

# Hypoferremia in Mice and Its Application to the Bioassay of Endotoxin

PHILLIP J. BAKER<sup>1</sup> AND J. B. WILSON

*Department of Bacteriology, University of Wisconsin, Madison, Wisconsin*

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## ABSTRACT

BAKER, PHILLIP J. (University of Wisconsin, Madison), AND J. B. WILSON. Hypoferremia in mice and its application to the bioassay of endotoxin. *J. Bacteriol.* 90:903-910. 1965.—The ability of endotoxin to induce hypoferremia in mice was used for the bioassay of endotoxin. A marked depression in the serum-iron levels of mice occurred 12 hr after the intraperitoneal injection of 0.01 to 100  $\mu\text{g}$  of *Escherichia coli* endotoxin; similar results were obtained with 1.0 to 100  $\mu\text{g}$  of *Brucella abortus* endotoxin. This biological response to endotoxin appeared to be specific, reproducible, and dose-dependent. As heat-killed cells of *B. abortus* and *E. coli* were also able to induce hypoferremia, this bioassay could be employed for the determination of the endotoxin content of killed-cell preparations. Treatment of endotoxin by acid hydrolysis, acetylation, or pyridine-formic acid greatly diminished the hypoferremic response as well as its lethality for mice. Pretreatment of mice with Thorotrast had little effect upon the ability of endotoxin to induce hypoferremia; however, a stimulation of the activity of the reticuloendothelial system (RES) by treatment of mice with triolein markedly reduced the ability of endotoxin to induce hypoferremia. The relationship between the hypoferremic response to endotoxin and alterations in the activity of the RES are discussed.

These investigations are a continuation of work concerning factors related to virulence in *Brucella abortus* (Wilson and Dasinger, 1960). In particular, we were interested in determining whether strains of *B. abortus* of high virulence have a greater endotoxin content than do strains of low virulence. This necessarily requires a reliable and sensitive assay for endotoxin, as well as one which is suitable for use with a large number of samples.

It has been our experience (Baker, 1962) and the experience of others (Ribi et al., 1964) that it is difficult to extract endotoxin quantitatively from intact-cell preparations, even if the extraction process is repeated several times. Therefore, it would be desirable to use a system of bioassay in which killed-cell preparations as well as purified endotoxin preparations could be employed. Thus, one could determine the endotoxin content of killed-cell preparations directly and eliminate the need for quantitative extraction.

In prior experiments (Wilson, Kolbye, and Baker, 1964), mouse LD<sub>50</sub> values were determined with heat-killed and acetone-killed cells of *B. abortus* 2308 (high virulence) and *B. abortus* 11

(low virulence). Although no differences in endotoxin content were noted, we felt that a more sensitive method of bioassay might reveal small differences in endotoxin content. An evaluation of commonly used methods for the bioassay of endotoxin has been prepared by Braude (1964), but few of the listed procedures were suitable for our purposes since we desired to use mice, which could be obtained in large numbers and at a low cost. We attempted to use the mouse urinary nitrogen excretion assay for endotoxin as described by Berry and Smythe (1961); however, we had difficulty obtaining a uniform response to adrenocorticotrophic hormone (ACTH) in several strains of mice (Wilson et al., 1964.)

Kampschmidt and Upchurch (1962) had reported that a single injection of 0.0001  $\mu\text{g}$  of *Escherichia coli* endotoxin produced significant hypoferremia in rats. A maximal decrease in serum or plasma iron occurred within 8 to 16 hr after the administration of endotoxin, and the degree of hypoferremia appeared to be dose-dependent. It occurred to us that these findings might be applied in an extremely sensitive system for the bioassay of endotoxin with mice as the experimental animal. In the studies to be described, the specificity of the response for endotoxin, the range

<sup>1</sup> Present address: Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, Bethesda, Md.

of sensitivity of the proposed system of bioassay, and the relationship between the hypoferremic response to endotoxin and its lethal effects in mice were investigated.

#### MATERIALS AND METHODS

**Cultures.** Two smooth strains of *B. abortus*, 11 (low virulence) and 2308 (high virulence), were used in these studies. These were obtained from the Bureau of Animal Industry, U.S. Department of Agriculture, and have been maintained and preserved for about 20 years in this laboratory, in subculture or in a lyophilized condition, without change in virulence or changes in cultural characteristics. The virulence of the two strains, as assessed in guinea pigs, shows  $ID_{50}$  values of 224 viable cells for strain 2308, and  $10^8$  viable cells for strain 11 (Dasinger, 1960; Baker, 1962). The acriflavine test of Braun (1947) and the crystal violet test of White and Wilson (1951) were used as tests for colonial morphology.

Cultures of *E. coli* H-52, *E. coli* Gratia, *E. coli* B, and *Bacillus cereus* G were obtained through the courtesy of W. B. Sarles, Department of Bacteriology, University of Wisconsin. Cultures of *E. coli* Rolf and *E. coli* ICR were isolated by conventional methods from the Rolfsmeier Swiss Webster and the A. R. Schmidt ICR strains of mice, respectively.

Trypticase Soy Broth and Trypticase Soy Agar (BBL) were used exclusively for the cultivation and maintenance of all cultures.

**Experimental animals.** Swiss Webster female mice, weighing 22 to 25 g, obtained from the Rolfsmeier Rat and Mouse Farm, Madison, Wis., were used exclusively. The mice were fed Purina Laboratory Chow and were given water ad libitum. The temperature of the animal rooms was maintained at 25 C.

**Cultivation of cells for the extraction of endotoxin.** As the source of cells, 48-hr broth cultures of *B. abortus* and 12-hr broth cultures of *E. coli* were used. The cells were cultivated on a rotary shaker at 37 C. The cells were harvested by centrifugation at  $10,000 \times g$  for 20 min at 4 C. The harvested cells were washed three times with cold saline and then were suspended in distilled water for the extraction of endotoxin.

**Heat-killed cell preparations.** Freshly harvested and washed viable cell suspensions were adjusted turbidimetrically and by direct microscopic count to contain  $10^9$  cells per milliliter. This cell suspension was kept at 65 C for 1 hr, after which time it was cooled to room temperature, diluted, and stored at 4 C until used. Viability tests were made with Trypticase Soy Agar to determine the effectiveness of the heat-killing process.

**Preparation of Boivin (TCA) endotoxins.** These endotoxins were prepared by the method of Boivin and Mesrobian (1935) with minor modifications (Baker, 1965).

**Preparation of Westphal (PW) endotoxins.** These endotoxins were prepared from strains of *B.*

*abortus* by extraction with hot phenol-water as described in detail by Redfearn (1960). Redfearn reported that only fractions 3 and 5, obtained by this method, had biological activity, and these were the only fractions considered in our studies. The extraction procedure (Redfearn, 1960) is given below.

About 5 g (dry weight) of cells of *B. abortus* were suspended in 170 ml of distilled water. The cell suspension was heated to 66 C in a water bath. A 90% (w/v) phenol reagent was prepared, and 190 ml of the phenol reagent were heated to 66 C and then added to the cell suspension. This resulted in a phenol-water ratio of 1:1 (w/w). The mixture was agitated vigorously with the aid of a power stirrer for 15 min. The mixture was then dispensed among eight 50-ml Lusteroid centrifuge tubes and rapidly cooled to 5 C by immersion in an ice bath. The mixture was centrifuged at  $13,000 \times g$  for 15 min at 4 C, causing the formation of four layers in the tubes. The top layer consisted of a phenol-saturated water layer. The interface precipitate separated the top layer from the water-saturated phenol layer, and the sedimented cell residue, at the bottom of the tube, constituted the fourth layer.

The upper phenol-saturated water layer (aqueous phase) was drawn off carefully with the aid of a capillary pipette attached to a vacuum line. An amount of 120 ml of phenol-saturated water was dispensed equally among the eight centrifuge tubes, and the centrifugation step was repeated. The aqueous phase was drawn off and added to the previous aqueous-phase sample. The pooled aqueous phases were dialyzed against several changes of distilled water and were concentrated to about 50 ml by pervaporation at room temperature.

The lower water-saturated phenol layer (phenol phase) and the precipitate formed at the interface were separated by filtration through Whatman no. 42 filter paper under slight negative pressure. To the filtered phenol phase were added 3 volumes of cold methanol reagent (methanol SA). The methanol reagent was prepared by adding 1 part of methanol saturated with sodium acetate to 99 parts of methanol. The solution was mixed for 5 min and was then held at 4 C for 1 hr to allow for maximal precipitation. The precipitated material was collected by centrifugation at  $6,000 \times g$  for 15 min at 4 C and kept. The supernatant liquid was discarded.

The precipitate was suspended in 80 ml of distilled water and agitated overnight with the aid of a magnetic stirrer. The suspension was centrifuged at  $10,000 \times g$  for 30 min at 4 C, and the supernatant liquid was collected. The precipitate was resuspended in 80 ml of distilled water, and the mixture was agitated for 1 hr. After centrifugation, the process was repeated an additional time. The three supernatant liquids were pooled and dialyzed against several changes of distilled water. After dialysis, the solution was pervaporated at room temperature to a final volume of 50 ml. Then

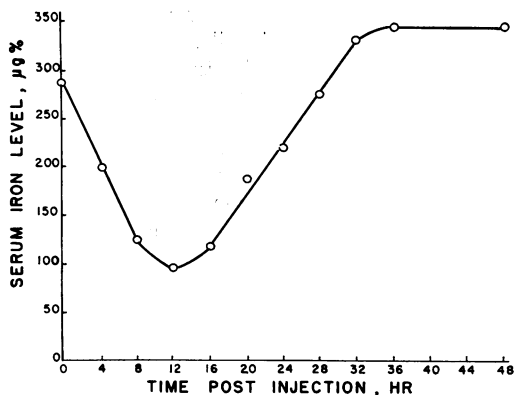


FIG. 1. Serum-iron levels (micrograms per 100 ml) of mice at various times after the intraperitoneal injection of 10 µg of *Escherichia coli* O55:B5-PW endotoxin.

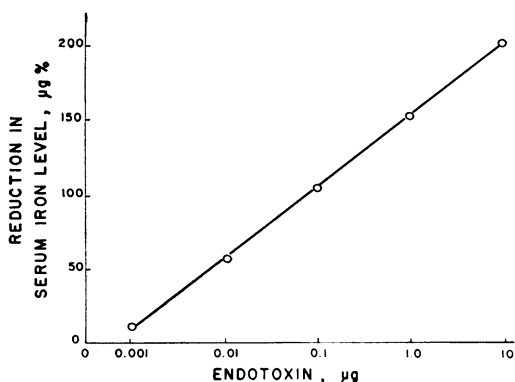


FIG. 2. Reduction (micrograms per 100 ml) in the serum-iron level of mice given various doses of *Escherichia coli* O55:B5-PW endotoxin.

2 volumes of cold methanol SA were added, and the mixture was allowed to stand for 2 hr to complete precipitation. The precipitate was recovered by centrifugation at  $10,000 \times g$  for 15 min and was dissolved in 20 ml of distilled water. The resulting material was lyophilized (fraction 5).

To the dialyzed and pervaporated aqueous phase were added 5 volumes of methanol SA, slowly and with stirring. The mixture was allowed to stand for 1 hr and was then centrifuged at  $10,000 \times g$  for 15 min at 4 C. The gel-like precipitate was dissolved in 25 ml of distilled water, and the solution was clarified by centrifugation at  $10,000 \times g$  for 30 min. The supernatant liquid was removed, and the product was reprecipitated by the addition of 4 volumes of methanol SA. The procedure was repeated an additional time after which the final clear solution was lyophilized (fraction 3).

**Commercially available endotoxin.** Two commercially available endotoxin preparations

(Difco) were also used in these studies. These were Boivin and Westphal endotoxin preparations obtained from *E. coli* O55:B5. The preparations will be referred to as *E. coli* O55:B5-TCA (Difco Catalog No. 3923) and *E. coli* O55:B5-PW (Difco Catalog No. 3120), respectively.

**Determination of serum-iron levels in mice.** Serum-iron levels in mice were determined according to the 2,2',2''-terpyridine method described in detail by Schade et al. (1954). The results are expressed either as the reduction in the serum-iron level or as the serum-iron level in micrograms of iron per 100 ml of serum.

**Collection of mouse serum.** Pooled blood samples from five mice were used to obtain a sufficient amount of serum (1.0 ml) to be used for a single serum-iron determination. The blood samples

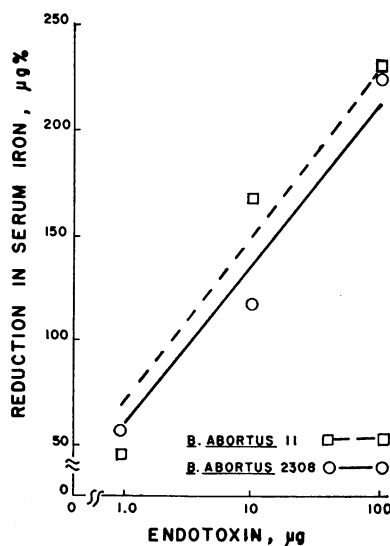


FIG. 3. Reduction (micrograms per 100 ml) in the serum-iron level of mice given various doses of *Brucella abortus* endotoxin (fraction 5).

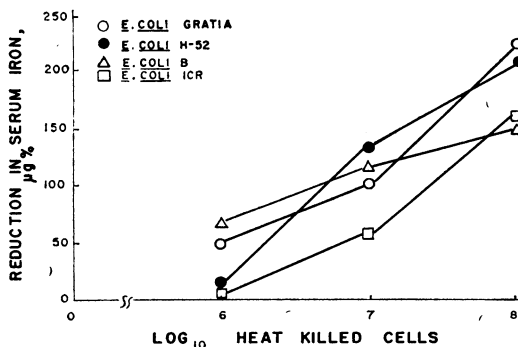


FIG. 4. Reduction (micrograms per 100 ml) in the serum-iron levels of mice given various doses of heat-killed cells of strains of *Escherichia coli*.

TABLE 1. Serum iron levels (micrograms per 100 ml) of mice 6 and 12 hr after receiving various test materials\*

Test material and dose	6 hr	12 hr
Untreated mice.....	310	308
Pyrogen-free saline (0.2 ml)....	312	304
Crystalline egg albumin (10 µg) ..	302	319
Glucose (10 µg).....	319	304
<i>Bacillus cereus</i> G (100 µg).....	273	342
<i>Brucella abortus</i> 11, TCA endotoxin (10 µg).....	223	197
Heat-killed <i>B. abortus</i> 11 (100 µg) ..	289	166
Heat-killed <i>B. abortus</i> 2308 (100 µg)	244	113
Heat-killed <i>Escherichia coli</i> ICR (100 µg).....	193	141

\* A single sample of pooled serum, obtained from five mice given a stated dose of test material, was used to determine the serum-iron level.

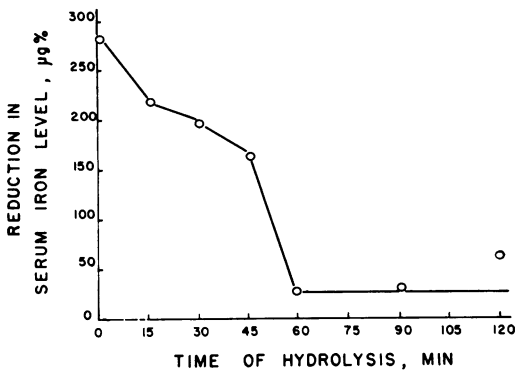


FIG. 5. Effect of acid hydrolysis of *Escherichia coli* O55:B5-PW endotoxin with 0.1 N acetic acid on its ability to induce hypoferremia in mice.

were collected from ether-anesthetized mice by cutting the brachial artery and the subclavian vein and then aspirating the blood with a Pasteur pipette. About 0.5 to 1.0 ml of blood could be obtained from a 22 to 25-g mouse by this procedure. The serum from pooled blood samples was collected after clotting and centrifugation of the samples. To reduce hemolysis, the blood samples were collected with as little agitation as possible and were allowed to clot in an ice bath.

## RESULTS

*Time after the intraperitoneal administration of endotoxin at which there is a maximal depression of the serum-iron level of mice.* A maximal depression of the serum-iron level occurred 12 hr after the intraperitoneal administration of 10 µg of *E. coli* O55:B5-PW endotoxin (Fig. 1). After 12 hr, the serum iron appeared to increase progressively. Since 12 hr postinjection was the time at which a

maximal depression in the serum-iron level occurred, blood samples in all subsequent experiments were collected at this time. Similar results were obtained with *B. abortus* endotoxin (fraction 5).

*Relationship between reduction in serum iron and dose of endotoxin preparations.* The relationship between the reduction in the serum-iron level and the logarithm of the dose of *E. coli* O55:B5-PW endotoxin is linear within the range of 0.001 to 10 µg of endotoxin (Fig. 2). By use of Westphal endotoxin preparations (fraction 5) obtained from *B. abortus* 2308 and 11, a reasonably linear dose-response curve was obtained within the range of 1.0 to 100 µg (Fig. 3).

TABLE 2. Lethal effects for mice of *Escherichia coli* Rolf-TCA endotoxin before and after treatment with acetic anhydride or pyridine-formic acid\*

Dose	Untreated endotoxin	Acetylated endotoxin	Pyridine formic acid-treated endotoxin
µg			
200	2/5	0/5	0/5
400	5/5	0/5	0/5
1,000	5/5	0/5	2/5

\* The figures listed represent number of deaths/total number of mice used.

TABLE 3. Reduction (micrograms per 100 ml) in the serum-iron levels of mice after receiving 100 µg of *Escherichia coli* Rolf-TCA endotoxin before and after treatment with acetic anhydride or pyridine-formic acid\*

Untreated endotoxin†	Acetylated endotoxin	Pyridine-formic acid-treated endotoxin
210 (3)	137 (5)	213 (5)
201 (3)	106 (5)	210 (5)
197 (4)	115 (5)	157 (5)
225 (3)	130 (5)	197 (5)
—	111 (5)	177 (5)
Mean 208	119	190

\* A single sample of pooled serum, obtained from the number of mice given in parentheses, was used to obtain the serum-iron level. This value was then subtracted from the mean serum-iron level of the saline control group (308 µg per 100 ml) to determine the reduction in the serum-iron level.

† Since 7 of 20 of the mice given the untreated endotoxin died, the survivors were redistributed at random into groups of two or four mice to obtain pooled serum samples for analysis.

*Relationship between reduction in serum-iron level and dose of heat-killed cells.* The results of a typical dose-response experiment conducted with heat-killed cells of the cultures of *E. coli* used in these studies are presented in Fig. 4. There appeared to be differences in the activities of these heat-killed cell preparations which suggest differences in endotoxin content, assuming no significant differences in average cell size between the strains; however, the data also suggest qualitative differences in the biological effect of the endotoxins of the strains. Heat-killed cells of *E. coli* B produced the greatest hypoferrmia of the strains tested at  $10^6$  cells; at  $10^8$  cells, they were the least effective. Similarly, differences in biological activity were also shown between the endotoxins of *E. coli* and *B. abortus* on the basis of the slopes of the dose-response curves (Fig. 2 and 3). The principal significance of these dose-response curves, obtained with endotoxins and with heat-killed cells, is that the degree of hypoferrmia is dose-related and linear within a given range; however, the endotoxins of various strains or species of bacteria may differ in biological activity.

*Specificity of the induction of hypoferrmia.* To determine the specificity of the induction of hypoferrmia, endotoxins, materials containing endotoxin, and materials thought to be free from endotoxic activity were used (Table 1). Only endotoxin or materials containing endotoxin, namely, heat-killed cells of *B. abortus* or *E. coli*, produced a

TABLE 4. Effect of pretreatment of mice with Thorotrast on the ability of *Escherichia coli* O55:B5-PW endotoxin to induce hypoferrmia

Pretreatment	Serum-iron levels	Mean serum-iron level	Mean reduction
No pretreatment plus saline	253, 331, 300, 298, 360	308	—
Saline plus 0.1 $\mu$ g of endotoxin	227†	227	81
Thorotrast plus saline	228, 225, 192	215	93
Thorotrast plus 0.1 $\mu$ g of endotoxin	137, 110, 142	129	179

\* The values listed represent the actual serum-iron levels obtained after a stated treatment. The mean reduction in serum-iron level was obtained by subtracting the mean serum-iron level for each experimental group from the mean serum-iron level obtained for the group that received no pretreatment.

† This value was obtained from the dose-response curve experiment (Fig. 2).

TABLE 5. Effect of pretreatment of mice with triolein on the ability of *Escherichia coli* O55:B5-PW endotoxin to induce hypoferrmia\*

Pretreatment	Serum-iron levels	Mean serum-iron level	Mean reduction
No pretreatment plus saline	253, 331, 300, 298, 360	308	—
Saline plus 0.1 $\mu$ g of endotoxin	227†	227	81
Triolein plus saline	300, 311, 325, 338	319	—
Triolein plus 0.1 $\mu$ g of endotoxin	285, 346, 237	289	19

\* The values listed represent the actual serum-iron levels obtained after a stated treatment. The mean reduction in serum-iron level was obtained by subtracting the mean serum-iron level for each experimental group from the mean serum-iron level obtained in the group that received no pretreatment.

† This value was obtained from the dose-response experiment (Fig. 2).

hypoferrmia at 12 hr postinjection. The fact that no hypoferrmia occurred with heat-killed vegetative cells of *Bacillus cereus* G indicates that the hypoferrmia is not dependent upon merely the particulate nature of some of the test materials employed, but probably is due to a more specific effect of endotoxin.

*Effect of acid hydrolysis on the ability of endotoxin to induce hypoferrmia.* The effect of acid hydrolysis upon the ability of *E. coli* O55:B5-PW endotoxin to induce hypoferrmia in mice is shown in Fig. 5. Loss in the ability of endotoxin to induce hypoferrmia began within 15 min of acid hydrolysis, after which time the activity of the endotoxin preparation progressively diminished until little if any ability to induce hypoferrmia remained after 60 min of acid hydrolysis.

*Effect of acetylation and pyridine-formic acid treatment upon the lethal effects of endotoxin and its ability to induce hypoferrmia.* The effects of acetylation and pyridine-formic acid treatment on the lethal effects of *E. coli* Rolf (TCA) endotoxin are summarized in Table 2. Whereas the mouse LD<sub>50</sub> of the untreated parent endotoxin preparation was between 200 and 400  $\mu$ g, the LD<sub>50</sub> of the acetylated and pyridine-formic acid-treated preparations appeared to be greater than 1,000  $\mu$ g. The fact that a few mortalities were observed with the pyridine-formic acid preparation suggested that this preparation was partially detoxified, whereas the acetylated preparation could be considered to be completely detoxified.

When the above three preparations were tested for their ability to induce hypoferrremia (Table 3), the parent endotoxin preparation had a high hypoferrremic activity, whereas the acetylated endotoxin preparation showed a loss in hypoferrremic activity. The fact that a substantial amount of hypoferrremic activity was demonstrable in endotoxin preparations, which appeared to be partially or almost completely devoid of lethal activity after the above treatments, probably can be explained on the basis of the relative sensitivities of the two systems of bioassay.

*Effect of pretreatment of mice with Thorotrast on the ability of endotoxin to induce hypoferrremia.* To determine the effect of pretreatment with Thorotrast, 0.2 ml of Thorotrast was given 6 hr before either pyrogen-free saline or 0.1  $\mu\text{g}$  of *E. coli* O55:B5-PW endotoxin (Table 4). The hypoferrremia obtained when endotoxin was given alone (81  $\mu\text{g}$  per 100 ml) and when Thorotrast was given alone (93  $\mu\text{g}$  per 100 ml), when added together, was nearly equal in magnitude to the hypoferrremia obtained when both materials were given in combination (179  $\mu\text{g}$  per 100 ml).

*Effect of pretreatment of mice with triolein on the ability of endotoxin to induce hypoferrremia.* Triolein itself had little effect on the serum-iron level (Table 5); however, in mice that had been given 0.1 ml of a 20% (v/v) suspension of triolein intravenously and 24 hr later were given 0.1  $\mu\text{g}$  of *E. coli* O55:B5-PW endotoxin, the effect of endotoxin on the serum-iron level was somewhat reduced.

#### DISCUSSION

Kampschmidt and Schultz (1961) and Kampschmidt and Upchurch (1962) reported the development of hypoferrremia in rats 12 to 16 hr after the administration of 0.0001 to 0.1  $\mu\text{g}$  of *E. coli* endotoxin. Since this biological response to endotoxin appeared to be dose-related and could be elicited with relatively small amounts of endotoxin, we felt that this response might be used in an extremely sensitive system for the bioassay of endotoxin in mice. Because virtually all of the nonheme iron found in plasma is bound to transferrin (Bothwell and Finch, 1962), serum-iron levels rather than plasma-iron levels were determined in our studies.

We found that hypoferrremia was maximal 12 hr after the intraperitoneal injection of 10  $\mu\text{g}$  of *E. coli* O55:B5-PW endotoxin. Similar results were obtained with 100  $\mu\text{g}$  of *B. abortus* fraction 5. Therefore, serum-iron levels were determined at 12 hr postinjection in our studies. Our results with mice are in general agreement with the findings of Kampschmidt and Schultz (1961) and Kampschmidt and Upchurch (1962) with rats.

