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Alteration and Restoration of Endotoxin Activity after Complexing with Plasma Proteins

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ABSTRACT

RUDBACH, JON A. (The University of Michigan, Ann Arbor), AND ARTHUR G. JOHNSON. Alteration of endotoxin activity after complexing with plasma proteins. J. Bacteriol. **92**:892–898. 1966.—A substantial decrease in the ability of endotoxin to be precipitated by homologous antiserum and to cause fever occurred after incubation with human plasma or human plasma, Cohn fraction IV-1. The endotoxin, thus altered, also displayed decreased lethality for rabbits. These alterations in endotoxin activity could be restored when the endotoxin-plasma protein mixture was treated with a proteolytic enzyme, and the endotoxin was precipitated with ethyl alcohol. Inactivation of the antigenic and toxigenic properties of the endotoxin molecule by plasma is discussed as resulting from complexing with plasma proteins rather than from enzymatic degradation.

The nature of the reaction by which human plasma alters biological and physical properties of endotoxin is unknown. Stauch and Johnson (16) showed that an endotoxin-altering factor (EAF) in serum reduced the ability of endotoxin to precipitate with homologous antiserum and caused the endotoxin to display a decreased rate of sedimentation during high-speed centrifugation. Yoshioka and Johnson (19) fractionated human plasma by the cold ethyl alcohol method of Cohn et al. (5) and demonstrated that this EAF was contained in fractions IV-1 and III-0. These plasma fractions were capable of diminishing the pyrogenicity of endotoxin in addition to reducing the capacity of endotoxin to be precipitated by homologous antiserum. Subsequently, it was shown (13) that the loss in ability of endotoxin to be precipitated by homologous antiserum was not associated with release of the major antigenic determinant sugar; rather, immunodiffusion analysis revealed that a major antigen of endotoxin was dispersed with the appearance of an antigen which formed a junction of continuity with the O-polysaccharide hapten of endotoxin.

These results, along with the work of others [*see reviews* (1, 9, 15)], suggested that the endotoxin polymer was degraded, possibly by a plasma enzyme, to a smaller aggregate size with concomitant loss of biological activity. However, our attempts to isolate the degraded products of endotoxin resulted in evidence that the detoxification of endotoxin by plasma was reversible, i.e., the ability to precipitate with homologous antiserum and to stimulate fever in rabbits could be restored quantitatively (14). The data recorded herein detail and extend the earlier observations on the reversibility of the loss in properties of endotoxin detoxified by human plasma.

MATERIALS AND METHODS

Endotoxins. As described previously (13), endotoxin was extracted from whole cells of *Salmonella typhosa* 0–901 by the method of Boivin and Mesrobeanu (2). This endotoxin was fractionated further by high-speed centrifugation in an attempt to eliminate some of the haptenlike components present in the preparation. The precipitate after centrifugation for 2 hr at 10,000 × *g* was diluted in distilled water, a sample was removed for dry-weight determination, and the remainder was distributed into vials and frozen at −30 C.

Radioactive endotoxin was prepared according to the procedure of Braude et al. (3). Briefly, 30 mg of lyophilized endotoxin was incubated with 600 μc of $NaCr^{51}O_4$ (Chromitope sodium; lot control no. 59 Z8; specific activity, 16.6 mc/mg; E. R. Squibb & Sons, New York, N. Y.), and the mixture was dialyzed at 4 C against daily changes of phosphate buffer until the dialysate was free of significant radioactivity. The solution was then centrifuged at 20,000 × *g* for 2 hr to obtain a constantly sedimentable fraction, and the re-

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sultant precipitate was dissolved in 6 ml of distilled water. The Cr^{51}-labeled endotoxin had a specific activity of 9.75 μc/mg. All counts were made in a Nuclear Chicago DS-3 well-type scintillation detector and recorded on a Nuclear Chicago Ultrascaler (model 192; Nuclear-Chicago Corp., Des Plaines, Ill.). Counting was carried out in the Geiger-Müller region at 1,150 v, with a counting efficiency of 8%.

