5. *Effect of immunization of potassium methylate RE, boron trifluoride RE, RE, and HP vaccine preparations in mice against a 10 LD₅₀ challenge of Salmonella typhimurium**

Treatment, 0.5 ml, sc†	Immunizing dose	Cumulative deaths						
		1 day	2 days	3 days	4 days	5 days	6 days	7 days
Potassium methylate RE	100 μ g	2	3	5 (S: <.01) ‡ (HP: >.05)	8	11	12	13 (S: .01) (HP: >.05)
Boron trifluoride RE	100	7	12	16 (S: >.05) (HP: <.01)	19	20	20	20 (S: >.05)
RE	100	2	11	11 (S: <.01)	15	17	18	18 (S: >.05)
HP	—	1	1	2 (S: <.01)	5	8	13	15 (S: .05)
Saline	—	16	18	20	20	20	20	20

* Challenged ip 7 days from last injection with approximately 2.95×10^7 organisms per 0.5 ml. Each treatment was administered to 20 mice.

† Two injections, 7 days apart.

‡ P value.

TABLE 6. *Effect of immunization with various concentrations of a potassium methylate RE vaccine preparation in mice against a 10 LD₅₀ challenge of Salmonella typhimurium**

Treatment, 0.5 ml, sc†	Immunizing dose	Cumulative deaths						
		1 day	2 days	3 days	4 days	5 days	6 days	7 days
Potassium methylate RE	10 μ g	8	11	18 (S: >.05) ‡ (HP: <.01)	19	19	19	19 (S: >.05)
Potassium methylate RE	50	3	5	9 (S: <.01) (HP: .05)	14	18	19	19 (S: >.05)
HP	—	0	0	2 (S: <.01)	7	10	12	13 (S: .01)
Saline	—	16	18	19	19	19	20	20

* Challenged ip 7 days from last injection with approximately 2.78×10^7 organisms per 0.5 ml. Each treatment was administered to 20 mice.

† Two injections, 7 days apart.

‡ P value.

after challenge, the 10- μ g immunizing dose of the potassium methylate RE vaccine preparation gave some protection for a 48-hr period after challenge. However, the 50- μ g immunizing dose of this preparation yielded a definite protective effect in mice during the first 3 days of the observation period. In addition, these latter results appear to compare favorably with the results obtained with HP vaccine-immunized mice challenged with the same 10 LD₅₀ dose. Chi-square analysis of these data revealed that, 72 hr after challenge, the difference in mouse protection between the potassium methylate RE and HP vaccine preparations was highly significant (HP:

< 0.01) with the 10- μ g immunizing dose whereas the 50- μ g dose was significant just at the 5% level (HP: < 0.05). These data (Tables 5 and 6) suggest that the minimal immunogenic dose of the potassium methylate RE vaccine preparation is approximately 50 μ g. However, as previously shown and elaborated in the Discussion, these results suggest a fivefold discrepancy between the minimal pyrogenic dose and the minimal immunogenic dose.

DISCUSSION

The data reported show that treatment of a crude endotoxin prepared by the RE procedure

from a strain of *S. typhimurium* with the various chemical procedures described yielded certain significant changes in biological activity. Others have reported similar data with a more purified endotoxin as the starting material. That is, with the exception of the potassium periodate RE preparation, the results obtained with our endotoxoids compared favorably with those prepared by others with regard to yield, pyrogenicity, lethality, and possibly mouse immunogenicity. Where differences do occur, it may be due to the various gram-negative organisms employed as well as the "purity" of the respective endotoxins used as starting materials. Although the RE method represents primarily denaturation of bacterial protein, the results in our studies with the RE preparation as the parent endotoxin suggest further that the molecular site or sites were not altered or exposed to the same extent as compared with the bacterial cell walls which were extracted with the many procedures capable of yielding a much "cleaner" preparation of endotoxin. Therefore, the differences reported that do exist between the endotoxoids studied by us and those reported by others are not of great magnitude.

