Isolation From Human Serum of an Inactivator of Bacterial Lipopolysaccharide

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By a series of chromatographic procedures involving precipitation by salt, gel filtration, anionic exchange, and hydroxyapatite elution, a protein—termed the *lipopolysaccharide inactivator* (LPS-I)—has been isolated from normal human serum. As a result of treatment of bacterial lipopolysaccharide (LPS) by LPS-I, the treated LPS loses its toxicity for mice and reactivity in the *Limulus* assay and appears to be irreversibly disaggregated. The inactivation of the LPS by the purified LPS-I is temperature and time dependent and is not blocked by the addition of irreversible inhibitors of serine esterases. The LPS inactivator migrates as an α-globulin in whole serum and has a sedimentation velocity of approximately 4.5S. Characteristics of the inactivated LPS are briefly described using internally labeled LPS. (Am J Pathol 88:559–574, 1977)

THE INDUCTION OF SHOCK in humans subsequent to endotoxemia represents a serious and major problem in clinical medicine. Although it is now thought that humoral rather than cellular (the macrophage phagocytic system) mechanisms account for the ability of the host to resist and recover from endotoxemia (see Skarnes and Rosen [1] for review), the precise manner by which interaction of the bacterial endotoxin (lipopolysaccharide, LPS) with plasma/serum occurs is presently unknown. The alteration in LPS following incubation with serum has been associated with a change in the LPS referred to as "detoxification." As a result, the LPS loses its ability to induce fever in rabbits, kill actinomycin D–treated mice, induce tumor regression, and react with the *Limulus* extract.[1] Detoxification has also been associated with a change in the antigenic composition of the LPS.[2-6] Although binding of the polysaccharide portion of LPS with antibody can also result in the blocking of biologic activity,[7] detoxification of LPS in most studies appears to involve interaction of LPS with nonimmunoglobulin factors of human serum.

The nature of the detoxifying activity in serum is unclear. On the one hand, reversible interaction of serum factor(s) with LPS, resulting in disaggregation, has been suggested as a mechanism.[7] Another study suggests interaction of LPS with a serum lipase, although no direct evidence for this mechanism has been obtained.[8] In yet another series of studies,

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two proteins, both α-globulins, have been implicated in the detoxification reaction.[9,10] In this situation, LPS is thought to react with a heat-stabile serum esterase that causes disaggregation of the LPS. Following this, a second α-globulin (organophosphate-resistant and heat-labile) presumably reacts enzymatically with the disaggregated LPS to render it nontoxic.[9,10] Finally, the fifth (C5) and sixth (C6) components of complement have been incriminated as playing some indirect role in the detoxification of LPS.[11]

These studies describe the isolation from human serum of a single protein that has the ability to irreversibly inactivate LPS. Some characteristics of the inactivated LPS are also described.

Materials and Methods

Endotoxin Preparations

Lipopolysaccharide (LPS) was obtained by the hot aqueous phenol extraction method [11] from either a rough strain of *Salmonella typhimurium* (G30) or from *Escherichia coli* strain 0111:B4 (purchased from the Difco Laboratories, Detroit, Mich.). The former preparation was internally labeled by growing the bacteria in medium containing tritium-labeled galactose prior to extraction of the LPS. The resulting LPS from *S. typhimurium*, by chemical analysis, contained <1% nucleic acid and <1% nitrogen by weight. Unless otherwise indicated, 5 μg LPS was incubated with a sample to be tested for LPS-I activity. The incubation was carried out at 37 C for 3 hours, followed by bioassay or other analysis. The LPS was dissolved in phosphate-buffered saline (pH 7.4).[11] Where used, trace quantities (100 ng) of ^{3}H-labeled LPS were also present. Details of the LPS preparations are given elsewhere.[12] Most experiments listed in this paper were carried out with LPS from *S. typhimurium* because of the availability of the labeled preparation. However, when the LPS from *E. coli.* was used, results similar to those obtained from the *S. typhimurium* LPS preparation were also obtained.

Limulus Assay for Lipopolysaccharide

Except where radioactivity or bioassay in mice was used as a method of analysis, the extract from *Limulus* was employed as the rapid and sensitive method for the analysis of LPS activity. The preparation of the *Limulus* extract is described elsewhere.[13] The extract had a sensitivity for detecting 5 ng LPS, indicating an ability to measure as little as 0.1% of the remaining LPS after its inactivation.

Mouse Lethality Assay

Outbred Swiss-Webster adult male mice weighing approximately 50 g were used. These animals were pretreated with actinomycin D to increase the sensitivity of the assay. Under these conditions, 20 ng LPS/mouse will kill all mice within 24 hours; Five ng killed 9 of 10 mice in the same period of time. This assay is described in greater detail elsewhere.[11]

Ultracentrifugal Separation of Lipoproteins

The density of normal human serum was adjusted to 1.06 and 1.22 with the addition of potassium bromide and sodium bromide salts.[14] The serum was then placed in a swinging bucket rotor and was subjected to 105,000*g* in a Beckman ultracentrifuge for 20 hours at 12

C. The floatation layer, consisting of several millimeters of the top of the fluid in each tube, was removed. The remaining upper third of the volume of the tube was discarded. The lower two thirds of the tube was retained for testing. Serum which was not altered by the addition of salts was also tested, with the density of this preparation assumed to be 1.005.