Human plasma. Healthy volunteers were bled by venipuncture into the anticoagulant solution ACD [trisodium citrate·$2H_2O$ (22.09 g); citric acid (8.09 g); glucose·H_2O (24.5 g) in 1,000 ml of water], with 15 ml of ACD per 100 ml of blood. Tests for endotoxin-altering activity were started within 3 hr after the blood had been collected.

Plasma fraction IV-1. Human plasma fraction IV-1, prepared by the method of Cohn et al. (5), was generously supplied by Cutter Laboratories, Berkeley, Calif., from lot no. 21378.

Preparation of antiserum, quantitative precipitation assay, and immunodiffusion tests. These procedures have been described in a previous publication (13). When the test sample for the quantitative precipitation assay contained whole plasma, 10 units of heparin were added to the sample before the antiserum, to prevent clotting.

Pyrogenicity determinations. For determining the pyrogenicity of endotoxin preparations, New Zealand albino rabbits of both sexes, 11 to 16 weeks old, were placed in metal stocks on the day before the experiment for a period of 2 to 4 hr to accustom them to confinement. On the day of the experiment, their rectal temperatures were recorded by use of indwelling thermistor probes connected to a 12-channel Telethermometer (model 44RF; Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). The desired amounts of test materials were injected into the marginal ear vein in a volume of 1 ml, and the temperatures were recorded at 0.5-hr intervals for 5 hr thereafter.

Incubation system and assay procedure for EAF. Unless stated otherwise, the procedure for testing the degree of inactivation of endotoxin by fraction IV-1 or by whole plasma was the following. Fraction IV-1 (6.25 mg/ml) or whole human plasma (1 ml) was incubated with endotoxin (100 μg/ml) for 4 hr at 37 C. The amount of recoverable endotoxin in a test sample was determined by quantitative precipitation with anti-*S. typhosa* serum on a standard curve (16) or by its ability to cause fever in rabbits.

Restoration of altered endotoxin. The method for the restoration of altered endotoxin has been described previously (14). Briefly, it consisted of digesting the endotoxin-plasma mixture with Pronase (lot no. 502177; Calbiochem) for 18 hr at 37 C, and then precipitating the endotoxin from the digest with 6 volumes of ethyl alcohol. The restored endotoxin was taken up in the same volume of water as the original mixture of altered endotoxin.

RESULTS

Temporal course of the reaction of endotoxin with fraction IV-1 and plasma. An initial increase followed by a decrease in precipitation was obtained when endotoxin was incubated with samples of human plasma for varying time intervals, and the samples were analyzed for recoverable endotoxin by the quantitative precipitation procedure (Fig. 1). It may be seen that incubation of endotoxin with citrated human plasma for 15 to 30 min increased the serological reactivity of the endotoxin with homologous antiserum, i.e., 15 to 42% more endotoxin appeared to be recoverable from these samples than was originally added. Incubation of endotoxin with fraction IV-1 for the same time period resulted in a lesser degree of enhancement, i.e., values ranged from 92 to 125% recoverable endotoxin. The addition of citrate to fraction IV-1 in amounts equivalent to that found in ACD plasma did not increase these values.

The pyrogenicity of the various samples of endotoxin incubated with human plasma was tested by injecting intravenously into rabbits 1 ml of each sample, diluted to contain 0.1 μg of original endotoxin. The curves, plotted in Fig. 2, revealed no enhanced pyrogenicity of the endotoxin in the sample which showed increased ability to precipitate with homologous antiserum after incubation for 0.5 hr in human plasma. Instead, there was a progressive decrease in the pyrogenicity of the preparation.