The pyrogen test responses of the potassium periodate RE preparation indicated $> 100 < 200$ -fold reduction in pyrogenicity when compared with similar doses of parent RE and Boivin preparations. Moreover, it is of interest to note that the pyrogen test results with the potassium periodate RE preparation compare favorably with those reported for the Acet-RE preparation (9). On the other hand, the results obtained with this same periodate preparation indicated a $> 10,000 < 50,000$ -fold reduction in rabbit pyrogenicity when compared with the periodate-treated *E. coli* lipopolysaccharide of Neter and his associates (13). These workers reported that oxidation of their *E. coli* lipopolysaccharide with sodium periodate, prepared by the phenol-water method, yielded a compound of low toxicity and marked pyrogenicity (minimal pyrogenic dose, $0.0013 \mu\text{g/kg}$). Although the possibility of using potassium periodate instead of sodium periodate as the oxidizing agent cannot be discounted entirely, the apparent wide discrepancy between the two endotoxin preparations, at least with regard to minimal pyrogenic dose, suggests that the parent RE preparation responds more readily to the periodation procedure. The difference in "purity" of endotoxin starting material in this particular case apparently determines whether the molecular site or sites responsible for fever in rabbits will be altered as a result of chemical oxidation.

Under similar conditions, the potassium methylate RE preparation showed a reduced fever effect in rabbits, whereas the boron trifluoride RE preparation showed no reduction in pyrogenicity when compared with the parent RE preparation. Johnson and Nowotny (5) noted in their pyrogen studies that $1\text{-}\mu\text{g}$ amounts of their preparations would eliminate only the second peak in the biphasic fever responses, with their boron trifluoride preparation causing the most drastic reduction in this parameter. In contrast, employing the USP rabbit pyrogen test, our data indicate that the potassium methylate RE preparation reduced pyrogenicity from $1 \mu\text{g}$ (parent RE compound) to $10 \mu\text{g}$ per rabbit. However, the BF_3 preparation showed no reduction in pyrogenicity ($< 1 \mu\text{g}$) as per USP interpretation. Although our results compare favorably with those of Johnson and Nowotny (5), the differences that do exist in rabbit pyrogenicity with the various endotoxoids may be due once again to the "purity" of the respective parent endotoxins.

No enhancement or diminution in mouse lethality of the potassium periodate, potassium methylate, and BF_3 preparations was noted when compared with the parent RE preparation. That is, none of these preparations approached a mouse LD_{50} with the amounts tested ($2,500 \mu\text{g}$). These results show general agreement with those reported by Neter et al. (13) and those of Johnson and Nowotny (5). Neter and his co-workers (13) found that a mouse LD_{50} value for their sodium periodate preparation was approximately 30 mg/kg (or $600 \mu\text{g}$ per 20-g mouse), whereas Johnson and Nowotny (5) reported respective LD_{50} values for their potassium methylate and BF_3 preparations of $> 4,000 \mu\text{g}$ and $> 2,000 \mu\text{g}$ per mouse. However, the respective parent endotoxin mouse LD_{50} values were 3.0 mg/kg , or $60 \mu\text{g}$ per 20-g mouse (13), and $180 \mu\text{g}$ per mouse (5), as compared with a mouse LD_{50} of $> 2,500 \mu\text{g}$ for the RE preparation. These results indicated to Neter et al. (13), as well as Johnson and Nowotny (5), that their respective procedures were capable of reducing the mouse lethality of their parent purified endotoxins. Of interest, however, is the finding that the mouse LD_{50} of both our Acet-RE and RE preparations were also $> 2,500 \mu\text{g}$ (9), whereas Freedman and Sultz (3), by acetylating a purified endotoxin, showed data similar to those reported by the above authors. All of these findings give support to the idea that the molecular site or sites of the RE preparation responsible for mouse lethality are apparently "masked," whereas the purified endotoxins have their site or sites exposed to such an extent that

comparatively small (microgram) amounts are capable of inducing equivalent LD₅₀ results in mice.

