THE ALTERATION OF BACTERIAL ENDOTOXIN BY HUMAN AND RABBIT SERUM

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INTRODUCTION

Living and dead *Salmonella typhosa* cells and the endotoxin obtained therefrom provoke varied pathologic findings and physiological effects in the host. The methods of destruction of these cells or detoxification of their endotoxin have stimulated much interest.

Most of the progress attained in the study of the interaction of bacterial endotoxins with host substances has been made in elucidating the changes in the host following endotoxin administration. Little has been done to show whether or not the host exerts any effect upon endotoxin. Beeson (1) reported in 1946 that endotoxin injected intravenously into rabbits was rapidly removed from the circulation by the reticulo-endothelial system (R.E.S.). Braude *et al* (2) in 1955 and Cremer and Watson (3) in 1957 augmented this information by demonstrating that the majority of the injected endotoxin was taken up by the R.E.S. and granulocytes within five minutes after injection. Animals pretreated with cortisone were able to remove endotoxin from the circulation at the same rate. However, where as endotoxin disappeared from the R.E.S. after 10 hours in normal rabbits, the endotoxin in cortisone treated rabbits was retained by the R.E.S. for periods up to ten days. These studies demonstrated that mechanisms of the host were able to remove endotoxic substances from the circulation, but no evidence was obtained that the endotoxin was changed after uptake by the R.E.S.

Several *in vitro* studies have demonstrated that endotoxin could be

altered by normal serum. In 1955 Cluff (4) found that a beta globulin in serum was capable of complexing with Shigella endotoxin thereby altering its immunological activity. Employing agar gel diffusion procedures Cluff was able to show that three separate zones of precipitate (indicating at least three antigens) were formed when Shigella endotoxin was tested. However, only one diffuse zone of precipitate was produced when Shigella endotoxin, which has been incubated with normal human or rabbit serum, was employed as the antigen. In 1956 Rowley (5) reported the presence of a serum phosphatase that was capable of splitting phosphate from endotoxin. Rowley employed endotoxin of Escherichia coli labeled with P^{32} and found that after incubation with normal rat serum, labeled phosphate could be recovered by dialysis.

The purpose of this investigation was to determine whether or not normal serum was capable of altering purified endotoxin of S. typhosa. Also, if normal serum was found to alter endotoxin, then, the physical conditions affecting the alteration would be investigated; characterization of the alteration would be attempted; and serum would be fractionated by various methods in an attempt to determine the serum factor involved in the alteration. Inasmuch as this endotoxic substance is considered to be synonymous with the O-antigen, any change in the O-antigen after incubation with serum would be reflected in its precipitating ability with specific antiserum.

HISTORICAL

With Koch's unequivocal proof of the role of bacteria in disease, the mode of action in the causation of symptoms of disease led to much speculation. Many interesting theories in cause of death were offered. These included capillary plugging, production of anoxia in the host, utilization of the host's vital foods, red blood cell destruction and the production of bacterial poisons. Brieger's (6) search for bacterial poisons led to the discovery of ptomaines which at best were only capable of causing mild distress. Roux and Yersin (7) were the first to show that bacterial toxins existed and that they were found in the cell-free filtrates of cultures of the diptheria bacillus. Subsequent investigations revealed that only a few bacteria were capable of producing these toxins while the majority of the disease producing bacteria were not associated with bacterial toxins. The interest in toxins was spurred on by the revelation of von Behring and Wernicke (8) that toxins injected in sublethal doses protected the host against the classical disease. Another type of toxin was found by Koch and by Rowland and Macfadyen when the tubercle bacillus was ground up. This toxin was intimately associated with the bacterial cell and required disruption of the cell before it could be demonstrated. Hence, the two types of toxins were called exotoxins, those secreted by the bacterial cell, and endotoxins, those released when the bacterial cell was disrupted.

The investigation of toxins has proceded along three lines, one, their mode of action and fate in the host, second, their immunogenic response and, last their composition. The mode of action of the

lipoplysaccharide toxins of endotoxins is obscure. Recent work has answered many questions concerning their fate in the host, their composition, and the immunogenic response.

Endotoxins cause numerous physiological responses when injected into a host. However, the pathologic changes are varied and inconsistant. Following a single intravenous injection into a rabbit a sufficient dose of endotoxin to cause death, small punctate hemmorrhages are found in the thymus, abdominal lymph nodes, lungs and intestinal wall. Also, there may be small areas of necrosis in the liver, spleen, lymph nodes and myocardium. The presence of small thrombi attached to the endothelium of the veins in the liver, heart and brain may be encountered. (9). Zenker's hyaline necrosis has been observed in striated muscles and fibrinoid was observed beneath the endothelial lining of the coronary arteries (10). The pathologic alterations following two spaced injections of endotoxin into rabbits include bilateral renal cortical necrosis. This has been designated the generalized Shwartzman reaction (11). Brunson, Thomas and Gamble (12) have shown the generalized Shwartzman to be due to the occlusion of the renal arterioles by fibrinoid causing a hemorrhagic infarct. More recently Thomas and coworkers (13) have shown that there is an increase in arteriolar permeability due to the liberation of epinephrine in the kidneys.

Delauney and co-workers (14) were able to show that, following an intravenous injection of endotoxin, waves of vasoconstriction and dilation of the arterioles occured with increasing severity until death. The Capillary beds and venules were not notably affected. Tachycardia

and a diminished systolic force were also noted, but, the arterial pressure was not affected.

Numerous investigators as reviewed by Burrows (15) have shown that a hyperglycemia occurs one to two hours after injection of endotoxin, but hypoglycemia developes prior to death.

The production of fever of endotoxin has stimulated the interest of many workers. Bennett and Beeson (16) presented a comprehensive review of the pyrogenic effect of Gram-negative bacteria and their endotoxins, and indicated that no single mechanism adequately accounts for the fever production. Two main mechanisms are apparent. Heat may be retained due to the vasoconstriction of the surface arterioles, or in the case of humans where the peripheral vasoconstruction is little noted, the fever may be caused by the over production of heat.

The results of work of numerous investigators indicate that endotoxin <u>per se</u> is not an active pyrogenic agent, but that the endotoxin either forms a hemic complex which is pyrogenic or that it stimulates the tissues to elaborate an endogenous substance which is pyrogenic. The main basis for this reasoning is that the onset of fever is delayed from 90 to 120 minutes after an intravenous injection of endotoxin. Menkin (17) was able to show a correlation between the action of pyrexins in the host with a bacterial pyrogen but, indicated that they were not the same. Bennett and Beeson (18, 19) were not able to transfer the pyrogenic activity from tissues of animals injected with endotoxin. However, the granulocytes and the exudate contained a heat labile fraction causing fever with a shortened latent period. Atkins

and Wood (20) were able to show by means of passive transfer of serum from rabbits injected with typhoid vaccine that an "endogenous pyrogen" appeared in the host after 60 minutes and remained circulating for 120 minutes. Grant and Whalen (21) were able to demonstrate that the latent period for production of fever was approximately one hour in rabbits given endotoxin. The latent period could be reduced to 20 minutes when rabbits were injected with endotoxin which was incubated at 37°C for three hours with blood or plasma, which indicated an endotoxin activation of some factor in blood. Gerbrandy, Cranston, and Snell (22) reduced the latency of fever in humans by injection of endotoxin which had been incubated with plasma. More recently they found (23) that endotoxin which was incubated with blood rich in leucocytes caused an enhanced fever production and a shortened latent period. In contrast, a mixture of endotoxin and blood poor in leucocytes caused no demonstrable activity. Goodale *et al* (24) showed that endotoxin lost its ability to trigger pyrexia in humans when it was incubated with normal serum. Hegemann (25) demonstrated that both serum and plasma were capable of neutralizing the pyrogenic activity attributed to endotoxin. He also showed that the leucocytosis stimulating effect of endotoxin was lost after incubation with either serum or plasma.

Pillemer and co-workers (26) demonstrated that endotoxin injected intravenously into mice combined with properdin and caused a temporary decrease in the circulating properdin level, but after 24 hours the properdin level returned to a higher level.

Braude and co-workers in 1955 (2) using Cr^{51}-labeled endotoxin were able to show that endotoxin had a great affinity for reticulo-endothelial tissues. More recently Cremer and Watson (3) using fluorescein-tagged antibody demonstrated that large doses of endotoxin injected into a rabbit were rapidly adsorbed by blood platelets and absorbed by granulocytes. As a result of the accumulation of leucocytes in the lungs after the injection of endotoxin, leucopenia developed. Both of these methods showed that endotoxin was adsorbed by the histiocytes within twenty minutes after injection of the endotoxin and was no longer present there after ten hours. However, rabbits treated with cortisone retained the endotoxin in their phagocytes which were unable to destroy or dispose of it. Ribble and co-workers (27) using Cr^{51}-labeled *E. coli* endotoxin demonstrated that cortisone-treated mice had an increased splenic uptake of endotoxin while the hepatic uptake was impaired.

All of these studies showed that endotoxin could combine with or alter blood constituents. However, little investigation has been done relative to the possible change in endotoxin during any of the above processes. While this present study was in progress two investigators having made a different approach reported results showing alteration of endotoxin by normal serum. Cluff (4) found that normal rabbit serum, normal human serum or the beta globulin fraction of normal serum incubated with *Shigella* endotoxin altered the immunological reaction of the endotoxin as shown by the gel diffusion techniques. One diffuse band of precipitate developed when an incubated mixture of endotoxin and serum was used as the antigen while three distinct

more dense than the saline-endotoxin controls. Moreover, Cluff demonstrated that the serum of rabbits tolerant to S. marcescens had the same capacity for altering the immunological activity of endotoxin. The fraction of serum to which this activity was attributed was found to be heat stable at 56°C for 30 minutes. In contract the findings of Goodale and co-workers (23) and Hegemann (25), Cluff was also able to show that incubation of endotoxin with normal rabbit serum caused a fever of greater magnitude than endotoxin alone. However, the latent period was not appreciable shortened.

Rowley (5) using E. coli endotoxin labeled with P^{32} found that a heat labile fraction of normal human, rat, mouse and rabbit sera were capable of liberating dialyzable P^{32} from the endotoxin. He also found the optimum pH for the reaction to be about 8. The activity was greatly decreased when divalent cations were removed from the serum by ethylene diamine tetra-acetic acid.

The purpose of this study was to investigate the effect of normal serum in producing immunochemical or biological changes in endotoxin per se. The endotoxin of S. typhosa is synonymous with the somatic antigen, and therefore, if a known amount of endotoxin was added to serum or saline and incubated, one can assay these mixtures by the quantitative precipitin technique as adapted to measurement of antigens. Recovery of 100% of the endotoxin by the quantitative precipitin reaction would indicate no alteration, while recovery of less than 100% of the endotoxin would indicate some change in the endotoxin manifested by its loss of combining ability with antibody.

MATERIALS AND METHODS

1. Production of Bacterial Endotoxin

The endotoxin of S. typhosa 0-901 was employed throughout this entire study with one exception where rabbits were rendered tolerant to endotoxin by Serratia marcescens endotoxin*. A culture of S. typhosa 0-901 was obtained from the Walter Reed Army Medical Graduate School. The organism was maintained in the departmental culture collection by periodic transfer on tryptacase soy agar. Before the organism was used in the production of endotoxin, it was plated out on SS medium to ascertain its purity and checked by Kliger iron agar cultures and gram stain reactions. A typical smooth colony was picked from the SS medium and cultured in 50 ml. of trypticase soy broth for 18 hours at 37°C. This culture was used as inoculum for seed cultures which were prepared by adding 3 ml. of incoulum to 25 ml. of trypticase soy broth, and incubated at 37°C for 18 hours. Stainless steel trays (18"x16"x6") were used for mass culture. They were fitted with tops made of paper layered with cotton and Reynolds wrap aluminum and sterilized. Trypticase soy agar, 1800 ml. was placed in the rabbit trays which gave an agar depth of about $\frac{1}{2}$ inch. The trays were autoclaved, cooled, and incubated to check for sterility. One seed culture was used as inoculum for each tray which was poured onto the agar at one corner and then the tray was tilted back and forth to distribute the inoculum over the surface of the agar. The mass cultures were propagated at 37°C for 18 hours after which, the cells were scraped from the surface with a rubber

*Supplied by Difco Corporation

spatula and washed off of the agar with cold phosphate saline buffer pH 7.0. Endotoxin was prepared according to the method of Boivin (27) or Webster and co-workers (28) employing trichloracetic acid and ethanol fractionations. Figure 1. shows the procedure followed in the preparation of lots Boivin 2 (B2), Boivin 3 (B3) and Boivin 5 (B5) endotoxin. Using this procedure the yield of lyophilized endotoxin was 43.5 mg. (B2), 414.5 mg. (B3) and 998 mg. (B5). Because of the unwieldy nature of using rabbit trays for mass culture, a simplier method was sought to grow large numbers of cells. It was decided to employ the method of Tyrrell, MacDonald and Gerhardt (30) for concentrating and increasing the yield of cells. The precedure employs the principle of biphasic media, a solid agar base, trypticase soy agar, overlaid with trypticase soy broth with a ratio of 4:1. The culture media were prepared in two liter Erlenmeyer flasks and after inoculation with the organisms were grown at 37°C for 48 hours with aeration on a New Brunswick rotary shaker at a setting of five. Using this method lots B11, B12 and B13 endotoxin were prepared. The results of plate counting showed that a yield of 10 times the number of cells was obtained as prepared in a flask containing only nutrient broth. The lyophilized endotoxin represented a yield of 452 mg. (B11), 100 mg. (B12), and 537 mg. (B13). These yields show that the same ammount of endotoxin can be prepared from one flask of biphasic media (750 ml.) as from one rabbit tray utilizing solid medium (1800 ml.).

B3 and B5 endotoxins were further purified by two methods. Figure 2 indicates the first method employed using the ultracentrifuge.

Because of the polydisperse nature of endotoxin a constant sedimentable fraction was obtained in the following manner. Endotoxin suspended in distilled water was centrifuged at 10,000 x G for 2 hours. The supernate was then centrifuged at 20,000 x G for 2 hours and again the resultant supernate was centrifuged at 35,000 x G for 2 hours. This procedure yielded four fractions, the 10,000 x G, 20,000 x G., 35,000 x G., and supernate fraction. After lyophilization, the various fractions represented 60 mg., 20 mg., 12 mg. and 5 mg respectively. Endotoxin, 500 mg., was further purified according to the method of Webster and coworkers (29) employing ethanol fractionation in the presence of high salt concentration, ammonium sulfate fractionation, and ethanol fractionation without salt. The various fractions obtained were designated lipopolysaccharide (5L1) to lipopolysaccharide (5L10). 223 mg. of this purified lipopolysaccharide (5L10) was obtained. Figure 3. represents the procedure employed in the purification of the Boivin antigen.

B2, B3, B5, and 5L10 endotoxin were compared for biological activity with an endotoxin received from Walter Reed Army Medical Graduate School. These endotoxins were tested for pyrogenicity, antigenicity, and toxicity by localized Shwartzman activity, and they compared favorable with the standard preparation. B11, B12, and B13, endotoxin were compared with B5 endotoxin and were equally favorable.

2. Preparation of O-Polysaccharide

The haptenic moiety of lipopolysaccharide was prepared by two methods. Both methods are described in detail by Staub and Combes (31). The method of Mesrobeanu (32) was first employed which involved the acid

Seed culture - 25 ml. T.S.Broth

Mass Culture - 1800 ml T.S.Agar - 18 hr/37°C

Cells harvested (scraped & washed off agar) 4°C

Cell suspension centrifuged 15,000 x G/30 min.

Cells washed twice (ice Cold PBS* pH 7.0)

Cells weighed (wet weight)

4 ml. ice cold water/gm. cells

Equalvolume ice cold 0.5M TCA**

Agitate for 3 hours/4°C

Centrifuge 15,000 x G/15 min.

Supernate plus 2 vol. ice cold abs. ethanol

Mix well - let stand 18 hrs./-2°C

Centrifuge 2000 RPM/-2°C/60 min.

Dissolve precipitate in cold dist. water

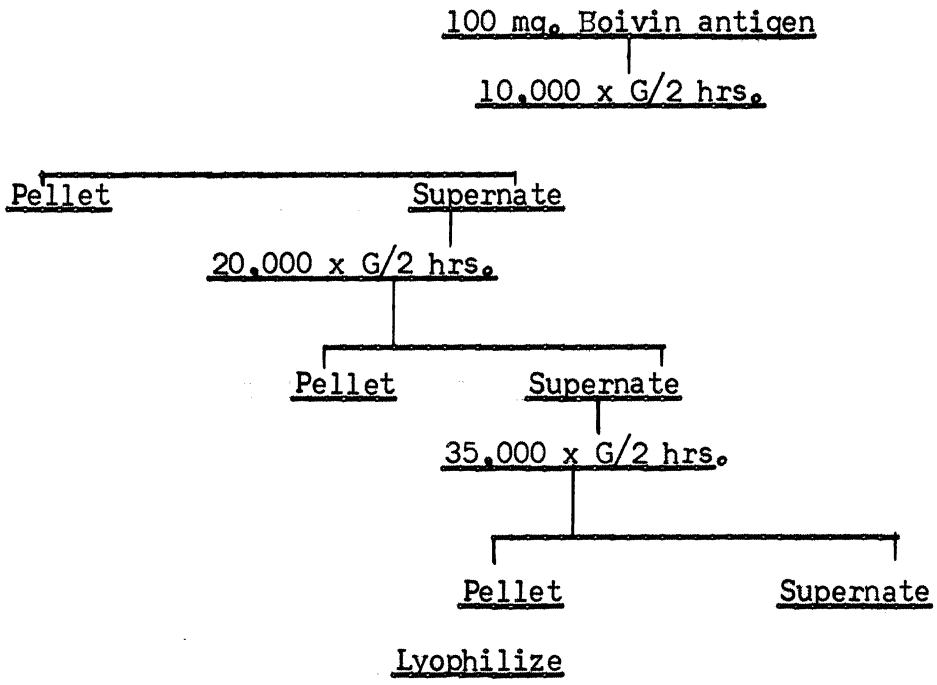
Dialize against dist. water/4°C

Lyophilize

* PBS - phosphate buffered saline

** TCA - trichloracetic acid

Figure 1. Flow sheet for preparation of Boivin antigen



Yield 60 mg	20 mg	12 mg	5 mg

Figure 2. Fractionation of Boivin Antigen by Ultracentrifugation

hydrolysis of the somatic antigen. 110 mg. of B11 endotoxin was suspended in 100 ml. of distilled water to which 5 ml. of 1N acetic acid was added. This mixture was heated in a boiling water bath for 1 hour. The mixture was allowed to cool and the resultant precipitate was removed by centrifugation at 2500 RPM for 30 minutes (International refrigerated centrifuge). The O-polysaccharide was precipitated from the supernate upon addition of four volumes of absolute ethanol. The resulting precipitate was removed by centrifugation at 2500 RPM for 30 minutes, and dissolved in 11 gm. of distilled water. The O-polysaccharide was again reprecipitated with six volumes of absolute ethanol. Since Staub states that O-polysaccharide prepared by this procedure is contaminated by immunologically inactive substances, it was decided to further purify by Freeman's procedure (33) employing glacial acetic acid fractionation. After the O-polysaccharide was reprecipitated in 6 volumes of absolute ethanol and removed by centrifugation, it was dissolved in 11 gm. of distilled water. The O-polysaccharide was carried through three ethanol precipitations at 46% and 86% ethanol by weight. The 45% fractions were discarded. The final precipitate was dissolved in 11 gm. of distilled water and to this 86% glacial acetic acid was added by weight, and the resultant precipitate was discarded. The O-polysaccharide was precipitated when the concentration of glacial acetic acid in the supernate was increased to 91.8% by weight. The precipitate was dissolved in 11 gms. distilled water and refractionated with glacial acetic acid at 86% and 91.8% by weight two more times. The glacial acetic acid was eliminated from the precipitate by reprecipitation

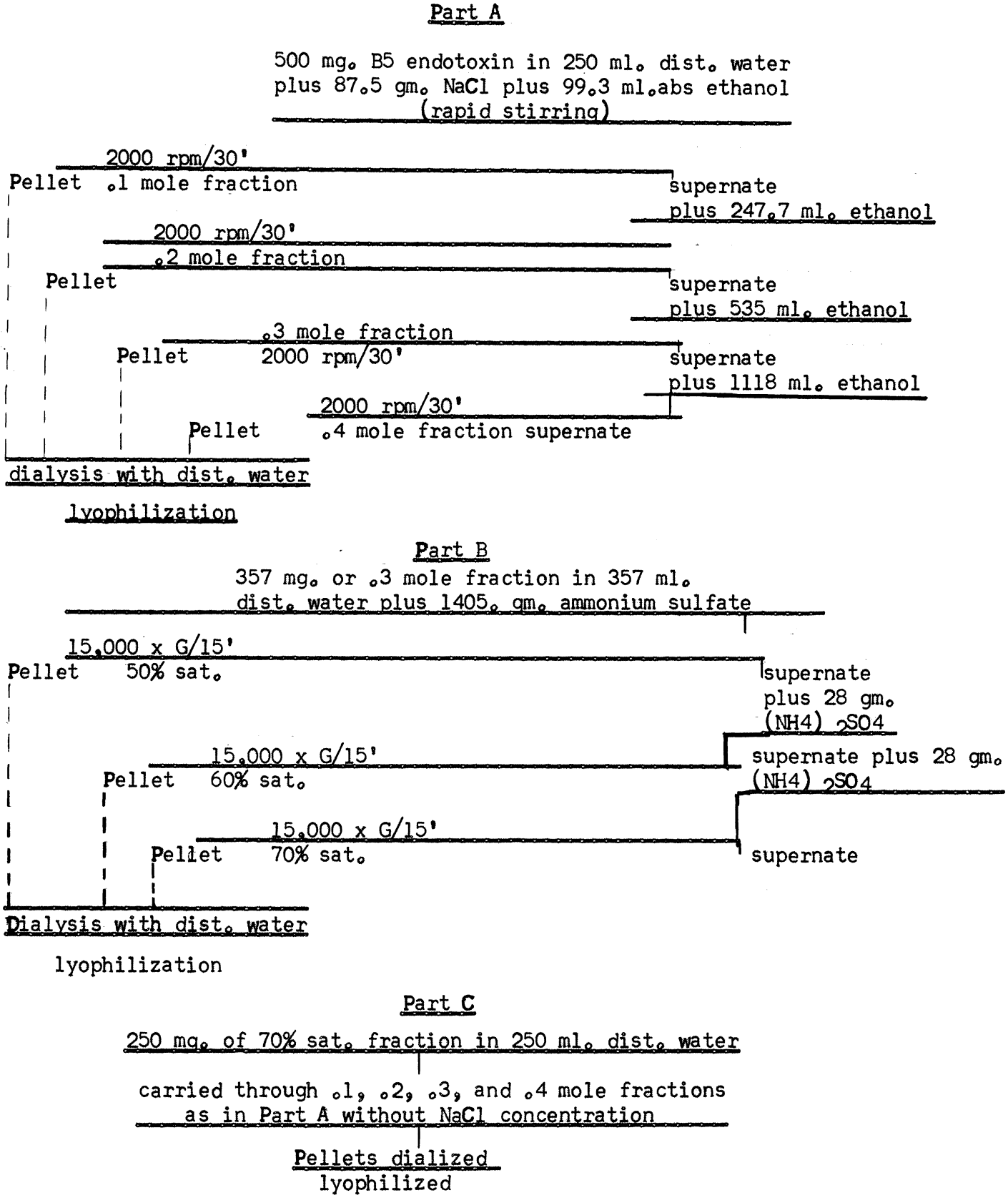


Figure 3. Purification of Boivin Antigen by Ethanol and Ammonium Sulfate Fractionation

twice with 6 volumes of absolute ethanol. The O-polysaccharide was twice washed with absolute alcohol and twice washed with ether. It was finally dried *in vacuo* over phospherous pentoxide. The yield was 34 mg. per 110 mg. of endotoxin.

The second method of preparation of O-polysaccharide that of Freeman (33) employing the direct hydrolysis of *S. typhosa* O-901 cells. The organisms were grown in the biphasic media as previously described using six flasks. After the cells were twice washed with PBS pH 7.1, 34 gm. wet weight of cells were obtained. The cells were suspended in 100 ml. distilled water. To this 1 N acetic acid was added so that the final concentration was N/5 acetic acid, and the resultant mixture was refluxed for two hours in a boiling water bath. After cooling and centrifuging at 2000 rpm/30 minutes in the International refrigerated centrifuge, the supernatant was concentrated at 50°C to 1/8 of its original volume. It was then neutralized with sodium bicarbonate to a pH of 6.5 and 1 volume of absolute ethanol was added. The resultant precipitate was discarded and the O-polysaccharide was precipitated by addition of 5 volumes of absolute ethanol to the supernate. The precipitate was removed by centrifugation and dissolved in a smaller volume of distilled water. The two alcoholic fractionations were repeated five more times. After addition of the alcohol, the precipitate was allowed to develop by standing at 4°C for four to five hours. When the precipitate would not develop after the 5 volume fractionation, 1 to 2 ml. of glacial acetic acid was added to aid the formation of the precipitate. After the sixth 5 volume precipitation, the partially purified O-polysaccharide

was dissolved in a small volume of distilled water and weighed. The O-polysaccharide was precipitated upon addition of 94% glacial acetic acid by weight. The precipitate was removed by centrifugation at 10,000 x G for 30 minutes. The 94% glacial acetic acid fractionation was repeated 3 times. Finally the glacial acetic acid was removed by reprecipitation with 6 volumes of absolute ethanol. The O-polysaccharide was twice washed with absolute ethanol and twice washed with ether, and dried *in vacuo* over phosphorus pentoxide. The yield was 246 mg. per 34g. cells wet weight.