Lethality of endotoxin for rabbits after alteration by fraction IV-1. Inasmuch as reduction of the pyrogenicity of endotoxin in rabbits was observed concurrently with the antigenic alteration of endotoxin by fraction IV-1, a correlative study was undertaken to determine whether the incubation of endotoxin with fraction IV-1 also reduced the lethality of endotoxin for rabbits. *S. typhosa* endotoxin (400 μg ml) was incubated with fraction IV-1 (25 mg/ml), or alone with buffer, for

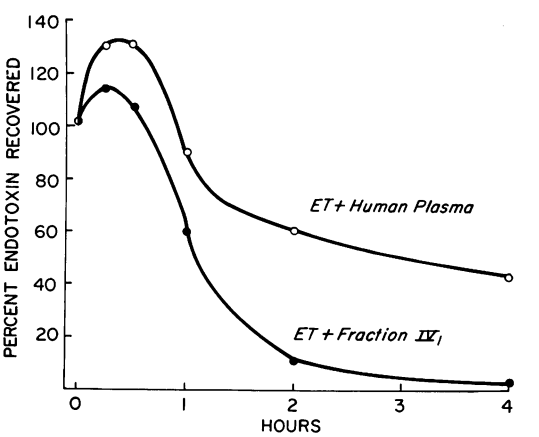

FIG. 1. *Increased and decreased serological activity of endotoxin (ET) after incubation for various time intervals in human plasma (○) or fraction IV-1 (●).*

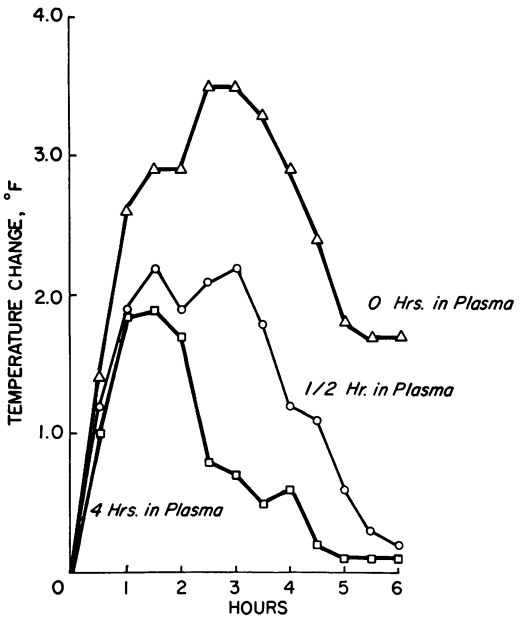

FIG. 2. *Depression of pyrogenic activity of endotoxin after incubation in human plasma for 0.5 hr* (○) *or 4 hr* (□).

4 hr at 37 C. Dutch-belted rabbits then were injected intravenously with 1- or 2-ml amounts of the incubation mixtures, which corresponded to 400 or 800 μg of endotoxin, respectively. Deaths were recorded at 6 hr, 12 hr, and daily thereafter for 4 days, as indicated in Table 1. The groups of animals receiving endotoxin incubated with fraction IV-1 suffered half as many deaths as the groups of animals receiving unaltered endotoxin. This finding supports our previous results in mice where 50% of the animals receiving endotoxin incubated in human plasma or fraction III_o (but not fraction IV-1) were protected (19).

Restoration of endotoxin activity. Indirect evidence was accumulated from several laboratories that endotoxin was degraded to a smaller size by a plasma enzyme with concomitant loss of biological activity. However, Oroszlan et al. (12) showed that the detoxification of endotoxin by liver extracts was a reversible reaction, and it was probably due to the binding of endotoxin by liver proteins. In a preliminary report, evidence was presented that similarly a reversible binding of proteins to endotoxin might be responsible for the alterations of endotoxin by plasma described above (14). This conclusion was based on data showing that endotoxin altered by fraction IV-1, when treated with Pronase and ethyl alcohol, regained pyrogenicity as well as the ability to precipitate quantitatively with homologous antiserum.

TABLE 1. *Reduction in lethality for rabbits of endotoxin after incubation with fraction IV-1*

Material	Amt injected[a]	Endotoxin recovered	Cumulative dead/total			
			6 hr	12 hr	1 day	4 days
	μg	%				
Endotoxin incubated in PBS[b]	400	100	1/9	2/9	6/9	6/9
Endotoxin incubated with IV-1[c]	400	7	0/9	0/9	2/9	3/9
Endotoxin incubated in PBS	800	100	4/9	8/9	8/9	8/9
Endotoxin incubated with IV-1	800	5	0/9	4/9	4/9	4/9

[a] Amount injected was calculated from the original amount of endotoxin present in the incubation mixture.