Chromatographic Isolation of Lipopolysaccharide Inactivator

Preliminary analysis of serum fractionated by the addition of ammonium sulfate indicated that the LPS-I remained in the supernatant fluid when ammonium sulfate was present at 40% of saturation, whereas, at 60% saturation, the inactivator was found in the precipitate. Accordingly, 1-liter volumes of normal human serum were first treated with ammonium sulfate at 40% saturation, the precipitate discarded, and then the precipitate was obtained when the concentration of ammonium sulfate was raised to 60% of saturation. The resulting precipitate was redissolved in distilled water. This material was dialized in phosphate buffer, pH 7.2, ionic strength 0.05, and then fractionated by Sephadex G 150 column chromatography. Resulting pools of LPS-I were then obtained, concentrated, and fractionated by ion exchange chromatography in DE52 cellulose. The zone containing LPS-I was then concentrated and chromatographed in Sephadex G 200. To this point, the fractionation procedures were similar to those used for the chemotactic factor inactivator.[15] The resulting material was subsequently fractionated by hydroxyapatite chromatography. This final preparation of LPS-I was then analyzed by analytical acrylamide disc gel electrophoresis as well as by immunoelectrophoresis.[15] In two experiments, preparative electrophoresis in acylamide gel or Pevikon was also employed.[15]

Disaggregation and Attempted Reaggragation of ^{3}H-Lipopolysaccharide

In one experiment, 5 μg LPS (with 1 μg ^{3}H-LPS) was incubated in a 100-μl volume with either 0.15 μl LPS-I (approximately 5 μg protein) or with 100 μl of 0.4% sodium dodecyl sulfate (Eastman Scientific Co., Rochester, N.Y.). The samples were maintained at 37 C overnight, and then subjected to ultracentrifugation in a sucrose density gradient (5 to 40% sucrose in 0.05 M phosphate, pH 7.2). This resulted in a main peak of radioactivity near the albumin marker (which was present in a separate reference gradient tube). The fractions containing the peak of radioactivity were dialyzed against phosphate buffer (pH 7.2; ionic strength, 0.05) which contained 25 mM $MgCl_2$ as a reaggregating agent. The material was then recentrifuged in another sucrose density gradient to determine if reaggregation of the LPS had occurred.

Esterase Assay

Fifty-microliter samples of material to be tested were incubated with 1 ml β-napthyl acetate (containing 1 mg/ml distilled water), to which was added 0.75 ml phosphate-buffered saline (pH 7.4). The mixture was incubated for 2 hours at 37 C, following which 2 ml distilled water was added and the solution read spectrophotometrically at 328 mm.

Results

Behavior of Lipopolysaccharide Inactivator at Various Specific Densities

Previous studies have suggested a lipoprotein-like nature for at least one component of the serum factors that alter LPS.[9,10] Accordingly, the specific density of serum was studied at values of 1.005 (unaltered serum), 1.06, and 1.22. By ultracentrifugal treatment, a flotation versus non-flotation position of the LPS-I was assessed. Quantities of 0.1 ml from the

ultracentrifuged samples were incubated with 5 μg of LPS and inactivation of the LPS assessed by the *Limulus* method. As shown in Table 1, even at a density of 1.22 the LPS-I was present in fluid beneath the floatation layer. These studies indicate that the LPS-I does not behave as a lipoprotein, on the basis of its lack of floatation in serum adjusted to high specific density.

Electrophoretic Behavior of Lipopolysaccharide in Normal Human Serum

Normal human serum was electrophoretically fractionated at pH 8.6 in Pevikon. The fractions were dialyzed against phosphate-buffered saline (pH 7.4) and then incubated with LPS. Each fraction was assessed for protein content by the Lowry reaction and also for LPS-I by the *Limulus* method following incubation with 5 μg LPS. As shown in Figure 1, the LPS-I activity appeared in a zone on the cathode side of the albumin peak. Under these conditions the LPS-I behaves electrophoretically as an α-globulin.

Chromatographic Isolation of Lipopolysaccharide Inactivator

As will be described in detail below, and as shown in Table 2, a series of chromatographic procedures was used for the isolation of the LPS-I. The last step varies in its necessity, depending upon state of purity of the LPS-I in the hydroxyapatite chromatographic step.

The redissolved precipitate obtained from whole human serum (following sequential addition of ammonium sulfate at 40% and then 60% of saturation) was chromatographically separated in Sephadex G-150. As shown in Figure 2, the LPS-I was found in an area from Fractions 52 to 100 which represented a valley between two major peaks of protein. The zone of LPS-I was concentrated and further fractionated by anionic exchange chromatography in DE-52 cellulose. A linear and increasing salt gradient of sodium chloride was used. Several separate peaks of protein were eluted. The LPS-I was found in the ascending shoulder of the main protein peak (Figure 3, Fractions 50 to 80). The pool of LPS-I was concentrated and applied to a Sephadex G-200 column, resulting in the

Table 1—Lipopolysaccharide Inactivator Activity

	LPS-I activity†	
Density of serum*	Flotation layer	Nonflotation layer
1.005 (unadjusted)	Absent	Present
1.06	Absent	Present
1.22	Absent	Present

* Adjusted with potassium chloride and potassium bromide salts.
† See Materials and Methods regarding details of sample selection and assay

