EFFECTS OF BACTERIAL ENDOTOXINS ON METABOLISM

III. Nitrogen Excretion after ACTH as an Assay for Endotoxin*

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Endotoxins can be assayed on the basis of their toxicity for experimental animals, their pyrogenicity, their ability to necrotize tumors, and their antigenicity. Another property, observed in the course of recent experiments, makes possible a new assay. The initial observation on which the assay is based was obtained when an intraperitoneal injection of appropriate numbers of heat-killed cells of *Salmonella typhimurium* was found to prevent completely the elevation in urinary nitrogen excreted by mice given adrenocorticotropic hormone (ACTH) subcutaneously (1). The same number of heat-killed cells had no apparent effect on the total protein catabolized in response to concurrently injected cortisone as judged by the increase in urinary nitrogen eliminated under these conditions. From these observations, endotoxins seem to prevent certain physiological manifestations normally elicited by ACTH but do not influence the metabolic activity of exogenous glycocorticoid. Since there is an inverse proportionality between the quantity of endotoxin injected and the quantity of urinary nitrogen excreted following ACTH administration (2), this relationship has been used to develop the assay. In order to establish the validity of such an assay, endotoxin alone must be shown to produce the effect on which the assay is based. The extent to which such proof is possible is, of course, limited but within these limits the evidence to be presented is convincing. It is also necessary to determine the sensitivity of the assay before its usefulness can be related to other methods. This report is concerned with results obtained with these aims in mind.

Method

Endotoxin.—Seven different preparations were used. A saline suspension of a known number of *Salmonella typhimurium*, strain SR-11, were killed by pasteurization at 60°C. for 30 minutes. Lack of growth on subculture in brain-heart infusion broth (Difco) was taken as proof of sterilization. Usually the suspension had to be heated a second or third time before no growth was observed. The final material was stored at 5°C. in sealed vials. The dry weight of three different preparations was found to be about ¾ mg. per 10^9 heat-killed cells. Comparatively crude endotoxin was derived as acetone and ether-extracted cells of

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S. typhimurium according to the technique previously described (3). A purified lipopolysaccharide from *Escherichia coli* O_2:K_1, lot No. 3712:92a, was generously supplied by Dr. C. W. deWitt, Upjohn Co., Kalamazoo. Another lipopolysaccharide from *E. coli* 0127:B8, control 444793, was purchased from Difco Laboratories. Results obtained with *Brucella abortus* lipopolysaccharide, control No. 114612, and with *Serratia marcescens* lipopolysaccharide, control No. 117799, both Difco Laboratories, Detroit, were used for comparative purposes along with another *S. marcescens* lipopolysaccharide generously provided by Dr. Maurice Landy, National Institutes of Health, Bethesda.

Endotoxin injections were given intraperitoneally as suspensions freshly prepared in isotonic pyrogen-free sodium chloride solution (Baxter Laboratories, Morton Grove) such that the desired amount was contained in a volume of 0.5 ml. In one set of experiments the endotoxin was injected intravenously in a volume of 0.2 ml. of saline.

Urinary Nitrogen Determinations.—The technique previously described (1) for collecting mouse urines was used in all experiments. Samples, each from two mice, were collected under toluene during an overnight period (about 17 hours). The mice were fasted throughout this time. Nitrogen was determined by micro Kjeldahl analysis.

Hormones.—Armour's acthar gel was used exclusively as the source of ACTH after it was observed that a lyophilized preparation gave smaller and more erratic urinary nitrogen yields. The gel was administered subcutaneously in a dose of two units (0.05 ml. of the 40 unit material).

Immunization of Mice against S. typhimurium.—Mice were immunized against *S. typhimurium* by weekly intraperitoneal injection of acetone ether-extracted cells. 0.5 mg. was given the 1st week and on each of two succeeding weeks, 1.0 mg. was administered. Two mg. of this material was about 90 per cent lethal for 20 gm. mice. One week following the third injection an agglutination titer of 1:1024 was found with pooled serum from three mice tested against heat-killed cells as antigen.

Miscellaneous.—The reticulo endothelial system (RES) of mice was "blocked" by the intravenous injection of 0.1 ml. saccharated iron oxide (proferrin of Merck, Sharp & Dohme, Rahway) 2 hours before endotoxin was to be administered.