[b] Endotoxin (*Salmonella typhosa*), 400 μg/ml, incubated in phosphate-buffered saline for 4 hr at 37 C.

[c] Endotoxin, 400 μg/ml, incubated with fraction IV-1, 25 mg/ml, for 4 hr at 37 C.

On the other hand, when fresh human plasma was used, quantitative estimation by the precipitin reaction of endotoxin after the restorative procedure was prevented by the appearance of a precipitate, probably containing plasma proteins denatured by the procedure. However, qualitative restoration of such altered endotoxin was shown through a regaining of pyrogenicity, as well as through the precipitation pattern in immunodiffusion plates. To measure quantitatively restoration of endotoxin altered by whole plasma, endotoxin externally labeled with hexavalent Cr^{51} by the method of Braude et al. (3) was utilized. This endotoxin was treated in the manner outlined previously for alteration by fraction IV-1 and fresh human plasma, and for restoration of the altered endotoxin. Samples of the altered and restored radioactive-endotoxin preparations were subjected to the quantitative precipitation test for antigen as previously outlined (14), except that quantitation of the precipitates was achieved by radioisotope counting rather than by nitrogen analysis. When the counts per minute precipitated (corrected for decay and background) were plotted against the volume of sample used in the quantitative precipitation tests, the relationships shown in Fig. 3 and 4 were obtained. If the counts per minute of unaltered endotoxin treated with Pronase and ethyl alcohol were taken as the 100% recoverable value, than endotoxin which had been altered by incubation with fraction IV-1 was only

recoverable to the extent of 5% (Fig. 3). Nevertheless, subsequent Pronase and ethyl alcohol treatment of this altered endotoxin permitted recovery of 100% of the endotoxin as measured by the quantitative isotopic precipitation procedure. Figure 3 also shows on the basis of isotopic measurements that Pronase and ethyl alcohol treatment reduced somewhat the ability of unaltered endotoxin to precipitate with homologous antiserum. This effect was not demonstrable when the precipitates were quantitated by their nitrogen content, as indicated previously.

The quantitative restoration of Cr-^{51}-labeled endotoxin, altered by incubation in fresh human plasma, is shown in Fig. 4. Incubation of the endotoxin in plasma resulted in an alteration of the endotoxin such that less than 10% was recoverable by quantitative precipitation. Pronase and ethyl alcohol treatment of this altered endotoxin restored the serological activity to 74% of that seen when unaltered control endotoxin preparation was treated with Pronase and ethyl alcohol. Unaltered isotopically labeled endotoxin treated with Pronase and ethyl alcohol again precipitated with antiserum to a lesser degree than did untreated endotoxin.

The data documenting the restoration of pyrogenicity of endotoxin altered by either whole plasma or fraction IV-1 were reported previously (14). In this regard, it should be noted that Pronase digestion alone, without ethyl alcohol treatment, of endotoxin altered by fraction IV-1 did not result in restoration of the pyrogenicity of the endotoxin.

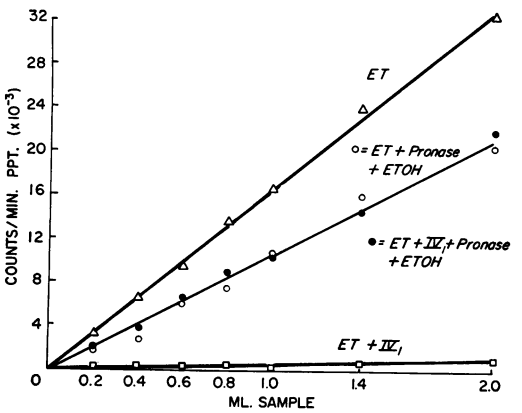

FIG. 3. *Restoration of precipitating activity of endotoxin incubated in fraction IV-1. The counts per minute of Cr^{51}-labeled endotoxin precipitated by anti-Salmonella typhosa antiserum were measured as a function of the volume of sample withdrawn from the various incubation mixtures. Endotoxin incubated in saline, △; endotoxin incubated in saline followed by treatment with Pronase and precipitation with ethyl alcohol, ○; endotoxin incubated in fraction IV-1 followed by treatment with Pronase and precipitation with ethyl alcohol, ●, endotoxin incubated in fraction IV-1 without restoration by Pronase and ethyl alcohol, □.*