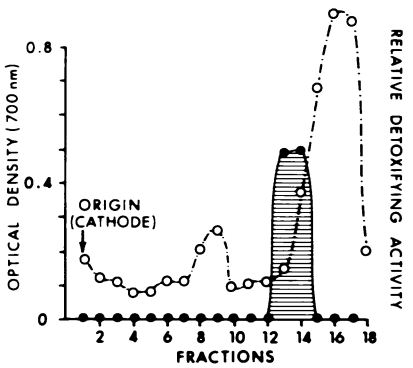

Figure 1—Electrophoretic separation of human serum and localization of LPS-I activity. (Relative detoxifying activity, *solid circles*; protein, *dotted line*; zone of LPS-I, *shaded area*)

appearance of LPS-I coincident with the major peak of protein (Figure 4, Fractions 60 to 100). The area of LPS-I obtained from the G-200 column was concentrated to a volume of 5 ml and dialyzed against 0.05 M potassium phosphate buffer and subsequently applied to a hydroxyapatite column, and elution was carried out by an increasing salt gradient. Two peaks of protein were found, but the LPS-I was only associated with the second peak (Figure 5, Fractions 80 to 105). This behavior of LPS-I in hydroxyapatite was consistent, and the results shown in Figure 5 were similarly obtained in four other experiments. For all subsequent studies to be herein described, the pooled fractions of LPS-I defined in Figure 5 were used.

Yield of Lipopolysaccharide Inactivator During Purification

Using a titration analysis and comparing each pool of LPS-I with a reference of the original sample of serum (and adjusting volumes of samples from the various pools so that they would represent the 100 μl reference of original serum), virtually complete recovery was found in the LPS-I activity through the first three chromatographic steps. This would suggest that most, if not all, of the inactivator activity was associated with the LPS-I pools. By the time LPS-I was eluted from hydroxyapatite, although it was highly active (>70% of LPS-I activity from the preceding

Table 2—Summary of Steps in Preparative Isolation of Lipopolysaccharide Inactivator

1. Precipitation of LPS-I from human serum by sequential addition of ammonium sulfate, proceeding from 40 to 60% of saturation; precipitate is then redissolved and dialyzed in 0.05 M phosphate buffer, pH 7.2
2. Gel filtration in Sephadex G-150
3. Anionic exchange chromatography in DE-52 cellulose
4. Gel filtration in Sephadex G-200
5. Elution from hydroxyapatite
6. Preparative electrophoresis in acrylamide gel

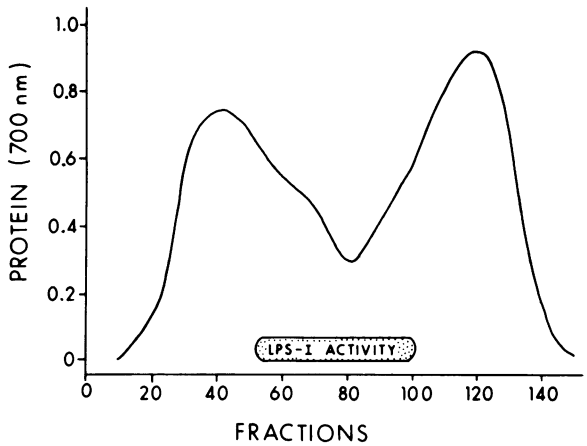

Figure 2—Elution of LPS-I from Sephadex G150. The material added to the column was the redissolved precipitate obtained by ammonium sulfate precipitation from human serum.

step was recovered), it demonstrated great lability at this point. Highly active preparations frequently become inactive within 48 hours while being maintained at 5 C. Reasons for this lability are unclear.

Analysis of Purified Lipopolysaccharide Inactivator for Physicochemical Characteristics

The LPS-I obtained from hydroxyapatite chromatography (Figure 5) was analyzed in analytical acrylamide disc gel electrophoresis (pH 8.6). An unusually large amount (100 μg) of LPS-I was applied to the disc gel in order to increase the likelihood of detecting contaminants, and for purposes of elution. As shown in Figure 6, the LPS-I appeared as a single band approximately midway in the gel. Two very faint bands were seen near the anode. For comparative purposes, a companion gel to which was

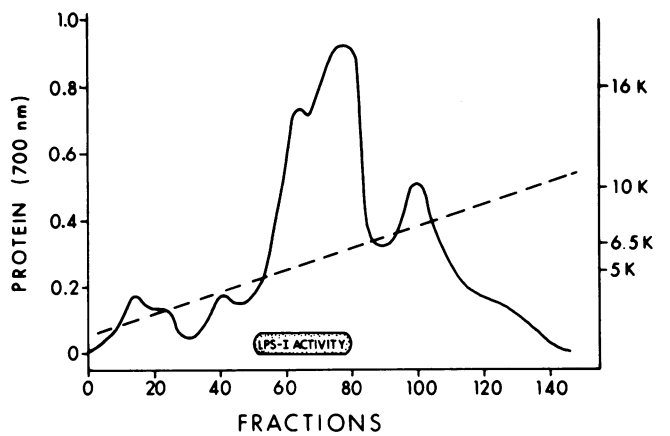

Figure 3—Ionic exchange chromatography of LPS-I from DE-52 cellulose. (Protein, *solid line*; conductance, *dotted line*)