A compound known to protect mice against the lethal effects of endotoxin (4) 3,4-dichloroisoproterenol (DCI)[1] (generously supplied by Dr. I. H. Slater, Eli Lilly Co., Indianapolis) was injected intraperitoneally at a dosage level of 10 mg./kg. 1 hour before challenge. This substance is reported to block inhibitory adrenergic responses in the vascular bed (5) and the cardiac stimulant effect of adrenergic materials (6).

Animals.—CF-1 female mice (Carworth Farms, New City, New York) and, in some experiments, Detwiler female mice weighing 20 ± 2 gm. were used as experimental animals. Previous tests, including the types described above, have failed to reveal any important differences in the metabolic responses of these and other strains of mice to endotoxin (3). They were housed 10 mice per cage in stainless steel cages with pine shavings as bedding. The animal room was maintained at 25 ± 2°C. They were fed Dietrich and Gambrill mouse food *ad libitum* and water was available at all times.

RESULTS

Effect of Graded Doses of Heat-Killed S. typhimurium on Urinary Nitrogen Excretion in Mice Injected with ACTH.—The first column of Table I shows the number of heat-killed salmonellae injected intraperitoneally into mice also given, at the same time, 2 units of ACTH subcutaneously. The dry weight in

[1] DCI, 3,4-dichloroisoproterenol.

micrograms of the number of cells shown is given in parentheses. The mice excrete the quantities of urinary nitrogen shown in the second column of Table I. While 5×10^7 cells are required to lower urinary nitrogen significantly (5 per cent level of probability by rank order test (7)), the administration of larger numbers of bacteria results in the elimination of even smaller quantities of urinary nitrogen. Mice given no ACTH excrete 13.3 mg. nitrogen (1). It is

TABLE 1

Urinary Nitrogen Excretion during a 17 Hour Period of Fasting in Mice Given a Subcutaneous Injection of 2 Units of ACTH and an Intraperitoneal Injection of Heat-Killed S. typhimurium

Results with normal mice are compared with those for mice previously immunized against *S. typhimurium*. Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

No. of heat-killed salmonellae injected (dry weight in μg.)	Urinary nitrogen excreted in	
	Control mice	Immune mice
	mg./mouse/17 hrs.	*mg./mouse/17 hrs.*
0	29.9±3.2 (8)	27.0±4.8 (8)
5×10^6 (0.38)	29.5 ± 3.3 (8)	
5×10^7 (38)	24.6 ± 4.1 (12)	
2.5×10^8 (190)	17.0 ± 3.8 (22)	
5×10^8 (380)	11.3 ± 1.8 (24)	23.5 ± 2.0 (8)
5×10^9 (3800)		17.8 ± 1.8 (8)

apparent, therefore, that 5×10^8 cells prevent completely the augmentation of nitrogen loss induced by the injection of ACTH.

These data, when plotted as milligrams nitrogen *versus* log number of heat-killed cells give almost a straight line. This relationship forms the basis of a possible bio-assay for endotoxin.

Effect of Immunization on Nitrogen Excretion Following Injections of Heat-Killed Cells and ACTH.—The first line of the third column of Table I shows that immune animals excrete normal amounts of urinary nitrogen in response to ACTH alone. They are, however, about 10 to 20 times as resistant to the

heat-killed cells as control mice as judged by the results presented. Thus the nitrogen eliminated by control mice given 5×10^7 cells is approximately the same as that for immune animals injected with 5×10^8 salmonellae. Similarly, control mice inoculated with 2.5×10^8 cells excrete a quantity of urinary nitrogen equivalent to that derived from immune mice given 5×10^9 cells. These findings suggest that endotoxin is the primary factor involved in suppressing nitrogen excretion under the conditions of the experiment.

TABLE II

Urinary Nitrogen Excreted by Mice Injected Subcutaneously with 2 Units of ACTH, with Varying Numbers of Heat-Killed S. typhimurium, and with and without 0.1 ml. of Saccharated Iron Oxide Given Intravenously

Each value is the mean ± the standard deviation for the number of determinations shown in parentheses.