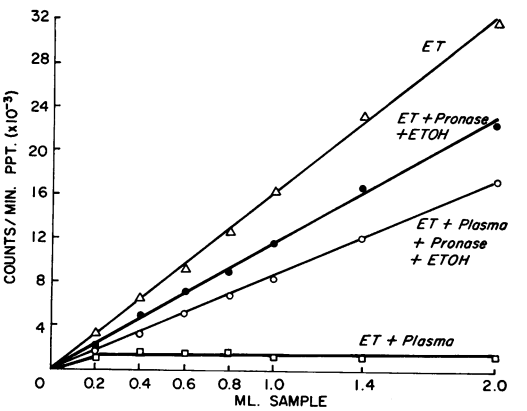

FIG. 4. *Restoration of precipitating activity of endotoxin incubated in human plasma. The counts per minute of Cr^{51}-labeled endotoxin precipitated by anti-Salmonella typhosa antiserum were measured as a function of the volume of sample withdrawn from the various incubation mixtures. Endotoxin incubated in saline, △; endotoxin incubated in saline followed by treatment with Pronase and precipitation with ethyl alcohol, ●; endotoxin incubated in human plasma followed by treatment with Pronase and ethyl alcohol, ○; endotoxin incubated in human plasma without restoration by Pronase and ethyl alcohol, □.*

The immunodiffusion pattern of altered endotoxin also could be restored by Pronase and ethyl alcohol treatment to a pattern which exhibited close similarity to the immunodiffusion pattern of native endotoxin. This is shown in Fig. 5, a photograph of immunodiffusion plates where the center wells contained antiendotoxin serum and the peripheral wells contained various endotoxin preparations. Note that the restored endotoxin precipitates with antiserum close to and concave toward the antigen well, in a pattern resembling the typical precipitin arc of native endotoxin. On the other hand, the altered endotoxin formed a straight line of precipitate equidistant between the antigen and antiserum wells.

The results of a single experiment showing restoration of lethality in endotoxin detoxified by fresh plasma are presented in Table 2. *S. typhosa* endotoxin was altered by incubation in fresh citrated human plasma and restored by Pronase and ethyl alcohol treatment. Incubation of endotoxin with plasma altered the endotoxin to the extent that only two of eight rabbits were killed by the detoxified product, whereas native endotoxin killed seven of nine rabbits. Pronase and ethyl alcohol treatment, however, partially restored

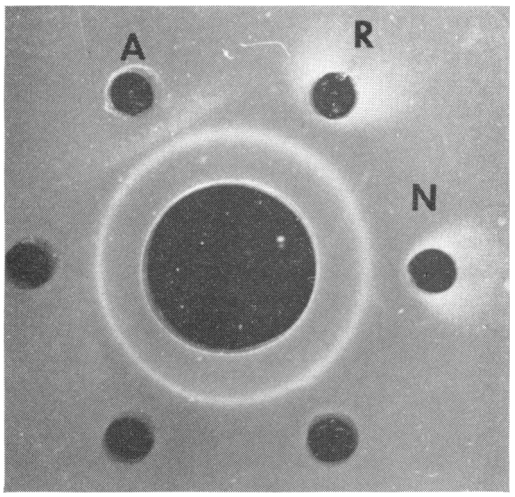

FIG. 5. *Precipitin pattern as seen in the Ouchterlony agar diffusion test of native endotoxin incubated in saline (N); endotoxin altered after incubation in fraction IV-1 (A); and endotoxin incubated in fraction IV-1 after restoration of biological activity by treatment with Pronase and ethyl alcohol (R).*

TABLE 2. *Restoration of lethality for rabbits of endotoxin detoxified by incubation in fresh human plasma*[a]

Material	Cumulative dead/total		
	6 hr	18 hr	5 days
Endotoxin incubated in PBS	3/9	7/9	7/9
Endotoxin incubated in plasma	1/8	1/8	2/8
Endotoxin incubated in plasma; restored	4/8	5/8	5/8
Plasma alone; restored	0/4	0/4	0/4

[a] Pooled human plasma collected into ACD solution [Trisodium citrate·$2H_2O$ (22.09 g); citric acid (8.09 g); glucose·H_2O (24.5 g) in 1,000 ml of water] and used within 3 hr. *Salmonella typhosa* endotoxin (200 μg/ml) was incubated with the fresh human plasma (1 ml) or in phosphate-buffered saline (PBS) for 5 hr at 37 C. The altered endotoxin was restored by digesting the plasma proteins with Pronase and precipitating the endotoxin with ethyl alcohol. Each rabbit received 4 ml of the solutions intravenously, which corresponded to 800 μg of endotoxin in the control solution.

toxicity so that five of eight died. A control preparation containing plasma plus Pronase and ethyl alcohol was nonlethal.

Detection of human plasma proteins bound to endotoxin. If plasma proteins were binding to the endotoxin, the protein-endotoxin complex should be detectable in solutions of altered endotoxin. To test for such complexes, rabbit antiserum directed against whole human plasma was added to samples of endotoxin after incubation in fraction IV-1. The precipitate was collected, washed, dissociated in 15% NaCl, and tested for the presence of endotoxin by immunodiffusion against anti-endotoxin serum. This technique revealed that altered endotoxin was, in fact, precipitated by antiserum to human plasma, presumably as a result of the endotoxin being bound to human serum proteins. However, it was possible that this was due to a nonspecific coprecipitation of endotoxin.

Accordingly, with a reversed approach to test for plasma protein-endotoxin complexes, altered endotoxin was precipitated from incubation mixture with anti-*S. typhosa* serum, and the precipitates were tested for the presence of human plasma proteins by immunodiffusion against rabbit antiserum to whole human plasma. Endotoxin was incubated with human plasma, fraction IV-1, or phosphate-buffered saline (PBS) at 37 C. Samples were withdrawn at various periods of time and anti-*S. typhosa* serum was added (*see* Fig. 1). The precipitates were collected, washed twice, and dissociated in 16% NaCl or in 1 N NaOH. The results of these tests showed that the endotoxin which had been incubated with human plasma for 0, 0.25, or 0.5 hr, i.e., those time intervals in which increased nitrogen was precipitated (Fig. 1), appeared to be bound to human plasma protein, inasmuch as the precipitates contained an antigen which formed a line in the immunodiffusion plates with antihuman plasma. The samples of endotoxin which had been incubated with fraction IV-1 or PBS for the same time intervals appeared negative in this respect. Thus, it was demonstrated that under certain conditions plasma proteins can be shown to be bound to the endotoxin polymer.

Demonstration of antibody activities in fraction IV-1. Fraction IV-1 at concentrations up to 10 mg/ml would not agglutinate *S. typhosa* 0-901. When tested by the more sensitive passive hemagglutination technique, it was found that fraction IV-1 at a concentration of 1.25 mg/ml would agglutinate endotoxin-coated erythrocytes. After treatment with 0.1 M 2-mercaptoethanol, no hemagglutination of endotoxin-coated red blood cells could be demonstrated at concentrations of fraction IV-1 up to 6.25 mg/ml, whereas the fraction IV-1 control without mercaptoethanol showed hemagglutination at 1.56 mg/ml. However, both the reduced and the control solutions of fraction IV-1 were capable of diminishing the ability of endotoxin to precipitate with homologous anti-

serum to the 5% level. Consequently, it is not likely that the hemagglutinin for endotoxin-coated red blood cells demonstrable in fraction IV-1 is identical with EAF.