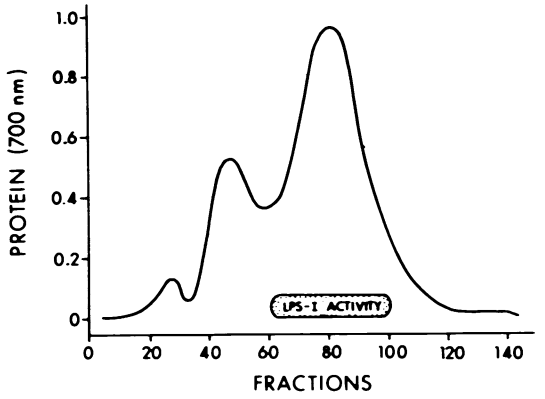

Figure 4—Elution of LPS-I from Sephadex G200.

applied normal human serum is also shown. A gel identical to the one in Figure 6 containing the LPS-I was cut into 3-mm sections and elution experiments carried out; this gel was not stained. The various sections were each suspended in 0.2 ml of buffered saline and crushed with a small grass rod. After standing overnight at 5 C, the fractions were tested for protein content (Lowry method) as well as for LPS-I content. The data in Figure 7 shows that the LPS-I was indeed associated with the main peak of protein. Immunoelectrophoresis of the LPS-I preparation (described in Figures 5 and 6) revealed that the purified material behaved as a β-globulin when compared with LPS-I in whole human serum (Figure 8).* The ultracentrifugal behavior of the LPS-I (described in Figures 5–7) was also assessed in a sucrose density gradient. Companion tubes contained reference protein markers. The LPS-I was present in Fractions 5 to 7 and

* The isolated LPS-I has a β-globulin position (Figure 8), although when serum is fractionated, as in Figure 1, the electrophoretic position is α. These differences can be accounted for by interactions of the inactivator with other serum proteins.

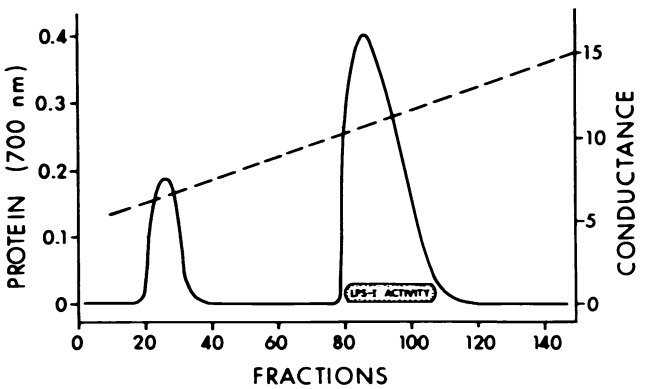

Figure 5—Elution of LPS-I from hydroxyapatite, using a linear salt gradient. (Protein, *solid line*; conductance, *dotted line*)

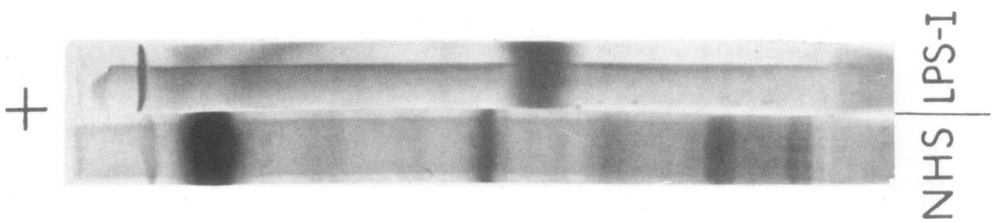

Figure 6—Analysis of LPS-I (obtained as described in Figure 5) in polyacrylamide gel electrophoresis. Reference gel (*NHS*) contains proteins from whole human serum.

was associated with a single peak of protein, indicating ultracentrifugal homogeneity of the LPS-I (Figure 9). The LPS-I was examined immunochemically by double diffusion in gel, using antibody to each of the nine complement components. (These antibodies were the gift of Drs. C. Arroyave and H. J. Müller-Eberhard.) No precipitin bands were found in these gels after 48 hours of diffusion at 5 C.

Effects of Various Compounds on Lipopolysaccharide Inactivator Activity

The purified LPS-I was tested in the presence and absence of a series of chemicals. Only at concentrations of 10^{-1} and 10^{-2} M did ionic calcium interfere with the LPS-I activity. No enhancement of LPS-I was found with citrate (sodium) at 0.05 M or with 0.05 M ethylenediaminetetraacetate. No effect on LPS-I activity was found with 0.05 M phosphate (monosodium and disodium).

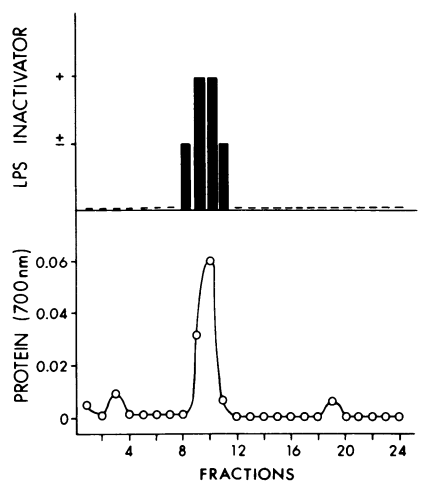

Figure 7—LPS-I eluted from gel described in Figure 6. Protein and LPS-I activity coincide.