No. of heat-killed *S. typhimurium* injected	Urinary nitrogen excreted in	
	Control mice	Proferrin-injected
	mg./mouse/17 hrs.	*mg./mouse/17 hrs.*
0	28.3 ± 3.8 (8)	25.0 ± 2.6 (8)
5×10^6	27.0 ± 5.6 (10)	18.7 ± 6.3 (8)
1×10^7	24.8 ± 2.5 (7)	17.0 ± 4.0 (8)
5×10^7	24.6 ± 4.1 (12)	10.5 ± 3.0 (7)

Effect of Reticulo-endothelial "Block" on the Nitrogen Excretion Following Injections of Heat-Killed Cells and ACTH.—The ability of various reticulo-endothelial blocking agents to sensitize experimental animals to bacterial endotoxins is well established (8–10). It was considered worthwhile, therefore, to test the effect of one of these, saccharated iron oxide, on the nitrogen excretion test. The data of Table II show that the number of heat-killed cells of *S. typhimurium* (5×10^7) which barely lowers the urinary nitrogen output in control animals lowers it to the same level in mice treated with saccharated iron oxide as that obtained when no ACTH is injected (13.3 mg. as given in reference 1). Moreover, only 3 of 16 mice injected with both the colloid and 5×10^7 heat-killed cells survived while 12 of 12 animals survived this dose of heat-killed cells alone. All deaths occurred between the end of the 17 hour period of urine collection and 48 hours postinjection. Thus, from the data of Table II, intravenous saccharated iron oxide, which alone fails to alter signifi-

cantly the urinary nitrogen excretion in ACTH-treated mice, sensitizes the animals to numbers of heat-killed cells that are without effect in its absence.

Effect of Heat-Killed Staphylococcus aureus on Nitrogen Excretion Following ACTH.—In order to observe the effect of injections of large numbers of heat-killed gram-positive bacteria on urinary nitrogen excretion in ACTH-treated mice, the experiments leading to the data reported in Table III were carried out. It can be seen that 5×10^9 *Staphylococcus aureus*, strain Giorgio, failed to alter urinary nitrogen excretion. This is the approximate number of viable cocci that can be cultured from mice at the time of death from experimental infections with this organism (11, 12). The fact that it is highly virulent is shown by the 100 per cent mortality obtained 5 days after 20 mice were infected intravenously with 10^8 cells of an overnight broth culture of the Giorgio

TABLE III

Urinary Nitrogen Excretion in Mice Injected Intraperitoneally with Heat-Killed Staphylococcus aureus and with 2 Units of ACTH Subcutaneously

Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

No. of heat-killed *Staph. aureus* injected	Urinary nitrogen excreted
	(mg./mouse/17 hrs.)
0	28.3 ± 3.8 (8)
5×10^9	29.0 ± 8.0 (8)

strain. Moreover, no change was observed in the urinary nitrogen excreted by these infected mice in response to ACTH during the first night postinfection. This is in contrast to the findings with mice inoculated with growing cells of *S. typhimurium*, previously reported (2), in which evidence for detectable amounts of endotoxin was obtained the first night postinfection and again during the terminal stages of the disease, about the 5th day under the conditions of the experiment.

Effect of E. coli Lipopolysaccharide on Nitrogen Excretion Following ACTH.—From the data of Table IV it can be seen that both 25 and 50 μg. of purified *E. coli* lipopolysaccharide (Upjohn Co.) give significant reductions in the urinary nitrogen excreted in response to ACTH. Injection of 75 μg. of the same material prevents completely the rise in nitrogen output and is lethal for 4 of 16 mice. 50 μg. killed none of the animals. 100 μg., on the other hand, was lethal within 24 hours for 11 of 12 mice also given 2 units of ACTH.