These two products were designated P1 and P2. They were tested for pyrogenicity, antigenicity, and toxicity by local Shwartzman activity. They caused no elevation of fever, produced no antibody and did not cause a localized Shwartzman reaction. Standard quantitiative precipitable antibody nitrogen curves were determined for further quantitation.

3. Preparation of S. typhosa Whole Cell Vaccine

Vaccines of two types were prepared. Acetone-killed-dried (AKD) cells were prepared. The cells were grown by shake culture for 18 hours at 37°C. The cultures were centrifuged at 2°C for two hours at 2100 rpm in the International refrigerated centrifuge. The cells were twice washed with cold PBS pH 7.0, and finally packed. Half of the cells were treated with three volumes of acetone and allowed to stand over night at 2°C. The acetone was decanted and the cells were dried at 37°C. After grinding the cells by mortar and pestle, the yield was 2.0709 grams. To prepare the vaccine the AKD cells were suspended

in phosphate saline buffer pH 7.0. A stock concentration of 2 mg. per ml. was thusly prepared. This vaccine was used to stimulate the production of antibody in rabbits.

The other half of the cell mass was heat killed at 56°C for one hour. After testing for sterility in nutrient and thioglycollate broth, the organisms were diluted with phosphate saline solution pH 7.0 to a concentration of 1 x 10^9 cells per ml. Standardization was accomplished by the Klett colorimeter after checking the tubes against a direct count of S. typhosa cells. Figure 4. represents the standard curve for the McFarland tubes with a 540 um. filter. 2.9 liters of this vaccine were prepared and tested with antiserum of high titer (1:25,600) by the agglutination procedure. This preparation is a standard antigen suspension for use in the agglutination reaction.

4. Preparation of Anti-O Rabbit Serum

Five batches of antisera were prepared employing 44 rabbits. The same general pattern was employed for each batch of antiserum. The antisera was prepared employing AKD cells of S. typhosa O-901 according to the protocol in Table 1. Rabbits were bled on the 7th and 8th day after the last immunizing dose. A total of 100 ml. of blood was taken from each rabbit by incising the marginal ear vein. After the second bleeding the rabbits were given 50 ml. of 10% dextrose-saline intraperitoneally to restore blood volume and prevent death due to loss of blood. The sera from all rabbits were titered by the agglutination reaction employing the standard heat killed S. typhosa antigen. 0.5 ml of serum was serially diluted to 1:25,6000 with either PBS pH 7.0 or

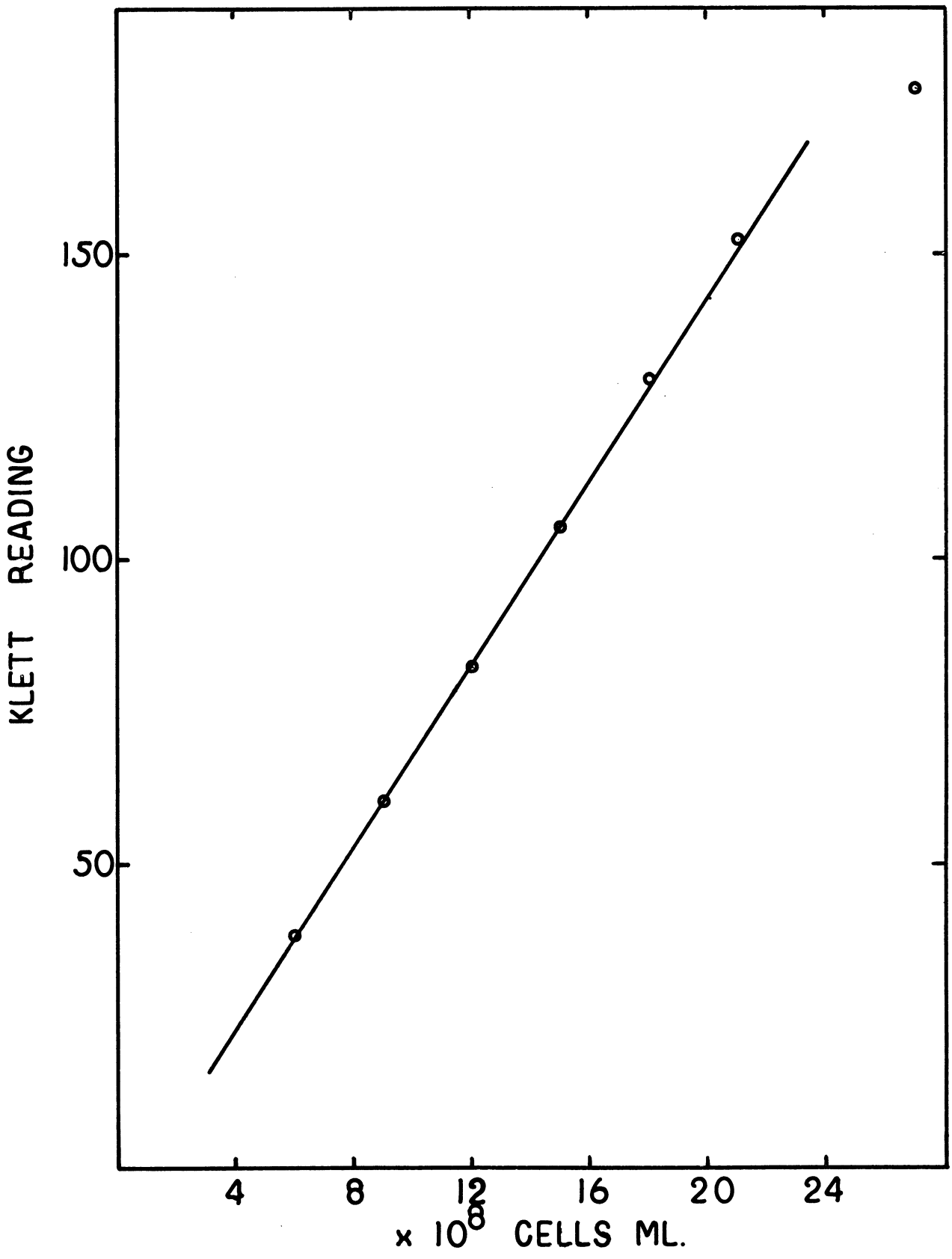


FIGURE 4. QUANTITATION OF CELLS OF S. TYPHOSA BY TURBIDITY (540 Mμ)

TABLE I

Program of Injection of Antigen for the Production of Antiserum

Day	Route	Amt. injected	Bled
1	Subcutaneous Intraperitoneal	100 ug. 100 ug.	
3	Intravenous	500 ug.	
5	Intravenous	1 mg.	
8	Intraperitoneal	1 mg.	
9	Intravenous	2 mg.	
10	Intravenous	2 mg.	
17			Bled
18			Bled
32	Intravenous	1 mg.	
39			Bled
40			Bled

veronal saline pH 7.4. To each tube 0.5 ml. of antigen suspension was added and the reaction was incubated at 52°C for 18 hours. The last tube showing macroscopic evidence of agglutination just different from the control was designated the titer of the serum. All sera with a titer of 1:3200 or less were pooled, frozen in a separatory funnel and thawed. There were three apparent layers in the funnel. Each layer was removed and tested with the O-901 standard antigen by the aforementioned agglutination reaction. The bottom layer gave a titer of 1:12,800; the second layer a titer of 1:1,600; and the third or top layer was 1:800. The heavy, high-titer layer was pooled with the other sera. The rabbits were rested 2 weeks, bled and injected with 1 mg. of AKD O-901 cells intravenously. On the 7th and 8th day after the booster dose the rabbits were bled again as previously described and the sera tested as described. The two pools of sera were combined into one lot and complement was inactivated by an unrelated antigen-antibody reaction using the bovine serum albumin and antibovine serum albumin system. The reaction occured while incubation at 37°C for two hours and for 48 hours at 4°C. After this reaction occured and the resulting precipitate was removed by centrifugation, the batch of antiserum was distributed in 20 ml. amounts and stored in the frozen state at -20°C until used. Agglutination titers and quantitative precipitable antibody nitrogen curves were established for future quantitation of endotoxin.

5. Normal Rabbit Serum

Normal rabbits were bled from the marginal ear vein. The blood was allowed to clot and the serum removed. The serum was tested for the presence of specific O-agglutinins by the agglutination reaction. All rabbit serum used as normal rabbit serum had a titer of less than 1:10 since this was the serum dilution in the first tube. These sera were used for incubation with endotoxin and fractionation.

6. Normal Human Serum

Human donors in good health were bled by syringe from the cephalic vein. The blood was allowed to clot and the serum was removed. The serum from all donors was tested for the presence of specific O-agglutinins as described be fore and only sera with a titer of less than 1:10 was used as normal human serum. These sera were used for incubation with endotoxin and fractionation.

7. Versene Treated Serum

Versene treated sera were prepared by adding 0.05M and 0.5M per ml. of tetrasodium ethylene diamine tetraacedic acid (EDTA) to normal rabbit and human sera. EDTA was added to these sera in excess for the removal of magnesium ions. According to Levine and co-workers (34) only 0.0073M EDTA is necessary to chelate all the magnesium ions present in serum. These sera were used to incubate with endotoxin to see if magnesium ions are necessary for the alteration of endotoxin.

8. Resin Treated Serum

Resin treated sera was prepared by adding an equal volume of the sodium form of Amberlite IRC - 50 to fresh normal rabbit and human

sera and gently mixed for ten minutes. The treated serum was then pipetted from the resin. Amberlite IRC - 50 was converted to the salt phase according to the method described by Lepow and co-workers (35). This method of removing magnesium ions from serum was employed by Pillemer and co-workers (36). Resin treated serum was prepared to support the evidence obtained from versene treated sera, that magnesium ions are not necessary for altering endotoxin.

9. Zymosan Treated Serum

Zymosan, the insoluble polysaccharide obtained from yeast cell walls by extraction, was added to fresh normal human sera (3 mg. per ml.). The mixture was gently mixed periodically during the 30 minute incubation at 24°C. The zymosan was removed from the serum by centrifugation in the Serval centrifuge at 10,000 x G for 15 minutes. According to Pillemer and co-workers (37) zymosan complexes with properdin during this procedure and removes the properdin from serum (RP) upon centrifugation. These sera were used to incubate with endotoxin to determine whether or not properdin is involved in the alteration of endotoxin.

10. Heat Inactivated Serum

Fresh human and rabbit normal sera were inactivated by heating in the water bath at 56°C for 30 minutes. It has been well documented by numerous investigators that the heat labile components of complement are inactivated by this technique. Also Pillemer and co-workers (38) have shown that properdin is inactivated by this procedure. Heat treated sera were used for incubation with endotoxin to determine if complement

was involved in the alteration of endotoxin and to support the evidence that properdin was not involved in the alteration of endotoxin.

11. Tolerant Serum

Since it has been shown by numerous investigators that heterologous endotoxins can render rabbits tolerant to other endotoxins, rabbits were treated as shown in Table 2. with S. marcesens endotoxin which was supplied by the Difco Corporation of Detroit, Michigan. The sera of these animals were tested by the agglutination test employing S. typhosa O-901 standard antigen suspension and no anti-typhosa O-901 agglutinins were present. These sera were used to incubate with S. typhosa endotoxin to determine whether or not they were capable of altering it. Sera were collected from tolerant rabbits one day, 8 days, 15 days, 22 days and 29 days after their last injection of endotoxin and used to study their activity on endotoxin. In order to further investigate the serum activity in tolerance, sera were collected from four rabbits while tolerance was being produced. The sera were collected before the 1st injection, 4, 8, 12, 24 hours after the first injection, 24 hours after the second injection, 24 hours after the third injection, 24 hours after the 7th injection, 24 hours after the 10th injection, 14 and 21 days after the 10th injection.

12. Fluoride Treated Serum

Sodium fluoride was added to normal human serum so that the final concentration was 0.01M or 0.1M fluoride ions. In addition 0.1M magnesium ions and 0.1M fluoride ions were added to serum. These sera were

TABLE 2

Program for Injecting Endotoxin To Render Rabbits Tolerant

Day	Amt. injected intravenously	Degrees F. change in temperature (R19)
1	1 ug.	3.3
2	2 ug.	1.4
3	2 ug.	0.5
4	5 ug.	2.0
5	5 ug.	1.6
6	5 ug.	1.1
7	5 ug.	0.7
8	10 ug.	2.9
9	10 ug.	0.7
10	1 ug.	0.3
11	Bled	

used to test the phosphatase inhibiting activity of serum on the alteration of endotoxin.

13. Mercury Treated Serum

Mercuric acetate was added to normal sera so that the final concentration was 0.01M or 0.1M mercuric ions. The precipitate that developed was removed by centrifugation at 15,000 x G for 15 minutes. These sera were used to study the effect of enzyme inhibition in serum on the alteration of endotoxin.

14. Fractionation of Serum

Fresh normal human and rabbit sera were fractionated by three methods. These employed the use of ammonium sulfate, alcohol in the cold, a combination of these two, or zinc ion fractionation in the cold. Rabbit serum was fractionated in the cold utilizing ammonium sulfate concentrations of 50% and 100%. Fresh human and rabbit sera were fractionated utilizing alcohol in the cold as described by Deutsch (39). Figure 5. illustrates the procedure employed. The supernate to precipitate A contains the albumins and alpha globulins. After dialysis in the cold, these were lyophilized. Precipitate A contains the beta globulins, gamma globulins and conalbumins. The beta globulins (precipitate B) are removed by changing the pH to 5.1 with 0.05M acetic acid and precipitated with 50% ethanol to a final concentration of 10% at -2°C. The gamma globulins are removed from the supernate to precipitate B by changing the pH from 5.1 to 7.4 with 0.5M sodium bicarbonate and precipitated upon addition of an equal volume of 50% ethanol at -6°C. The supernate to precipitate C contains the conalbumins.

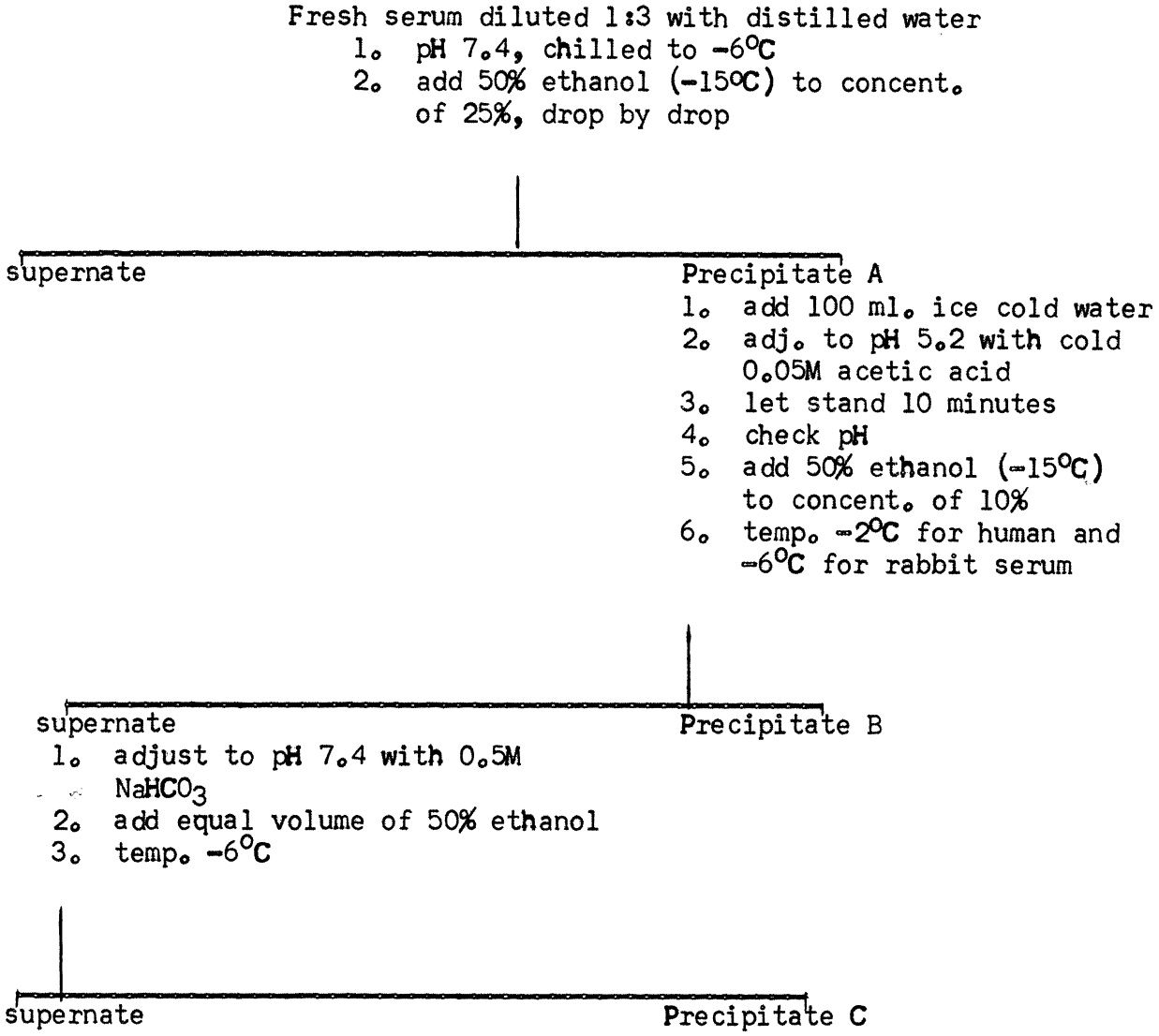


Figure 5. Flow sheet for the alcohol fractionation of serum.

The various fractions were recovered by dialysis and lyophilization.

The albumin-alpha globulin fraction was further fractionated with ammonium sulfate first using 50% and 100% saturation, and then 40%, 50%, 60%, 70%, 80%, 90% and 100% saturation. All of the various serum fractions were used for incubation with endotoxin in an attempt to locate the serum component which was responsible for alteration of endotoxin.

The third method of fractionation employed zine ions according to the method of Cohn et al (40). After the pH of fresh serum was adjusted to pH 7.4 with 1N HCl, it was cooled to -5°C in the alcohol-dry ice bath. While the serum was being agitated, zinc acetate was added slowly so that the final concentration of zinc ions was 0.02M. The precipitate was allowed to develop for 15 minutes and was removed by centrifugation at 10,000 x G for 20 minutes. The precipitate was solubilized in 0.05M EDTA. The solubilized precipitate and the supernate were dialyzed in the cold with distilled water. The fractions were recovered upon lyophilization, and were used to test their activity on endotoxin.

15. Normal Rat Serum

Blood was taken by cardiac puncture from six rats. After the serum was separated from the clots, the sera were pooled. Rat serum was used to test its ability to alter endotoxin.

16. Preparation of White Blood Cells and White Blood Cell Lysate

White blood cells were collected by two methods. They were collected from the peritoneal exudate of rabbits according to the

method of Hirsh (4). The rabbits were injected intraperitoneally with 250 ml. of saline containing 250 mg. of glycogen. Four hours after the injection the rabbits were sacrificed by air embolism, and 200 ml. of citrate saline was injected intraperitoneally. The abdomen was kneaded and opened to the right of the midline. The exudate was pipetted off. 780 ml. of exudate was collected from four rabbits. The exudate was maintained at room temperature while a total and differential while blood cell count was made. There were 2.3×10^6 cells per ml. or a total of 1.9×10^9 cells. 95% of the cells were granulocytes with neutrophiles predominating. The exudate was centrifuged at 1400 rpm for 10 minutes at room temperature (International #2, in 250 ml. bottles). The fluid was carefully drained and any red blood cells present were lysed on the addition of 20 ml. of hypotonic saline solution (lysis solution). After 10 minutes at room temperature 20 ml. of hypertonic saline solution (neutralizing solution) was added. The cell suspension was placed in centrifuge tubes and spun at 1200 rpm for 10 minutes at room temperature. The cells were twice washed with citrate saline. The white blood cells were suspended in a buffered salt solution (intracellular salt solution) at a concentration of 1×10^9 cells per 5 ml.

White blood cells were collected from human heparinized blood after centrifuging at 1200 rpm for 10 minutes. The buffy coat was removed and suspended in citrate saline solution. By repeated washing most of the red blood cells were eliminated. Those remaining were lysed as in the previous method. After the cells were twice washed

with citrate saline, they were suspended in intracellular salt solution at a concentration of 1 x 10^9 cells per 5 ml.

To prepare a white blood cell lysate, rabbit and human cells suspended in a buffered salt solution were frozen 3 times in an acetone-dry ice bath (-90°C) for 10 minutes and just thawed in a 38°C water bath. The lysate was placed in 50 ml. centrifuge tubes and spun at 3000 rpm for 15 minutes. The supernatant was stored at -20°C.

The white blood cells and the white blood cell lysates were incubated with endotoxin and with serum and endotoxin in order to see if they were active in altering endotoxin or enhanced the activity of serum in altering endotoxin.

17. Standard Quantitative Precipitable Antibody Nitrogen Curve

Inansmuch as endotoxin is synonymous with the O-somatic antigen, a standard quantitative precipitable antibody nitrogen curve can be prepared. Since endotoxin is a phosphorylated lipopolysaccharide, all the nitrogen precipitated will be antibody nitrogen. If a constant amount of anti-serum is added to various concentrations of endotoxin, a curve can be plotted for the amounts of antibody nitrogen precipitated. This curve then can be used for quantitation of unknown amounts of endotoxin. One ml. of high titered antiserum (1:12,800 or higher by agglutination reaction) was added to various amounts of endotoxin by weight in PBS pH 7.0. The system was incubated at 37°C for two hours and at 4°C for 18 hours. The resultant precipitates were centrifuged in the cold and the supernates were tested to determine the zone of activity, such as the zone of antibody excess, equivalence or

antigen excess. To determine this activity the supernates were divided into two samples. To one sample, 10 micrograms of endotoxin were added and to the other 1 drop of antiserum. After incubation the tubes were centrifuged and inspected for the presence of precipitate. If a precipitate occured in the tubes where endotoxin was added, this indicated the zone of antibody excess. If precipitates formed in both of the paired tubes, this was evidence of the equivalence zone and when precipitates formed in only those tubes where antiserum was added, this would demonstrate antigen excess. The precipitates for the antibody nitrogen curve were washed twice with cold PBS pH 7.0, and then digested with 1 ml. of kjeldahl digestion mixture plus a few crystals of potassium sulfate. The digestion mixture was heated until it was colorless. The digestion mixtures were chilled and diluted with distilled water, made alkaline with saturated sodium hydroxide, distilled into saturated boric acid solution, and titrated with N/70 hydrochloric acid using methyl purple as an indicator. The milligrams of nitrogen can be calculated using the factor of 0.2 times the number of milliliters of acid used. Table 4. shows the amounts of endotoxin employed, the results of the nitrogen determination, and the supernate analysis. The determinations were done in duplicate and the results averaged. The blank was subtracted from the average values and these were plotted. Figure 6. is the resultant curve using S1 antiserum and B3 endotoxin.