DISCUSSION

Landy et al. (10) noted an increase in the amount of antityphoid O antibody precipitated by 2.5 to 30 μg of endotoxin after incubation for 1 hr in human defibrinated plasma as contrasted to incubation in saline. These results were interpreted by them as the release of haptenic polysaccharide by a factor in plasma, with the increase in precipitable antibody nitrogen being attributable to the well-known ability of the hapten to precipitate more antibody per unit weight than the complete antigen. This contrasted with our finding that incubation for 4 hr of 400 μg of endotoxin with human plasma, or fractions derived therefrom, decreased the amount of antibody precipitated in vitro by endotoxin (19). However, the latter authors (*unpublished data*) noted also an increase in ability to precipitate antibody if the incubation time was shortened to 15 to 30 min. For example, brief incubation of endotoxin in three samples of human plasma resulted in recovery of 169, 145, and 150% of the endotoxin added. This was observed routinely, not only after incubation in plasma, but also when endotoxin was incubated for 4 hr in saline containing citrate. In the latter experiments, 400 μg of endotoxin was added to 4 ml of PBS, *p*H 7.0 (or physiological saline, *p*H 7.0) which also contained sodium citrate in a concentration of 1.1×10^{-2} M. The endotoxin recovered after such incubation for 4 hr at 37 C, ranged from 124 to 143% in 13 tests. An explanation for these results was advanced by Yoshioka et al. (18), who suggested that recovery of more than 100% endotoxin after brief incubation in plasma was the result of a nonenzymatic citratelike effect, involving disaggregation of the endotoxin which uncovered previously inaccessible determinant groups.

Hegemann (8) observed that endotoxin preparations neutralized by plasma gave skin reactions or elicited typical fever patterns when injected into some, but not all, patients. From these observations, he concluded that substances which could reverse the endotoxin-altering reaction were present in certain individuals. However, this point of view was largely superseded by the theory that alterations in endotoxins were due to cleavage of the molecule by a plasma enzyme (1). Recently, the latter hypothesis has received further support from the experiments of Skarnes (Ann. N.Y. Acad. Sci. *in press*). However, if it were assumed that cleavage was due to a hydrolytic enzyme, effective reversal of the reaction should be precluded. The findings presented here suggest that, on incubation with human plasma or fraction IV-1, the endotoxin polymer first is opened up, or dispersed, with the appearance of a greater number of antigenic and pyrogenic sites than are present in unaltered endotoxin. This is evidenced by the ability of endotoxin incubated with plasma for short periods of time to precipitate increased amounts of antibody and to stimulate greater febrile responses than unaltered endotoxin. Although enhanced pyrogenicity was not demonstrable in the present series of experiments, it has been observed by others (4, 6). Yoshioka et al. (18) have come to similar conclusions regarding the initial dispersion of endotoxin. That this initial dispersion may be due to bile salts present in plasma is suggested by the recent data of Tarmina et al. (Bacteriol. Proc., p. 51, 1966). After dispersion, both the antigenicity and pyrogenicity of the endotoxin and plasma (or fraction IV-1) mixture are decreased, presumably by plasma proteins binding to the endotoxin disaggregated by bile salts. In certain instances, this plasma protein-endotoxin complex could be detected, and the use of a proteolytic enzyme for the restoration of altered endotoxin, also, is suggestive evidence that altered endotoxin is bound to protein. The experiments of Oroszlan et al. (12) showing binding between endotoxin and rabbit liver protein, as well as the dissociation of endotoxin to a smaller particle size by plasma factors with surfactant activities (11), support this hypothesis. Additional supportive data have been presented elsewhere (Rudbach et al., Ann. N.Y. Acad. Sci., *in press*).

The nature of this endotoxin-altering protein still remains obscure. The data presented above indicate that it is not O antibody. Previous work (19) had categorized the active serum factor as lipoprotein in nature. It is of interest that the binding of lipids (endotoxin?) to protein often depends on the same factors as the combination of enzymes to substrates; in addition, large molecular weight anionic polysaccharides are some of the most specific reagents for precipitation of certain classes of lipoprotein (7). Although human hemoglobin has been shown by Van Vunakis et al. (17) to interact with endotoxin and to reduce its capacity to act as an antigen in the complement-fixation test, several experiments in our laboratory indicated that hemoglobin was not active in the endotoxin-altering system described by us.

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