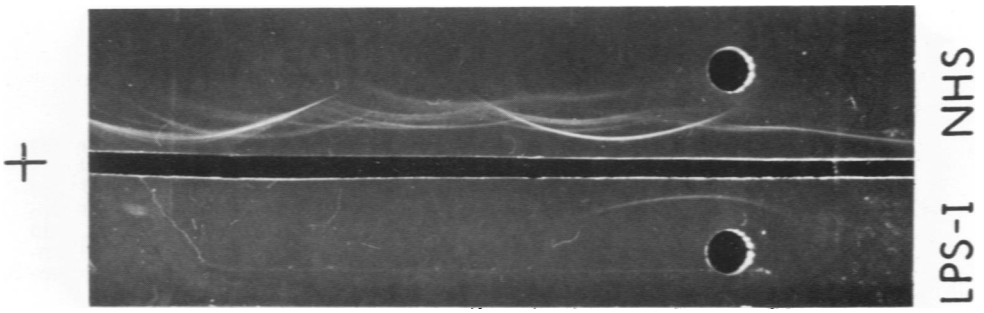

Figure 8—Immunoelectrophoresis of LPS-I, obtained in Figure 5, using anti-whole human serum. Whole serum (*NHS*) is shown as a reference.

Dissociation Between Esterase Activities and Lipopolysaccharide Inactivator

Because of the studies (cited above) which have suggested that an esterase activity is correlated with serum-associated inactivation of LPS,[9,10] two different experiments were carried out to examine the LPS-I preparation. The first approach involved the use of an LPS-I preparation obtained after the third chromatographic step (Sephadex G200). The LPS-I preparation was then fractionated by ultracentrifugation in a sucrose density gradient. Fractions were examined for content of protein, LPS-I (using the *Limulus* assay), and β-naphthylacetate esterase activity. The results are shown in Figure 10. As expected, the LPS-I activity sedimented in the position of the albumin marker. The β-naphthylacetate esterase activity was rather widely disbursed. The bulk of the activity was in Fractions 5 to 8, but a small peak of activity was present in Fraction 10 (Figure 10, middle frame). These results indicate that, if the esterase activity is associated with LPS-I, this represents only a small fraction of the total β-naphthylacetate esterase activity in the preparation.

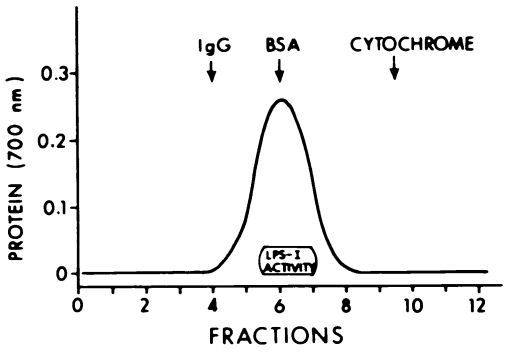

Figure 9—Density gradient analysis of LPS-I obtained in Figure 5.

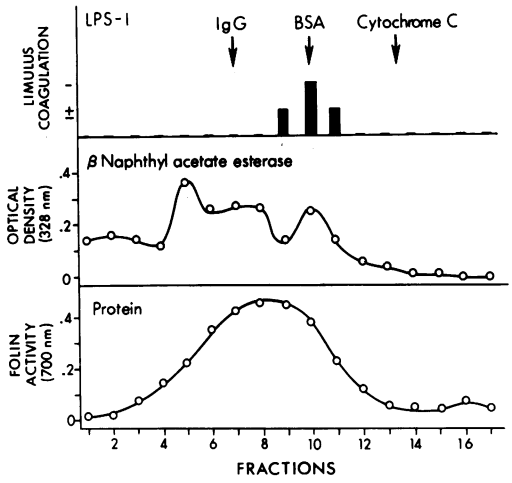

Figure 10—Analysis in sucrose density gradient ultracentrifugation of LPS-I (obtained at the Sephadex G200 step) for distribution of protein and β-naphthyl acetate esterase activity, and endotoxin-inactivating activity. Numbers in x-axis represent fraction numbers.

A second series of experiments were carried out using irreversible inhibitors of serine esterases. Samples of LPS-I (50 μl) were mixed with equivalent volumes of the inhibitors and then LPS was added, the mixture incubated at room temperature for 15 minutes and subsequently assayed (by *Limulus* extract) for inactivation of LPS. The following inhibitors were used: diisopropylfluorophosphate at final concentrations of 10^{-2}, 10^{-3}, and 10^{-4} M; and 2×10^{-4} M final concentrations of the ω-chlorobutyl-, ω-chloropentyl- and ω-chlorohexyl-*p*-nitrophenylethyl phosphates. (All of these serine esterase inhibitors were kindly supplied by Dr. Elmer Becker.) In spite of the treatment of LPS-I with these irreversible serine esterase inhibitors, no interference with the LPS-I activity was seen. The LPS was rendered inactive by LPS-I, according to the *Limulus* assay. These results suggest that the interaction of LPS-I with LPS does not require a serine esterase activity.