Once this curve has been established it was used as a reference for quantitating unknown amounts of endotoxin so long as the pre-

TABLE 4

Results of Quantitative Precipitable Antibody Nitrogen Determination Employing S1 Anti-O Serum and B3 Endotoxin

Tube	Antigen Added	Antibody Precip. A.		Antibody Precip. B		Average Antibody N_2
		Ml. N/70 HCl	mg. N_2	Ml. N/70 HCl	mg. N_2	
1.	0	.05	.010	.05	.010	0.10
2.	15	.185	.037	.205	.041	.039
3.	30	.35	.070	.335	.067	.069
4.	50	.47	.094	.47	.094	.094
5.	75	.57	.114	.58	.116	.115
6.	100	.70	.140	.105	.141	.141
7.	150	.90	.180	.83	.166	.173
8.	200	.95	.190	.90	.180	.185

Table 4 (Continued)

Supernate Tested for excess A.		Supernate Tested for excess B.	
Antibody	Antigen	Antibody	Antigen
////	-	////	-
////	-	////	-
////	-	////	-
///	-	///	-
///	-	///	-
///	-	///	-
//	/	//	/
//	/	//	/

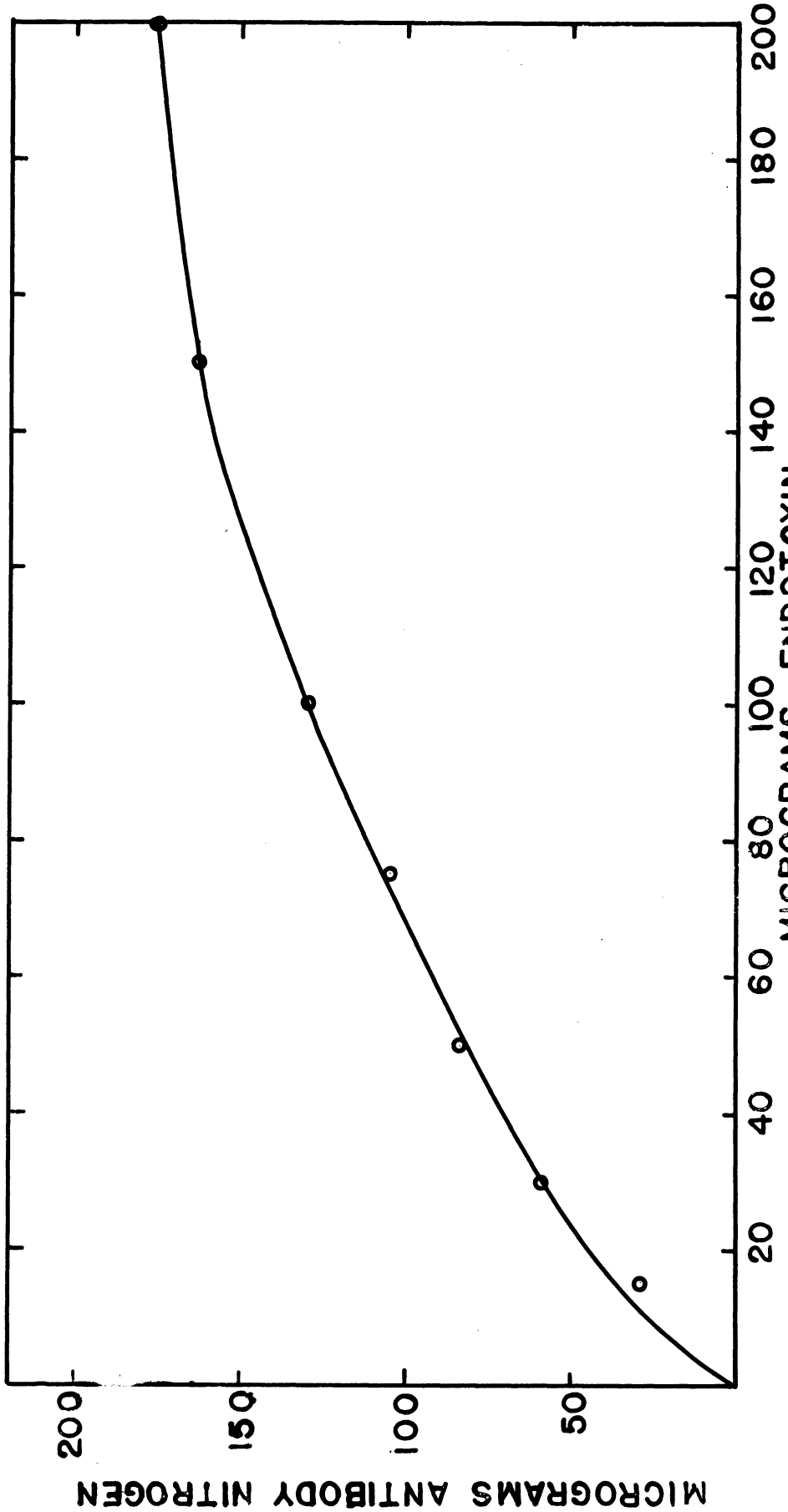


FIGURE 6. QUANTITATIVE PRECIPITABLE ANTIBODY NITROGEN CURVE WITH SI ANTISERUM AND B3 S. TYPHOSA ENDOTOXIN

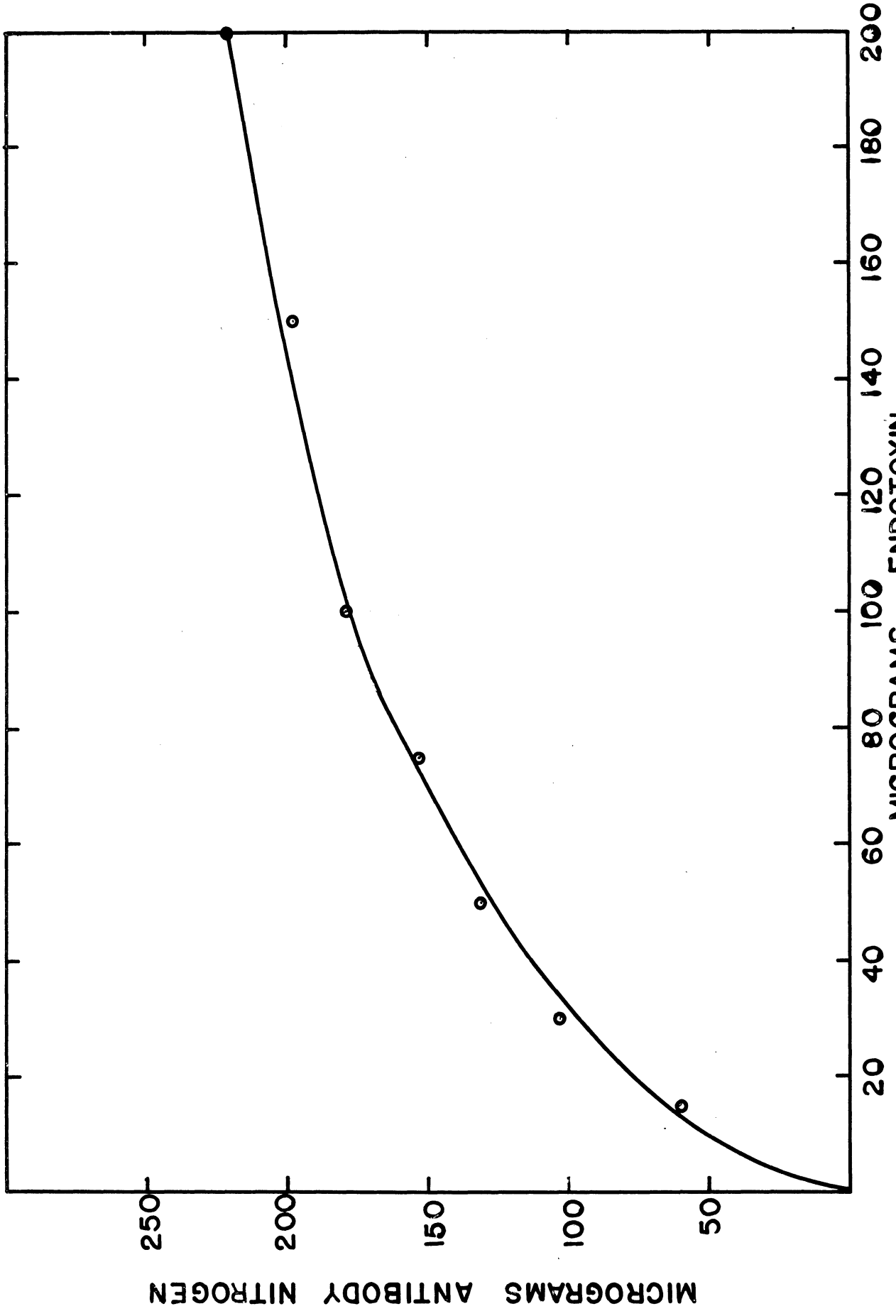


FIGURE 7. QUANTITATIVE PRECIPITABLE ANTIBODY NITROGEN CURVE WITH S2 ANTISERUM AND B3 S. TYPHOSA ENDOTOXIN

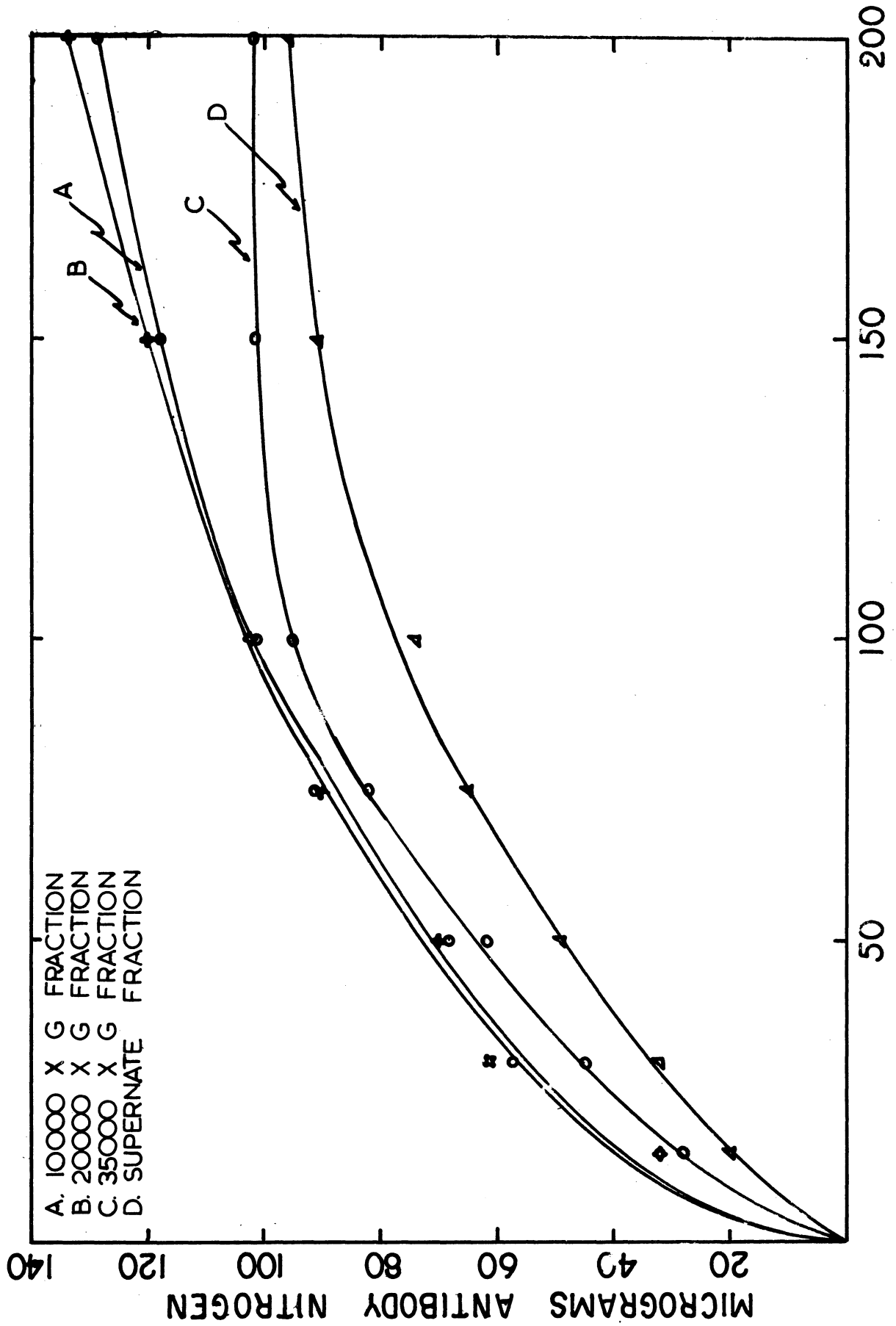


FIGURE 8. QUANTITATIVE PRECIPITABLE ANTIBODY NITROGEN CURVES FOR ULTRACENTRIFUGED FRACTIONS OF B3 ENDOTOXIN

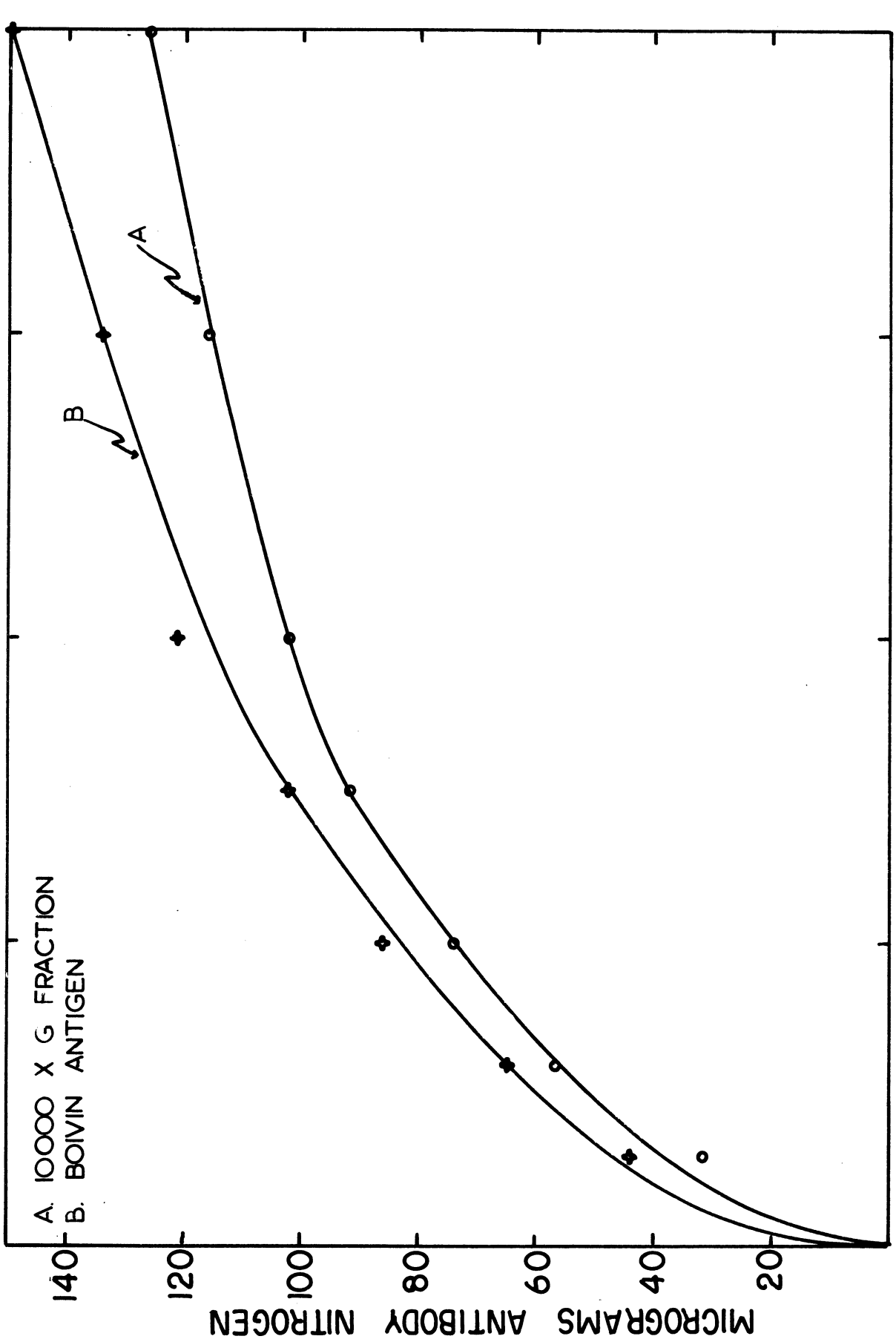


FIGURE 9. QUANTITATIVE PRECIPITABLE ANTIBODY NITROGEN CURVES FOR B3 10,000 X G. FRACTION AND BOIVIN ANTIGEN

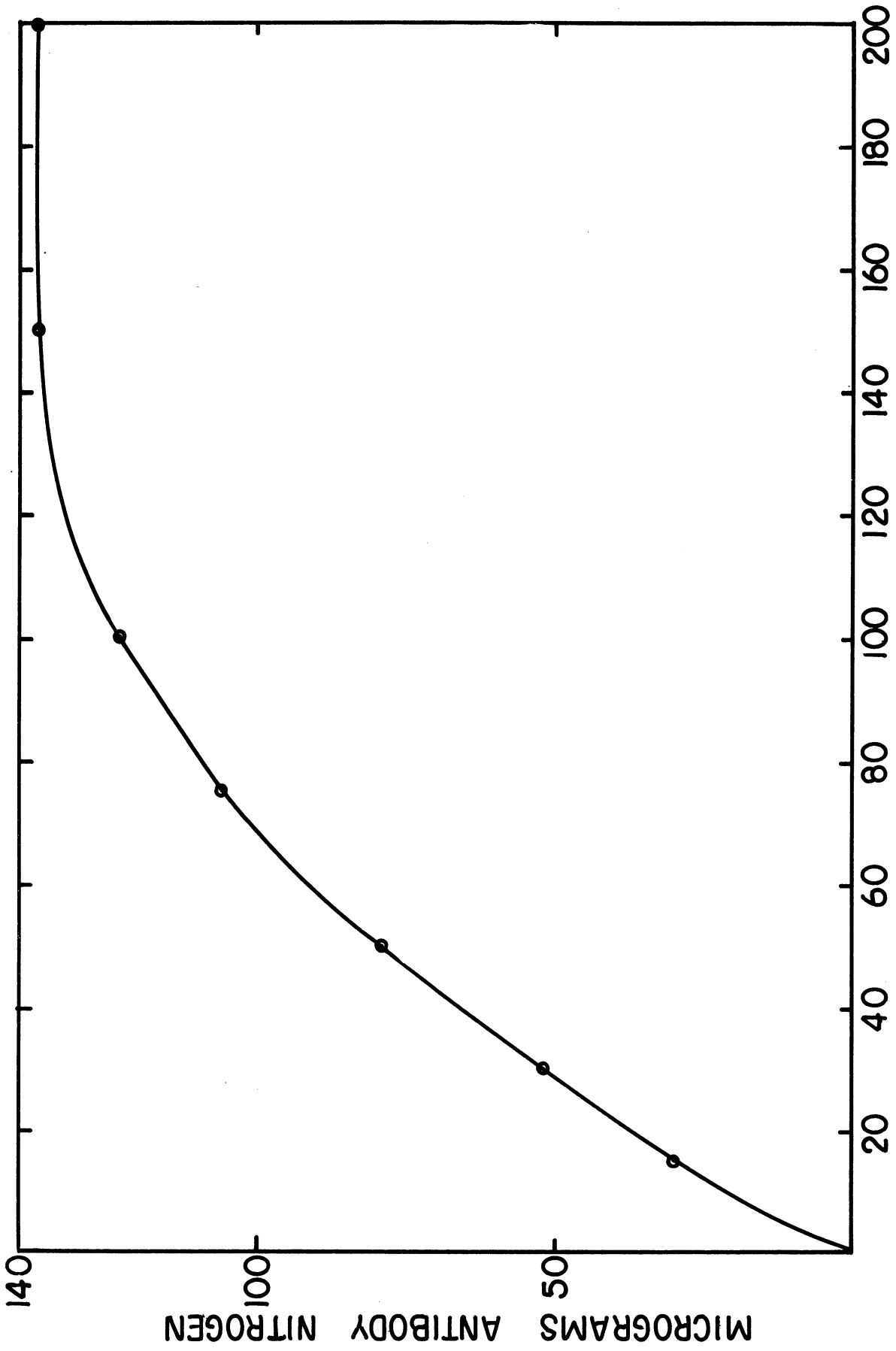


FIGURE 10. QUANTITATIVE PRECIPITABLE ANTIBODY NITROGEN CURVE FOR S3 ANTISERUM AND B5 ENDOTOXIN (10000 X G. FRACTION)

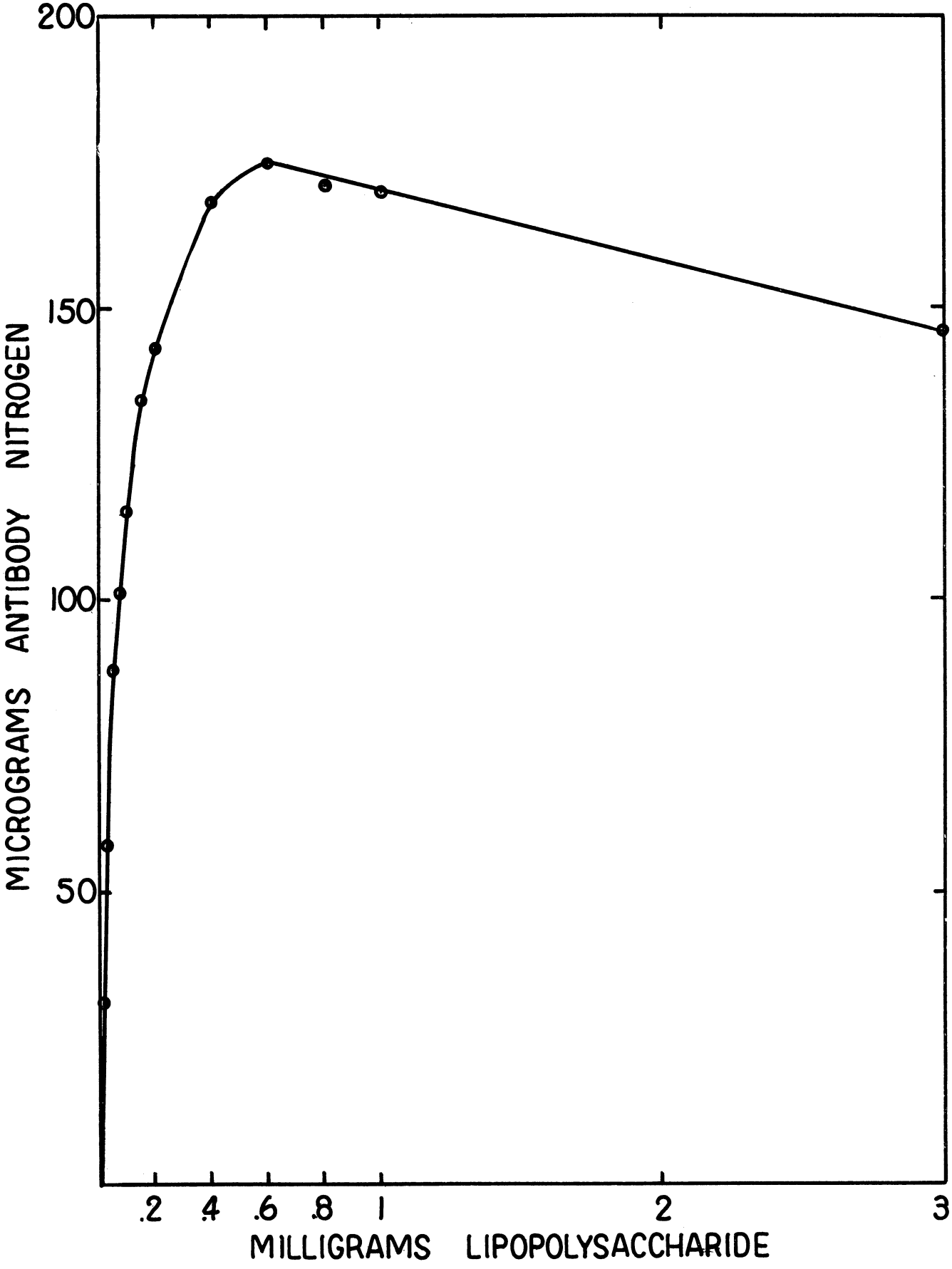


FIGURE 11. QUANTITATIVE PRECIPITABLE ANTIBODY NITROGEN CURVE FOR S3 ANTISERUM AND PURIFIED S. TYPHOSA LIPOPOLY-SACCHARIDE