Influence of Serum "Endotoxin-Detoxifying Component" (EDC) and DCI on the Assay for E. coli Endotoxin.—As Landy and collaborators have reported in a

series of publications (13–16), calcium-free serum from a number of animal sources is capable of detoxifying endotoxin as judged by its loss of (*a*) tumor-necrotizing action, (*b*) toxicity for experimental animals, and (*c*) antigenicity. With this work as a background, the effect of calcium-free mouse serum on

TABLE IV

Urinary Nitrogen Excreted by Mice Given an Intraperitoneal Injection of Graded Doses of Purified E. Coli Lipopolysaccharide (Upjohn) and 2 Units of ACTH Subcutaneously, Compared to Values Obtained after the Endotoxin Had Been Incubated 1 Hour in Normal and in Citrated Mouse Serum

Also shown is the effect of endotoxin on mice pretreated with dichloroisoproterenol. Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

Treatment given the mice	Urinary nitrogen excreted
	mg./mouse/17 hrs.
Controls	28.3 ± 3.8 (8)
25 μg. *E. coli* endotoxin	19.5 ± 3.5 (10)
50 μg. *E. coli* endotoxin	15.8 ± 5.8 (6)
75 μg. *E. coli* endotoxin	12.5 ± 3.9 (13)
75 μg. *E. coli* endotoxin incubated 1 hour in normal serum	12.5 ± 1.5 (10)
75 μg. *E. coli* endotoxin incubated 1 hour in citrated serum	19.8 ± 3.6 (10)
75 μg. *E. coli* endotoxin 1 hr. after mouse pretreatment with DCI (10 mg./kg.)	21.0 ± 4.5 (11)

the toxicity of *E. coli* lipopolysaccharide as judged by urinary nitrogen excretion following injection of ACTH was determined. The results shown in lines five and six of Table IV were obtained. Incubation of 75 μg. of *E. coli* endotoxin for 1 hour at 37°C. with normal mouse serum altered in no way its effect on nitrogen excretion. The same quantity of endotoxin incubated an hour in serum containing 2.4 mg. of sodium citrate per ml. and then introduced into mice gave the same amount of urinary nitrogen excretion following ACTH as that found after 25 μg. of endotoxin. According to this test, about 50 μg. of

endotoxin was destroyed or inactivated during the 1 hour period of contact with calcium-free serum. Therefore, this effect of calcium-free serum on endotoxin strongly suggests that endotoxin is the essential substance in the assay. It also shows that the assay can be used as a measure of EDC activity.

The ability of DCI to protect mice against the lethal effects of endotoxin is made evident by the fact that only 8 of 20 control mice survived an injection of crude *S. typhimurium* endotoxin survived by 17 of 20 mice treated with DCI 1 hour in advance. The probability of this occurring by chance, according to the rank correlation method (17), is about 1 per cent. The last line of Table IV establishes another relationship of interest. Here it can be seen that mice treated with DCI 1 hour prior to the injection of 75 μg. *E. coli* endotoxin excrete more nitrogen than animals given 25 μg. of the lipopolysaccharide alone. Apparently, therefore, DCI not only protects against the toxic effects of endotoxin but also reduces the ability of endotoxin to diminish the urinary nitrogen excreted in response to ACTH. The physiological and pharmacological bases for these results is unknown, but it was established that animals injected with DCI alone excrete normal amounts of nitrogen.

Comparison of Endotoxins from Different Sources.—In order to present additional evidence that endotoxin is responsible for the "block" in urinary nitrogen excreted in response to ACTH, seven different endotoxins were compared with respect to their nitrogen content (determined by micro Kjeldahl), lipid content (determined gravimetrically as the chloroform-soluble extract after acid hydrolysis), "blocking" dose, and LD_{50} as calculated by the method of Reed and Muench (18). The ratio of the two dosages was also calculated. The results are presented in Table V. A study of the data makes it apparent that a proportionality exists between the toxicity of an endotoxin and its ability to "block." This is seen in the constancy of the ratio for all preparations, including the acetone-ether-extracted salmonellae. The smaller ratio obtained with heat-killed cells may or may not have significance. Since endotoxins are of interest, in large measure, because of their toxic manifestations, it is important to have an assay correlate so well with this property. It is questionable whether any of the other currently employed tests for endotoxin are as reliable in this respect.

The data of Table V show that endotoxins vary extensively in their nitrogen and lipid content. In general, the smaller the nitrogen and the greater the lipid, the more toxic the preparation but neither generalization holds in detail. It is impossible, from the results presented, to confirm or specifically deny, therefore, Westphal's contention that lipid is the toxic component of endotoxin (19). The nitrogen found in endotoxins is believed to be due to attached proteins and to their content of hexosamine (19–21). The three Difco lipopolysaccharides possessed more nitrogen than materials of this type should, as evidenced by the low nitrogen content of the potent *E. coli* endotoxin of Upjohn. Nevertheless, the Difco preparations are more toxic on a weight basis, *B.*

abortus excepted, than the acetone-ether-extracted salmonellae. The comparatively massive dose of *B. abortus* lipopolysaccharide that failed to "block" nitrogen excretion clearly establishes its almost total lack of toxicity. In addition, its large nitrogen content hardly justifies calling the material a lipopolysaccharide. The lipid analysis for this preparation was omitted because of an

TABLE V

Comparison of Nitrogen Content, Lipid Content, "Blocking" Dose, LD_{50} and the Ratio of the Two Doses for Seven Different Endotoxin Preparations

Type of endotoxin	Nitrogen	Lipid	"Blocking" dose	LD_{50}	Ratio: $\frac{\text{block}}{LD_{50}}$
	per cent	*per cent*			
Heat-killed *S. typhimurium* SR-11	16	8	5×10^8 cells (0.4 mg.)	2×10^9 cells (1.5 mg.)	0.3
Acetone-ether dried *S.typhimurium*	8	2	0.6 mg.	1.5 mg.	0.4
E. coli (Upjohn) lipopolysaccharide	2.5	15	0.075 mg.	0.175 mg.	0.4
E. coli (Difco) lipopolysaccharide	9.0	6	0.15 mg.	0.36 mg.	0.4
S. marcescens (Difco) lipopolysaccharide	5.7	11	0.10 mg.	0.25 mg.	0.4
S. marcescens (Landy) lipopolysaccharide	1.2*	10*	0.75 to 1.0 mg.	—	—
B. abortus (Difco) lipopolysaccharide	14.8	—	10 mg. No block	10 mg. Not toxic	—

* Data supplied by Dr. Landy.

inadequate supply. The *Serratia marcescens* endotoxin supplied by Dr. Landy is highly purified, as judged by its low nitrogen content, but is no more toxic than the acetone-ether-dried *S. typhimurium*, as indicated by the data of Table V. This same material, according to Landy *et al.* (13–16), is a potent pyrogen, however, and is effective in tumor necrosis. The Upjohn preparation of *E. coli* lipopolysaccharide stands out, on the other hand, as the most active material so far tested by the urinary nitrogen assay and is unusually lethal for mice.

Least Amount of E. coli Lipopolysaccharide Detectable by Urinary Nitrogen

Assay.—Fever is produced in experimental animals by smaller doses of endotoxin when the doses are administered intravenously rather than by intramuscular, subcutaneous, or intraperitoneal routes (21). The minimum quantity of our most potent material (*E. coli* lipopolysaccharide of Upjohn) detectable by the urinary nitrogen assay was determined after intraperitoneal and intravenous inoculations in mice sensitized by an injection of saccharated iron oxide. In all experiments the colloid was given 2 hours before the endotoxin. The results are summarized in Table VI. Intraperitoneally, 5 μg. of endotoxin is readily detectable with good statistical reliability while intravenously, 0.25 μg. gives an equally significant value. A smaller amount, 0.1 μg., is without apparent influence as judged by nitrogen excretion. The assay is capable of measuring, therefore, 10 to 100 times the quantity of endotoxin detected by immunological procedures (16) but the present method does not require knowledge of the antigenic makeup of the organism from which the endotoxin is derived.

The intravenous route of injection requires, therefore, approximately $\frac{1}{20}$ the endotoxin for assay as that needed for an intraperitoneal test (5 μg. *versus* 0.25 μg.). Moreover, mice sensitized with saccharated iron oxide excrete about the same amount of nitrogen after 5 μg. of *E. coli* endotoxin, given intraperitoneally, as that found when 50 μg. are injected in mice without the RES "block" (compare results in Tables IV and VI). Similar results were also obtained with heat-killed *S. typhimurium* given to mice with and without RES "block" as the data of Tables I and II show.