There was a reasonable linear relationship between the logarithm of the dose of endotoxin and the degree of hypoferrremia induced in mice. With *E. coli* O55:B5-PW endotoxin, the relationship was linear within the range of 0.001 to 10  $\mu\text{g}$ . Fraction 5 endotoxin preparations of *B. abortus* 2308 and 11 gave similar dose-response curves within the range of 1.0 to 100  $\mu\text{g}$ . Thus, the response obtained with *E. coli* endotoxin exceeded that obtained with *B. abortus* endotoxin preparations.

A reasonable linear relationship between the logarithm of the number of heat-killed cells of *E. coli* and the degree of hypoferrremia produced was also observed within the range of  $10^6$  to  $10^8$  heat-killed cells. Since heat-killed cells as well as purified endotoxin preparations were capable of inducing hypoferrremia, this response could be employed for the determination of the endotoxin content of killed-cell preparations.

In preliminary experiments, only endotoxin or materials containing endotoxin induced hypoferrremia. Heat-killed vegetative cells of *Bacillus cereus* G did not induce hypoferrremia, although a modest hypoferrremia, which was well within experimental error, occurred at 6 hr postinjection.

Acid hydrolysis progressively reduced the ability of endotoxin to induce hypoferrremia, in a manner similar to the loss of other biological properties of endotoxin under the same conditions (Ribi et al., 1961). In our studies and in those of Ribi et al. (1961), nearly complete inactivation of endotoxin was noted after 60 to 90 min of hydrolysis. These data indicate that, in respect to the ability of endotoxin to induce hypoferrremia as with other biological properties of endotoxin, an endotoxin complex of a particular size is essential for biological activity.

When *E. coli* Rolf-TCA endotoxin was detoxified by the pyridine-formic acid method (Nowotny, 1963) and by acetylation (Freedman, Sultzer, and Kleinberg, 1961), there was a loss in the ability of the detoxified preparations to induce hypoferrremia. Although these data suggest a relationship between the ability of endotoxin to induce hypoferrremia and toxicity, the two systems of bioassay differ greatly in sensitivity. Undoubtedly, amounts of endotoxin that were sublethal but were sufficient to induce hypoferrremia still remained in the detoxified preparations.

Biozzi, Benacerraf, and Halpern (1955) and Benacerraf and Sebastyen (1957) reported that, soon after the administration of endotoxin to mice or rabbits, there is a depression of the phagocytic activity of the reticuloendothelial system (RES). A maximal depression occurred 12 hr after the administration of endotoxin, after which time the activity of the RES increased until, at 24 to 36 hr

postinjection, normal activity was restored. With regard to time sequence, these observations coincide remarkably well with our observations and those reported by Kampschmidt and Schultz (1961) concerning the hypoferremia induced by endotoxin. It appears that the hypoferremia might be related to the transient depression of the activity of the RES after the administration of endotoxin, since the cells of the RES play an important role in iron metabolism (Bothwell and Finch, 1962; Vannotti, 1957). One would expect that alterations in the activity of the RES would lead to corresponding changes in the serum-iron level. Although the mechanism of the hypoferremic response to endotoxin is unknown, Kampschmidt and Arredondo (1963) noted an accumulation of iron in the cells of the RES at the time of hypoferremia.

When mice were given Thorotrast to suppress RES activity (RES blockade), a marked hypoferremia occurred. Although the mode of action of Thorotrast is not known, it does possess some of the toxic properties of endotoxin, namely, the ability to produce fever and to prepare animals for the Schwartzman reaction (Petersdorf and Shulman, 1964). Whether Thorotrast acts solely by producing RES blockade or whether it possesses additional pharmacological effects is open to question. The fact that Thorotrast per se is capable of inducing hypoferremia and that the combined effect of Thorotrast and endotoxin is additive suggests that, in respect to their ability to induce hypoferremia, they may have a common mode of action; however, the possibility that some Thorotrast preparations might be contaminated with endotoxin still remains to be investigated.

Cooper and Stuart (1961) noted that, 24 hr after the intravenous administration of triolein to mice, there occurred a marked increase in the phagocytic activity of the RES, and the LD<sub>50</sub> for triolein-treated mice was 175  $\mu$ g of *E. coli* endotoxin as opposed to 325  $\mu$ g for nontreated mice. The authors suggested that endotoxin activity is dependent upon its rate of uptake by cells of the RES. In our experiments, triolein treatment nearly eliminated rather than increased hypoferremic response to endotoxin. Our data on this point are difficult to reconcile with those of Cooper and Stuart (1961); perhaps the great difference in amount of endotoxin accounts for the difference in results.

#### ACKNOWLEDGMENTS

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