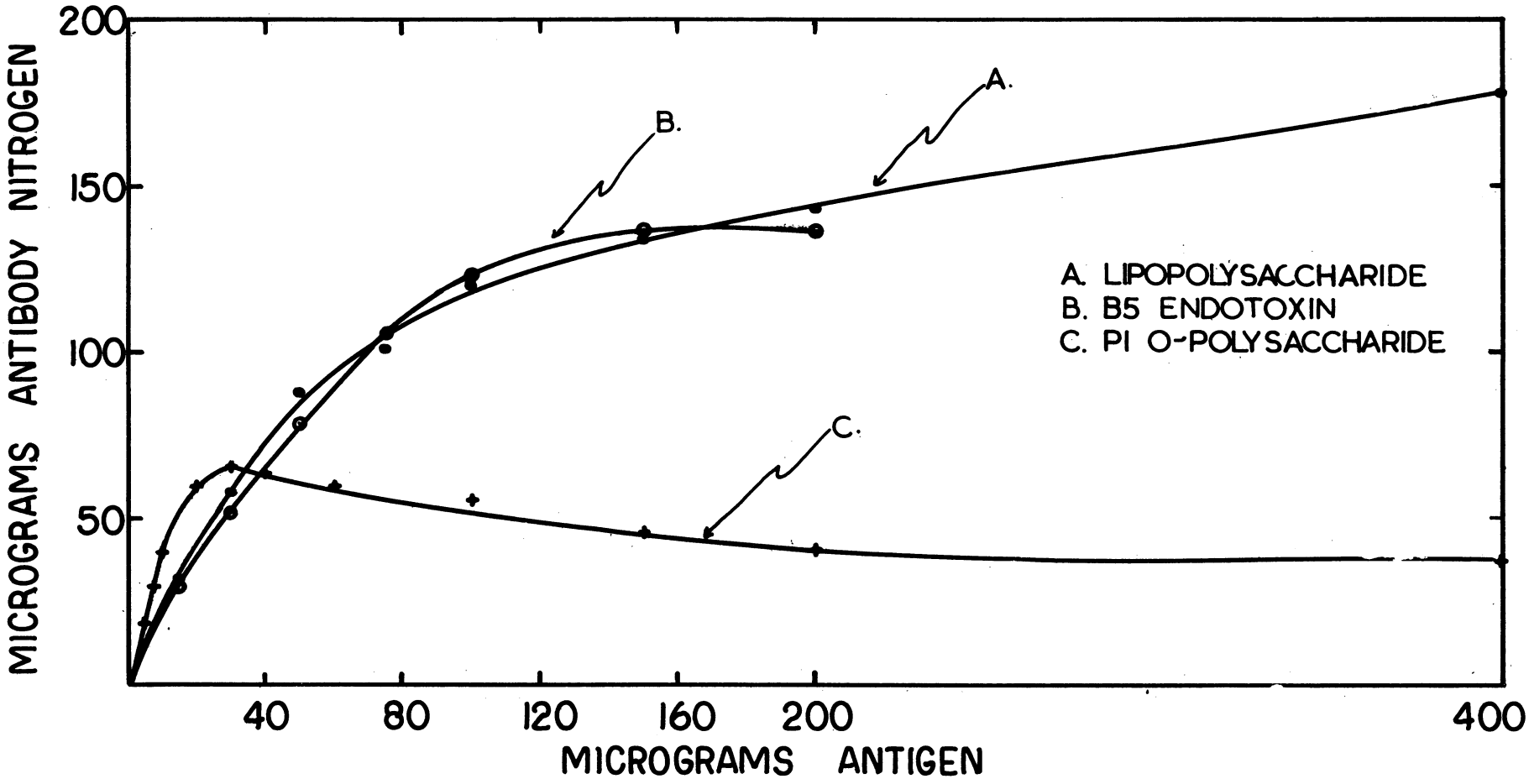


FIGURE 12. COMPARATIVE QUANTITATIVE PRECIPITABLE ANTIBODY NITROGEN CURVES FOR S3 ANTISERUM AND P1 O-POLYSACCHARIDE, PURIFIED LIPOPOLY-SACCHARIDE AND B5 ENDOTOXIN (10000 X G. FRACTION)

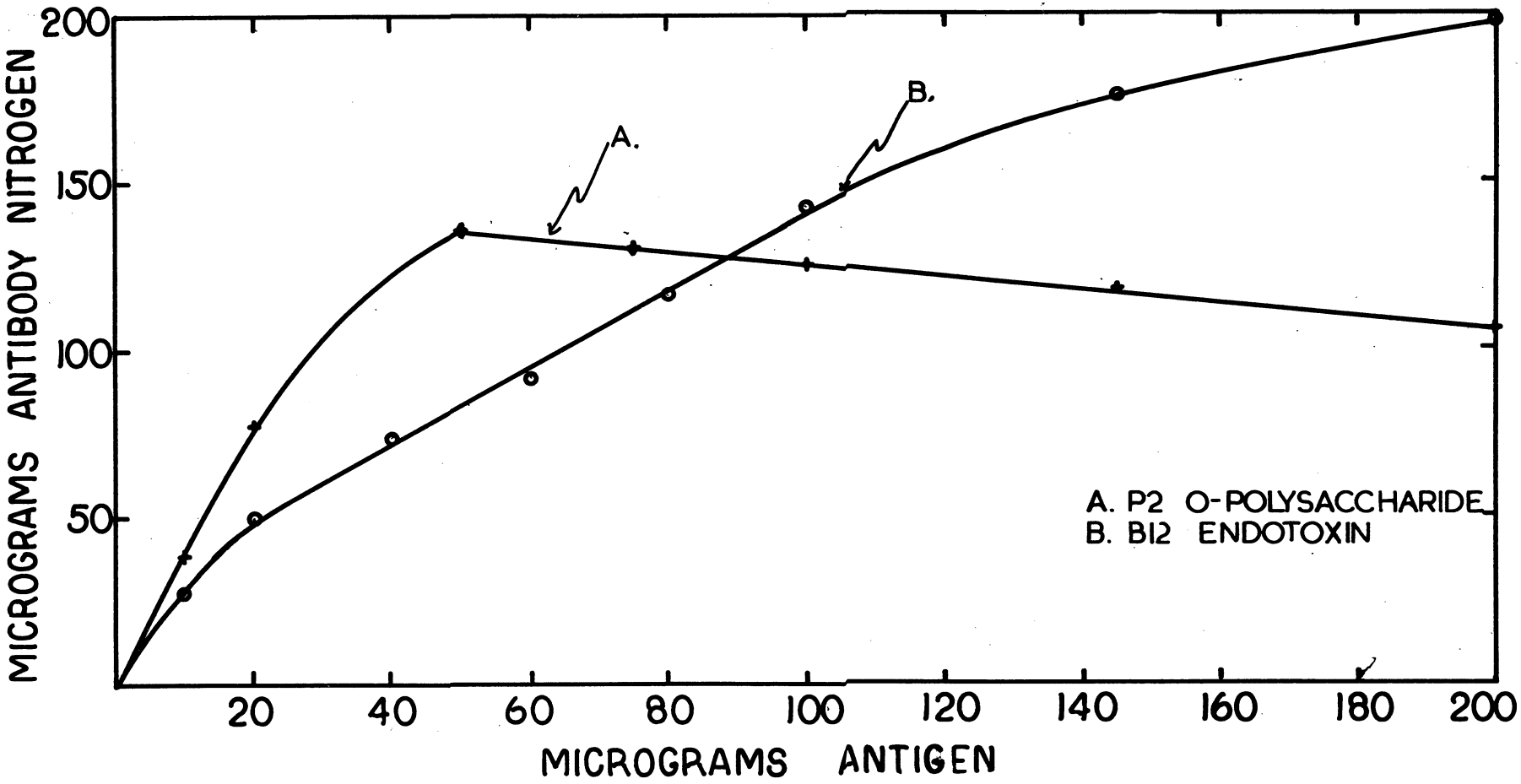


FIGURE 13. QUANTITATIVE PRECIPITABLE ANTIBODY NITROGEN CURVES FOR S4 ANTISERUM WITH P2 O-POLYSACCHARIDE AND B12 ENDOTOXIN (10000 X G. FRACTION)

cipitin reactions occur in the zone of antibody excess, and as long as these two reagents, S1 antiserum and B3 endotoxin, were employed. If either of these reagents were changed, a new curve was made. Accordingly curves were established for S1 antiserum and B3 endotoxin, S2 antiserum and B3 endotoxin, S2 antiserum and B3 endotoxin ultracentrifuge fractions, S2 antiserum and B5 endotoxin 10,000 x G fraction, S3 antiserum and B5 endotoxin 10,000 x G fraction, S3 antiserum and purified lipopolysaccharide (5L10), S3 antiserum and O-polysaccharide (P1), S4 antiserum and B12 endotoxin 10,000 x G fraction, and S4 antiserum and O-polysaccharide (P2). Figures 6. to 13 are the resultant curves.

18. Serum Incubation Test System

The system employed to test the effect of fresh normal sera, treated sera, serum fractions, white blood cells and white blood cell lysate on endotoxin was basically the same. Figure 14. represents diagramatically the procedure employed. In all tests a saline-endotoxin control was run along with the test substance and endotoxin. The test substance, such as 2.5 ml. of normal serum was added to a known amount of endotoxin. The amount of endotoxin was 2 mg. in the initial tests but 1 mg. was later used as the standard amount. The mixture was frequently shaken during incubation at various temperatures for various lengths of time. The standard conditions selected in the test procedure was 37°C and the time of incubation was 4 hours for rabbit serum and 6 hours for human serum. After incubation the mixture was subjected to ultracentrifugation at 35,000 x G for two

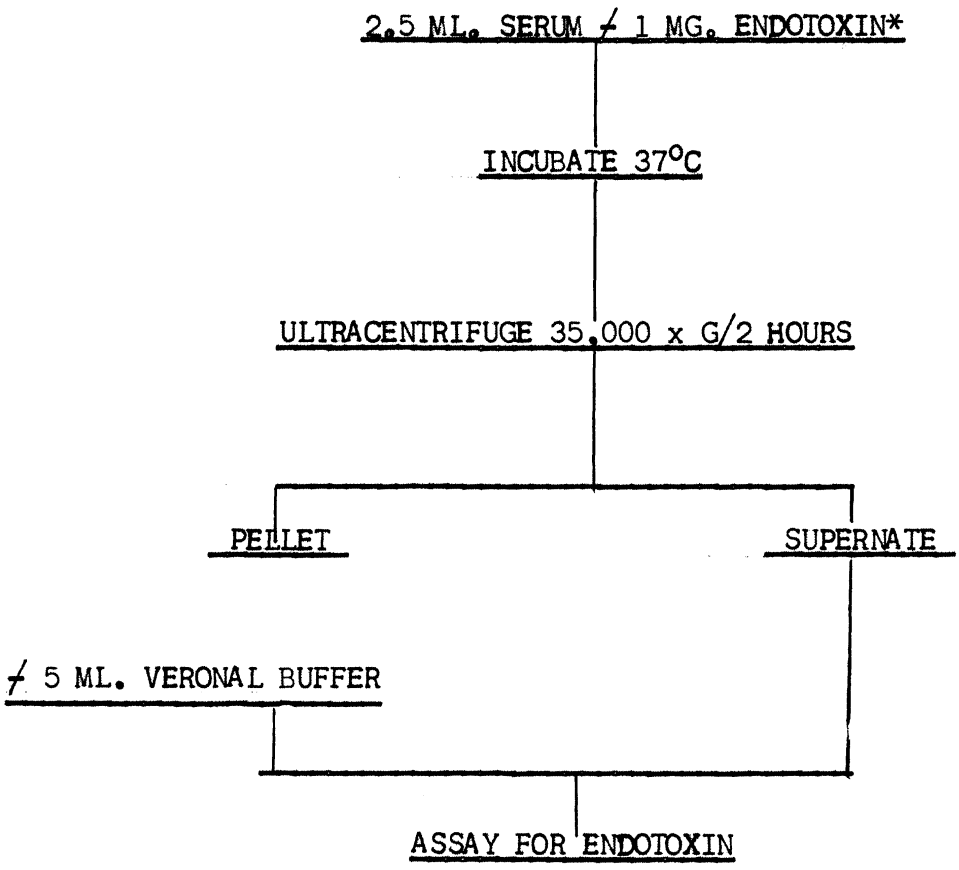


Figure 14. Procedure for Studying Reaction Between Serum and Endotoxin

* Suspended in 0.5 ml. veronal buffer

hours. After this the supernate was carefully collected and the pellet was solubilized in 5 ml. of veronal buffered saline pH 7.4. Any insoluble material present was removed by centrifugation at either 4000 rpm for 5 minutes or 2000 rpm for 10 minutes. Aliquots of the solubilized pellet and the supernate were assayed for the presence of endotoxin. Inasmuch as this endotoxic substance is the O-antigen, it will combine with its specific antibody. Accordingly, the aliquots were added to 1 ml. of antisera (S1, S2, S3 or S4) and the amount of endotoxin present was analysed by the quantitative precipitable antibody nitrogen procedure. The amount of endotoxin was determined by interpolation from the standard curves. The amount of endotoxin recovered in the saline-endotoxin control was designated 100%. Therefore, if 100% of the endotoxin was recovered after treatment with the test substance, then the endotoxin was not affected.

EXPERIMENTAL RESULTS

1. Action of Normal Serum on Endotoxin.

A. Normal Rabbit Serum.

Initial serum incubation experiments employing endotoxin derived by the technique of Boivin (28) were unsatisfactory for assay by the quantitative precipitin technique because more endotoxin was recovered in both the saline-endotoxin control and serum-endotoxin mixture then was added in the test. These data, as well as a detailed tabular account of the assay, can be seen in Table 5. From these results it is apparent that the Boivin antigen suspension could not be quantitated by this procedure so that the amount of endotoxin recovered from saline equaled the amount of endotoxin put in the mixture. It is known that endotoxin is a polydisperse mixture of molecules varying widely in molecular weight. Therefore in order to obtain a constant sedimentable fraction of endotoxin, it was submitted to differential ultracentrifugation as previously described (Figure 2.). After the quantitative precipitable antibody nitrogen curves were prepared for each of the various fractions of endotoxin (Figure 8.), 1 ml. of each fraction was incubated with 2.5 ml. of normal serum at 37°C for two hours. Table 6 shows the results of this experiment. The amount of endotoxin recovered from saline controls was approximately 100% except

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In this study the term "altered endotoxin" means that per cent of endotoxin which was unable to combine with antibody as compared to that amount of endotoxin precipitated by antibody in the saline control. The term "recovered endotoxin" means that per cent of endotoxin precipitated by antibody as compared to the amount of endotoxin precipitated by antibody in the saline control.

TABLE 5

Assay of Endotoxin after Incubation with Normal Rabbit Serum

Sample	Saline + 2 mg. endotoxin		Serum + 2 mg. endotoxin	
	Assay of		Assay of	
	Pellet	Supernate	Pellet	Supernate
Ml. of antibody	1	1	1	1
Ml. of sample	.5	.2	.5	.2
Ml. of buffer	1.5	1.8	1.5	1.8
Reaction occur in Ab. excess	+	+	+	+

Incubate 37°C/2 hrs., overnight 4°C

Ml. of N/70 HCL	.765	.345	.665	.715
X 0.2 = ug. Ab. N	153	79	133	143
µg. of endotoxin*	116	38	92	100
X dil. factor** mg. of endotoxin	2.784	.560	2.208	1.590
Total recovered mg. of endotoxin	3.344		3.798	
Per Cent Recovered	167		189	

Incubation time, 2 hours at 37C
* Interpolation from Figure 6.
** Supernate dilution factor 1:15, pellet dilution factor 1:25

TABLE 6

Activity of Normal Rabbit Sera on Ultracentrifuge Fractions of B3 Endotoxin*

Endotoxin fraction	Mg. endotoxin recovered			
	saline		serum	
	Pel.	sup.	Pel.	sup
10,000 x G.	.475	.525	.538	.322
20,000 x G.	.263	.810	.263	.450
35,000 x G.	113	1.005	.175	.698
Sup. to 35,000 x G.	163	1.380	.138	1.140

*Incubation for 2 hours at 37°C

Table 6 Continued

Total endotoxin recovered		Per cent recovered	
saline	serum	saline	serum
1.00	.860	100	86
1.073	.713	100	66
1.118	.873	100	78
1.543	1.278	100	83

in the supernate fraction following 35,000 x G. The amount of endotoxin recovered from the serum-endotoxin mixtures was in all cases less than that recovered in the saline controls, indicating some activity by the serum on the endotoxin which prevented it from combining with its specific antibody. This experiment was repeated twice and similar results were obtained. Despite the fact that alteration appeared less when using the fraction sedimenting at 10,000 x G., it was employed for all further studies inasmuch as it contained the bulk of the whole endotoxin and allowed 100% recovery when incubated with saline.

B. Normal Human Serum

Thirteen human sera were prepared from blood of donors and incubated with B3 and B5 endotoxin at 37°C for various lengths of time. The results of the experiments are presented in Table 7. In this series of experiments incubation of endotoxin with saline resulted in essentially 100% recovered endotoxin. A comparison was made of the amount of endotoxin recovered in the supernate to that recovered in the pellet. In saline controls the supernate: pellet ratio was 0.1 to 0.3 or in other words 70-90% of the endotoxin recovered was found in the pellet. However, incubation of endotoxin in serum resulted in a shift of the amount of endotoxin recoverable from the pellet to the supernate, with a supernate = pellet ratio of 2 to 3, in other words only 20-35% of that recovered was found in the pellet. The supernate: pellet ratios in this experiment are fairly consistent falling in the range of

TABLE 7

Per Cent Recovery of Endotoxin after Incubation with Normal Human Serum

Serum	Time Incubated Hours/37C	Mg. Endotoxin Recovered	Ratio Supernate To Pellet	Per Cent Endotoxin Recovered
H1	2	.642	1.3	62
H2	3	.502	0.9	46
H3	3	.423	0.95	46
H4	4	.442	0.7	41
H5	4	.352	0.25	33
H6	4	.400	0.7	38
H7	4	.445	0.8	43
H8	4	.337	1.2	33
H9	4	.533	2.7	52
H10	4	.428	9.0	41
H11	4	.419	18.0	42
H14*	6	.289	1.2	28
H16°	6	.650	2.0	62

* Diabetic Serum (child)
° Serum Stored 20 Days 4°C

1 to 3.* These data also show that, in general, human sera were capable of altering a greater percentage of endotoxin than rabbit sera.

2. Activity of Serum on Purified Lipoplysaccharide.

The endotoxin used in the previous experiment is the Boivin complex of polysaccharide, lipid and protein (29). The protein moiety initially present in this product can be removed by purification, employing the method of Webster et al (29). This procedure results in a product that retains complete antigenic and serological activity. Accordingly, a sample of endotoxin (B5) was purified by this method as described in Materials and Methods. After the quantitative precipitable antibody nitrogen curve was prepared for the purified lipopolysaccharide, 1 mg. of this endotoxic substance (#5L10) was incubated with normal rabbit and human sera and assayed as previously described. Table 8 gives the results of four such experiments. These data indicate that normal serum has the ability to alter purified, protein-free lipopolysaccharide, and that the extent of alteration is essentially the same as that observed with the 10,000 x G. fraction of the Boivin antigen. Because of the greater ease in preparation, the Boivin antigen was used in all further experiments, except where noted.

*The two exceptions (H10 & H11) can be explained. When endotoxin was prepared in suspension, one large batch was made so that uniform results would be obtained. It was observed that constant freezing and thawing of such an endotoxin suspension caused a shift from the pellet to the supernate of the endotoxin when incubated in the saline control. In these two cases such results with saline control were obtained. For later experiments endotoxin suspensions were prepared in a large batch but stored in small quantities. This eliminated the freezing and thawing effect on the supernate: pellet ratio.

TABLE 8

Comparative Activity of Normal Sera in the Alteration of either Purified Lipopolysaccharide or Boivin Complex*

	Purified lipopolysaccharide		
Serum sample	Total recovered saline	Total recovered serum	Per cent recovered serum
R 90	1.190	.735	62
H 18	.940	.512	54
R 86	1.001	.708	71
R 93	1.190	.752	63

	Boivin complex		
Serum sample	Total recovered saline	Total recovered serum	Per cent recovered serum
R 90	1.097	.772	70
H 18	.910	.505	55
R 86	- -	- -	-
R 93	- -	- -	-

*Boivin complex (B5, 10,000 x G_o fraction)

3. The Influence of Time of Incubation on the Alteration of Endotoxin by Normal Serum.

In order to determine the effect of time of incubation on the loss of endotoxin, a sample of endotoxin was incubated with normal rabbit serum at 37°C for 0.5, 2, 4, 6, 12 and 18 hours. After assay for endotoxin it was found that the amount of loss of endotoxin was a function of time with a maximum alteration occuring after four hours. Figure 15 is the curve showing the percentage of endotoxin recovered. Two human sera were also studied with reference to the incubation time on the alteration of endotoxin. One normal serum was fresh while the other had been stored for twenty days at 4°C. Figure 16 shows the results of these two experiments. The fresh normal serum had a maximum activity at six hours incubation when only 22% of the endotoxin was recovered. The stored serum at 18 hours incubation had altered 58% of the sample. The latter two curves do not represent a comparison of stored and fresh sera inasmuch as they were different serum samples.

4. The Influence of Temperature of Incubation on the Alteration of Endotoxin.

H17 serum and endotoxin were incubated at various temperatures for six hours, and the data are presented in Figure 17. At 0°C and 4°C no alteration occured, but as the temperature was increased, the amount of alteration increased from 37% alteration at 56°C to 98% at 65°C. H18 serum and endotoxin were incubated in a similar manner and like results were obtained except that 55% of the endotoxin was altered at 56°C.