DISCUSSION

The quantity of urinary nitrogen excreted by control mice injected with 2 units of ACTH varies with the size and age of the animals. The range of values presented in the top line of Tables I and II and the last line of Table VI is about as great as one encounters in mice within about 1 gm. of 20 gm. body weight. Younger and smaller mice excrete less nitrogen than larger, older animals. Environmental variables and certainly the dietary regimen must be controlled for reproducible results. To the extent possible, all experimental procedures and conditions, including size of mice, room temperature, time of day at which injections are given (to avoid the complications of periodicity effects reported by Halberg and Howard (22)), and the precise timing of collection periods, etc., should also be established. During summer months more erratic values are likely to be obtained. This may be the result, at least in part, of various stresses to which animals are subject during the warm months of the year. Since response to ACTH occupies a key role in the assay, it is apparent that the use of unstressed animals is important.

The evidence in this report implicating endotoxin as the necessary substance in the assay is, inevitably, inferential. It must remain inferential until

chemically defined endotoxins are available. For the present, one can merely correlate, as well as possible, data obtained from procedures known to alter response of animals to endotoxin. Modification of response may be produced by a change in either the animal or the endotoxin. Both techniques have been employed and the results are consistent with endotoxin being the material assayed. Toxicity for mice has been the only reference criterion employed in

TABLE VI

Urinary Nitrogen Excreted during a 17-Hour Period of Fasting in Mice Given a Subcutaneous Injection of 2 Units of ACTH and an Intravenous Injection of Proferrin

Results with mice given an intraperitoneal injection of *E. coli* lipopolysaccharide (Upjohn) 2 hours after the proferrin are compared to results with mice given the endotoxin intravenously. Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

Quantity of lipopolysaccharide injected	Urinary nitrogen excreted in mice injected	
	Intraperitoneally	Intravenously
μg	*mg./mouse/17 hrs.*	*mg./mouse/17 hrs.*
10	7.9 ± 1.6 (6)	
5	18.2 ± 3.2 (4)	
1	25.8 ± 1.8 (4)	10.2 ± 2.4 (8)
0.25		15.4 ± 7.7 (9)
0.1		27.5 ± 3.2 (4)
Control	25.0 ± 2.6 (8)	25.0 ± 2.6 (8)

these studies but pyrogenic effects and tumor-necrotizing properties will be used for comparison in future.

The physiology underlying the assay is inadequately understood. There are at least three major points at which endotoxin could act to produce the effects observed. (*a*) It could prevent in several ways the ACTH from stimulating adrenal cortical secretion of glycocorticoids. (*b*) It could block the biochemical action of endogenous glycocorticoids released in response to the ACTH such that protein deamination (or transamination) is suppressed. (*c*) It could alter kidney function so as to reduce in proportion to dose the clearance of nitroge-

nous wastes. A number of experiments aimed at discriminating among these possibilities has substantially eliminated (*a*), made (*b*) appear unlikely, and pointed to (*c*) as most probably involved. This evidence is now in preparation for publication.

SUMMARY

There exists an inverse proportionality between number of heat-killed cells of *Salmonella typhimurium* injected intraperitoneally into mice and the quantity of urinary nitrogen the animals excrete during a 17 hour period following the subcutaneous administration of 2 units of ACTH. This relationship has been developed into an assay for bacterial endotoxin. Mice immunized against *S. typhimurium* require 10 to 20 times the number of cells needed by control animals to suppress urinary nitrogen excretion to the same extent. Intravenous saccharated iron oxide sensitizes animals so that fewer heat-killed salmonellae can be detected. Heat-killed cells of *Staphylococcus aureus* are without effect in the assay. Several lipopolysaccharides derived from Gram-negative bacteria are effective in preventing the rise of urinary nitrogen excreted in response to ACTH and the amount required, compared to the LD_{50}, is in the same ratio for all of them. Citrated mouse serum partially inactivates the endotoxin during *in vitro* incubation for 1 hour at 37°C. while normal serum does not. Dichloroisoproterenol protects mice against the lethal effects of lipopolysaccharide and it lowers its effectiveness in the assay. The minimum amount of endotoxin reliably determined by the test is 0.25 μg. of an *E. coli* preparation that was given intravenously in mice in which the reticuloendothelial system had been "blocked" with saccharated iron oxide.