Disaggregation of Lipopolysaccharide by Lipopolysaccharide Inhibitor

Five micrograms of LPS with trace amounts of ^{3}H-LPS (see above) were incubated with 5 μg LPS-I (described in Figure 5) for 4 hours at 37 C, and then fractionated in a sucrose density gradient by ultracentrifugation. A control gradient contained untreated LPS, while a third tube contained protein markers. Fractions from each gradient containing LPS were analyzed for radioactivity, and calculations were made to determine the approximate amount (or equivalent) of LPS contained in each peak of radioactivity. This was done by reference to the original, total amount of radioactivity in the starting preparation. As shown in Figure 11, the untreated LPS incubated with saline showed relatively little radioactivity

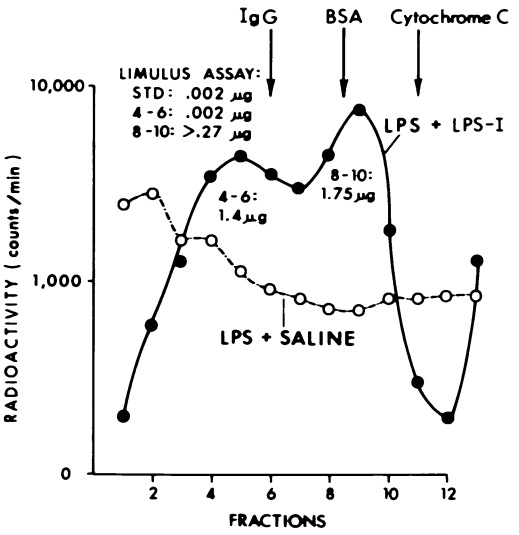

Figure 11—Disaggregation of LPS by treatment with LPS-I. Radioactivity of galactose-labeled LPS is shown. The biologic activity of the LPS peak, expressed according to the estimated amounts of LPS (determined by radioactivity), is given. The two peaks of disaggregated LPS, represented by Fractions 4–6 and 8–10, show approximately 1.5 μg LPS each. Bioassay by the *Limulus* assay reveals Fractions 4 to 6 to be as active as the untreated standard (cutoff point in coagulation of *Limulus* extract is approximately 2 ng), whereas Fractions 8 to 10 show evidence of 100-fold loss of biologic activity.

in the gradient, since most was concentrated in a pellet at the bottom and was not recovered in the gradient fractions (only 8% of the total radioactivity was recovered in the gradient). In contrast, nearly 70% of radioactivity remained in the gradient when the LPS was first treated with LPS-I. The pattern of radioactivity revealed two peaks, one present in Fractions 4 to 6 and the second in Fractions 8 to 10 (Figure 11). It was calculated that the first peak contained the equivalent of 1.4 μg of LPS, while the second peak contained the equivalent of 1.75 μg of LPS. The total amount of LPS originally used was 5 μg. The gradient peaks were each tested by two different assays for evidence of inactivation of the LPS. For reference purposes, *Limulus* testing using the original, unaltered LPS preparation revealed an end point of LPS reactivity at the level of 2 ng. When the pool of Fractions 4 to 6 was tested, full *Limulus* reactivity was found, and the cut-off in activity remained at 2 ng. This indicated no loss of LPS activity in Fractions 4 to 6. In contrast, when the pool of Fractions 8 to 10 was tested in the *Limulus* assay, no reactivity was found when the equivalent of >270 ng of LPS was tested. This would indicate a more than 100-fold loss of LPS activity as a result of interaction of LPS with the LPS-I. Mouse toxicity assays were also carried out, and the following findings were noted: an equivalent of 80 ng of LPS (calculated on the basis of the radio active marker) from Pool 4 to 6 caused 19 of 20 mice to die. In contrast, from Pool 8 to 10 the equivalent of 80 ng of LPS caused only 5 of 20 mice to die.

These data indicate that LPS-I induces a biphasic zone of dis-

aggregation of LPS. One zone is fully reactive in two bioassay systems, while a second, disaggregated product shows evidence of substantially reduced biologic activity.

Time and Temperature Dependence of Lipopolysaccharide Inactivator

Using the same preparations of LPS and LPS-I described above, and in the same ratios, samples were incubated at 37 C for 0, 5, 10, and 15 minutes as well as 1 hour, and also at 4 C for 1 hour. Each sample not only was tested for calculation of amounts needed to coagulate the *Limulus* extract, but also analyzed in a sucrose density gradient and by ultracentrifugation to assess the degree of disaggregation of the LPS. This was determined by measuring the amount of radioactivity of LPS in the density gradient over and above what the untreated LPS control showed (described in Figure 11). At zero time or at 4 C, after 1 hour, only 5 to 6% of the LPS was found in the gradient, and *Limulus* testing revealed a cut-off in reactivity at ≤ 7 ng, similar to the untreated control (Table 3). In contrast, at 5 and 15 minutes, and 1 hour the amount of disaggregation varied from 65 to 77% and a greater than tenfold reduction in reactivity of the LPS was found. These data indicate a time and temperature dependency for the disaggregation and detoxification of LPS by LPS-I.

Reaggregation of Dissociated Lipopolysaccharide

To determine to what extent LPS had been disaggregated by treatment with LPS-I or with the detergent sodium dodecyl sulfate (SDS) and to what extent the disaggregated LPS could be reaggregated in the presence of magnesium salt, 5 μg of LPS with trace amounts of ^{3}H-LPS was first

Table 3—Dependence of Lipopolysaccharide Inactivator on Temperature and Duration of Incubation

Reaction conditions	Percent disaggregation* (^{3}H-LPS)	Amount LPS (ng) needed to coagulate *Limulus* extract†	Relative detoxification of LPS‡
37 C, 0 time	6.2	$\leq$ 7	None
37 C, 5 min	64.7	>70	>10 ×
37 C, 15 min	77.4	>70	>10 ×
37 C, 1 hr	72.8	>70	>10 ×
4 C, 1 hr	5.2	$\leq$ 7	None

* Seven micrograms purified LPS-I was incubated with 5 μg LPS containing trace amounts of ^{3}H-LPS. Disaggregation is defined as the amount of radiolabeled LPS present in the sucrose gradient over and above the amount found in the gradient containing the untreated, and macromolecular LPS.