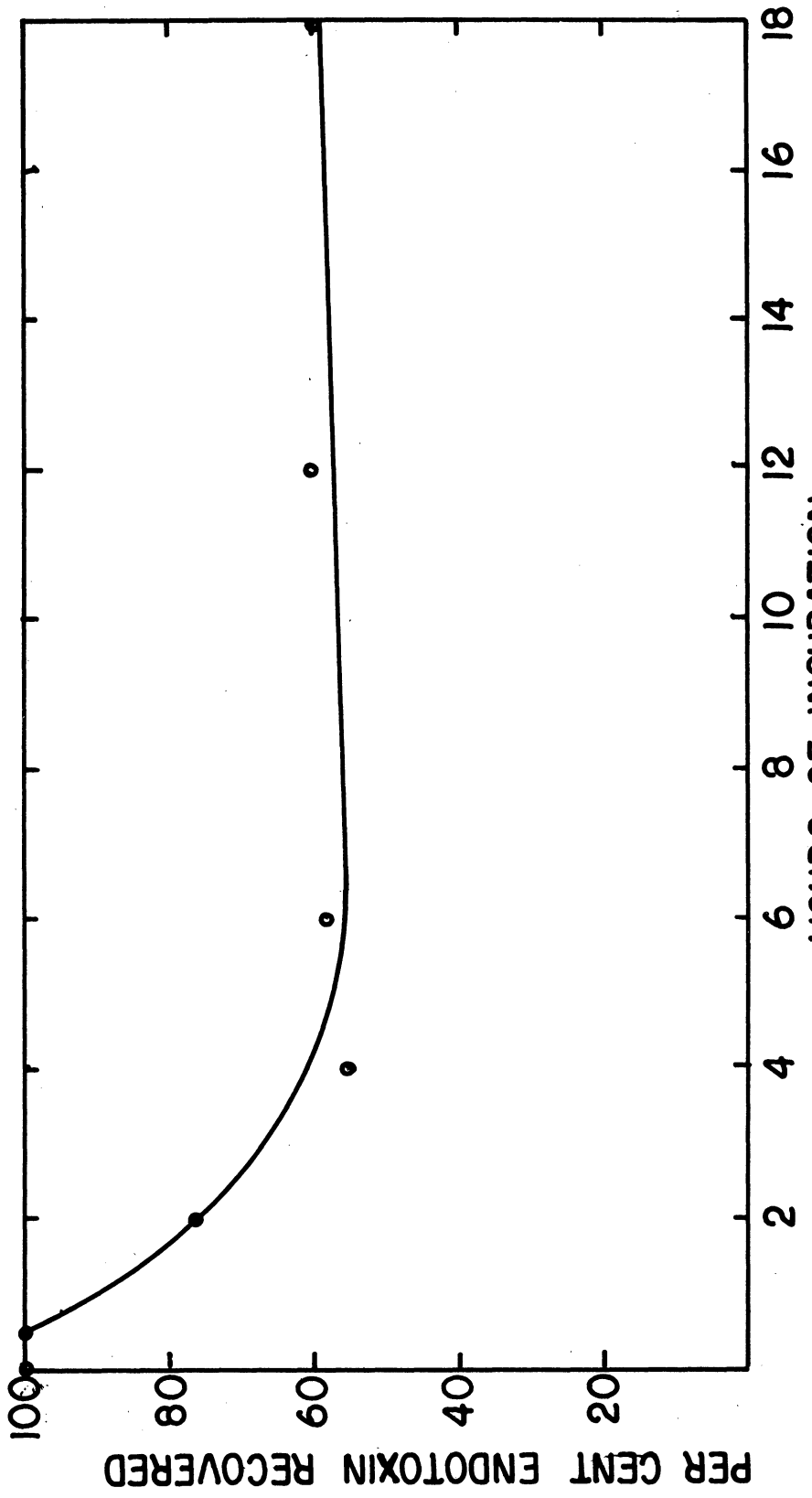

FIGURE 15. RABBIT SERUM INCUBATED WITH ENDOTOXIN AT 37C FOR VARIOUS LENGTHS OF TIME

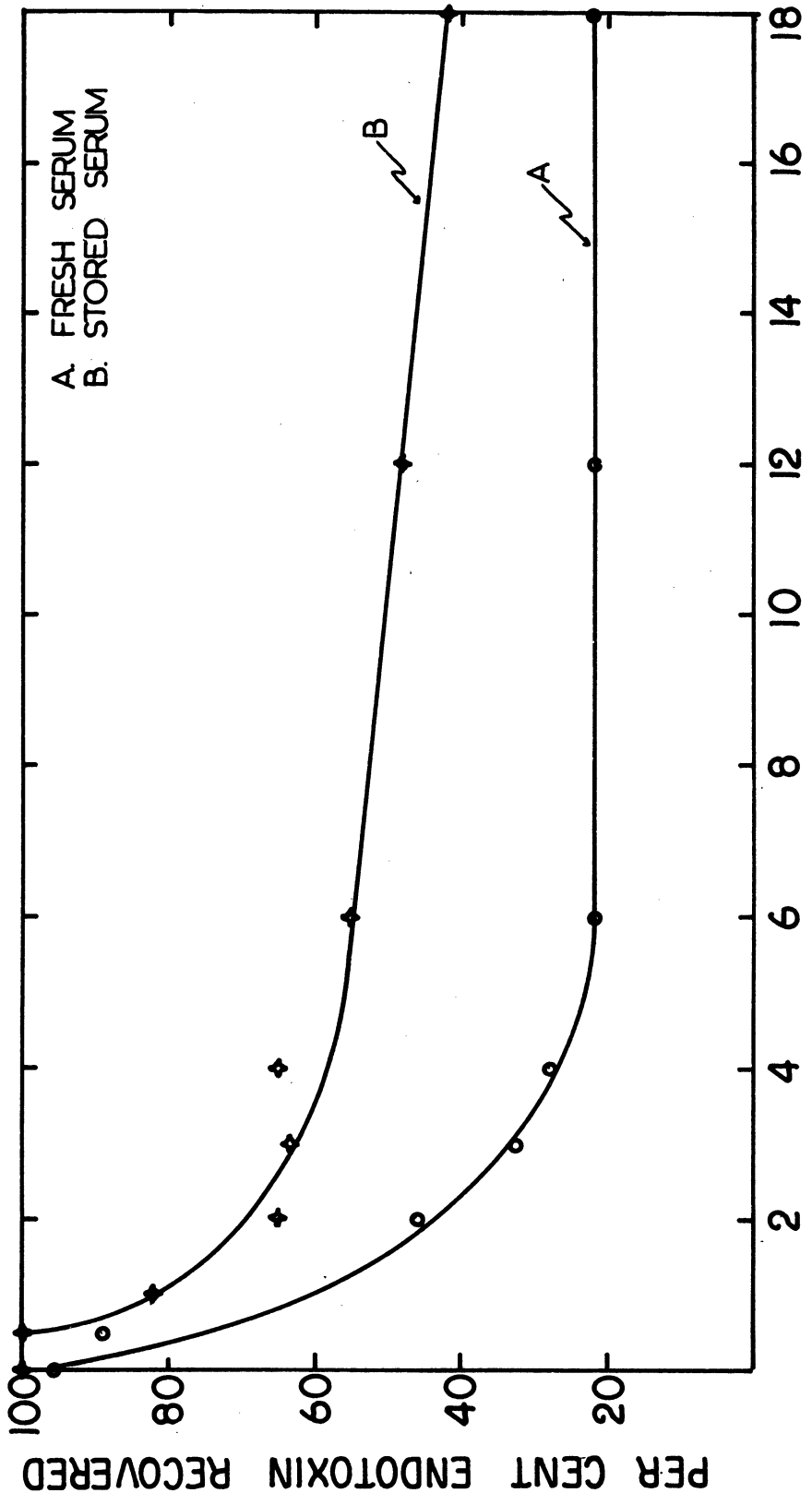

FIGURE 16. HUMAN SERUM (H9-FRESH AND H16-STORED 20 DAYS/4C) INCUBATED WITH ENDOTOXIN AT 37C FOR VARIOUS LENGTHS OF TIME

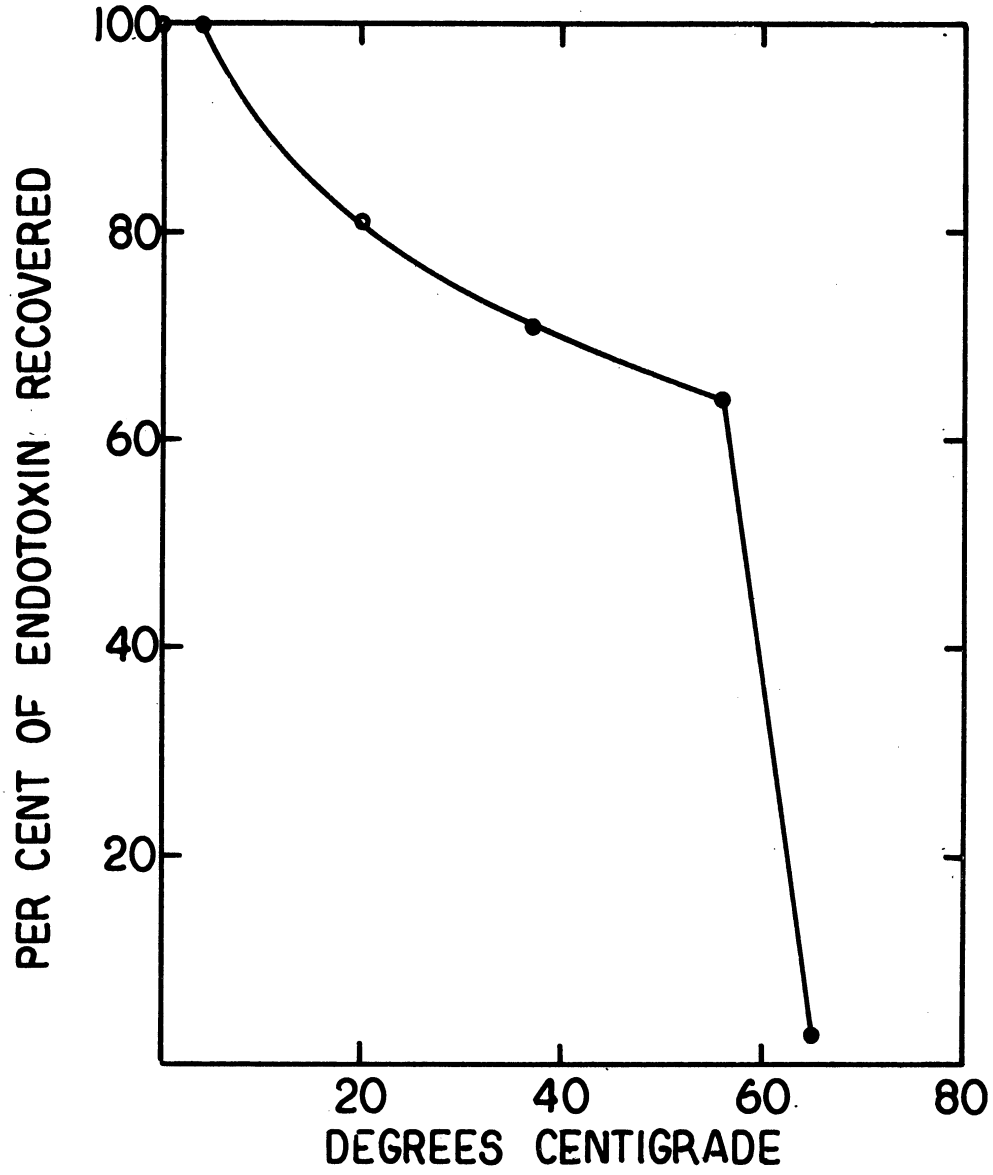

FIGURE 17. HUMAN SERUM INCUBATED WITH ENDOTOXIN AT VARIOUS TEMPERATURES

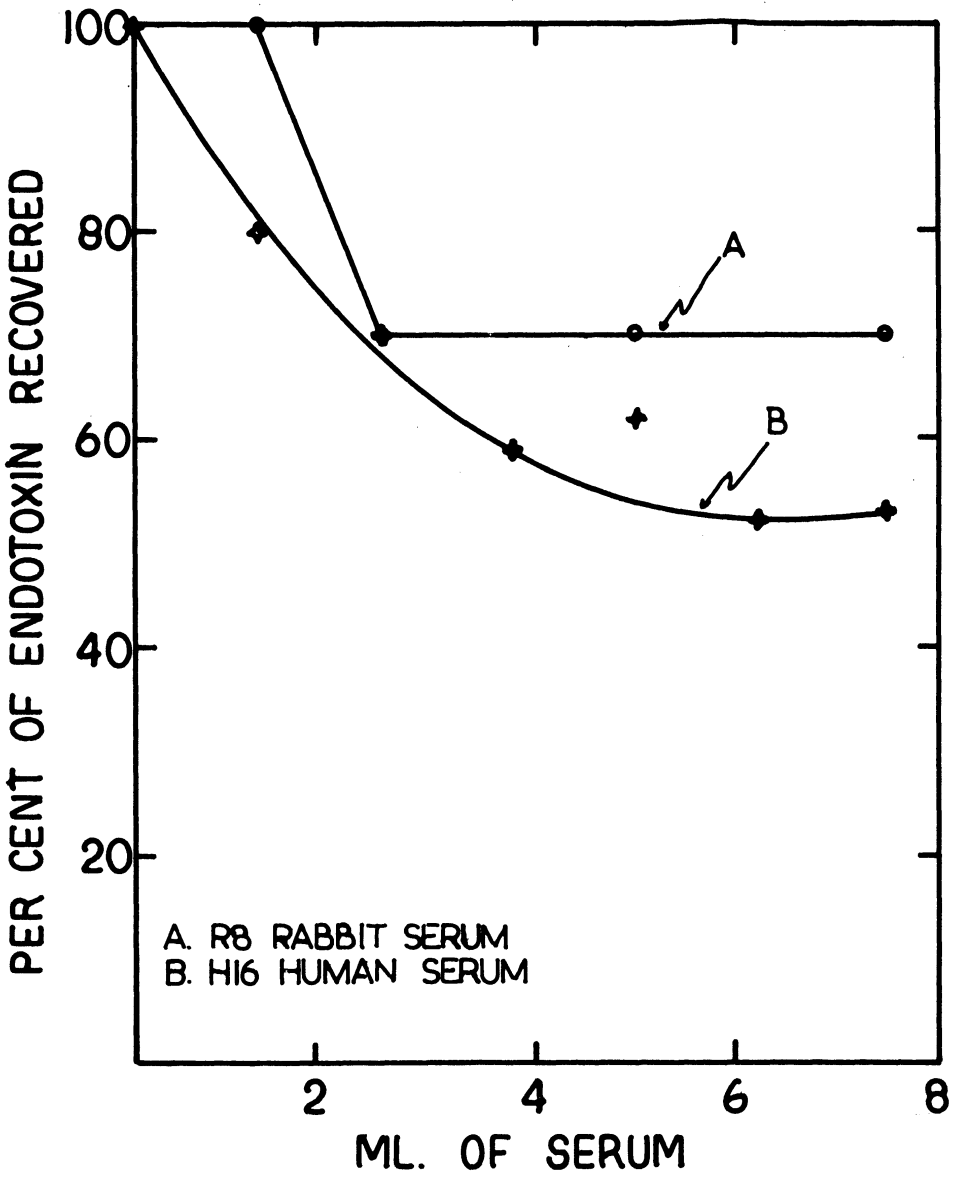

FIGURE 18. THE INFLUENCE OF VARIOUS AMOUNTS OF SERUM ON THE ALTERATION OF A CONSTANT AMOUNT OF ENDOTOXIN (1MG.)

Again, no endotoxin was recoverable at 65°C. Saline-endotoxin controls incubated at the same temperature resulted in 100% recovery of the endotoxin.

5. The Effect of Various Amounts of Serum on the Alteration of a Constant Amount of Endotoxin.

The effect of volume of rabbit and human serum incubated with a constant amount of endotoxin at 37°C for six hours was investigated. No activity of rabbit serum was observed until a concentration of 2.5 ml. was reached and it remained constant with increasing volumes of serum. However, as the concentration of human (H17) serum was increased, the amount of endotoxin altered increased until the maximum alteration of 48% occured with 6.25 ml. of serum. H21 serum and endotoxin was incubated in the same manner and the curve was essentially the same except that 55% of the endotoxin was altered with 6.25 ml. of serum.

6. Effect of Concentration of Endotoxin on the Altering Capacity of Human Serum.

In order to study the influence of a constant amount of human serum (2.5 ml.) on varying amounts of endotoxin, 0.5 mg. to 4 mg. of the latter were incubated at 37°C for six hours. Figure 19 presents the results and indicates that serum is capable of altering a greater percentage of endotoxin if a smaller amount of endotoxin is incubated with it, since 50% of the endotoxin was altered when 0.5 mg. was incubated and the percentage decreased to 18% when 4 mg. was used.

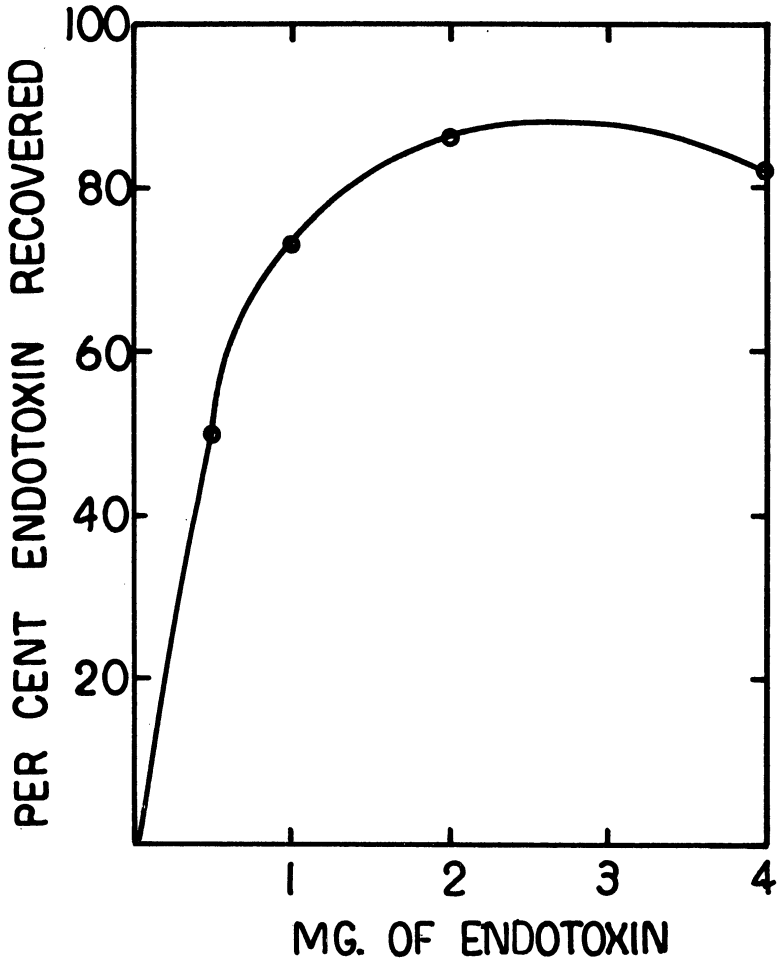

FIGURE 19. THE INFLUENCE OF VARIOUS AMOUNTS OF ENDOTOXIN ON ITS ALTERATION BY A CONSTANT AMOUNT OF HUMAN SERUM (2.5 ML.)

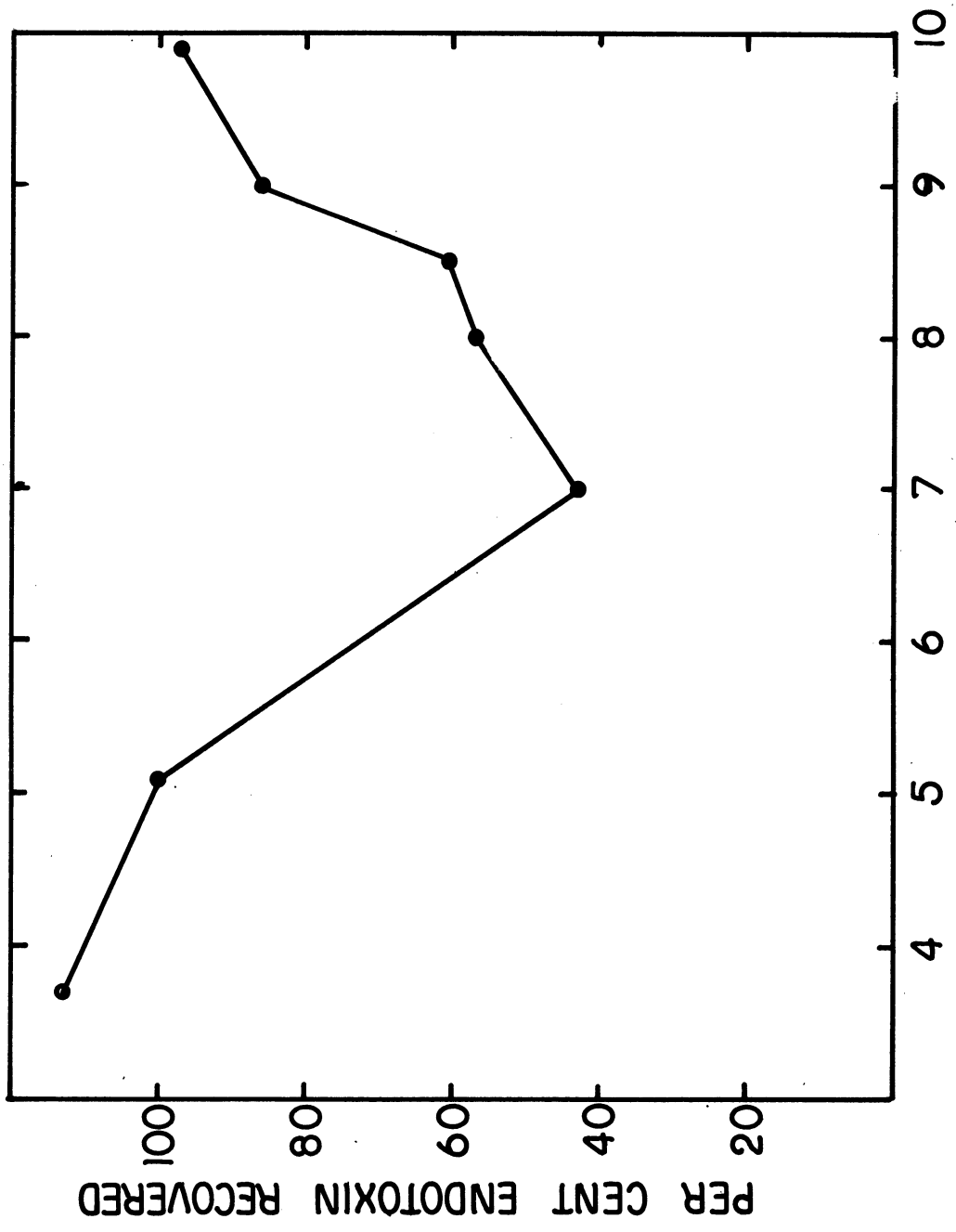

FIGURE 20. THE EFFECT OF pH OF NORMAL SERUM ON THE ALTERATION OF ENDOTOXIN

7. Effect of pH on the Alteration of Endotoxin

Samples of normal human serum were adjusted to pH 4.0 to 10.0 with .1N HCl and 0.1N NaOH. Saline controls were similarly adjusted. To each of these, 1 mg. of endotoxin was added and the mixture incubated for six hours at 37°C. The pH's of the supernates were rechecked after the mixtures were centrifuged at 35,000 x G. for two hours and the supernates removed. The saline-endotoxin controls showed 100% recovery of the endotoxin. Figure 20. shows the per cent of endotoxin recovered as a function of pH of the supernates after centrifugation. At pH 3.7, 5.1 and 9.8 normal human serum exerted no effect, since essentially 100% of the endotoxin initially added was recovered. This experiment was repeated with the same serum and the pattern was essentially the same with maximum alteration at pH 7.1.

8. The Effect of Storage of Serum on Its Endotoxin Altering Capacity.

Human serum stored at 4°C for various lengths of time was tested for capacity to alter endotoxin. The results of this experiment showed that there was no loss in this activity when stored for 10 days. At 18 days the altering capacity had diminished 18% and at 25 days it had diminished by 25%. A second experiment employed a human serum sample which originally altered 37% of the endotoxin, but after 4 months at 4°C the altering capacity was completely lost. Human serum stored at -24°C for a period of at least 30 days did not show a loss of its endotoxin altering capacity.

Summary of the Phenomenon of Alteration of Endotoxin by Normal Serum.

It has been shown that normal human and rabbit sera are capable of altering endotoxin that has been purified either by ultracentrifugation at 10,000 x G. for two hours or according to the method of Webster et al. The amount of alteration is manifested by the loss of ability by the endotoxin to combine with its specific antibody. It was found that the amount of alteration of endotoxin by serum was a function of time of incubation, temperature of incubation, pH of the mixture, and concentration of serum and endotoxin. The serum component responsible for the alteration was relatively stable when serum was stored at -24°C. A slight loss in activity was observed on standing at 4 to 6°C for several weeks.

The following experiments were designed to investigate the nature of the serum factor and to gain insight into its mechanism of action on endotoxin.

9. The Properdin System in Relation to the Alteration of Endotoxin by Serum.

Pillemer et al (26) found that endotoxin is capable of combining with properdin in the presence of complement. Magnesium ions (36) were found essential for this reaction. In order to determine whether serum properdin was responsible for the alteration of endotoxin observed in the experiments reported herein, resin (IRC-50) treated serum and versene (EDTA)-treated serum were prepared as previously described and incubated with endotoxin. Tables 9 and 10 show that not only did these sera have the same ability to alter endotoxin as untreated sera, but

TABLE 9

Effect of Resin* on Normal Serum

in Alteration of Endotoxin

	Total Mg. Endotoxin Recovered	
Serum Used	Untreated	Treated
Rabbit 8	.532	.554
Human 10	.428	.298
Human 16	.652	.481

*Resin - IRC-50, sodium form

TABLE 10

Effect of Versene on Normal Serum

in Alteration of Endotoxin

	Total Mg. Endotoxin Recovered		
Serum Used	Untreated	0.05M/Ml. Treated	0.5M/Ml. Treated
Rabbit 7	.758	.734	
Human 4	.442	.409	.323
Human 6	.400	.463	.322
Human 16	.650	.743	.536
Saline	1.035	.982	.913

TABLE 11

Effect of Zymosan Adsorption on Normal Serum in Alteration of Endotoxin

	Total Mg. Endotoxin Recovered	
Serum Used	Untreated	Treated
Human 16	.457	.437
Human 17	.502	.494

they were capable of altering a slightly greater percentage in most cases. Properdin was removed from serum by treatment with zymosan as previously described, and its effect on endotoxin tested. Table 11 demonstrates that zymosan adsorbed sera had the same altering capacity as untreated sera. In addition, nine sera were heat treated to 56°C for 30 minutes to inactivate complement, and then, incubated at 37°C with endotoxin. The data in Table 12 indicates that there was a partial loss of the sera's ability to alter endotoxin; however, two sera, R6 and R4, had an increased activity when they were heat treated. For the most part it would appear that heat treatment of serum causes a partial inactivation of the serum component responsible for altering endotoxin. The mean diminished activity of the nine sera was found to be 11% $\pm$ 4.6. Three sera heated to 65°C for thirty minutes and tested for endotoxin altering activity showed the following results. One serum had the same altering capacity as unheated serum while the other two showed a diminished activity. In one case there was a 20% loss in altering capacity and in the other there was a 40% loss.

10. The Effect of Normal Rat Serum on Endotoxin.

Rats have been found to be highly refractory to the pyrogenic, Shwartzman, and lethal effects of endotoxin. In addition, rat serum contains a high level of properdin. It was therefore of interest to study the effect of their sera upon endotoxin. Sera from young rats were pooled and incubated with endotoxin at 37°C for six hours. After assay for endotoxin, 100% of the endotoxin was recovered as in the saline controls. There was, however, a shift of the supernate:pellet

TABLE 12

The Effect of Heat Treated Serum*

on the Alteration of Endotoxin

Serum	Mg. of Endotoxin Recovered with Unheated Serum	Mg. of Endotoxin Recovered with Heat Treated Serum	Mg. of Endotoxin Recovered in Saline Control
R6	.635	.460	.951
H2	.502	.688	.951
H3	.423	.638	.927
H4	.442	.414	1.088
H6	.400	.509	1.040
H7	.445	.638	1.040
H8	.337	.442	1.035
H9	.533	.703	1.035
H10	.428	.685	1.043

Mean diminished activity 11% ± 4.6

*Serum heated to 56°C/30 minutes but incubated with endotoxin at 37C.

ratio and essentially all of the endotoxin recovered was in the supernate in contrast to the saline control. Serum from another group of older rats was similarly tested with endotoxin. This sera did have a small amount of activity since only 89% of the endotoxin was recovered. A similar supernate:pellet ratio shift was seen here.

11. The Effect of Leucocytes and Leucocytic Extract on the Alteration of Endotoxin.

Since Braude and co-workers (2), Cremer and Watson (3) and Beeson (1) were able to show that endotoxin injected in vivo was rapidly absorbed by reticulo-endothelial cells and granulocytes, it was decided to study the effect of leucocytes and leucocytic extract, either alone or added to serum, for endotoxin altering activity. Accordingly, rabbit and human leucocytes were incubated with a saline solution of endotoxin and with a mixture of serum and endotoxin. Table 13 presents the results of this experiment. The addition of leucocytes to the serum-endotoxin mixtures resulted in essentially no change in the serum's altering ability when the mixtures were incubated at 37°C. However, the combination of leucocytes, serum and endotoxin when incubated at 4°C resulted in 15% alteration in one case and 17% alteration in another. This is of interest inasmuch as it had been shown previously (Figure 17) that serum incubated with endotoxin at 4°C. resulted in no loss of endotoxin.

