Detoxified Bacterial Endotoxins

I. Preparation and Biological Properties of an Acetylated Crude Endotoxin from *Salmonella typhimurium*¹

WILLIAM J. MARTIN² AND STANLEY MARCUS

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

Received for publication 20 November 1966

ABSTRACT

MARTIN, WILLIAM J. (University of Utah, Salt Lake City), AND STANLEY MARCUS. Detoxified bacterial endotoxins. I. Preparation and biological properties of an acetylated crude endotoxin from *Salmonella typhimurium*. J. Bacteriol. 91:1453-1459. 1966.—Acetylation of a crude endotoxin prepared by the Roschka-Edwards (RE) procedure from a strain of *Salmonella typhimurium* yields a product with reduced pyrogenicity in rabbits as well as one which is nontoxic in mice. A reduction in pyrogenicity of approximately 100 times was noted with this acetylated crude endotoxin when compared with the parent RE preparation. A comparison was made of immunogenicity with mice of Boivin, RE, and acetylated Roschka-Edwards (Acet-RE) preparations with a heat-killed, phenol-preserved (HP) vaccine prepared from the same strain of *S. typhimurium*. Less than pyrogenic doses of all vaccines were not protective. The least pyrogenic preparation (Acet-RE) was immunogenically effective in about five times the minimal pyrogenic dose. The data suggest that the Acet-RE preparation should be considered further in the search for enteric fever vaccines with lowered potential for undesirable systemic responses.

The toxicity of available vaccines against *Salmonella typhosa* and other enterobacteriaceal organisms is a problem, particularly for international travelers and for those under military conditions, where the loss in personnel time due to immunization reactions may be significant. Attempts to provide protection against salmonellae and *Salmonella*-like enteric diseases with polyvalent vaccines must also take into account the additive nature of the toxicities of the component organisms. A procedure that would minimize side reactions would be to reduce the amount of each component until the toxicity of the individual components was within tolerable limits. Since the antigenic potential of a bacterial vaccine is influenced by concentration of bacteria, however, this procedure might result in use of less than immunogenic concentrations of vaccine. In

recent years, the procedure of chemically modifying the endotoxin molecule by acetylation with recovery of a preparation showing reduced pyrogenicity and lethality without the apparent destruction of immunogenicity has been reported by Treffers (13), Treffers et al. (Federation Proc. 5:255, 1946), and Freedman and his associates (2, 3, 12). In this paper we describe the acetylation of a crude endotoxin material obtained by the Roschka-Edwards (RE) method from a strain of *Salmonella typhimurium*. Results indicate that the preparation obtained had reduced pyrogenicity in rabbits, although no apparent reduction in mouse lethality was observed when compared with the parent RE preparation. In addition, data are shown concerning immunogenicity in mice of a Boivin vaccine preparation, a RE vaccine preparation, and the acetylated RE vaccine preparation compared with a heat-killed, phenol-preserved vaccine prepared from the same strain of *S. typhimurium*. The hypothesis examined is that a nonpyrogenic, nontoxic product may still retain its immunogenicity, i.e., act as an "endotoxoid." An ultimate objective of such work is to use this product as a less toxic enteric fever vaccine.

¹ This paper is part of a thesis submitted by W. J. Martin to the Department of Microbiology, University of Utah, in partial fulfillment of the requirements for a Ph.D. degree, and was presented in part at the Annual Meeting of the American Society for Microbiology, Atlantic City, N.J., April 1965.

² Present address: Enteric Bacteriology Unit, Communicable Disease Center, Atlanta, Ga.

MATERIALS AND METHODS

Experimental animals. Testing for pyrogenicity was done in rabbits (albino *Oryctolagus cuniculus*) that had been properly "screened" by methods previously reported (6). In brief, previously sham-tested male and female rabbits (3 to 5 kg) obtained from a local source were caged individually in a constant-temperature room. Pyrogen tests were carried out according to techniques described in *The United States Pharmacopeia XVI*, with the following exceptions: all glassware was boiled in detergent solution, then rinsed with nonpyrogenic distilled water, and sterilized by heating in an oven at 250 C for 2 hr. Pyrogen-free (PF) saline solutions to be tested were at room temperature rather than at 37 C; total volumes of PF saline solutions injected were 33 ml per rabbit instead of the usual 10 ml/kg of rabbit weight; temperature recordings were taken at 1 hr and immediately prior to injection, and at 15 min, 1, 2, and 3 hr after injection. The results were interpreted as per *The United States Pharmacopeia XVI*. Rabbits which yielded positive fever results, and which were used in more than one experiment involving pyrogenicity, were "rested" for at least 2 weeks before being reused.