† Material was collected from density gradients and calculations made, based on radioactivity, to estimate the amount of LPS present in the disaggregated form. The material was then titrated for reactivity in the *Limulus* assay. The minimal amount of untreated LPS to react in the *Limulus* assay was 2 ng.

‡ Calculated according to the *Limulus* assay.

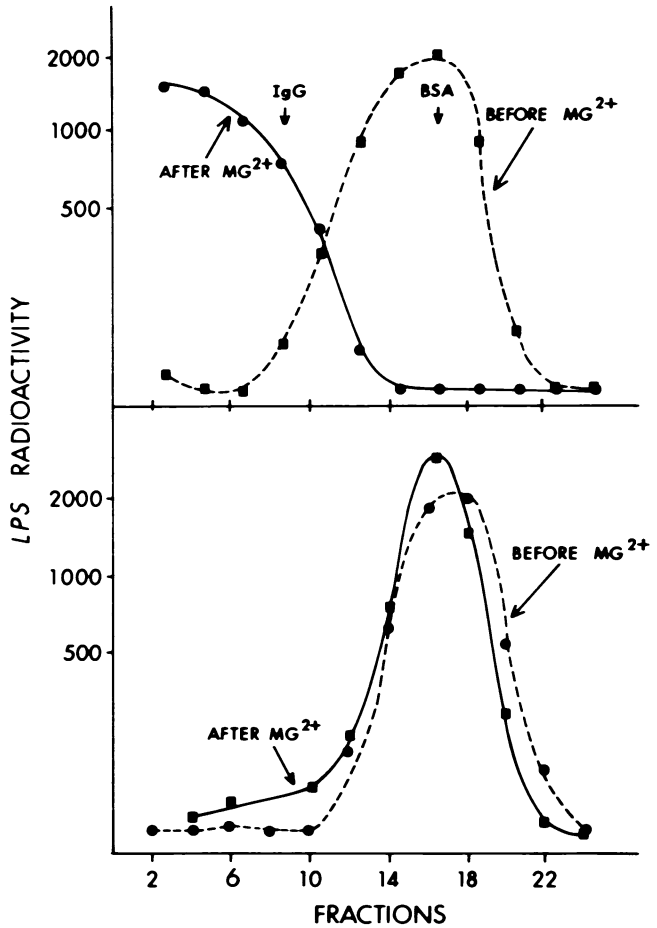

Figure 12—LPS treated with SDS (top) can be reaggregated in presence of Mg^{2+}, whereas the LPS-I-treated LPS (bottom) cannot be reaggregated.

treated with one of these two reagents, as described in the Materials and Methods section. Both reagents lead to disaggregation of approximately 70% of the LPS, and in each case, a peak of radioactivity was found in the upper portion of the gradient near the 4S position (Figure 12, upper and lower frames).

Dialysis of the SDS-treated peak and treatment with $MgCl_2$ lead to an abrupt repositioning of the LPS into the lower portion of the gradient, indicating evidence for reaggregation (Figure 12, upper frame). In contrast to the findings with SDS, treatment of the disaggregated LPS with the LPS-I failed to result in any repositioning of the radioactivity (Figure 12, lower frame). These findings suggest that LPS which has been disaggregated by SDS undergoes reversible changes, whereas LPS that has been disaggregated into LPS-I cannot be reaggregated under similar conditions.

An additional experiment was designed to explore the reversibility of disaggregation of treated LPS. In this study, the LPS-I (approximately 5 μg) was incubated with 1 μg pronase either before or after incubation with 5 μg LPS (containing trace radioactivity). Controls included LPS with pronase of LPS alone. All samples were then examined in sucrose density gradient ultracentrifugation (similar to the experiments described in Figure 12). Pretreatment of LPS-I with pronase resulted in no effect on LPS; the latter sedimented to the bottom of the gradient. However, when the LPS–LPS-I mixture was first incubated and then pronase was added, the LPS sedimented into a position similar to that seen in Fractions 15 to 19 (Figure 12, lower frame). These results indicate that the continuing presence of LPS-I is not needed once LPS is inactivated and disaggregated. These findings add support to the theory that LPS-I induces an irreversible state of disaggregation in LPS.

Discussion

The isolation from human serum of a single protein that has the ability to irreversibly inactivate LPS should permit a detailed investigation into the nature of this interaction and might permit determination of any physical and/or chemical changes that account for loss of toxicity of the LPS. Although the inactivation of LPS is time and dose dependent, as demonstrated in this paper, no direct evidence for an enzymatic alteration of LPS has been obtained. Certainly, the bulk of evidence would suggest that the modification of the LPS occurs in the lipid A portion of the LPS molecule, since this part of the LPS molecule has been implicated in most studies as the toxic moiety.[16] However, a phosphodiesterase activity or enzyme directed at the core or lipid portions of the polysaccharide could be responsible for the inactivation.[8-10] Alternatively, it might also be possible to bring about inactivation of the LPS by extensive disaggregation of the macromolecule, based on a nonenzymatic action. None of the evidence presented in this paper differentiates between an enzymatic and a nonenzymatic mechanism. The recent reports by Braude *et al.* suggest that susceptibility of rabbits to lethal challenge by LPS can be prevented by the presence of antibody to the polysaccharide side chain or, perhaps more interestingly, by antibody directed against polysaccharide present in the core portion of the LPS molecule.[17] For several reasons, it seems unlikely that the LPS-I described in this report would be an antibody. In the first place, one would expect extensive aggregation, not disaggregation, to be caused by any such interaction with LPS. Secondly, LPS-I is not recognizable as an immunoglobulin on the basis of its electrophoretic position and its sedimentation velocity.