Rabbit leucocytic lysate incubated at 37°C with saline-endotoxin solution in the absence of serum caused a 12% loss in one sample and a 31% loss in another. However, when the leucocytic extract was incubated

TABLE 13

The Effect of White Blood Cells and White Blood Lysate on the Alteration of Endotoxin*

Endotoxin Incubated in	Temperature of Incubation	Mg. of Endotoxin Recovered	Per Cent of Endotoxin Recovered
R6 Serum	37	.585	60
R6 Serum ≠ wbc'	37	.648	66
R6 Serum	4	1.027	100
R6 Serum ≠ wbc	4	.810	83
Saline ≠ wbc	37	.880	90
Saline ≠ wbc	4	.785	80
H2 Serum	37	.495	49
H2 Serum ≠ wbc'	37	.533	53
H2 Serum	4	.927	93
H2 Serum ≠ wbc	4	.860	85
Saline ≠ wbc	37	1.007	100
Saline ≠ wbc	4	.905	90
H3 Serum	37	.447	45
H3 Serum ≠ wbc"	37	.433	44
Saline ≠ wbc	37	1.000	100
Rabbit wbc Lysate ' " ≠ Saline	37	.925	88
Rabbit wbc Lysate ≠ Saline	4	.080	8
Rabbit wbc Lysate ≠ Saline (Stored 6 month/-24C	4	.560	53

Continued next page

TABLE 13 Con't.

Rabbit wbc Lysate ≠ Saline	37	.697	69
Rabbit wbc Lysate ≠ Saline	4	.168	16
Human wbc Lysate ≠ Saline (Stored 6 month/-24C)	37	1.140	100
Human wbc Lysate ≠ Saline (Stored 6 month/-24C)	4	.168	17

* All tests in each block run concurrently

' 1.8×10^4 cells added

" 2.3×10^6 cells added

' " wbc Lysate equivalent to 5.0×10^8 cells

at 4°C with endotoxin and saline, there was essentially a complete loss of endotoxin. One rabbit leucocytic extract, stored for 6 months at -24°C and tested for its ability to cause a loss of endotoxin retained 47% activity i.e.: only 53% endotoxin was recoverable. A human leucocytic extract also stored for 6 months at -24°C was still extremely active at 4°C incubation with only 17% recoverable endotoxin. In contract, 100% of endotoxin was recoverable when this same mixture was incubated at 37°C.

12. The Effect of Chemical Inhibitors.

In an attempt to inhibit the reaction between serum and endotoxin, fluoride ions in 0.01M and 0.1M final concentration, fluoride ions and magnesium ions in 0.1M final concentration, mercuric ions in 0.01 M and 0.1M final concentration, and para-chloromercuric benzoate in 0.1M final concentration were added to normal serum as previously described in materials and methods. The sera so treated were added to endotoxin and incubated at 37°C for 4 to 6 hours. Mercuric ions in a 0.1M concentration caused precipitation of the serum proteins. The resultant supernate was unable to alter endotoxin since 100% of endotoxin was recovered as in the saline-endotoxin control. None of the other chemicals had any effect in reducing the serum altering capacity on endotoxin. The fluoride ions in 0.01M and 0.1M concentration in serum resulted in 50% recovery of endotoxin while the serum without fluoride ions resulted in 62% recovery of endotoxin. Serum containing 0.1M fluoride ions and 0.1M magnesium ions was able to alter 24% of the endotoxin while the untreated serum resulted in 18% alteration of

endotoxin. When para-chloromercuric benzoate was added to serum in 0.1M concentration and tested with endotoxin, it resulted in 84% recovery of endotoxin, and the untreated serum at the same time resulted in 87% recovered endotoxin.

13. The Effect of Boiled Serum on the Alteration of Endotoxin.

Since boiling of serum results in inactivation of nearly all enzymes, human serum was boiled for two minutes in a water bath to determine if such a treatment would inhibit the serum's effect on endotoxin. The jelled serum was centrifuged at 35,000 x G. for 30 minutes and the resultant supernate was incubated with endotoxin at 37°C for 6 hours. After assay for endotoxin the results showed that boiling serum causes essentially a complete loss in ability to alter endotoxin since 95% of the endotoxin was recovered while the same unboiled serum resulted in only 60% of the endotoxin recovered.

14. The Effect of Specific Enzymes on Endotoxin.

Because the reaction between serum and endotoxin appeared to be an enzymatic reaction, alpha amylase, lysozyme and wheat germ lipase were incubated with endotoxin to see if any of these enzymes were capable of altering endotoxin. Alpha amylase was chosen because it is a constituent of animal sera and is known to degrade glucose polysaccharides of alpha 1-4 linkages. Lysozyme (43) was employed since it has been used for the preparation of bacterial protoplasts from Gram negative organisms by degradating the polysaccharide in their cell walls. Wheat germ lipase was used since Kirsch (44) obtained evidence that it could detoxify endotoxin when it was incubated with this enzyme. Alpha

amylase was added to saline and endotoxin in vernal buffer pH 7.4 in 100 µg., 10 µg. and 1 µg. concentrations. The lysozyme and wheat germ lipase were added to saline and endotoxin in vernal buffer pH 7.4 in 0.1% concentration, and all tests were incubated at 37°C for six hours. The alpha amylase had some effect on the endotoxin in that 84% of the endotoxin was recovered from the 100 µg. concentration, 89% in the 10 µg. concentration and 92% in 1 µg. concentration of alpha amylase. The lysozyme and wheat germ lipase had no effect since the amount of endotoxin recovered was essentially the same as the saline control.

15. The Effect of Serum on O-polysaccharide.

Since the polysaccharide moiety of endotoxin is generally regarded as responsible for the serological activity of endotoxin, and since serum alters the precipitating activity of endotoxin, it was of interest to test the effect of normal serum on this haptenic substance. O-polysaccharide preparations, P1 and P2, were prepared as previously described and the quantitative precipitable antibody nitrogen curves were prepared (see Figures 12 and 13.). The latter were characteristic of the curves found by Landy et al (45) using a purified O-polysaccharide. O-polysaccharides P1 and P2, were incubated with normal rabbit and human sera in the same manner as when testing the activity with endotoxin. At the same time the same serum was tested with endotoxin. After centrifuging at 35,000 x G. for two hours a pellet did not form either in the O-polysaccharide-serum mixtures or in the O-polysaccharide-saline mixtures. Despite increasing the force to 154,000 x G. for 2 hours, no O-polysaccharide was deposited as a pellet. As a result

all of the precipitating activity was found in the supernates. Table 14 shows the results of these experiments and clearly illustrates that normal serum has no effect on the polysaccharide moiety of endotoxin. 100 µg. of alpha amylase in PBS pH 7.0 was incubated with P2 O-polysaccharide and saline, and this experiment resulted in 100% recovery of the hapten.

16. The Effect of Tolerant Serum on Endotoxin.

Since rabbits rendered tolerant to endotoxin are refractory to pyrexia and localized Shwartsman activity, it was of interest to see if the serum of such animals had any effect on endotoxin. Accordingly, three rabbits were rendered tolerant to S. marcescens endotoxin as previously described. The sera of these animals were collected 1, 8, 15, 22 and 29 days after the last injection of endotoxin. The sera from these animals were not tested before tolerance was begun. The sera while fresh were incubated with endotoxin for 4 hours at 37°C. Figure 21. represents the activity of the sera on endotoxin. The sera collected one day after the last injection were unable to alter endotoxin, while the sera collected 8, 15 and 22 days after the last injection had increasing activity in altering endotoxin until it was in the normal range.

Since these results suggested that the sera of tolerant rabbits are unable to alter endotoxin, it was of interest to see how the sera of rabbits would react as they were being rendered tolerant. Accordingly, four rabbits were made tolerant as previously described, and their sera were obtained before the first injection, 4, 8, 12 and 24

TABLE 14

The Activity of Normal Sera on O-Polysaccharide*

Compared with the Activity of Serum on Endotoxin

Experiment	Sample	1 mg. of O-Poly-saccharide	Per Cent Recovered	1 mg. of Endotoxin	Per Cent Recoered
1	Saline	P1	100	B5	100
	H18 Serum	P1	100	B5	56
2	Saline	P1	100	B5	100
	R11 Serum	P1	100	B5	73
	H19 Serum	P1	100	B5	54
3	Saline	P2	100	B12	100
	H20	P2	100	B12	60

*Incubation at 37C for 6 hours.

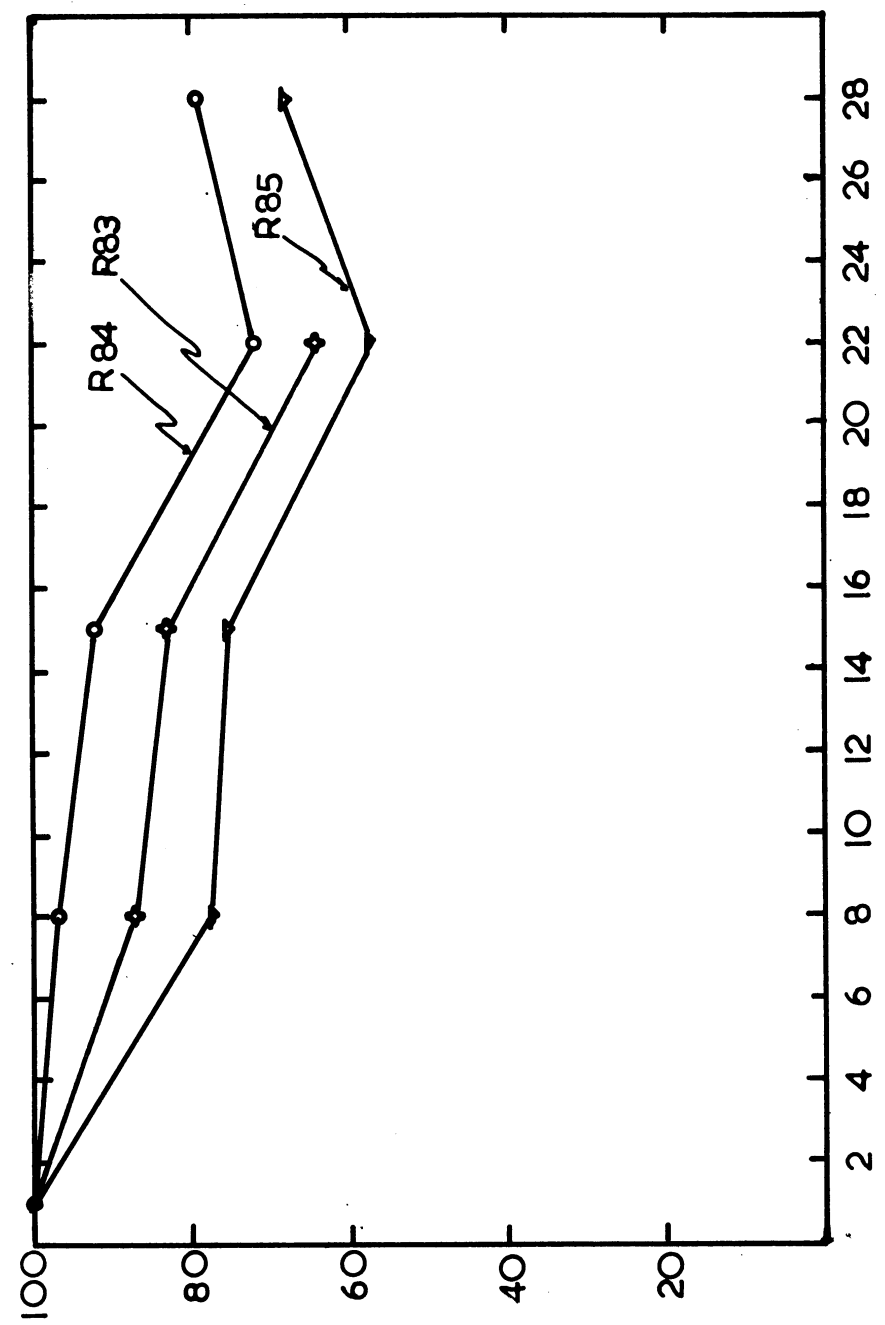

FIGURE 21. PER CENT OF ENDOTOXIN RECOVERED AFTER INCUBATION AT 37C/4 HRS. WITH SERA OF TOLERANT RABBITS

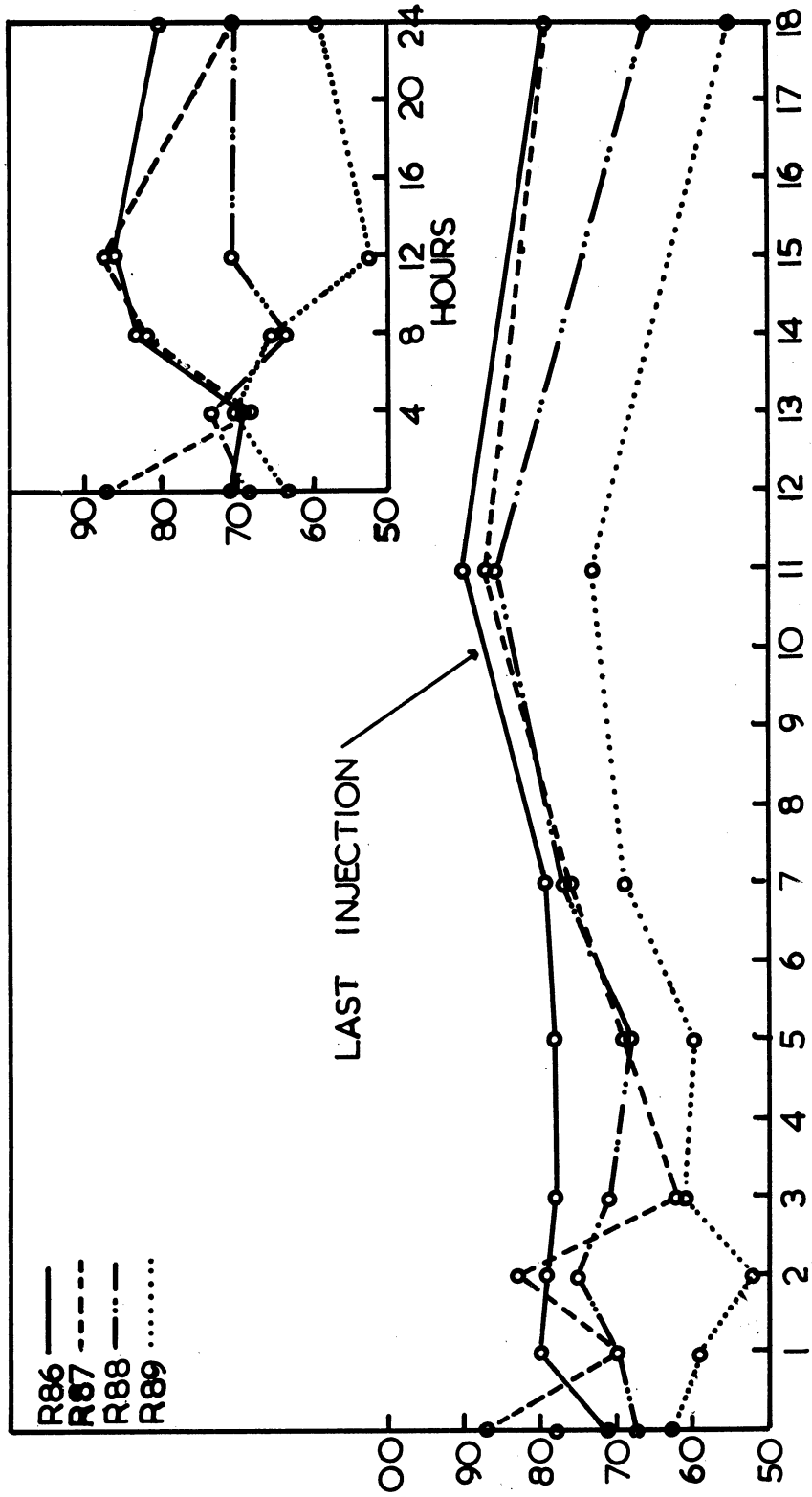

FIGURE 22. PER CENT OF ENDOTOXIN RECOVERED AFTER INCUBATION AT 37C/4 HRS. WITH SERA OF RABBITS DURING THE PRODUCTION OF TOLERANCE

hours after the first injection, 24 hours after the second, third, fifth, seventh and tenth injections, and then, at weekly intervals for three weeks. These sera were incubated with endotoxin to study their effect upon it. Figure 22 shows the results of this experiment. The activity of the serum during the first three days while tolerance was being developed showed considerable variation. However, after the third day there was a definite pattern showing a loss in ability to alter endotoxin. But, in no case did any of the sera show a complete loss as was seen in the preliminary experiment. After tolerance was established, the serum collected at weekly intervals showed an increasing activity to alter endotoxin until in the normal range.

Since the frequent bleeding of the rabbits may have had some effect on the reaction under study, a third experiment was done in the same manner as before employing three normal rabbits not injected with endotoxin and three rabbits which were rendered tolerant to endotoxin. Both the normal rabbits and the injected rabbits were bled at the same time and both sera were tested with endotoxin at the same time. Figures 23, 24 and 25 represent the results obtained after the samples were assayed for endotoxin. The sera of the normal and injected rabbits had essentially the same pattern of activity during the experiment. The sera collected during the first 24 hours were variable in their altering activity. The results of analysis of the sera collected on the second day are most interesting in that both groups caused a high amount of precipitable nitrogen to be recovered which would indicate upon interpolation from the standard curve that more endotoxin was present than