In each experiment, one group of rabbits injected with PF saline was included. This group served as a control for technique and other external factors such as temperature, humidity, and noise.

Mice. Tests for immunogenicity and lethality were performed in the morning with white mice (albino *Mus musculus*) weighing approximately 20 to 25 g; deaths were recorded for a period of 1 week.

Organism employed. A mouse-virulent strain of *S. typhimurium* was employed in all experiments and was originally obtained from the Utah State Department of Health. This organism has been maintained by transfer culture at monthly intervals on nutrient agar slants. Transfers were incubated at 37 C for 24 hr and were thereafter kept at room temperature.

Preparations for the RE procedure. Crude endotoxin was prepared according to the procedure of Edwards (1), which is primarily an alcohol-acetone treatment of bacterial cells. The sediment was dried at 37 C and then ground to a fine powder. The resulting dried 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 was disintegrated (Sonifier, Branson Instruments, Stamford, Conn.) for 15 min under sterile conditions. The suspension was then filtered and the precipitate was dried overnight at 37 C. This procedure of ultrasonic exposure produced no visible changes in yield or in alteration of the crude endotoxin. Hereafter, this product will be referred to as the parent RE preparation.

Preparations for acetylation procedure (2). To 500 mg of parent RE preparation were added 115 ml of acetic anhydride and 75 mg of anhydrous powdered sodium acetate as a basic catalyst. The reaction mixture was heated in a capped centrifuge bottle in a boiling-water bath for 4 hr with occasional mixing. After cooling, the acetic anhydride was removed as a

clear solution by decantation. The remaining insoluble material was freed of anhydride by drying at room temperature under a stream of nitrogen. Once dried, it was ground to a powder by mortar and pestle. The powder was washed three times with 100-ml portions of saline, and once with water. The washes were removed by centrifugation at low speed, yielding a clear supernatant solution which was discarded. The washed precipitate was then dried under nitrogen and again ground to a powder. This powder, amounting to approximately 65% of the starting material, was granular in appearance, light tan in color, and strongly lyophobic. The yield was 324 mg (64.8%) with some loss apparently due to manipulative procedure. Chemical analysis of the acetyl content (Australian Microanalytical Service, Univ. of Melbourne) as COCH_3 (methanolic NaOH used as the saponification agent) was 6.8%. A stock solution of 50 mg/10 ml was readily prepared by adding a small amount (0.5 to 1.0 ml) of saline and triturating thoroughly with a mortar and pestle to wet and disperse the solids. Saline was added in small samples as the lyophobic powder was worked into suspension. It should be noted that, as with the parent RE preparation, this stock solution should be mixed thoroughly before testing, since the suspension is rather dense and separates readily. Hereafter, this product will be referred to as the acetylated Roschka-Edwards (Acet-RE) preparation.

Attempts to recover an anhydride-soluble, water-insoluble material by pouring the anhydride solution into excess cold water and filtering it yielded a small amount of solids which accounted for less than 10% of our starting material. Since the yield was small, no attempt was made to test its biological effects in animals.

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 prepared to be pyrogen-free (PF) (6).

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

Heat-killed, phenol-preserved (HP) vaccine. A freshly prepared heat-killed, phenol-preserved (HP) vaccine of *S. typhimurium* was prepared in the following manner. Cultures (18 hr) of *S. typhimurium*, grown on tryptose-phosphate (Difco) agar, were harvested in saline. After the suspension was filtered through sterile glass wool, the organisms were heated in a 56 C water bath for 2 hr. After heating, the suspension was cooled to room temperature, and was standardized turbidimetrically by the McFarland method (4). Dilutions were prepared in PF saline to give a final concentration of 1.8×10^9 organisms per milliliter (McFarland #6) of saline containing 0.5% phenol. The vaccine was then put in sterile vials, fitted with the appropriate sterile rubber stoppers, and stored in a refrigerator until ready for use.

Method of challenge. The potency of the vaccines was, for the most part, determined by active-immunized mouse-protection tests as outlined by the Public Health Service, Division of Biologics Standards (8). In each test, groups of 20 mice were immunized

TABLE 1. Summary of pyrogen test responses in rabbits with three preparations of *Salmonella typhimurium*

Amt tested*	Fever responses with		
	RE	Boivin	Acet-RE
0.1	—†	—	—
1.0	+†	+	—
10	+		—
100			—
200			+
500			+
1,000			+

* Micrograms per 33 ml of PF saline.

† Negative or positive pyrogen test, USP XVI.

with two, 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) doses by the intraperitoneal (ip) route. This challenge dose was sufficient to kill about 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 obtained from the Division of Biologics Standards (DBS); the challenge numbers of organisms were estimated by subsequent viability counts. The mouse virulence of each challenge culture was ascertained by ip inoculation of 0.5 ml of the culture suspended in PF water. The LD₅₀ values of approximately 2.66×10^6 bacilli, as calculated by the methods of both Reed and Muench (11) and Miller and Tainte (7), were obtained.

RESULTS

Summary of the pyrogen test responses in rabbits with use of the Acet-RE, RE, and Boivin preparations. The reduction in pyrogenicity exhibited by the Acet-RE preparation is shown in summary form in Table 1. These pyrogen test results were interpreted by USP definition and suggest a minimal pyrogenic dose (MPD) of 100 µg in the rabbit. Thus, the data indicate a $> 100 < 200$ -fold reduction in pyrogenicity when compared with comparable doses of untreated RE and Boivin preparations. It is of interest to note that these results compared well with those reported by Freedman and co-workers (2) with use of acetylated Boivin preparations.

Lethality of Acet-RE, RE, and Boivin preparations injected intraperitoneally into mice. In Table 2 are shown the data from experiments compar-

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

Preparation	Dose*	No. of mice	Cumulative deaths			
			1 day	2 days	3 days	7 days
Acet-RE	µg 2.5	8	0	0	0	0
	25	8	0	0	0	0
	250	8	0	0	0	0
	500	8	1	1	1	1
	2,500	8	2	2	2	2
RE	2.5	8	0	0	0	0
	25	8	0	0	0	0
	250	8	0	1	1	1
	500	8	0	0	0	0
	2,500	8	0	0	0	0
Boivin	2.5	8	0	0	0	0
	25	8	0	0	0	0
	250	8	1	1	1	1
	500	8	1	2	4	4
	2,500	8	6	7	7	7
Saline controls	—	8	0	0	0	0

* All preparations given in 0.5 ml of saline.

ing the lethality by ip injection of a pool of five individual acetylated preparations with that of the parent RE "crude" endotoxin preparations and "pure" endotoxin (Boivin), respectively. Although 3 of 16 mice injected with 500 µg or more of the Acet-RE preparation died, the results of these experiments indicate that, for all practical purposes, the procedure of acetylation neither enhanced nor diminished the already existing nonlethal property of the parent RE preparation.

*Immunogenic effect of HP vaccine preparation in mice against an LD₅₀ and a 10 LD₅₀ challenge of *S. typhimurium*.* To evaluate the protective capacity of the HP vaccine, as well as the challenge procedure against both an LD₅₀ and a 10 LD₅₀ challenge of *S. typhimurium*, an immunization experiment was carried out (Table 3). Two groups of mice were immunized with HP vaccine by sc injection with two 0.5-ml doses containing 900×10^6 organisms per 0.5 ml at 7-day intervals. In addition, one group of mice served as controls, receiving only sc injections of saline. Seven days after immunization, one group of immunized mice, as well as the group of saline control mice, was challenged ip with approximately 10 LD₅₀ doses, whereas the second group of immunized mice received an LD₅₀ challenge dose. It can be seen in Table 3 that, by employing the

TABLE 3. *Effect of immunization with HP vaccine preparation in mice against a 1 LD₅₀ and a 10 LD₅₀ challenge of Salmonella typhimurium*

Treatment (0.5 ml, sc)*	No. of bacteria per mouse ip†	No. of mice	Cumulative deaths						
			1 day	2 days	3 days	4 days	5 days	6 days	7 days
Immunized (HP vaccine)	$3.73 \times 10^7/0.5$ ml (10 LD ₅₀)	20	0	0	1	14	16	18	19
Immunized (HP vaccine)	$2.66 \times 10^6/0.5$ ml (LD ₅₀)	17	0	0	0	0	0	8	13
Nonimmunized saline controls	$3.73 \times 10^7/0.5$ ml (10 LD ₅₀)	25	19	22	23	24	25	25	25
Nonimmunized saline controls‡	$2.66 \times 10^6/0.5$ ml (LD ₅₀)	20	3	8	11	16	17	18	18

* Two injections 7 days apart.

† Challenged 7 days from last injections.

‡ Reference LD₅₀ dose.TABLE 4. *Effect of immunization with Acet-RE, RE, Boivin, 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
Acet-RE.....	µg 100	20	10	13	13 (S: < .01) ‡ (HP: < .01)	15	18	18	18 (S: > .05)
RE.....	100	20	2	11	11 (S: < .01)	15	17	18	18 (S: > .05)
Boivin.....	100	19	3	5	7 (S: < .01)	11	15	16	17 (S: > .05)
HP.....	—	20	0	0	1 (S: < .01)	6	9	13	16 (S: > .05)
Saline.....	—	20	17	20	20	20	20	20	20

* Challenged ip 7 days from last injection with approximately 3.47×10^7 bacteria per 0.5 ml.

† Two injections 7 days apart.

‡ P value.

DBS criteria for recording mortalities over a 3-day period, both groups of immunized mice were protected against their respective challenges. However, the saline control mice show mortalities of greater than 90% in the same 3-day period, thus indicating good agreement with the estimate of a 10 LD₅₀ response. It is of interest to note that, after 3 days, the cumulative deaths of the immunized mice challenged with 10 LD₅₀ showed a marked increase on the 4th day, whereas the immunized mice challenged with a LD₅₀ were protected up through the 6th day.

Immunogenic effect of Acet-RE, RE, Boivin, and HP vaccine preparations in mice against a 10 LD₅₀ challenge of S. typhimurium. Since it was found (Table 3) that the 10 LD₅₀ challenge gave a reproducible end point within the 3-day observation period, it was decided to employ this dose for all challenge experiments involving the various

vaccine preparations. The data in the next two tables (Tables 4 and 5) show results of experiments with groups of mice immunized with the Acet-RE, RE, Boivin, saline, and HP vaccine preparations, respectively. Each animal received a total of 2 sc injections, the second 7 days after the first, with the amounts shown under the column headed Immunizing dose. The immunizing doses of 100 and 200 µg/0.5 ml for the various preparations shown in both tables were arbitrarily arrived at, since < 200 µg of the Acet-RE preparation was found to be nonpyrogenic in rabbits. By again employing the DBS criteria of recording deaths over a 3-day period, the HP vaccine provided the best protection when compared with the respective saline control mice as well as with the mice immunized with 100-µg dose amounts. Of the latter, as shown in Table 4, the mice immunized with the Boivin preparation

TABLE 5. *Effect of immunization with Acet-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
Acet-RE.....	200	20	1	2	7 (S: < .01)‡ (HP: > .05)	10	17	20	20 (S: > .05)
RE.....	200	19	1	1	4 (S: < .01)	9	12	15	17 (S: > .05)
HP.....	—	20	1	1	2 (S: < .01)	5	8	13	15 (S: > .01–< .05)
Saline.....	—	20	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.

† Two injections 7 days apart.

‡ P value.

TABLE 6. *Effect of immunization with two, weekly 500-μg immunizing doses (total 1,000 μg) of an Acet-RE vaccine preparation 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
Acet-RE.....	500	20	0	0	3 (S: < .01)‡ (HP: > .05)	8	9	11	13 (S: > .01)
HP.....	—	18	0	0	0 (S: < .01)	5	8	10	13 (S: > .01–< .05)
Saline.....	—	20	16	18	19	20	20	20	20

* Challenged ip 7 days from last injection with approximately 3.23×10^7 organisms per 0.5 ml.

† Two injections 7 days apart.

‡ P value.

show the greatest protection, followed by the RE and Acet-RE vaccine preparations, respectively.

The data presented in Table 4 and data from other active-immunized mouse protection experiments employing the MPD of 100 μg were essentially the same. However, as shown in Table 5, the deaths recorded over a 3-day period in mice immunized with two 200-μg (two times the MPD) doses with the same Acet-RE vaccine preparation indicate a good minimal immunogenic dose (MID) when compared with the saline-immunized mice after challenge. Moreover, these results correlated well with the results obtained with the HP vaccine-immunized mice challenged with the same 10 LD₅₀. That is, the HP vaccine protected 90% of the mice as compared with 65% protection in the 200 μg (Acet-RE)-immunized mice. Further evidence of this correlation between the Acet-RE and HP vaccine preparations was shown by chi-square analysis (5). Although both of these preparations show significant protection

at the 1% level when compared with the saline control group after 3 days, the difference in mouse protection between the Acet-RE and HP vaccine preparations was not significant (HP: > .05). Thus, under the conditions of these experiments, the 200-μg immunizing dose of the Acet-RE vaccine preparation, but not the 100-μg dose, yielded results not significantly different from those obtained with HP vaccine.

Immunogenic effect with two, weekly 500-μg immunizing doses (total 1,000 μg) of the Acet-RE vaccine preparation in mice against a 10 LD₅₀ challenge of S. typhimurium. Since all previous results, including results not reported here, indicated that the HP vaccine is more protective in mice against a 10 LD₅₀ challenge than the 100-μg Acet-RE immunizing dose employed (Table 4), the hypothesis was examined that the Acet-RE vaccine preparation is not as immunogenic as the HP vaccine. That is, rather than relying on the approximate MPD of the Acet-RE preparation

as the MID to employ in mouse-immunization experiments, the following considerations suggested other possibilities. Approximately 1 mg of wet bacteria contains 10^9 bacterial cells (10) or approximately 200 μg of solids (dry weight). The HP vaccine employed in our active mouse-immunization experiment contains approximately 900×10^6 bacteria per 0.5 ml. This value suggested that our mice were receiving anywhere from 200 to 500 μg per immunizing dose of HP vaccine. The Acet-RE preparations had been used only in 100 and 200 $\mu\text{g}/0.5$ ml immunizing doses, since the MPD of this preparation is approximately 100 μg . Thus, we may have been administering two to five times more of the HP vaccine than of the Acet-RE preparation. This possibility was tested experimentally (Table 6). Animals injected with 500- μg immunizing doses of the Acet-RE vaccine preparation were compared with mice similarly injected (twice) with HP vaccine; the results support the hypothesis that there is a factor of approximately 2 to 5 in vaccine strength between the two vaccines. That is, no significant difference in mouse mortality either at the 3-day or 7-day observation period occurs. These data suggest that the difference between the MPD (100 μg) and the MID (200 μg), as well as the effective immunogenic dose (EID) (500 μg), is approximately two to five in rabbits and mice, respectively. In other words, it takes approximately two to five times the MPD of the Acet-RE preparation to give an immunogenic dose equal to that of the HP vaccine.

DISCUSSION

It is apparent that acetylation of a crude endotoxin prepared by the RE procedure from a strain of *S. typhimurium* yielded a material which was nontoxic in mice and had a reduced fever effect in rabbits. That is, the results obtained with our endotoxoid compared favorably with those prepared by Freedman and Sultz (2) with regard to yield, pyrogenicity, lethality, and possibly mouse immunogenicity. Where differences do occur, they may be due to the various gram-negative organisms employed, as well as the "purity" of the respective endotoxins used as starting materials.

Since the RE procedure represents primarily an attack on and presumably denaturation of bacterial protein, these results suggest that the molecular site or sites of bacterial structure responsible for fever induction in rabbits are not altered to any great extent when the bacterial cell walls are extracted with trichloroacetic acid, as is done in the Boivin procedure. Moreover, studies employing purified enterobacterial lipopolysaccharides indicate that various biological activities

of bacterial endotoxins are affected differentially by different physical and chemical agents (9). A similar concept may be derived from our studies. The results reported here with the RE preparation as the parent product suggest the possibility that the molecular site or sites of this crude endotoxin were not altered or exposed to the same extent, as compared with the bacterial cell walls which were extracted by procedures (phenol-water, aqueous ether, trichloroacetic acid, etc.) capable of yielding a much "purer" preparation of endotoxin.

Although the experimental rabbit pyrogen data (Table 1) approximate the upper and lower limits of decrease in pyrogenicity under our conditions only, it is of interest to note that the pyrogen test results with the Acet-RE preparation compare favorably with those reported by Freedman and Sultz (2). These investigators noted a marked reduction in pyrogenicity with their acetylated endotoxin (fraction P) of 1,000-fold and 100-fold, respectively. Thus, any real differences in rabbit pyrogenicity that do exist with the respective endotoxoids may be due, as previously mentioned, to the "purity" of the respective parent endotoxins.

Of interest is the finding that the mouse LD_{50} of both the Acet-RE and the RE preparation was $>2,500$ μg , whereas Freedman and Sultz (2) reported an LD_{50} response in mice of 250 μg with their parent endotoxin derived from *S. typhosa*, while acetylation of this same endotoxin yielded a mouse LD_{50} of approximately 2,000 μg . These observations lend support to the idea that the molecular site or sites of the RE preparation responsible for mouse lethality are apparently "masked." That is, under our experimental conditions, anywhere from 10 to 50 times more of the parent RE preparation ($>2,500$ μg) is needed to produce the same LD_{50} response as those reported above for the Boivin endotoxin.

A critical evaluation of the immunogenicity of the vaccine preparations was obtained by employing the active-immunized mouse protection test. The reproducibility of the 10 LD_{50} challenge dose in each of the active mouse-protection tests has been remarkably consistent with these conditions of challenge. The saline control mice included in each experiment showed a reproducible 10 LD_{50} response within 72 hr after ip challenge with the test strain of *S. typhimurium*. Therefore, it was assumed that protection was dependent on the immunogen and the immunization schedule. Moreover, this was observed in mice with all of our endotoxoid preparations (Martin and Marcus, *in preparation*) when comparison was made with both the HP vaccine and the saline-treated mice.

The protection afforded mice by the HP vac-

cine as compared with two or more sc 100- μ g immunizing (nonpyrogenic) doses of the Acet-RE, RE, and Boivin vaccine preparations (Table 4) suggests that the HP vaccine is much more protective against a 10 LD₅₀ challenge of *S. typhimurium*. However, two sc 200- or 500- μ g immunizing (pyrogenic) doses of the Acet-RE preparation followed by 10 LD₅₀ challenge (Tables 5 and 6) indicate a minimal (MID) and an effective (EID) immunogenic dose, respectively, when compared with the HP vaccine. Moreover, the apparent similarity between the parent RE and Acet-RE vaccine preparations in their protective ability in mice suggests that acetylation has not immunogenically altered the antigenic sites to any great extent on the original RE preparations.

These data indicate that it takes approximately five times the MPD of the Acet-RE preparation to yield an approximate EID equal to that of the HP vaccine. However, the difference of approximately a 50- to 100-fold reduction in rabbit pyrogenicity over that of the parent RE preparation may be of critical significance. This observation suggests a less pyrogenic vaccine if it is used in immunogenic concentrations equal to the HP vaccine. These results suggest that the Acet-RE preparation should be considered further in the search for enteric fever vaccines with lowered potential for undesirable systemic responses.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants K6-AI-14,924 and CA 07302 from the National Institutes of Health.

During this investigation, William J. Martin was the recipient of a Public Health Service predoctoral fellowship 5 FLGM-18,194 from the Division of General Medical Sciences.

LITERATURE CITED

1. EDWARDS, P. R. 1951. Preparation of antisera for detection of the somatic antigens of *Salmonella* cultures. Public Health Rept. U.S. 66:837-839.
2. FREEDMAN, H. H., AND B. M. SULTZER. 1962. Dissociation of the biological properties of bacterial endotoxin by chemical modification of the molecule. J. Exptl. Med. 116:929-942.
3. 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.
4. KOLMER, J. A., AND F. BOERNER. 1945. Approved laboratory technic, 4th ed., p. 493-495. Appleton-Century-Crofts, Inc., New York.
5. MAINLAND, D., L. HERRERA, AND M. I. SUTCLIFFE. 1956. Statistical tables for use with binomial samples—contingency tests, confidence limits, and sample size estimates. New York Univ. Press, New York.
6. MARTIN, W. J., AND S. MARCUS. 1964. Studies on bacterial pyrogenicity. III. Specificity of the United States Pharmacopeia rabbit pyrogen test. Appl. Microbiol. 12:483-486.
7. MILLER, L. C., AND M. L. TAINTER. 1944. Estimation of the ED₅₀ and its error by means of logarithmic-probit graph paper. Proc. Soc. Exptl. Biol. Med. 57:261-264.
8. NATIONAL INSTITUTES OF HEALTH. 1953. Minimum requirements for typhoid vaccine. 2nd revision. National Institutes of Health, Bethesda, Md.
9. 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.
10. OGINSKY, E. L., AND W. W. UMBREIT. 1959. An introduction to bacterial physiology, p. 16. W. H. Freeman and Co., San Francisco.
11. REED, L. J., AND H. MUENCH. 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. 27:493-497.
12. SULTZER, B. M., AND H. H. FREEDMAN. 1962. The stimulation of nonspecific host resistance to infection by chemically modified endotoxin. J. Exptl. Med. 116:943-956.
13. TREFFERS, H. P. 1946. The detoxification by acetylation of soluble antigens from *Shigella dysenteriae* (shiga) and *E. typhosa*. Science 103:387-389.