Changes in LPS that are associated with its inactivation are not re-

flected by a single pattern of disaggregation. As shown in Figure 11, two zones of disaggregation are produced by treatment with LPS-I, but only one zone (the lighter one) is associated with inactive LPS. When treated with detergent, a single pool of disaggregated LPS is found, and this is easily converted to a polymer by dialysis and treatment with magnesium cations. Using a different LPS and a different detergent (sodium lauryl sulfate), Oroszlan and Mora have also found reversibility of disaggregation and retention or loss of biologic activity, depending on the state of aggregation.[18] However, our studies with pronase, which destroys LPS-I, suggest that removal of LPS-I by proteolytic digestion of the LPS-treated preparation does not restore the biologic activity or the aggregated state of the altered LPS. This is probably the most direct evidence that disaggregation by LPS-I is irreversible, although we cannot rule out the retention of a small pronase-resistant fragment in the LPS treated preparation.

The isolation of LPS-I as a single protein (this report) stands in contrast to the conclusions of Skarnes, who has found two α-globulins which are involved in the inactivation of LPS.[9,10] Our studies suggest that LPS-I is a single protein and that it is neither a lipoprotein nor a serine esterase. The reasons for these discrepancies are not apparent, although it must be emphasized that quite different methodologies and different endotoxins have been employed in the two laboratories, thus making comparisons very difficult.

The results in this paper do not readily explain our earlier observations that implicated a role for C5 and C6 in the inactivation of LPS.[11] LPS-I in its purified form was not reactive by double diffusion in agar gel when tested with antibodies to each of the nine classic components of complement. Furthermore, highly purified preparations of C5 and C6 failed to inactivate LPS. Experiments designed to test the enhancing effect of C5 and C6 on LPS-I failed to provide any evidence of an interaction between these proteins.[19] This suggests that C5 and C6 are not directly involved in the inactivation of LPS, but the results do not rule out the possibility that LPS may undergo a change as a result of its incubation with serum proteins, thereby making it more susceptible to inactivation by LPS-I. The most direct approach to the resolution of this question would probably require specific immunologic depletion of LPS-I from fresh human serum, so that interactions of LPS with serum factors other than LPS-I could be carefully assessed.

It has been suggested that the ability of serum to inactivate LPS increases as a result of exposure of rabbits to LPS.[9,10] Assuming that this increased ability of serum to detoxify is due to changes in LPS-I, it might be possible to alter serum levels of LPS-I in ways and under conditions

that would be therapeutically beneficial to humans. Alternatively, the approach using antibody to the core region of LPS is also appealing. An understanding of the mechanism of inactivation of LPS might lead to a new approach to the problem of combating in man the potentially lethal exposure to bacterial lipopolysaccharide.

References

1. Skarnes RC, Rosen FS: Host-dependent detoxification of bacterial endotoxin. Microbial Toxins: A Comprehensive Treatise, Vol 5. Edited by SJ Ajl, S Kadis, G Weinbaum. New York, Academic Press, Inc., 1971, pp 151–164
2. Rudbach JA, Johnson AG: Changes in serologic reactivity of endotoxin induced by fraction IV-1 (Cohn) of normal human serum. Proc Soc Exp Biol Med 111:651–655, 1962
3. Rudbach JA, Johnson AG: Alteration and restoration of endotoxin activity after complexing with plasma proteins. J Bacteriol 92:892–898, 1966
4. Landy M, Trapani R-J, Shear MJ: Inactivation of endotoxin by a humoral component. IV. Alteration in the immunological properties of typhoid endotoxin. J Exp Med 110:731–750, 1959
5. Skarnes RC, Rosen FS, Shear MJ, Sandy M: Inactivation of endotoxin by a humoral component. II. Interaction of endotoxin with serum and plasma. J Exp Med 108:685–699, 1958
6. Stauch JE, Johnson AG: The alteration of bacterial endotoxins by human and rabbit serum. J Immunol 82:252–263, 1959
7. Rudbach JA, Anacker RL, Haskins WT, Johnson AG, Milner KC, Ribi E: Physical aspects of reversible inactivation of endotoxin. Ann NY Acad Sci 133:629–643, 1966
8. Schultz DR, Becker EL: The alteration of endotoxin by postheparin plasma and its purified fractions. II. Relationship of the endotoxin detoxifying activity of euglobulin from postheparin plasma to lipoprotein lipase. J Immunol 98:482–489, 1967
9. Skarnes RC: The inactivation of endotoxin after interaction with certain proteins of normal serum. Ann NY Acad Sci 133:644–662, 1966
10. Skarnes RC: Host defense against bacterial endotoxemia: Mechanism in normal animals. J Exp Med 132:300–316, 1970
11. Johnson KJ, Ward PA: The requirement for serum complement in the detoxification of bacterial endotoxin. J Immunol 108:611–616, 1972
12. Osborn MJ, Rosen SM, Rothfield L, Horecker BL: Biosynthesis of bacterial lipopolysaccharide. I. Enzymatic incorporation of galactose in a mutant strain of Salmonella. