



The effects of purified *Salmonella enteritidis* endotoxin on the immune response of Balb/c mice  
by John Brian Barnett

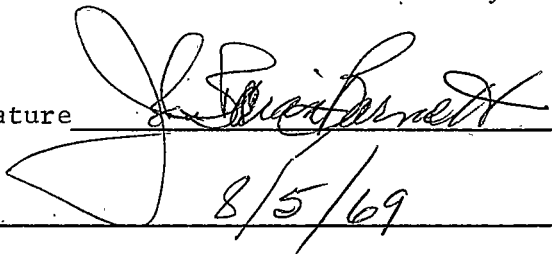
A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of  
MASTER GF SCIENCE in Microbiology  
Montana State University  
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**Abstract:**

Wasting disease is a syndrome characterized by ruffled fur, diarrhea, wrinkled and dry skin, a high stepping gait, failure to gain weight and, in some instances, death. Since the normal microbial flora has been implicated in the pathogenesis of many forms of wasting disease, endotoxins, as products of the normal flora, were used to induce wasting in neonatal Balb/c mice. The endotoxins prepared from *Salmonella enteritidis* cultures were chemically and biologically characterized to provide a potent endotoxin for the study. The three endotoxins used were an endotoxin (Se-ether) prepared by the aqueous ether method, an endotoxin (Se-dioxane) prepared by the dioxane method, and an endotoxin (Se-Ribi) furnished by Dr. E. Ribí, all of which possessed essentially the same biologic activity. Protein determinations showed 21%, 42%, and 21.5% Se-ether, Se-dioxane, and Se-Ribi, respectively. The percentage of carbohydrate for Se-ether was 38.7%, for Se-dioxane was 7.7%, and for Se-Ribi was 46%. Lipid content was determined to be 18% for Se-ether and 13.3% for Se-Ribi. It was shown that the U.V. spectra for Se-ether was essentially identical to that obtained for Se-Ribi. In comparing the infra-red spectra for all three endotoxins it was found that they also were virtually identical. A severe wasting disease was induced by injecting neonatal mice with an initial dose of 35 to 75  $\mu\text{gm}$  per gram body weight and a dose of 75 or 150  $\mu\text{gm}$  of endotoxin every third day until day 18. The degree of wasting was determined from the calculation of the ranting (weight loss) index. Mortality was determined to be approximately 46% and the mean survival time approximately 15 days. Hematologic data showed that an absolute neutrophilia and a leukocytosis developed by day 10 and persisted through day 30. The immunologic data indicated an impaired responsiveness to sheep erythrocytes existed at day 20 which was still evident at day 30. It was postulated that endotoxins affect the Immune response which, in turn, allowed the normal flora to invade the surrounding tissues and cause wasting disease.

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THE EFFECTS OF PURIFIED SALMONELLA ENTERITIDIS ENDOTOXIN  
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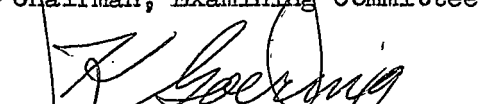
in

Microbiology

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August, 1969

ACKNOWLEDGEMENT

The author wishes to thank Dr. John W. Jutila for his inspiring attitude and guidance throughout this study. The author also wishes to express his gratitude to Mrs. Charlotte Staab for her technical assistance and especially to his wife for her patience and encouragement.

This investigation was supported by Public Health grants AI 06552-03 and 2TI AI 131-06 from the National Institutes of Allergy and Infectious Diseases.

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## ABSTRACT

Wasting disease is a syndrome characterized by ruffled fur, diarrhea, wrinkled and dry skin, a high stepping gait, failure to gain weight and, in some instances, death. Since the normal microbial flora has been implicated in the pathogenesis of many forms of wasting disease, endotoxins, as products of the normal flora, were used to induce wasting in neonatal Balb/c mice. The endotoxins prepared from Salmonella enteritidis cultures were chemically and biologically characterized to provide a potent endotoxin for the study. The three endotoxins used were an endotoxin (Se-ether) prepared by the aqueous ether method, an endotoxin (Se-dioxane) prepared by the dioxane method, and an endotoxin (Se-Ribi) furnished by Dr. E. Ribi, all of which possessed essentially the same biologic activity. Protein determinations showed 21%, 42%, and 21.5% Se-ether, Se-dioxane, and Se-Ribi, respectively. The percentage of carbohydrate for Se-ether was 38.7%, for Se-dioxane was 7.7%, and for Se-Ribi was 46%. Lipid content was determined to be 18% for Se-ether and 13.3% for Se-Ribi. It was shown that the U.V. spectra for Se-ether was essentially identical to that obtained for Se-Ribi. In comparing the infra-red spectra for all three endotoxins it was found that they also were virtually identical. A severe wasting disease was induced by injecting neonatal mice with an initial dose of 35 to 75 ugm per gram body weight and a dose of 75 or 150 ugm of endotoxin every third day until day 18. The degree of wasting was determined from the calculation of the runtting (weight loss) index. Mortality was determined to be approximately 46% and the mean survival time approximately 15 days. Hematologic data showed that an absolute neutrophilia and a leukocytosis developed by day 10 and persisted through day 30. The immunologic data indicated an impaired responsiveness to sheep erythrocytes existed at day 20 which was still evident at day 30. It was postulated that endotoxins affect the immune response which, in turn, allowed the normal flora to invade the surrounding tissues and cause wasting disease.

## INTRODUCTION

Wasting diseases have been produced in newborn mice by neonatal thymectomy (Miller, 1961) or by treatment of newborn with cortisol acetate (Schlesinger, 1964, Reed and Jutila, 1965), estradiol (Reilly et. al., 1967) or endotoxin (Keast, 1968, Keast and Walters, 1968). These syndromes are characterized by diarrhea, ruffled fur, wrinkled and dry skin, a high stepping gait, and failure to gain weight normally. The similarities of these diseases have caused many workers to postulate that common pathological or physiological events are responsible for the wasting and death of diseased animals.

There is considerable evidence that the microbial flora of the animal may play a major role in the development of symptoms of many forms of wasting disease. It was shown that germ-free (GF) mice were highly resistant to wasting disease induced with cortisol acetate (Reed and Jutila, 1965). Similarly, Wilson, Sjodin, and Bealmear (1964) and, independently, McIntire, Sell, and Miller (1964) showed that thymectomized germ-free mice failed to waste in contrast to severe wasting exhibited by conventionally-reared (CR) mice thymectomized at birth. Likewise, Ekstedt and Nishimura (1964) found that CR mice treated with multiple doses of heat-killed streptococci and staphylococci wasted whereas GF mice developed few or no symptoms of wasting disease.

The course of many forms of wasting disease has been mitigated by treatment with antibiotics. Hence, neonatally thymectomized mice treated with oxytetracycline (Azar, 1964) displayed less severe symptoms of wasting disease and a lower mortality from wasting disease than their untreated counterparts. Similarly, the antibiotic treatment of mice

injected neonatally with cortisol acetate improved the clinical course of wasting disease and reduced the incidence of death. Thus, these observations tend to incriminate microorganisms and/or their products in the development of pathologic events that contribute to symptoms of wasting disease.

Among the normal flora organisms isolated from tissues and organs of mice suffering from various forms of wasting diseases are several species of the Enterobacteriaceae (Jutila and Reed, 1967, Cantrell and Jutila, 1969). A common component of gram-negative enteric bacteria is endotoxin which is known to adversely influence the immune mechanism and produce pathologic changes similar to those observed in many of the wasting diseases. Although the precise mode of action of endotoxin is not known, the tissue and organ changes produced by an endotoxemia in experimental or natural infections or by the injection of purified endotoxins are well documented.

Rowlands and coworkers (1965) demonstrated a transient depletion of thymic tissue and an alteration of the morphology of the thymus in adult mice following treatment with a single dose of endotoxin. With the disappearance of distinction between the cortex and the medulla there appeared to be a decrease in thymocytes combined with an increase in pyroninophilic cells. Recovery from the effects of endotoxin occurred between 10 and 14 days post injection.

Keast and Walters (1968) conducted an extensive study on pathological changes which occur in young mice after treatment with multiple, large doses of endotoxin. Splenomegaly and thymic atrophy was observed shortly

after the initiation of treatment. When the liver was examined, large, grey, necrotic plaques were observed in addition to a fatty metamorphosis of the hepatic cells. By the ninth day, subcapsular eosinophilic necrosis was observed which eventually developed into a massive necrosis of the basophilic type by the first month. Keast and Walters (1968) also observed that by the first week the pancreas became edematous but only after 3 weeks of treatment were intestinal lesions observed. Macrophages, neutrophils, and lymphoid cells had infiltrated the muscle wall and villi with small necrotic foci appearing by one month. Lymphoid hyperplasia was also consistently observed.

Ekstedt and Hayes (1967) detected a striking decline in the immune response in the runt mouse when assayed by the Jerne plaque technique after an injection of 20 ugm of staphylococcal endotoxin every third day for 3 weeks. It was noted that the peak of this depression occurred four days after the immunization injection. However they saw no significant change in the mean survival time of skin allografts. It was their contention that the exposure of neonatal mice to large doses of bacterial antigens (endotoxins) altered the immune mechanism at the level of immunoglobulin synthesis but does not extend to the level of cellular immunity.

Bradley and Watson (1964) were able to demonstrate an immunosuppressive effect in adult mice treated with 400 ugm per gm body weight of a commercial nonpurified endotoxin. They showed that repeated injections of endotoxin subsequent to antigenic stimulation with actinophage MSP-8 impaired the early and late antibody response. Cessation of endotoxin

treatment permitted phage neutralizing activity to increase in the stimulated animals.

Another effect of endotoxin is that with the administration of multiple but sublethal doses of endotoxin, the effects of a lethal dose of endotoxin given some time later is largely mitigated i.e., tolerance to endotoxin has apparently been induced. Tolerance per se is much easier to induce in the newborn animal and may account for the observation that much greater doses may be used in neonates as opposed to adult animals (Petersdorf and Shulman, 1964, Louis Chedid et. al., 1964). Petersdorf and Shulman (1964) have postulated that tolerance may be due to one of the following: 1) increased reticuloendothelial system (RES) clearance, 2) inactivation by humoral factors in serum or tissue, 3) neutralization of endotoxin by specific antibody, 4) exhaustion of endogenous substances, 5) refractoriness of the C.N.S. to toxic stimuli and 6) adrenal unresponsiveness. Although it is clearly shown that the RES is stimulated by multiple sublethal doses of endotoxin, and presumably more functional, it is equally clear that it is not the complete picture of tolerance (Petersdorf and Shulman, 1964, Chedid et. al., 1964). There is evidence that humoral factors including specific antibody may interfere with the activity of endotoxin (Ritts et. al., 1964 and Greisman et. al., 1964).

The most commonly studied effect of endotoxin in the intact animal and in vitro systems is its adjuvant action. Although the mechanism for this is still undetermined, it has been shown that the administration of a very small dose of endotoxin, (1-5  $\mu\text{gm}$ ) a few days prior to or in conjunction with the injection of the antigen, causes an enhancement of

antibody production (Miler, Sterzl, Kostka, Lanc, 1964, A. G. Johnson, 1964). The mechanism which promotes this effect may be the increase in RES function after the injection of very small doses of endotoxin or increased cellular proliferation among the immunocytes (Biozzi et. al., 1955).

The primary purpose of this study was to demonstrate that endotoxin produces pathologic changes that are expressed as syndromes of wasting, immunosuppression, and, in some instances, death. This data, it is hoped, may serve to elucidate the relationship between wasting diseases and microorganisms of the normal flora.

## MATERIALS AND METHODS

### Mice

Inbred Balb/C mice were used throughout the study. The mice were originally obtained in 1966 from the National Cancer Institute (Bethesda, Maryland) in the germ-free state and were conventionalized six months later. The mice have since been maintained by successive brother-sister matings in our laboratory. All animals received Purina Laboratory Chow and water ad libitum.

### Preparation of Endotoxin

Endotoxin extracts were prepared from Salmonella enteritidis, strain S-795, (kindly supplied by Dr. E. Ribí) grown in tryptic soy broth (BBL). The endotoxin was prepared by either the dioxane or aqueous ether methods of Ribí, (1958) (1961).

The dioxane method involved the addition of 1,4 dioxane to an equal volume of 0.15 M saline containing 10 mg per ml of washed cells. The suspension was stirred at room temperature for 12 hours and the resulting extract clarified by centrifugation at 2500 g for 70 minutes. The supernatant fluid was dialyzed against several changes of distilled water for six days and then lyophilized.

The aqueous ether extraction was performed by resuspending freshly harvested and washed cells in saline on the basis of turbidity (scale reading of 770 in a Klett-Summerson colorimeter, filter number 540). Two volumes of precooled (6-12°C) diethyl ether were added to the cell suspension at the same temperature and the mixture shaken for 6 consecutive 10 minute intervals with precaution taken to release the pressure after each

interval. The suspension was left overnight at a temperature of 6-12°C, after which the aqueous phase was drawn off and the residual ether removed from it by bubbling air through the suspension. The remaining steps were performed at a temperature of 4-6°C. The residual cells were removed from the supernate containing soluble endotoxin by centrifugation at 2500g for 70 minutes. The supernate was dialyzed against daily changes of distilled water for 5 days. NaCl was then added to a final concentration of 0.15 M and the endotoxin precipitated by slowly adding absolute ethanol until a final concentration of 68% by volume was reached. After the suspension was allowed to stand at 6°C overnight, the precipitate was collected by centrifugation at 2000g for 45 minutes, dissolved in the same volume of 0.15 M NaCl, and reprecipitated with ethanol. Following centrifugation the precipitate was lyophilized. The lyophilized preparation, after rehydration, was autoclaved at 18 pounds pressure for 15 minutes.

#### Properties of Endotoxin

Total protein content was determined by Lowry's technique (Lowry, 1951) using a bovine serum albumin to establish a standard curve. The carbohydrate determination was done using the anthrone reagent method and glucose to establish the standard curve (Morris, 1948). To check for nucleic acid content, an ultraviolet absorption curve was obtained using a Beckman DB spectrophotometer. A peak at 260 mu was taken to indicate the presence of NA. Infra-red (IR) spectrographic analysis of different endotoxin preparations was conducted with a spectrophotometer.

Lipid content was determined by a modification of the gravimetric technique by Entenman, (1957). A dried and pre-weighed sample of endotoxin was mixed with 25 ml of chloroform-methanol (2:1). The mixture was stirred vigorously for 3 minutes and then placed on a rotator overnight at room temperature. The endotoxin preparation was filtered through a pre-dried and weighed sintered glass filter. The filter was allowed to dry at 80°C overnight and then weighed again. The difference between the weight of untreated endotoxin and the weight of the lipid-free endotoxin was used to establish the lipid content of the endotoxin preparation.

The LD<sub>50</sub> was determined by injecting one of four groups of mice in the tail vein with a given dose of endotoxin. Each group contained five adult Balb/C mice and were given the following doses. Group 1 received 100 ugm per gram body weight, group 2 received 50 ugm per gram body weight, group 3 received 25 ugm per gram body weight and group 4 received 12.5 ugm per gram body weight. The LD<sub>50</sub> was subsequently determined by a modification of the Reed-Muench technique described by Carpenter, (1956).

#### Production of Wasting Disease

Experimental litters were sized to contain between 5 and 8 neonatal mice of mixed sex. The average weight of these mice at the time of the initial injection was between 1.0 and 2.0 grams. The initial injection was given intraperitoneally (i.p.) to animals less than 24 hours old. The initial dose was 35 to 75 ugm of endotoxin. Subsequent doses of 75

or 150 ugm of endotoxin were given i.p. every day until day 18. The time of onset and severity of wasting was approximated by the use of a runting index (RI) described by Keast, (1968).

### Hematology

Blood for hematological studies was drawn from the tail or retro-orbital sinus. Differential leukocyte counts were performed on Wrights stained smears and recorded as lymphocytes, polymorphonuclear leukocytes (PMN) and monocytes. Total leukocyte counts were performed on a Model B Coulter Counter (Coulter Electronics, Hialeah, Florida). Hematocrit values were obtained by drawing blood into heparinized capillary tubes and centrifugation in an Adams Autocrit centrifuge (Clay-Adams Inc., New York).

### Organ Weights

Following sacrifice the spleen, liver, and thymus were removed and bathed in phosphate buffered saline (PBS) until weighed. After weighing, an organ index (O.I.) was calculated by a modification of the Simonsen method as follows (Simonsen, 1958):

$$O.I. = \frac{\text{organ weight}}{\text{body weight}} \times 100$$

### Immunization Schedule and Determination of Antibody Titers

All animals were injected with 0.1 ml of 10% suspension of thrice-washed sheep red blood cells (SRBC) at either 10, 20, or 30 days of age. At day 5 and 10 after immunization, 10 drops of tail or retroorbital blood was collected in 0.5 ml saline and the serum removed for titering.

The peak of 19S hemolysin production was presumed to occur at day 5, whereas the 10 day period approximated the peak of 7S hemagglutinating Ab production.

Hemagglutinin titers were determined by serial two fold dilutions of individual sera in saline. To a volume of 0.1 ml of serum, 0.1 ml of a 1% sheep red blood cell suspension was added. The tubes were incubated for 30 minutes at 37°C and stored at 4°C overnight. The tubes were centrifuged at 3500 rpm for 1 minute and the degree of agglutination scored. If agglutination occurred, the cells came up as one large clump (scored as 4), several large clumps (scored as 3), small clumps and loose cells (scored as 2), or mainly loose cells with some persistent clumps (scored as 1). In the absence of agglutination, there was a cloud of loose cells with no clumping (scored as negative). The titer was taken as the last serum dilution in the series to be scored as 1 or more.

Hemolysin titers were determined by adding 0.05 ml of guinea pig complement (BBL) diluted 1:5 to the standard hemagglutination system described above. The tubes were incubated at 37°C for 30 minutes and stored at 4°C overnight before reading. The last tube showing complete hemolysis was taken as the end point or the titer.

## RESULTS

Comparative chemical analysis of the various endotoxins employed in the study. Chemical analyses were performed on S. enteritidis endotoxin prepared by the dioxane method (designated Se-dioxane) and the aqueous ether method (Se-ether) described by Ribi (1958). The chemical composition of these endotoxins and the aqueous ether-extracted endotoxin obtained from Dr. Ribi is described in Table I.

The results show that the chemical composition of endotoxins prepared by the aqueous ether method, both Dr. Ribi's preparation and that prepared for this study, was essentially similar. A protein content of 21 and 21.5%, a hexose content of 38.7 and 47% and a lipid content of 18 and 13.3%, respectively, were determined for Barnett and Ribi endotoxin.

These results are contrasted with those obtained for Se-dioxane where protein estimates were increased to 42% and hexose content decreased to 7.7%. A lipid determination was not performed on Se-dioxane. The results of the U.V. scan on Se-ether shown in Figure 1 revealed no peak at 260 mu indicating the absence of nucleic acid. An identical U.V. scan was obtained for Se-Ribi (Fukushi, 1964).

The I.R. spectra for Se-dioxane, Se-ether and Se-Ribi are shown in figures 2, 3, and 4, respectively. Although the magnitude of the peaks apparently differ due to difference in the quantity of endotoxin employed in the spectral analysis, the position of the number of peaks are virtually identical for all three figures. This indicated considerable uniformity, qualitatively at least, in the chemical composition of the endotoxin preparations.

## TABLE I

Chemical composition of endotoxin prepared by the aqueous ether or dioxane method.

Endotoxin	% Protein	% Carbohydrate	% Lipid
Se-dioxane	42.0	7.7	N.A. <sup>a</sup>
Se-ether	21.0	38.7	18.0
Se-Ribi <sup>b</sup>	21.5	46.0	13.3

<sup>a</sup> Not available

<sup>b</sup> J. Bact. 87: 391

## Figure 1

Ultraviolet-absorption curve of S. enteritidis endotoxin (aqueous-ether)  
suspended at 100 $\mu$ gm/ml.

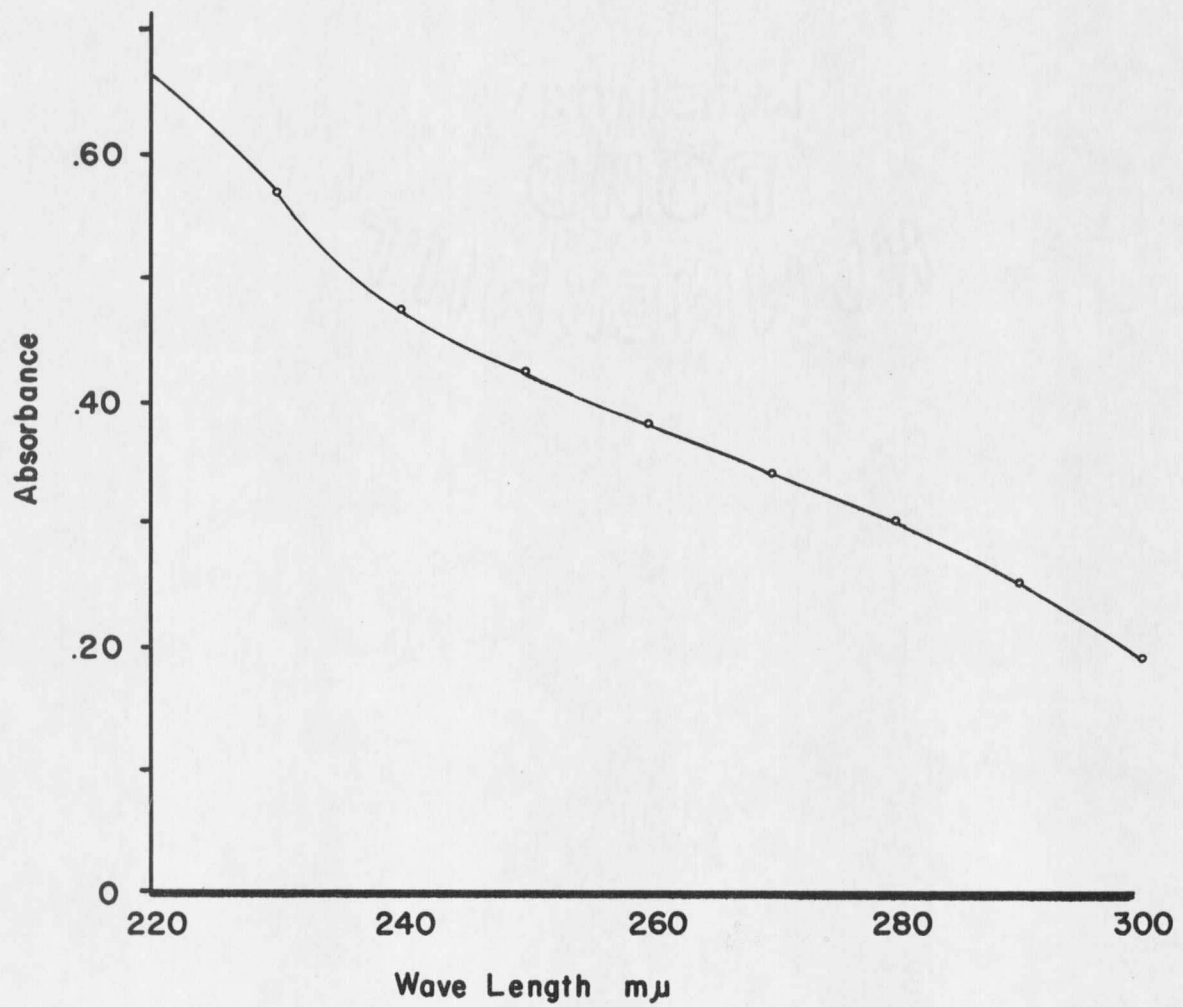


Figure 2

Infrared - absorption curve of *S. enteritidis* endotoxin (dioxane).

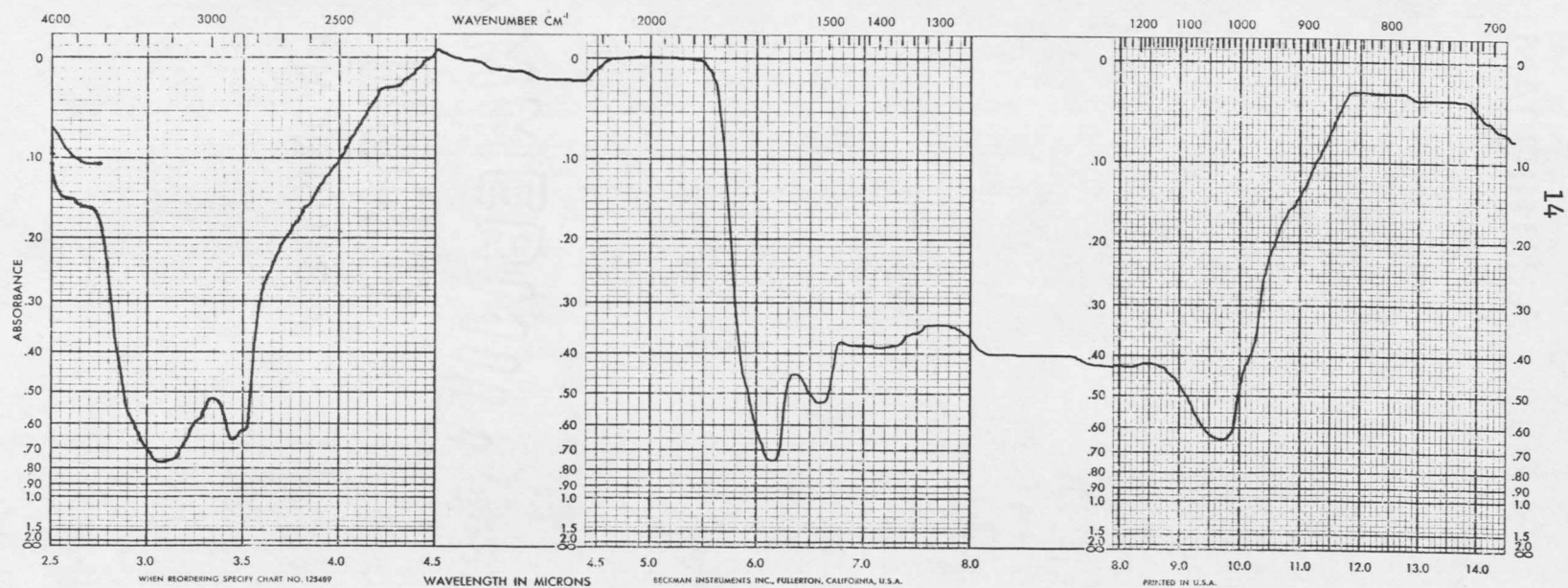


Figure 3

Infrared-absorption curve of *S. enteritidis* endotoxin (aqueous ether).

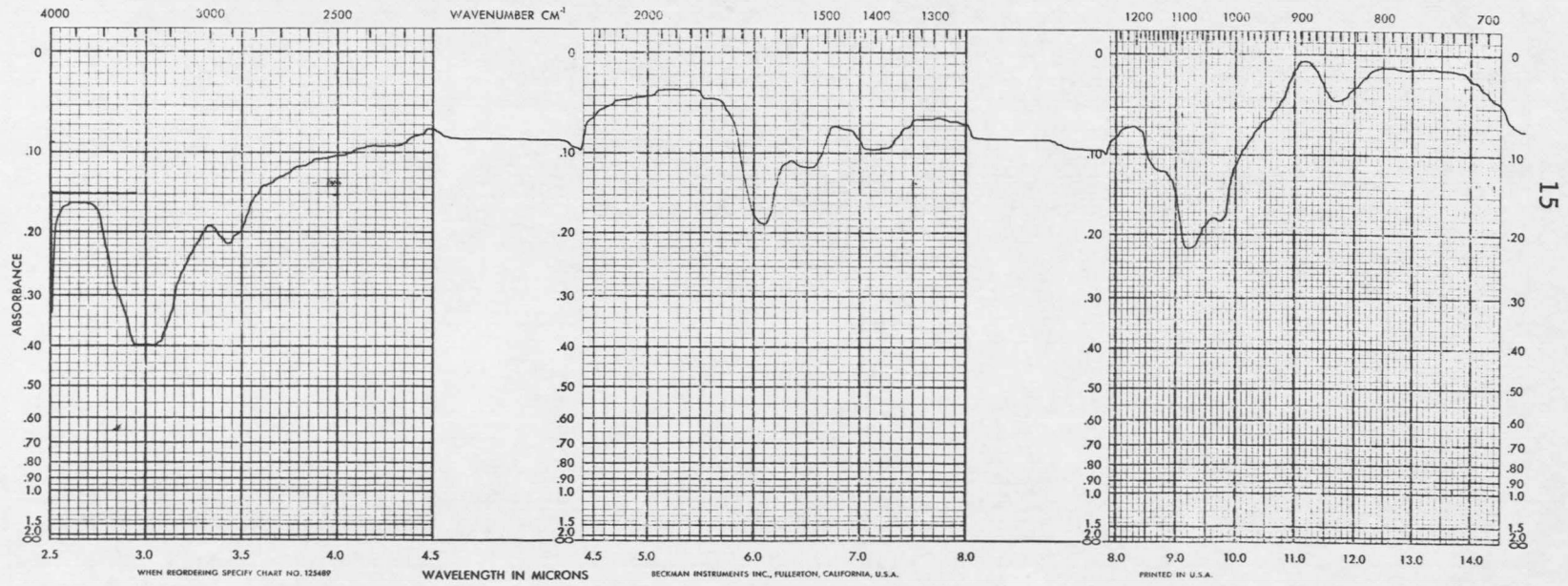
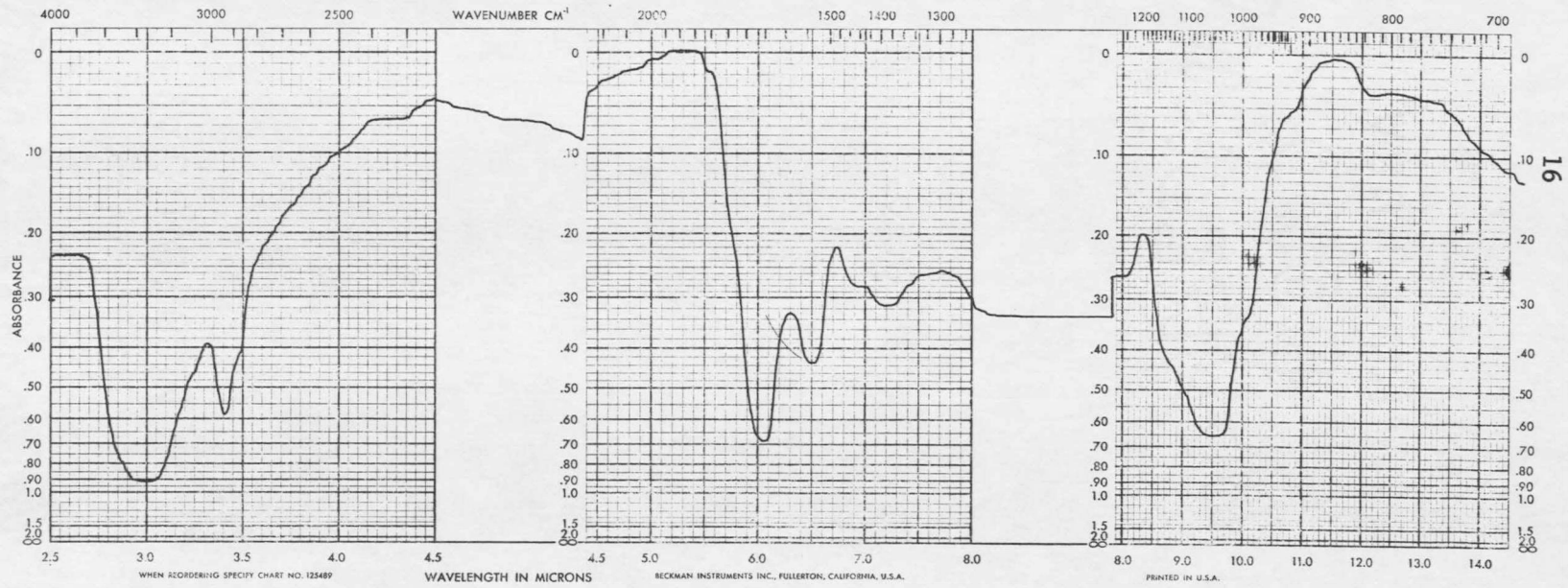


Figure 4

Infrared-absorption curve of *S. enteritidis* endotoxin (Ribi).



Characteristics of Wasting Disease. The LD<sub>50</sub> of Se-dioxane for adult Balb/c mice was found to be 22.9 ugm/gm body weight. Since newborn mice tolerated this dose with few toxic symptoms, the study employed an initial dose of 35 to 75 ugm into 1 to 2 gram mice followed by a dose of 150 ugm in the subsequent injections to induce wasting diseases.

The time of onset and severity of wasting disease was estimated by the runting (weight loss) index calculated at various times during treatment with endotoxin. The data shown in figures 5, 6, and 7 describe and, at the same time, compare the onset and severity to wasting disease induced with the 3 endotoxin preparations. The onset of wasting was evident within 3 days in every instance and marked failure to gain weight was observed over a 12 day period. In the case of Se-dioxane-induced wasting, weight gains were in a plateau phase between 12 and 20 days and then, following weaning, a marked failure to gain weight was again observed. Aqueous ether endotoxins generally produced a more pronounced effect on weight gain, and they were considered to be more potent inducers of wasting diseases. A regular finding was that a commercial endotoxin preparation E. coli lipopolysaccharide (Difco L.P.S.-B E. coli 0111:B4 #3922) failed to induce symptoms of wasting in spite of the injection of comparable doses of endotoxin.

The incidence of wasting disease was found to be 100% regardless of the endotoxin employed, as determined by the criteria defined in materials and methods (Table II). Although, the symptoms of diarrhea and high stepping gait were not as pronounced as those seen in other

Figure 5

Runting Index (RI) of Balb/C mice treated with S. enteritidis endotoxin (dioxane).

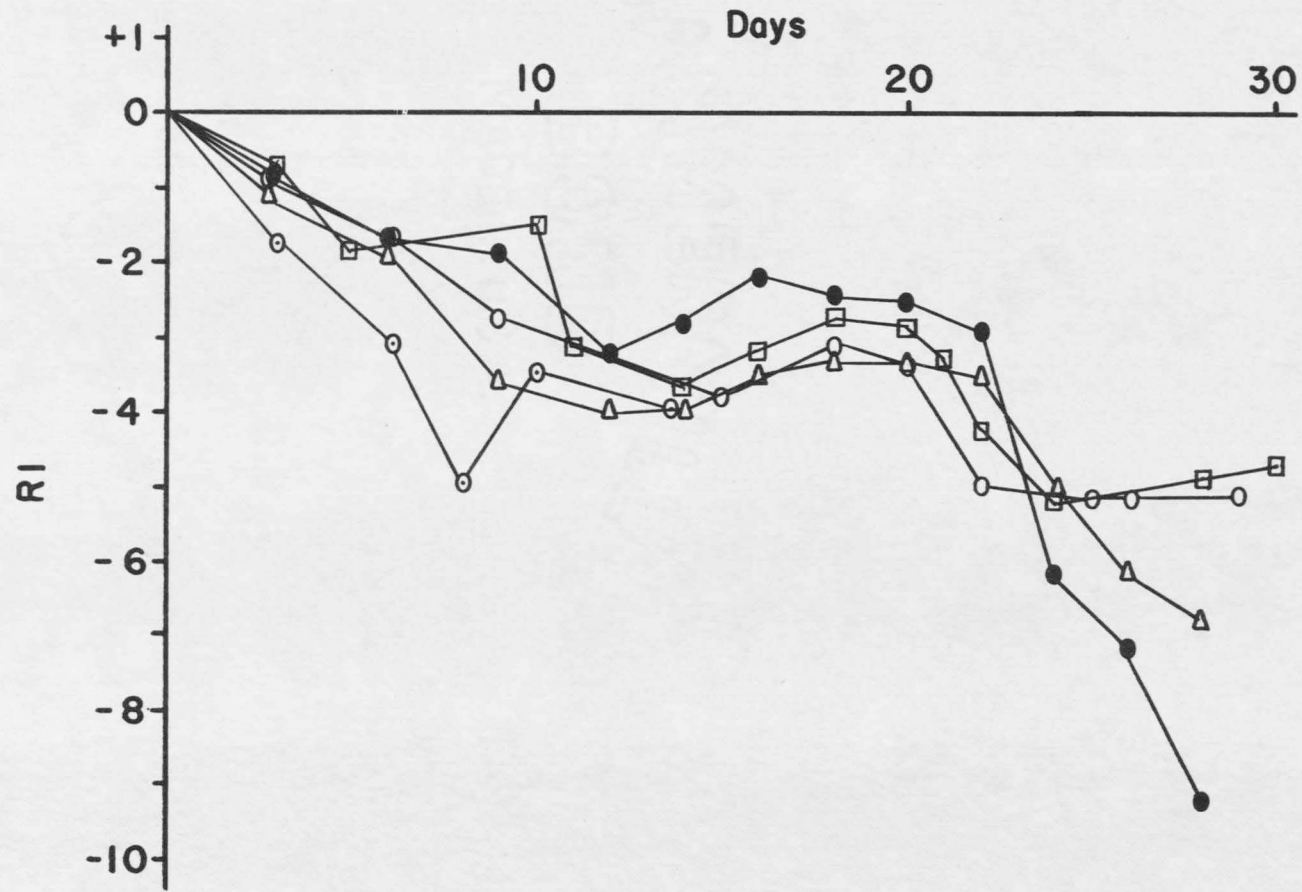


Figure 6

Runting Index (RI) of Balb/C mice treated with S. enteritidis endotoxin (Ribi)

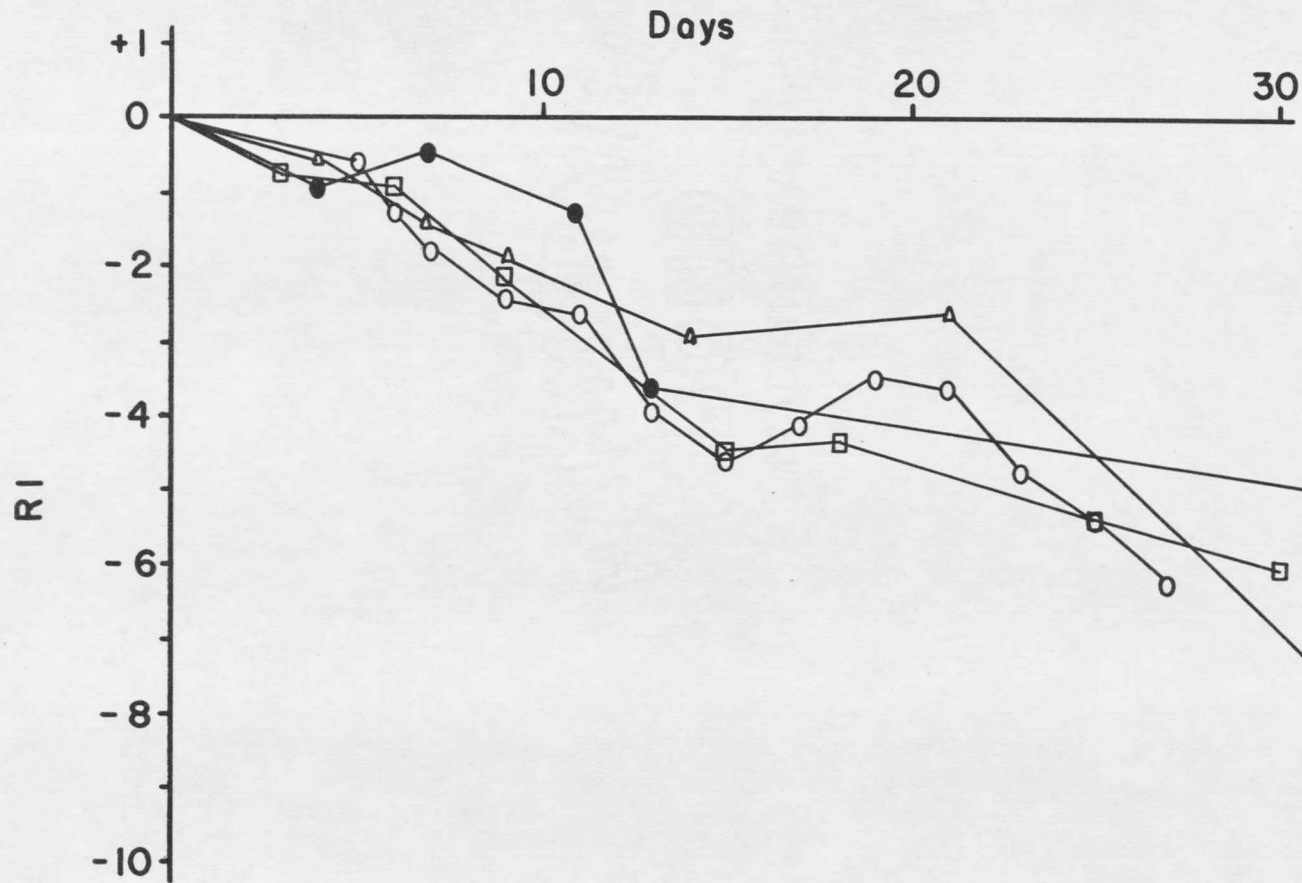
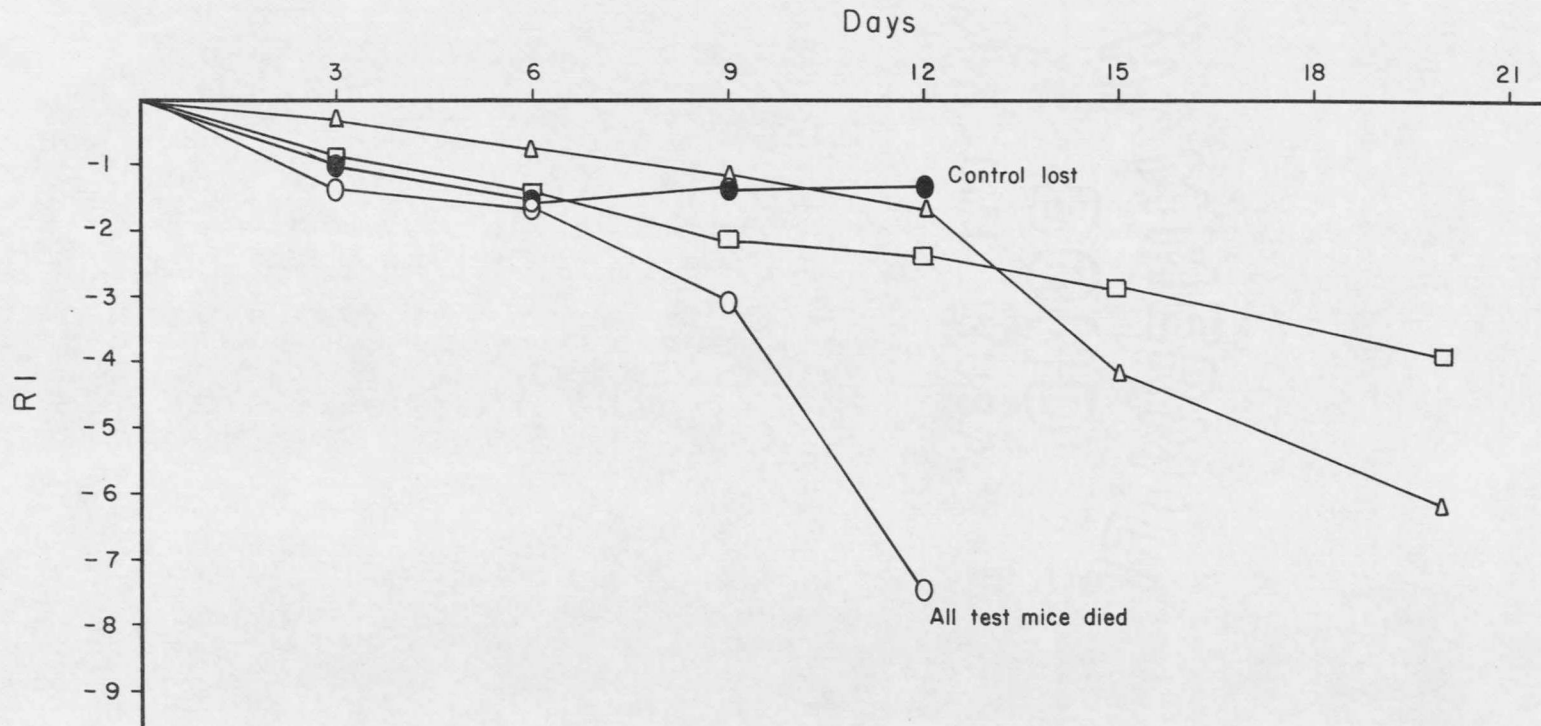


Figure 7

Runting Index (RI) of Balb/C mice treated with S. enteriditis endotoxin (aqueous ether)



## TABLE II

Incidence of wasting among Balb/C mice treated with endotoxin

Endotoxin	% Wasting	% Dying	Mean survival time (days)
Se-dioxane	100	38	17.8
Se-ether	100	60	12.7
Se-Ribi	100	42	14.8
E. coli (Difco)	0	0	—

forms of wasting disease, the failure to gain weight and other symptoms were severe. The mortality was calculated to be 38% with Se-dioxane treated mice, 60% in Se-ether and 42% in Se-Ribi treated mice. Only those mice dying later than 48 hours after the initial injection were considered as dying from an endotoxemia. Most deaths occurred during the interval day 2 to day 25. Many mice appeared to be recovering by day 40.

The effect of endotoxin on the hematology of Balb/c mice. The hematological changes during and following treatment with endotoxin are shown in Table III. The results indicate that a pronounced leukocytosis had developed as early as day 10 and represented an increase in neutrophils. The neutrophils persisted to day 30 but the degree of the shift to the left could not be ascertained because of the failure to obtain total WBC's at this time. No significant difference (Students t-test) in the hematocrit could be detected between the control and the experimental groups for each test period. The weights of thymus, spleen, and liver of mice treated with endotoxin were nearly identical to organ weights of control mice. The failure to obtain atrophy of thymus and spleen following treatment with endotoxin was correlated with little or no change in absolute lymphocyte counts.

The effect of endotoxin on the immunological response of Balb/c mice. The results of the immunological studies in mice treated with Se-dioxane are presented in Table IV. The endotoxin-treated mice demonstrated a depression in both the hemolysin and the hemagglutination response at day 20 and 30. It was observed that 68% of the experimental

## TABLE III

**Hematology of Balb/C mice treated with multiple doses of endotoxin**

Treatment <sup>a</sup>	Day of Test	Total WBC <sup>b</sup>	Hematocrit	Lymphocytes	PMNs	Monocytes
Endotoxin	10	6514	32±2.0 <sup>c,e</sup>	46±10.0	51±11.0	1.6±0.8
Control	10	4090	32±2.2	84±7.1	15±7.0	1
Endotoxin	20	9200	38±1.0 <sup>e</sup>	49±10.0	51±10.0	0
Control	20	7319	43±4.24	64±8.5	37±12.0	1
Endotoxin	30	N.A. <sup>d</sup>	45±2.75	53±8.4	44±8.2	3±1.5
Control	30	N.A.	50±1.26	81±5.5	14±2.3	5±0.25

<sup>a</sup> Test animals received injections of endotoxin every third day from birth to day 18.

<sup>b</sup> All values expressed as means

<sup>c</sup> Standard deviation

<sup>d</sup> Not available

<sup>e</sup> Not significant by the student t-test

TABLE IV

The effect of multiple administration of endotoxin on the hemolysin and hemagglutination response in young Balb/C mice

Treatment <sup>a</sup>	Days of Immunization <sup>b</sup>	Days of Titration <sup>c</sup>	Antibody Titers <sup>d</sup>					
			Hemolysin			Hemagglutination		
			No. responded <sup>e</sup>	Mean	Range	No. responded	Mean	Range
Endotoxin (Se-dioxane)	20	5	2/3	88	0-144	2/3	56	0-96
		10	6/9	33	0-80	5/9	52	0-160
Control	20	5	2/2	120	96-144	2/2	87	86-87
		10	2/2	75	70-80	2/2	360	80-640
Endotoxin	30	5	8/12	70	0-320	7/12	52	0-200
		10	8/11	130	0-320	9/11	149	0-320
Control	30	5	5/5	512	320-640	5/5	384	320-640
		10	5/5	240	80-320	5/5	448	320-640

<sup>a</sup>The mice received injections of endotoxin every third day from birth to day 18.

<sup>b</sup>Designates day after birth that mice were immunized with 0.1 ml of 10% sheep red blood cells i.p.

<sup>c</sup>Day mice were bled for antibody titrations.

<sup>d</sup>Titers are expressed as reciprocals of serum dilutions and the means are rounded off to the nearest whole number.

<sup>e</sup>The numerator designates those animals responding to immunization; the denominator the total number of animals immunized.

mice responded at day 20 to vaccination with sheep erythrocytes whereas 100% of the controls responded. Although the antibody titer of the 30 day endotoxin-treated mice had increased well above that observed for the 20 day old test group, it is apparent that a severe impairment of responsiveness to sheep erythrocytes still persisted.

## DISCUSSION

Wasting diseases have been induced in many ways such as by neonatal thymectomy (Miller, 1961), injection of cortisol acetate (Schlesinger and Mark, 1964, Reed and Jutila, 1965), or thymectomy together with the administration of rabbit anti-mouse thymocyte serum (RAMTS) (Cantrell and Jutila, 1969). In addition, it has been shown that wasting diseases can be induced with heat-killed bacterial cells or cell wall extracts rich in lipopolysaccharides (LPS) from these organisms (Eksedt and Nishimura, 1964, Eksedt and Hayes, 1967, Keast, 1968, and Keast and Walters, 1968). Many workers have demonstrated a reduction in the severity of wasting diseases by eliminating the normal flora either by use of antibiotics (Azar, 1964a) or germ-free mice (Wilson et. al., 1964, McIntire et. al., 1964, Eksedt and Nishimura, 1964, and Reed and Jutila, 1965). These data suggest that certain members of the normal flora and their endotoxins are major factors in the development of pathologic changes leading to death. Significantly, the pathologic effects of endotoxin mimic those described in other forms of wasting disease. These studies were designed to induce wasting in neonatal animals by treatment with endotoxin and to compare pathologic changes with those reported for other forms of wasting disease. To accomplish this refined endotoxin was prepared and chemically characterized to provide a material with potent biological activity and constant properties.

Although the chemical composition of the endotoxins prepared by the dioxane method and the aqueous ether method varied quantitatively (Table I), the I.R. spectra for each of the endotoxins were very similar (Figures 2, 3, and 4). Moreover, the biological activity of each

endotoxin preparation, as expressed by the I.R., was also essentially the same (Figures 5, 6, and 7). It was therefore assumed that, for these studies, the immunologic and hematologic effects of various endotoxins would be similar.

The incidence of wasting induced with purified Se-dioxane and Se-ether endotoxin was found to be very high (100%) and a mortality of 38% and 60%, respectively, was observed in the interval day 0 to day 25. This is in contrast to the failure to induce wasting disease with commercial endotoxin preparation (Difco LPS). The latter finding confirms those of Eksedt and Hayes, 1967, but conflicts with those of Keast and Walters, 1969. Although the latter investigators observed symptoms of wasting disease following multiple and large doses of commercial E. coli LPS, they reported no fatalities among wasted animals. The commercial LPS preparations apparently contain little endotoxin activity and data obtained with these preparations cannot be correlated with that obtained with purified endotoxin.

The symptoms of wasting disease induced with endotoxin were reminiscent of those observed with other forms of wasting disease most notably that induced with cortisol acetate or neonatal thymectomy. Hence, it could be concluded that some of the pathological events associated with wasting induced by these methods were produced, in part, by the effects of endotoxin. The incidence of deaths among mice treated with endotoxin (Table II) was similar to mortality among thymectomized mice treated with RMTS (Cantrell and Jutila, 1969) but significantly less than the mortality among cortisol acetate treated mice (Reed and

Jutila, 1965).

The hematological response of an animal treated with endotoxin was characterized by a neutrophilia which developed by day 10 and persisted through day 30 (Table III). A similar finding was made in mice treated with cell wall preparations rich in endotoxin (Eksedt and Hayes, 1967). In contrast, the hematologic picture differs in thymectomized mice given anti-lymphocyte serum (Monaco, 1967, Agnew, 1968, Cantrell and Jutila, 1969) and those treated with cortisol acetate (Reed and Jutila, 1967, Jutila, 1969). Thymectomized mice and neonates treated with cortisol acetate characteristically demonstrated a lymphopenia. In addition to this, Reed and Jutila, (1967) also observed an initial depression in the neutrophil counts after treatment with cortisol acetate but when infections developed a neutrophilia occurred. The discrepancy between the data obtained from mice treated with endotoxin and that obtained from thymectomized mice or mice treated with cortisol acetate may be explained by the fact that lymphocyte-producing organs such as thymus and spleen were unaffected by the treatment in one case and severely damaged or abated in the other. It seems particularly pertinent that a neutrophilia, as observed in this or other forms of wasting, is commonly associated with infection.

The immunological studies on endotoxin-treated mice (Table IV) closely parallel those seen in wasting mice following thymectomy or treatment with cortisol acetate (Jutila, 1969). A depression of responsiveness to sheep erythrocytes was evident at day 20 with slight recovery at day 30 after initiation of treatment. These results confirm

the findings of Eksedt and Nishimura (1964) and Eksedt and Hayes (1967) who also demonstrated an impaired antibody response in endotoxin-treated mice. Of interest is the observation by Eksedt and Hayes (1967) that although no circulating antibody to sheep erythrocytes could be detected, the skin graft rejection mechanism was not affected. This would indicate that although the humoral immune response was impaired, the cellular response was unaffected and emphasized the well-known and unexplained dichotomy between the two systems. The precise reasons for antibody suppression was not determined, however, it is postulated that this effect probably occurred on the afferent limb of the immune response. In support of this postulate it was noted that although the lymphocyte counts and morphology appeared to be unaffected, there was little antibody being produced. This may be explained by reasoning that the antigen was prevented from reacting with the Ag-sensitive cell. Precisely how this effect may be mediated is not known, however, it is proposed that it may occur in one of the following ways. The endotoxin may cause a hyper-reactivity of the adrenals which, in turn, provokes the production and release of abundant steroids (Hayes, 1969). The steroids, because of their predilection for lymphoid tissue, eliminate or damage the antigen recognition units (Miller, 1967, Mitchell and Miller, 1968). Alternately, steroids may inhibit the lysosome-mediated transformation of the responsive lymphocyte (Hischhorn et. al., 1967, Weissmann et. al., 1967). Another possibility which deserves investigation is that endotoxin, with its interaction with lymphoid cells may directly rather than through the indirect steroid pathway, interfere with or damage the antigen.

recognition unit.

Immunological suppression in wasting animals is postulated to be a necessary prerequisite to the events which eventually kill the animal. It has been demonstrated that symptoms of and death from wasting disease are caused by an infectious process following impairment of the immune mechanism of the animal (Cantrell and Jutila, 1969, Jutila and Reed, 1968, Jutila, 1969, and Azar, 1964a, 1964b). The depression allows the organisms of the normal flora to invade the body of the animal and subsequently cause wasting disease induced with endotoxin, it is concluded that many symptoms and death may have resulted from an infectious process.

## SUMMARY

Wasting disease was produced in neonatal Balb/c mice with purified Salmonella enteritidis endotoxins. The endotoxins were chemically characterized to provide the study with a potent endotoxin with constant biological activity. Neonatal mice received an initial injection of 35 to 75 ugm per gram body weight of endotoxin and a dose of 75 or 150 ugm of endotoxin every third day until day 18.

The degree of wasting was estimated by the runting (weight loss) index. Mortality was calculated to be 46% with a mean survival time of approximately 15 days. The hematologic studies conducted at day 10, 20, and 30 showed an absolute neutrophilia and leukocytosis by day 10 which persisted to day 30. Immunologic data indicates that the responsiveness to sheep erythrocytes was depressed to day 20 and continued to persist at day 30. It was postulated that endotoxin either directly or indirectly affects the immune apparatus which allows the normal flora of the animal to establish an infection which leads to wasting and death.

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