A critical evaluation of the immunogenicity of the respective endotoxoids was obtained by employing the active-immunized mouse protection test and using a readily reproducible method of challenge with a 10 LD₅₀ dose instead of the usual 1 LD₅₀ dose (9). The reproducibility of this 10 LD₅₀ challenge dose in each of the active-immunized mouse protection tests shown in Tables 4, 5, and 6 has been remarkably consistent with these conditions of challenge, and it was generally assumed that protection was dependent on the immunogen and the immunization schedule.

A comparison of the immunogenic effect in mice of the HP vaccine to two subcutaneous 100- μ g immunizing doses of the potassium periodate (Table 4) as well as the potassium methylate, BF₃, and RE preparations (Table 5) showed that the potassium methylate RE vaccine preparation gave the best protection. In contrast, both the BF₃ and the periodate preparations were the least protective. In addition, the potassium methylate preparation showed some protection in mice immunized with two weekly sc doses as low as 10 μ g when compared with the saline-immunized mice (Table 6). These data strongly indicate that the potassium methylate procedure when applied to an RE endotoxin preparation increases its antigenicity in mice, whereas the respective BF₃ and periodate treatments of the same parent endotoxin show almost identical decreased mouse antigenicity. However, Johnson and Nowotny (5) reported that, when compared with their parent endotoxin, both of their chemical methods (BF₃ and potassium methylation) resulted in decreased antigenicity in rabbits when each of these preparations was given intravenously in a single dose of 1 μ g. They further reported that the BF₃ procedure appeared to be more drastic in disrupting antigenicity than the potassium methylate procedure. These results were similar to those previously published by Nowotny (15), where it was shown that several injections of the BF₃-treated preparation into rabbits resulted in antibody levels lower than those of the potassium methylate preparation, as well as the control endotoxin. In addition, this investigator suggested that the BF₃ procedure destroyed the O antigen determinant group (carbohydrates), whereas the potassium methylate procedure appeared not to affect this component. Our data, employing these preparations as vaccines in mice, show general agreement with these observations.

The effect of decreased antigenicity of the potassium periodate RE vaccine preparation

suggests that oxidation of the parent RE preparation is also destroying or, at least, modifying the specific antigenic sites involved in mouse protection. Neter et al. (13) concluded from their *in vitro* studies that periodate-treated purified *E. coli* lipopolysaccharide had antigenic characteristics which were markedly altered. They suggested that the amount of periodate used up corresponds to one C—C cleavage of every fourth sugar unit, with each cleavage resulting in two titratable aldehyde groups. With this type of severe oxidation, it is not surprising that our potassium periodate-treated RE preparation is poorly antigenic in mice.

The exact nature of the antigenic site or sites disrupted by acetylation as well as the potassium periodate, potassium methylate, and BF₃ procedures, which leads to apparent detoxification, is as yet unknown. However, whatever the nature of the antigenic site or sites disrupted by these chemical procedures, the method of acetylation held the most promise for our purpose of trying to develop a less toxic enteric fever vaccine. Although the data indicate that the difference between the minimal pyrogenic dose (100 μ g versus 10 μ g) and the minimal immunogenic dose (200 μ g versus 50 μ g) is approximately the same for both the acetylated and the potassium methylate preparations, it was felt that the procedure of acetylation, which apparently has the greatest effect on reducing pyrogenicity, should be considered further (9). Moreover, results with immunodiffusion tests (Martin, Ph.D. Thesis, Univ. of Utah, Salt Lake City, 1965), support these observations. The acetylated as well as the potassium methylate RE vaccine preparations, in contrast to the BF₃ and periodate preparations, reacted specifically with the immune rabbit serum, thus indicating that antigenicity was intact. Although the above consideration of reducing rabbit pyrogenicity led us to select the Acet-RE vaccine preparation for further study in mouse protection, the observation that the potassium methylate RE vaccine preparation demonstrates twice the protective response in mice against the same 10 LD₅₀ challenge on a dose-response relationship must not be overlooked. The further work indicated to resolve these problems is being carried out at the present time.

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