BIBLIOGRAPHY

1. Berry, L. J., and Smythe, D. S., Effects of bacterial endotoxin on metabolism II. Protein-carbohydrate balance following cortisone. Inhibition of intestinal absorption and adrenal response to ACTH, *J. Exp. Med.*, 1959, **110,** 407.
2. Berry, L. J., and Smythe, D. S., Some metabolic aspects of host-parasite interactions in the mouse typhoid model, *Ann. New York Acad. Sc.*, in press.
3. Berry, L. J., and Smythe, D. S., Effects of bacterial endotoxin on metabolism. I. Carbohydrate depletion and the protective role of cortisone, *J. Exp. Med.*, 1959, **110,** 389.
4. McLean, R. A., and Berry, L. J., Adrenergic inhibition and lethal effect of bacterial endotoxin in mice, *Proc. Soc. Exp. Biol. and Med.*, 1960, **105,** 91.
5. Powell, C. E., and Slater, I. H., Blocking of inhibitory adrenergic receptors by a dichloro analog of isoproterenol, *J. Pharmacol. and Exp. Therap.*, 1958, **122,** 480.
6. Moran, N. C., and Perkins, M. E., Adrenergic blockade of the mammalian heart by a dichloro analog of isoproterenol, *J. Pharmacol. and Exp. Therap.*, 1958, **124,** 223.

7. White, C., The use of ranks in a test for significance for comparing two treatments, *Biometrics*, 1952, **8**, 33.
8. Beeson, P. B., Tolerance to bacterial pyrogens; role of the reticuloendothelial system, *J. Exp. Med.*, 1947, **86**, 39.
9. Good, R. A., and Thomas, L., Studies on the generalized Schwartzman reaction. II. The production of bilateral cortical necrosis of the kidneys by a single injection of bacterial toxins in rabbits previously treated with Thorotrast or trypan blue, *J. Exp. Med.*, 1952, **96**, 625.
10. Smith, R. T., and Thomas, L., Influence of age upon response to meningococcal endotoxin in rabbits, *Proc. Soc. Exp. Biol. and Med.*, 1954, **86**, 806.
11. Smith, I. M., Wilson, A. P., Hazard, E. C., Hummer, W. K., and Dewey, M. E. On death from staphylococci in mice. I. Route of inoculation and bacterial enumeration studies, *J. Infect. Dis.*, in press.
12. Houser, E., unpublished observations.
13. Landy, M., Skarnes, R. C., Rosen, F. S., Trapani, R. J., and Shear, M. J., Inactivation of biologically active ("endotoxic") polysaccharide by fresh human serum, *Proc. Soc. Exp. Biol. and Med.*, 1957, **96**, 744.
14. Skarnes, R. C., Rosen, F. S., Shear, M. J., and Landy, M., Inactivation of endotoxin by a humoral component. II. Interaction of endotoxin with serum and plasma, *J. Exp. Med.*, 1958, **108**, 685.
15. Rosen, F. S., Skarnes, R. C., Landy, M., and Shear, M. J., Inactivation of endotoxin by a humoral component. III. Role of divalent cation and a dialyzable component., *J. Exp. Med.*, 1959, **110**, 731.
16. Landy, M., Trapani, R. J., and Shear, M. J., Inactivation of endotoxin by a humoral component. IV. Alteration in the immunological properties of typhoid endotoxin., *J. Exp. Med.*, 1959, **110**, 731.
17. Wilcoxon, F., Some Rapid Approximate Statistical Procedures, New York, American Cyanamid Co., 1949.
18. Reed, L. J., and Muench, H., A simple method of estimating fifty per cent endpoints, *Am. J. Hyg.*, 1938, **27**, 493.
19. Westphal, O., and Luederitz, O., Chemical research on lipopolysaccharides of Gram negative bacteria, *Angew. Chem.*, 1954, **66**, 407.
20. Burrows, W., Endotoxins, *Ann. Rev. Microbiol.*, 1951, **5**, 181.
21. Bennett, I. L., Jr., and Cluff, L. E., Bacterial pyrogens, *Pharmacol. Rev.*, 1957, **9**, 427.
22. Halberg, F., and Howard, R. B., 24 hour periodicity and experimental medicine, *Postgrad. Med.*, 1958, **24**, 349.