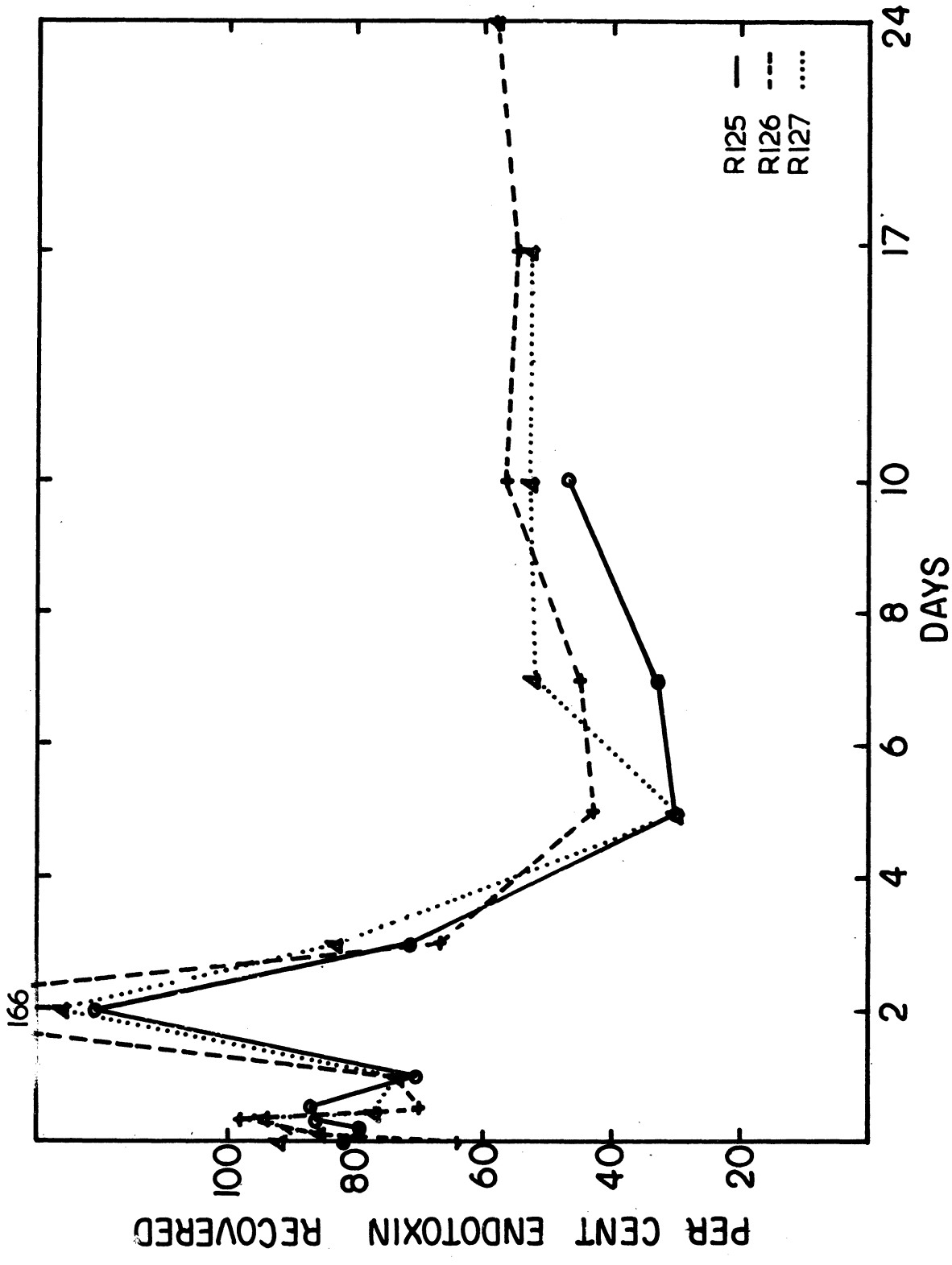

FIGURE 23. THE EFFECT OF NORMAL RABBIT SERA, BLED AND TESTED WITH ENDOTOXIN AS CONTROLS TO THE TOLERANT RABBITS

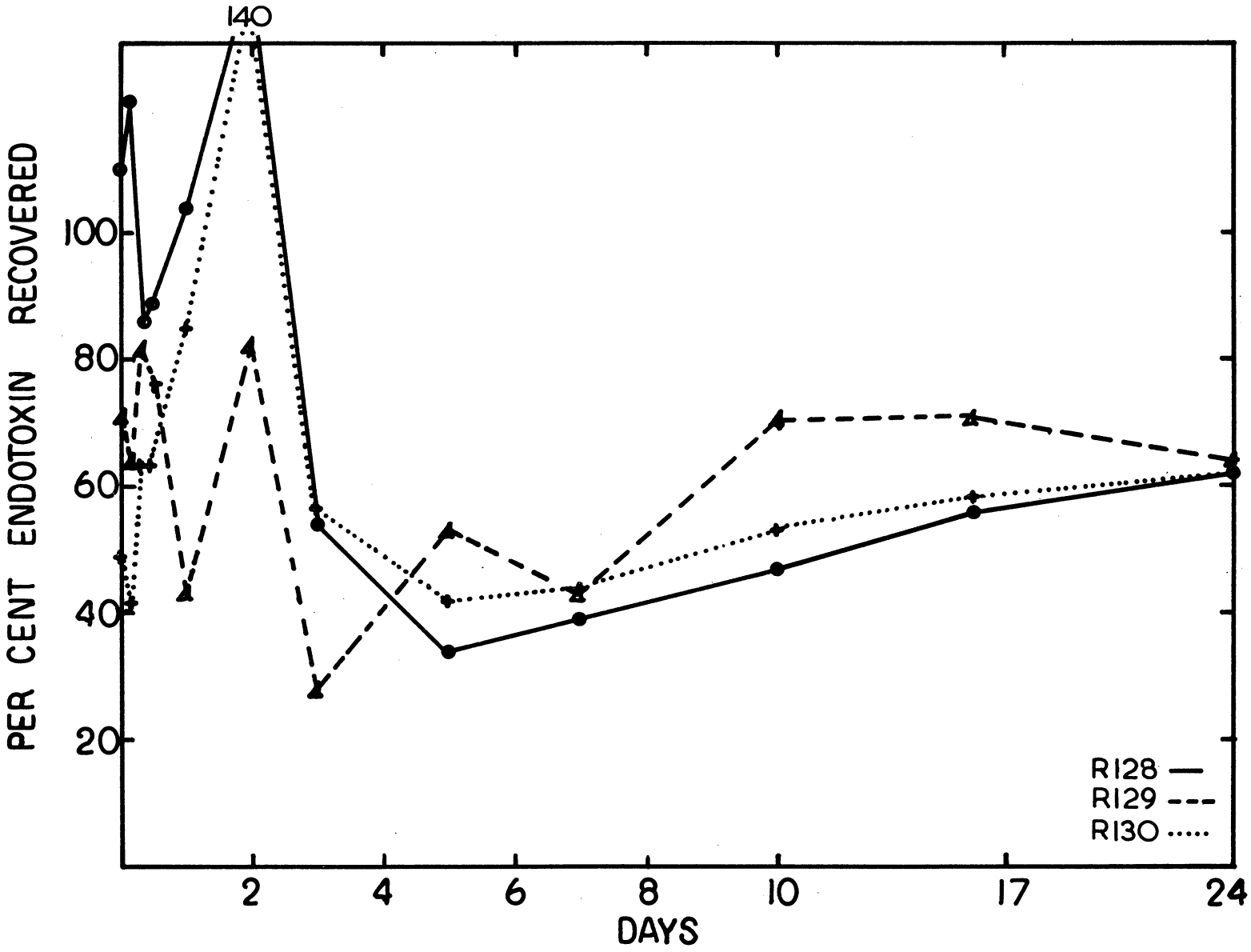

FIGURE 24. THE EFFECT OF SERA UPON ENDOTOXIN FROM RABBITS BEING RENDERED TOLERANT TO ENDOTOXIN

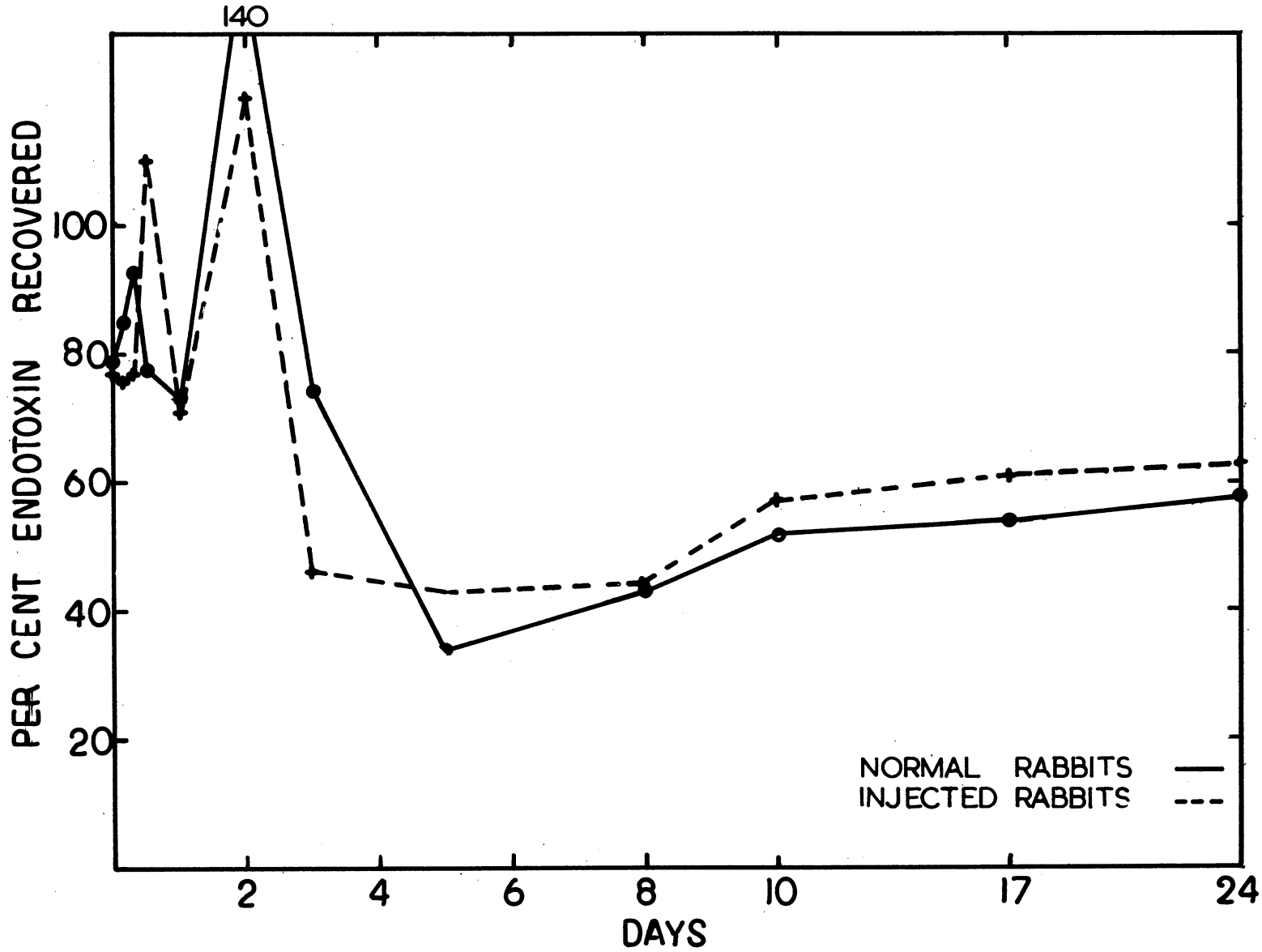

FIGURE 25. COMPARISON OF THE EFFECT OF NORMAL SERA AND SERA FROM RABBITS BEING RENDERED TOLERANT (MEAN OF 3 RABBITS; EXCEPT 17TH DAY, MEAN OF 2 N. SERA; AND 24TH DAY, 1 N. SERA; R125 & 127 DIED)

was employed in the test. It is possible that a compensating serum protein may be present that was capable of complexing with endotoxin, which, when combined with antibody, was responsible for the high amount of recovered precipitable nitrogen. After the second day, as Figures 23 and 24 show, the altering activity of the sera returned within the expected range, then, became hyperactive, and as the bleedings became less frequent there was a leveling off in the normal range. Figure 25 which is the mean of the three rabbits in each group shows that the two graphs parallel quite well. These results indicate that frequent bleeding may effect the endotoxin altering effect of the sera.

Summary of the Nature of the Serum Factor and Mechanism of Action on Endotoxin.

The evidence gathered in these experiments indicate that the properdin system is not involved in this reaction on endotoxin. A factor that is capable of altering endotoxin in the absence of serum to a greater degree at 4°C than at 37°C can be obtained from white blood cells. Of the known enzymatic chemical inhibitors employed only mercuric ions in 0.1M concentration caused a loss of the serum's altering capacity. However, boiling serum for two minutes also produced a similar effect. Of the three enzymes tested with endotoxin only alpha amylase had any effect, but this was minimal and due reservation must be exercised in implying that this is the factor in serum responsible for the altering activity. It was shown that serum is not active on the O-polysaccharide produced by acid hydrolysis of the lipopolysaccharide. Although an initial experiment indicated that the sera

from rabbits rendered tolerant to endotoxin was inhibited in the alteration of endotoxin, later experiments indicated that this finding may not be the general picture. Inasmuch as normal and injected animals showed a similar pattern in the altering activity on endotoxin indicates that the serum is affected by the frequent bleedings.

17. The Effect of Serum Fractions on Endotoxin.

Preliminary to the purification of the factor in serum involved in the alteration of endotoxin, serum was fractionated by several procedures in an attempt to place the factor in one of the major serum protein groups.

A. Ammonium Sulfate Fractions of Serum.

Rabbit serum was fractionated into two fractions by precipitation with 50% and 100% saturated ammonium sulfate. These fractions were reconstituted in the normal concentration calculated to be contained in 2.5 ml. of whole serum and incubated with one mg. of endotoxin at 4°C and 37°C for three hours. Table 15 shows the results of this experiment. The fraction precipitated at 100% saturation when incubated at 4°C had no activity but had 16% altering activity when incubated at 37°C. The samples of the 100% precipitable fraction that were heat treated to 56°C and 65°C for 30 minutes and incubated at 37°C also had approximately the same amount of activity. The 50% precipitable fraction incubated at 4°C had essentially no activity. However, the samples that were incubated at 37°C were interesting in that instead of causing a loss in precipitable nitrogen they gave a considerable increased amounts which would indicate 148% of endotoxin present. Also,

TABLE 15

The effect of ammonium sulfate fractions of normal rabbit serum on the Alteration of Endotoxin

Sample	Temperature of incubation	Total Recovered endotoxin	Ratio S:P	Per Cent endotoxin recovered
Saline	37	.881	2	100
100% fraction	4	.878	1	100
100% fraction	37	.740	2	84
100%(56C/30 Min)	37	.705	2	80
100%(65C/30 Min)	37	.680	2	77
50% fraction	4	.846	1	96
50% fraction	37	1.480	58	167
50%(56C/30 Min)	37	1.456	46	165
50%(65C/30 Min)	37	1.007	9	114
Saline	37	1.089	.1	100
40% fraction	37	.794	.25	73
60% fraction	37	1.143	3	105
80% fraction	37	1.010	.2	93
Supernate fract.	37	1.136	.13	104

essentially all of the precipitable nitrogen was in the supernate.

Human serum #21 was similarly fractionated into 50% and 100% ammonuim sulfate saturation fractions. After reconstitution as above, these fractions were incubated with 1 mg. of endotoxin at 37°C for 6 hours. Whole normal serum when tested with endotoxin, resulted in 45% alteration of the endotoxin. In contrast, the 100% ammonium sulfate fraction caused a loss of 16% of the endotoxin whereas the 50% fraction caused a 30% loss in endotoxin. Also, the supernate:pellet ratio for the 50% fraction was as normally seen with a ratio of 2 while the endotoxin-saline ratio was 0.1.

Another rabbit serum was fractionated into four fractions with 40% ammonium sulfate saturation, 40-60% saturation, 60-80% saturation and supernate to 80% saturation. These fractions were incubated at 37°C with 1 mg. of endotoxin for four hours. Table 15 gives the results of this experiment. The 40% fraction contained essentially all the activity causing a 27% alteration of endotoxin, however, the 80% fraction did cause a slight loss of endotoxin. The 60% fraction was of interest in that though this fraction resulted in 100% recovery of endotoxin it caused the shift of recovered endotoxin from the pellet to the supernate with a ratio of supernate:pellet of 3.0 compared to the saline ratio of 0.1.

B. Alcoholic Fractions of Serum

Serum fractions obtained by the alcoholic procedure in the cold as previously described were incubated with endotoxin at 37°C for 6 hours. The albumin-alpha globulin fraction was the only fraction that was able

to show any appreciable activity, though some of the other fractions showed a small amount of activity. Table 16 shows the results of this study. H19 and H21 albumin-alpha globulin fractions resulted in 45% and 38% alteration of endotoxin and caused a complete shift of the endotoxin from the pellet to the supernate with essentially all the recovered endotoxin in the supernate, while the saline control had 90% of the endotoxin in the pellet. One human serum (H20) was capable of 40% alteration of endotoxin, while all of the alcoholic fractions obtained from the serum had no altering ability of endotoxin. However, the albumin-alpha globulin fraction did cause a complete shift of the recovered endotoxin from the pellet to the supernate as seen with H19 and H21. It is possible that this serum suffered denaturation since the temperature rose from $-6^{\circ}C$ to $+2^{\circ}C$ during the initial fractionation step.

The sera from ten rabbits were pooled and fractionated by the alcoholic procedure. The whole sera when incubated with endotoxin resulted in 18% alteration of the endotoxin's precipitating activity. The albumin-alpha globulin fraction resulted in 19% alteration of the precipitating activity with again a complete shift of the recovered endotoxin from the pellet to the supernate. The other alcoholic fractions had essentially no activity with a supernate:pellet ratio like the saline control of 0.1.

C. Zinc Ion Fractions of Serum.

Because of the possibility of altering protein by the alcoholic procedure, it was decided to employ the zinc ion fractionation of Cohn. This procedure yields a precipitated fraction which is completely

TABLE 16

The Activity of Serum Fractions Obtained by Alcoholic Fractionation on the Alteration of Endotoxin

Serum	Endotoxin used	Per cent of endotoxin recovered when incubated with				
		Saline	Albumin Alpha globulin	Beta globulin	Gamma globulin	serum
H19	B5	100	55	100	79	46*
H20	B12	100	100	128	98	60
H21	B13	100	62	132	100	55
Pooled Rabbit	B5	100	81	113	94	82

* not done concurrently

soluble and unaltered in the presence of heavy metal chelating agents (40). The fractions were reconstituted to the normal serum concentration and were incubated with endotoxin at 37°C for 6 hours. The zinc ion soluble fraction resulted in 94% of the endotoxin recovered while the zinc ion precipitated fraction resulted in 84% of the endotoxin recovered. When the fractions were reconstituted in $\frac{1}{4}$ the normal concentration of serum and incubated with endotoxin, the zinc ion soluble fraction resulted in 100% recovery of endotoxin, while the zinc ion precipitable fraction resulted in 87% recovery. The zinc ion precipitable fraction was reconstituted to 1/8 the normal serum concentration and incubated with endotoxin. This resulted in 91% recovery of endotoxin.

Because human serum (H20) was capable of altering 40% of the endotoxin and the zinc ion precipitated fraction was only able to alter 16% of the endotoxin, it was through that the pH of the reaction should be studied since it might have some effect on the amount of endotoxin altered. Accordingly, 17 mg. of zinc ion precipitated protein (1/8 normal serum concentration) was suspended in buffers ranging from pH 4.0 to pH 10.0 and were incubated with 1 mg. of endotoxin at 37°C for 6 hours. Saline controls and endotoxin were similarly suspended in these buffers. All of the saline controls resulted in 100% recovery of endotoxin with the same supernate:pellet ratio of 0.1. Figure 26 represents the results obtained for the zinc ion precipitable fraction and show that at pH 7.0 there was 28% loss in precipitating activity of the endotoxin which is 2/3 of the altering capacity of the

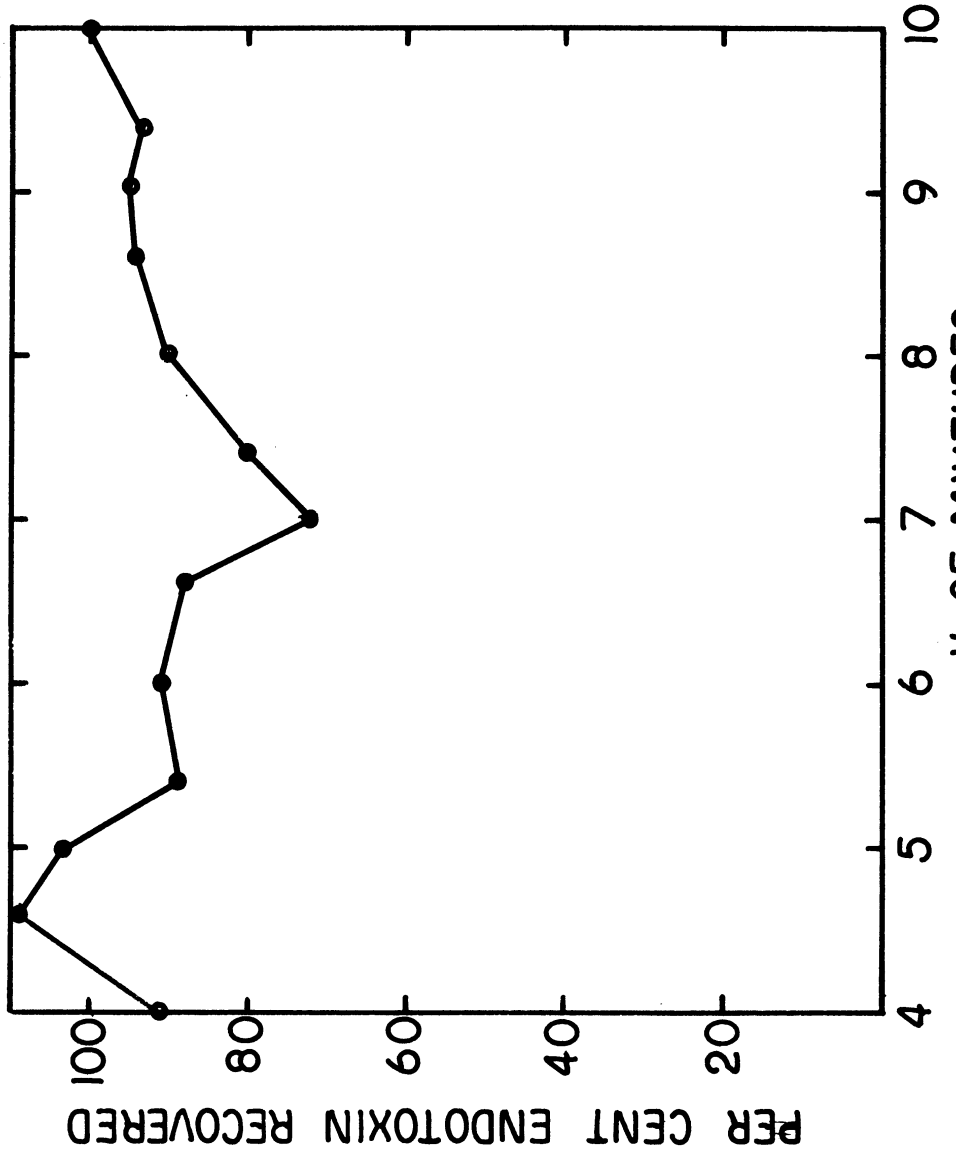

FIGURE 26. THE EFFECT OF pH OF ZINC ION PRECIPITATED PROTEIN (H_2O SERUM) ON THE ALTERATION OF ENDOTOXIN

normal serum. The tests carried out at the other pH values had less activity as seen in the figure, hence, pH 7.0 was considered to be the optimum for the altering activity of the zinc ion precipitated fractions of serum. This result compares favorably with the pH study on serum (see 9.)

Human serum 21 was similarly fractionated with zinc ions and the two fractions were reconstituted to the normal serum concentration in PBS pH 7.0. After the two fractions were incubated with endotoxin at 37°C for 6 hours, the zinc ion precipitated fraction resulted in 36% alteration of endotoxin while the zinc ion soluble fraction resulted in no activity since 99% of the endotoxin was recovered.

D. Mercuric Ion Fractions of Serum.

Since 0.1M mercuric ions caused a precipitation of the serum proteins and the resultant supernate was not active on endotoxin, H21 serum was treated with mercuric ions as previously described. The resultant precipitate was solubilized in versene (EDTA), dialyzed and lyophilized. The precipitate and soluble fractions were then tested for activity in altering endotoxin. The mercuric ion soluble fraction resulted in 100% recovery of endotoxin, whereas the mercuric ion precipitable fraction resulted in 30% alteration of the endotoxin.

Summary of the Effect of Alteration of Endotoxin by Serum Fractions.

The active factor responsible for the alteration of endotoxin was found in the fraction precipitated by 50% saturated ammonium sulfate and in the albumin-alpha globulin fraction by the alcoholic procedure. Zinc ions at 0.02M concentration at pH 7.4 precipitates alpha, beta

and gamma globulins, beta-lipoprotein and copper protein, while the soluble fraction contains albumins, metal-combining globulin, glycoprotein, amylase, iodoprotein and esterase. The endotoxin altering factor was present in the precipitated fraction. Mercuric ion fractionation at pH 7.4 in 0.1M concentration precipitated all protein, and the endotoxin altering factor was present in the precipitable fraction. These results indicate that the active factor responsible for endotoxin alteration is an alpha globulin.

18. Biological Activity of Blood Components Incubated with Endotoxin.

When serum or fractions of serum containing the active factor are incubated with endotoxin and assayed, there is generally a certain amount of endotoxin that is recovered in the supernate. It was of interest to see if the endotoxin that is recovered had been altered in any other manner since there is the possibility that endotoxin could be changed without altering its serological activity. The lipopolysaccharide could be degraded to phospholipid and O-polysaccharide, and in this case the O-polysaccharide would have equal precipitating activity as the complete antigen. It is generally considered that endotoxin must contain the complete molecule to be pyrogenic. Therefore, this activity was used as a method to denote such a degradation of the molecule of endotoxin other than involving its precipitating ability.

A. The Pyrogenic Activity of Blood and Serum Incubated with Endotoxin.

Grant and Whalen (21) have reported that when endotoxin is

incubated with rabbit whole blood at 37°C for three to four hours and then injected into rabbits, there was a reduced latent period in the subsequent production of fever. Cranston et al (23) reported that when human blood rich in leucocytes is incubated with endotoxin and injected into humans, the latent period is reduced and the extent of fever is increased, while blood poor in leucocytes is incubated with endotoxin, the latent period is not affected and the elevation in body temperature is decreased. Goodale and co-workers (24) and Hegemann (25) have reported that when normal human serum is incubated with endotoxin, there is a neutralization of the pyrogenic effect of endotoxin. In an attempt to reproduce these experiments with rabbits and to gain insight in the effects of blood components incubated with endotoxin 8 ml. of rabbit whole blood, blood rich in leucocytes (blood plus buffy coat), blood poor in leucocytes (blood minus buffy coat), normal rabbit serum and saline were each incubated with 4 µg. of S. typhosa endotoxin (B5, 10,000 x G. fraction) at 37°C for four hours. Following this, 1 ml. (calculated to contain 0.5 µg. of endotoxin of each sample) was injected into groups of two rabbits each. The rectal temperatures were taken every 30 minutes until the fever returned to normal. Figure 27 represents the degrees of fever produced by these samples. The graphs are the mean response of two rabbits. The whole blood gave the poorest fever response with only 1.9°F and the latent period was not reduced. The blood rich in leucocytes had a normal latent period but had a slightly increased fever compared to blood poor in leucocytes which had a delayed latent period

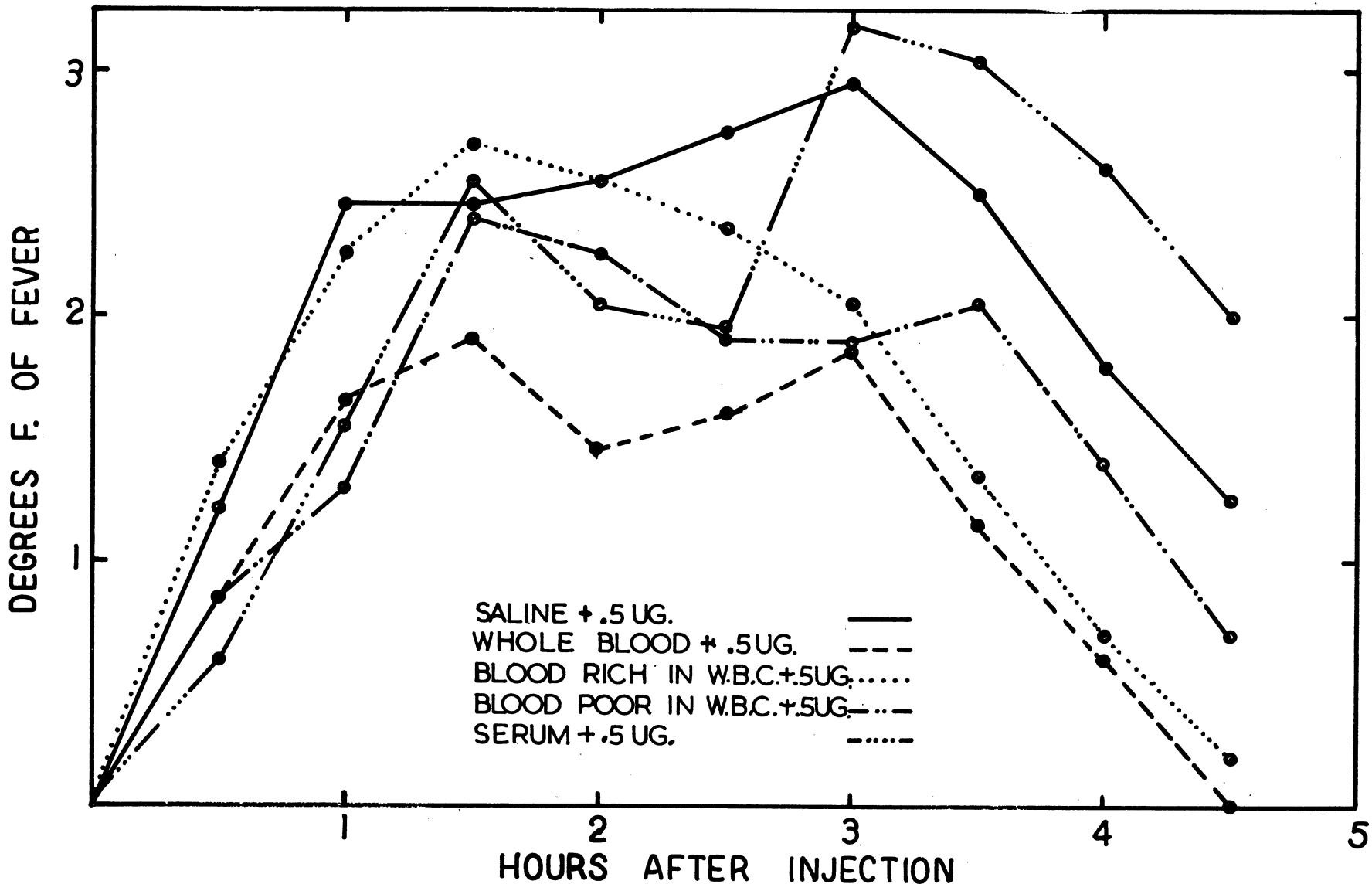

FIGURE 27. PYREXIA IN RABBITS CAUSED BY INJECTION OF VARIOUS BLOOD COMPONENTS INCUBATED 4 HR./37C WITH ENDOTOXIN

and a decreased fever response. The normal serum had a delayed latent period but gave the greatest fever response with 3.2°F and the most prolonged fever response. This is in contrast to the results of (21), (24) and (25).

B. The Pyrogenic Activity of the Supernate after Serum Incubation with 1 Mg. of Endotoxin.

In order to determine whether the endotoxin that is present in the supernate of serum after incubation has been altered, it was decided to investigate its pyrogenic activity. 2.5 ml. of normal human serum was incubated with endotoxin, centrifuged at 35,000 x G. for two hours, and the pellet and supernate assayed for endotoxin. The supernates were diluted so that 1 ml. contained 0.1 µg. of endotoxin. The saline-endotoxin supernate was similarly diluted. One ml. of these samples was injected I.V. into rabbits and the rectal temperatures recorded at 30 minute intervals. Figure 28 represents the degrees of fever produced by these samples. The results of this experiment shows that the endotoxin recovered in the supernate was not altered with respect to pyrogenic activity.

C. The Pyrogenic Activity of the Supernate after Albumin-Alpha Globulin Incubation with 1 Mg. of Endotoxin.

In studying the effect of the albumin-alpha globulin fraction of serum on the alteration of endotoxin, it was observed that all of the endotoxin present was found in the supernate. To determine if this endotoxin was altered by the fraction, its pyrogenic activity was studied. As with the serum supernate in the previous experiment, the

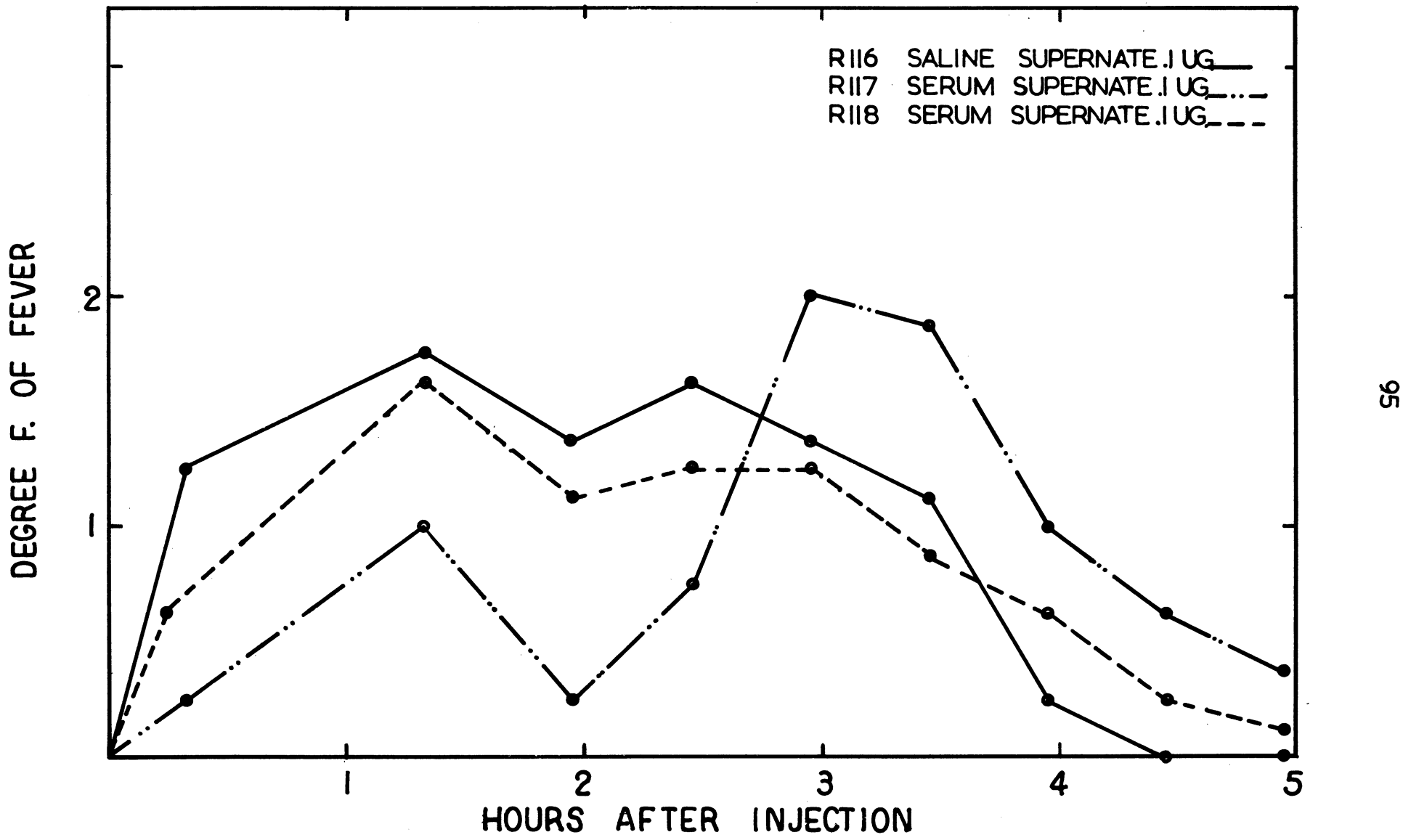

FIGURE 28. PYREXIA IN RABBITS INJECTED WITH SALINE AND H21 SERUM SUPERNATES DILUTED TO CONTAIN .1 UG. OF RECOVERED ENDOTOXIN

saline-endotoxin supernate and the albumin alpha globulin supernate were diluted so that 1 ml. contained 0.1 µg. of recovered endotoxin, and one ml. was injected I.V. into rabbits. Rectal temperatures were recorded at 30 minute intervals. Figure 29 represents the degrees of fever produced by these samples. The latent periods were not reduced by the albumin-alpha globulin supernates, but they caused a slightly increased and prolonged fever response than the saline control supernate. These results show that the endotoxin recoverable following incubation was not biologically or serologically altered by the albumin-alpha globulin fraction.

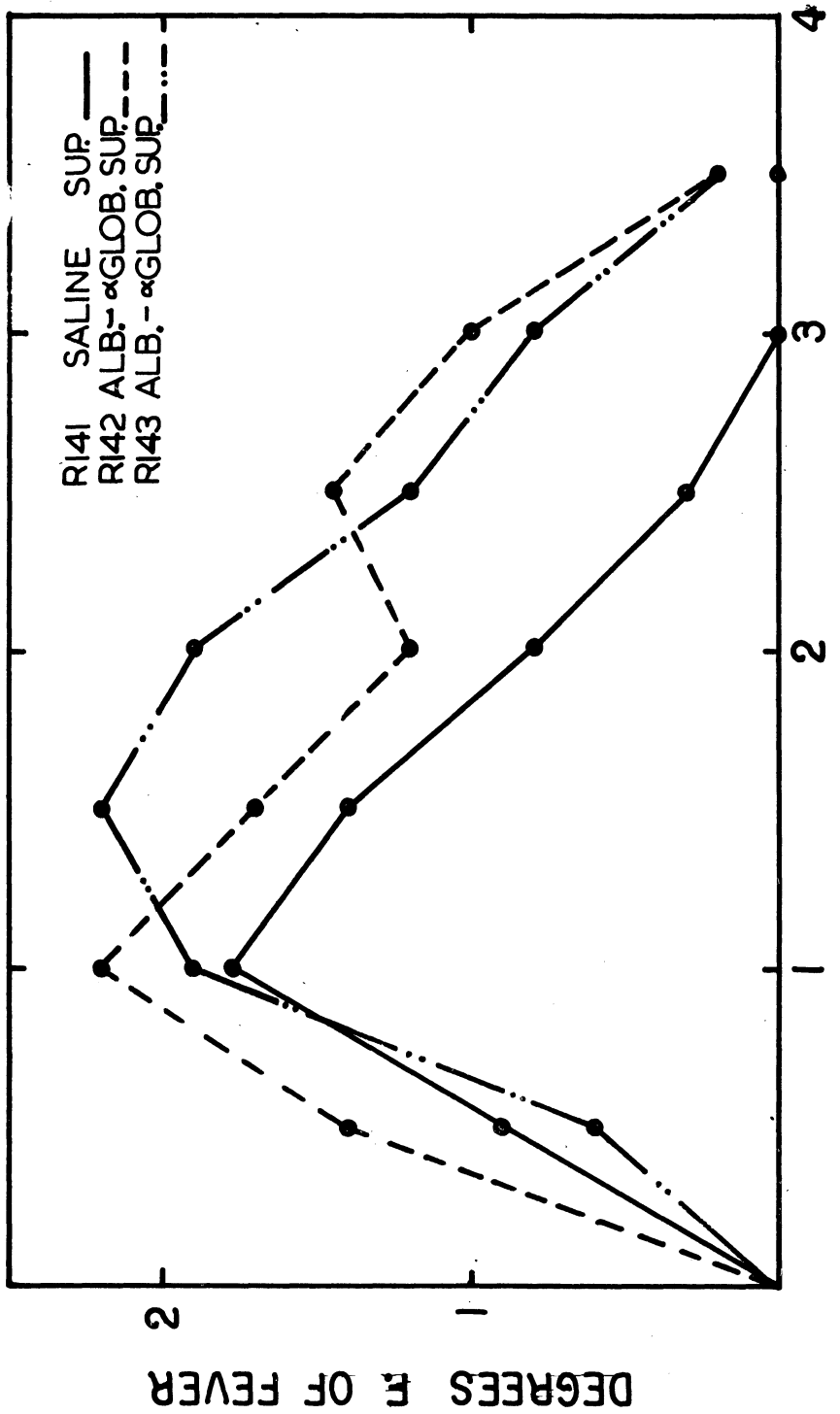

FIGURE 29. PYREXIA IN RABBITS INJECTED WITH SALINE AND H21 ALBUMIN- α GLOBULIN SERUM FRACTION SUPERNATES DILUTED TO CONTAIN .1 UG. OF RECOVERED ENDOTOXIN

DISCUSSION

It was found that normal human and rabbit sera were capable of altering the precipitating activity of endotoxin when incubated with it. The extent of alteration was found to be a function of time of incubation, temperature of incubation, pH, and concentration of serum and endotoxin. Along with these attributes, the fact that the endotoxin altering substance in serum deteriorates upon storage for four months at $4^{o}C$, and is inactivated upon boiling suggests that the reaction between serum and endotoxin may be an enzymatic one.

Rowley (5) working with the endotoxin of E. coli found that normal serum from rats and other mammals was capable of splitting lipopolysaccharide as demonstrated by the liberation of labeled phosphorous. The amount of recovered P^{32} was found to be a function of temperature of incubation, time of incubation and concentration of serum. In contrast to this study, Rowley found the optimal pH to be 8.2 and that heating the serum to $56^{o}C$ for twenty minutes completely inactivated the serum-splitting ability. Also, the addition of versene (EDTA) to serum reduced the splitting activity by sixty per cent. Moreover, Rowley found that rat serum was more active than human or rabbit serum, while in this study human serum was most active, then rabbit, and rat serum had little if any activity.

Rowley (5) distinguished between the properdin system and the phosphatase splitting activity by showing that human serum with the third component of complement removed (R3) had splitting activity

equal to the serum from which it was derived.

The properdin system was eliminated in this study on the alteration of endotoxin by removal of magnesium ions which are essential for the properdin system; the heat inactivation of complement; and the removal of properdin by zymosan adsorption. The sera from which magnesium ions were removed were capable of altering as much as or slightly more endotoxin then the same untreated sera. Heat treatment of serum to 56°C accomplishes inactivation of complement and properdin. The sera so treated had a partial inactivation for the alteration of endotoxin. The mean diminished serum activity for nine sera was 11% $\pm$ 4.6 (95% probability) of the untreated sera. The sera from which properdin was removed by adsorption with zymosan had equal endotoxin altering ability as the serum from which it was derived.

Two experiments which were done indicate that Rowley's splitting factor is not the primary action of the serum factor in this study. Normal sera that were treated with 0.01M and 0.1M fluoride ions and a combination of 0.1M fluoride ions and 0.1M magnesium ions were not inhibited by the presence of these ions, and fluoride ions in general inhibit phosphatase activity. Rats are highly refractory to the Shwartzman reaction, pyrogenicity, and lethality of S. typhosa endotoxin. Their sera showed little or no alteration of this endotoxin when incubated with it, while Rowley's splitting factor was most active in rat serum.

Cluff (4) studied the immunological activity of Shigella endotoxin following incubation with normal serum. Employing the agar-

diffusion techniques to denote any change manifested by the normal serum-endotoxin mixture. Cluff found that one diffuse zone of precipitate developed, while three distinct zones of precipitate developed in the saline-endotoxin control. The observed results indicate that each of the three different antigenic components of Shigella endotoxin contains a substance common to all three which is capable of combining with a serum component. Such a complex, then, would result in one diffuse zone of precipitation instead of the three zones seen in the saline-endotoxin controls. Cluff also found that human serum was equally capable of altering the immunological reaction of endotoxin and that by heating the serum to 56°C for thirty minutes prior to incubation with endotoxin did not change the capacity of serum for altering the endotoxin's immunological reactivity. Moreover, serum from rabbits made tolerant to S. marcescens endotoxin was equally capable of altering the endotoxin's serological activity. Following zonal electrophoresis in starch of normal rabbit serum, it was found that the beta globulin fraction was responsible for this activity on endotoxin.

In this study, in contrast to Cluff's procedure, various fractionation procedures were employed to determine the type of serum protein involved in the alteration of endotoxin. The results of these fractionations indicate that possibly an alpha globulin is associated with the endotoxin-altering activity. Hence, it is a different factor than Cluff's beta globulin altering component. Cluff's factor could cause an increase in recoverable precipitable nitrogen if an endotoxin-beta globulin-antibody complex was formed. There was some evidence of this

type of activity during the course of this study. Three different beta globulin fractions obtained by alcoholic fractionation resulted in increased amounts of precipitable nitrogen recovered compared to the saline-endotoxin controls. Cluff's factor may also explain the results obtained from the sera of normal animals and animals being rendered tolerant on the second day (Figures 23-25) where increased amounts of precipitable nitrogen were obtained. Perhaps, in compensating for the loss of blood volume, unusual levels of various serum proteins were produced, and a greater amount of this beta globulin which is able to combine with endotoxin was present on the second day.

In contrast to Cluff's results, the alteration of endotoxin by serum in this study demonstrated a loss in precipitating ability of endotoxin. Two experiments performed in this study indicate that the endotoxin altering component is different than Cluff's reported factor. Cluff performed his test employing approximately 1 ml. of normal rabbit serum which was incubated with endotoxin for only thirty minutes at 37°C. In contrast to Cluff's results, in this study essentially no endotoxin precipitating ability was lost when endotoxin was incubated for only thirty minutes at 37°C, and a volume of more than 1.25 ml. of rabbit serum was required to demonstrate any loss of endotoxin precipitating ability. In other words under similar conditions, there was no demonstrable alteration of endotoxin in this study. In testing the effect of concentration of endotoxin with a constant amount of serum, it was found that a greater percentage of

endotoxin lost its combining power when 0.5 mg. of endotoxin was used than when 4 mg. of endotoxin was used. Cluff employed 4 to 6 mg. of endotoxin in his test. Perhaps, in Cluff's work some of the endotoxin lost its combining ability, but since such an excess was employed, there was more than enough to present his observed results of one diffuse zone. Cluff did not investigate a quantitative effect on endotoxin *per se*, but only demonstrated that the endotoxin present was capable of combining with antibody, and as a result one diffuse zone of precipitate developed.

Braude *et al* (2) and Cremer and Watson (3) were able to show that endotoxin injected into rabbits was adsorbed by reticulo-endothelial cells and granulocytes within five to ten minutes after injection. The endotoxin was retained by these cells up to 10 hours, after which it could no longer be found.

In this study to determine whether or not leucocytes or leucocytic extract had any effect on endotoxin, these substances were tested with endotoxin. The results indicate that a factor is present in white blood cells that is capable of altering endotoxin in the absence of serum and that a greater extent of endotoxin is altered when incubated at 4°C than at 37°C. The rate of reaction for enzymes is in general slow at 4°C, but perhaps incubation of the mixture for 6 hours is long enough for the demonstrated activity to occur. Also, there is the possibility that a naturally occuring inhibitor is present in white blood cells that is able to limit the reaction at 37°C but is not inhibitory at 4°C. Moreover, the enzyme, if one is responsible for

the activity is more stable at 4°C than at 37°C. Whether or not this factor is able to operate in the host has not been studied and is yet to be determined.

In this study the serum-endotoxin mixture and the saline-endotoxin mixture were subjected to ultracentrifugation for two hours at 35,000 x G. in order to gain insight into the change, if any, in molecular size. The fact that endotoxin can be fractionated by differential ultracentrifugation into molecules of different size, is in keeping with the polydisperse nature of endotoxin. The endotoxin used for this study was a fraction obtained by ultracentrifugation at 10,000 x G. for two hours. This fraction contains the largest molecules, and when 1 mg. of this fraction was incubated in saline and subsequently centrifuged at 35,000 x G for two hours and assayed, 90% of the endotoxin was found in the pellet. However, when 1 mg. of this endotoxin was incubated with serum and similarly centrifuged and assayed, up to 90% of the endotoxin recovered was then found in the supernate instead of the pellet. Three experiments eliminated the specific density of serum as the cause for this shift from the pellet to supernate. Serum was mixed with 1 mg. of endotoxin and centrifuged at 35,000 x G. for two hours without incubation. Essentially 100% of the endotoxin was recovered and 90% of the endotoxin was found in the pellet like the saline control. Serum incubated with endotoxin at 4°C for 6 hours also resulted in 100% recovery of the endotoxin with the same ratio of 90% in the pellet after centrifugation. Moreover, serum stored for four months at 4°C and incubated with 1 mg. of endotoxin

resulted in 100% recoverable endotoxin and the same ratio existed with 90% of the endotoxin in the pellet. From this evidence one might conclude that serum changes the molecular size in the process of alteration of endotoxin. This would indicate that serum degrades the molecules to a size so that they are too small to be sedimented at 35,000 x G. for two hours, but are still active with antibody while the endotoxin that is lost is degraded to a point where it will not precipitate antibody.

The polysaccharide moiety is generally considered to be responsible for the serological activity of endotoxin. Therefore, since there was a loss in precipitating ability as well as a change in molecular size, it was thought that the serum component must be primarily directed against the polysaccharide moiety of endotoxin. However, when normal sera were incubated with O-polysaccharide, the polysaccharide moiety of endotoxin, there was no loss in precipitating ability of this haptenic substance. This indicates that the serum altering factor is not primarily directed against the O-polysaccharide serologically active site, but rather, it must degrade the molecule at some other linkage.

Landy et al (45) in studying the homogeneity of purified lipopolysaccharide found that it contained three serologically active components by the Oudin gel diffusion technique, while the hapten derived from it showed only one visible band. Also, O-hapten was only capable of precipitating 75% of the total antibody precipitable by the lipopolysaccharide. Therefore, the immunological role of lipopolysaccharide in the precipitin reaction is not wholly confined to the serological activity

of the polysaccharide moiety.

The observation of Landy *et al* (45) may, therefore, explain the lack of serum altering ability with the O-hapten. It may be that the serum altering component is primarily associated with the two other serologically reactive components of lipopolysaccharide that are absent from the polysaccharide moiety.

Since the endotoxin altering reaction by normal serum has been confined to *in vitro* studies, the role that this reaction may have *in vivo* on the destruction or detoxification of living *S. typhosa* cells, can only be postulated. Since endotoxin is rapidly removed from the circulation by the reticulo-endothelial (R.E.) system, it would appear that the endotoxin would not be in contract with the serum altering factor long enough to have any effect. However, Cremer and Watson (3) reported that the endotoxin disappears from the R. E. system after ten hours. Therefore, if the endotoxin returns to the blood and remains there for any period, perhaps, the serum altering factor may have importance. If such a sequence occurs, it must yet be determined.

SUMMARY

Normal human, rabbit and rat sera were studied for their ability to alter endotoxin when incubated at 37°C and assayed by the quantitative precipitin technique. It was found that human and rabbit serum were able to alter endotoxin as manifested by loss in precipitating ability with homologous antiserum. However, rat serum had little if any ability to alter the precipitating activity of endotoxin.

The ability of serum to alter endotoxin was found to be a function of time of incubation, temperature of incubation, pH, and concentration of serum and endotoxin. The altering component in serum was relatively stable when stored at 4°C and was able to withstand heating to 56°C for thirty minutes.

Evidence was presented that eliminated serum properdin as a possible factor responsible for the alteration of endotoxin in this reaction.

Fractionation of human and rabbit serum indicates that the endotoxin altering factor is a protein and is associated with the serum globulins.

White blood cells of human and rabbit contain a factor which in the absence of serum alters endotoxin to a greater extent when incubated at 4°C than at 37°C.

APPENDIX I

Reagent Solutions

The following reagent solutions were routinely used in this investigation.

a. Phosphate Buffered Saline Solution (PBS) pH 7.0

Na_2HPO_4	6.204 gm.
NaH_2PO_4	3.014 gm.
NaCl	52.605 gm.
Distilled water	6000 ml.
pH adusted to 7.0	

This saline was used as a general purpose saline solution in the washing of cells in the preparation of endotoxin, for resuspending the AKD cells in the preparation of the vaccine for antibody production, for resuspending the heat killed cells in preparation of the standard suspension antigen for the agglutination reaction, and as a diluent in the quantitative precipitin reaction.

b. Veronal Buffered Saline Solution pH 7.4

5,5 diethyl barbituric acid	5.75 gm.
Sodium 5,5 diethyl barbiturate	3.75 gm.
NaCl	85.00 gm.
Dist. water to make	2,000 ml.

The acid was dissolved in 500 ml. of hot distilled water and solutions of other components are added. This prepared a stock solution. A working solution of 1 part of stock solution was added to 4 parts distilled water and was prepared fresh for use. It was employed as a diluent for endotoxin that was used for incubation with serum and serum fractions and as a diluent of serum for the agglu-

tination reaction.

c. Saline Glycogen Solution

Saturated NaCl solution in distilled water	26 ml.
Glycogen	1 gm.
Distilled water	974 ml.

This saline solution was used to create an intraperitoneal inflammatiory condition in rabbits and 250 ml. was injected intraperitoneally to stimulate the accumulation of white blood cells in the exudate.

d. Citrated-saline

Saturated NaCl solution in dist. water	26 ml.
Sodium citrate(anhydrous)	4 gm.
Distilled water	974 ml.

This saline solution was injected (200 ml.) intraperitoneally in rabbits just prior to the harvest of peritoneal exudate. The citrate is in sufficient concentration to prevent the formation of a fibrin clot so that the white blood cells may be recovered.

e. Lysis Solution

Saturated NaCl solution (37g. NaCl/100 ml. dist. water)	7 ml.
Sodium citrate(anhydrous)	1 gm.
Distilled water	993 ml.
Adj. to pH 5.0 with 1N HCl	

This hypotonic saline solution was used to lyse any contaminating red blood cells in the white blood cell preparation. The white blood cells were suspended in 20 ml. of this saline and incubated at room temperature for ten minutes.

f. Neutralizing Solution

Saturated NaCl solution in distilled water	41 ml.
Sodium citrate (anhydrous)	7 gm.
Distilled water	959 ml.

This hypertonic saline solution was used to neutralize the hypotonic saline used to lyse red blood cells. This solution (20 ml.) was added to the hypotonic suspension of white blood cells.

g. Intracellular Salt Solution

KH_2PO_4	7.5 gm.
$MgSO_4$	2.46 gm.
Sodium citrate (anhydrous)	3.85 gm.
Saturated KCl solution in dist. water	11.4 ml.
Distilled water to make	1000 ml.

This solution was used to suspend the purified white blood cells. White blood cells were suspended in a concentration of 1×10^9 cells per 5 ml.

h. Kjeldahl Digestion Mixture

Conc. H_2SO_4	9 lb.
Saturated $CuSO_4$ solution in dist. water	40.0 ml.
Selenium	4.4 gm.

Heat the mixture until the components dissolve and turns from a green color to colorless. This solution is used to digest the washed antigen-antibody precipitates in the quantitative determination of precipitated antibody nitrogen.

i. N/70 Hydrochloric Acid

The barometric pressure was obtained from the weather office at Willow Run Airport and concentrated HCl was added to 1000 ml. of distilled water as indicated in Table 3.

TABLE 3

Preparation of Standard HCl from Constant Boiling HCl (42)
(After Foulk and Hollingsworth)

Barometer pressure mm Hg.	Per Cent HCl in acid	Grams of acid weighed in air, required for 1 liter of N/70 acid
770	20.197	2.577
760	20.221	2.574
750	20.245	2.571
740	20.269	2.568
730	20.293	2.565

The HCl solution was titrated against 0.01N NaOH with methyl red methylene blue indicator and corrected to give N/70 HCl. After correction the N/70 HCl was retitrated with 0.01N NaOH to ascertain the proper normality of the HCl solution. This solution was used to titrate the ammonia distilled over the microkjeldahl procedure. This normality of acid gives a factor of 0.2 times the ml. of N/70 HCl used to give mg. of protein nitrogen.

j. Boric Acid Solution

A saturated solution of boric acid in distilled water was prepared and 5 ml. was used to receive the ammonia distilled over in the microkjeldahl procedure.

k. Saturated Sodium Hydroxide

A saturated solution of sodium hydroxidein distilled water was prepared and the insoluble carbonate was removed by filtration. Five ml. of this solution was added to the Kjeldahl digestion mixture in the microKjeldahl aparatus to neutralize the sulfuric acid and liberate the ammonia so that it could be distilled over into the boric acid.

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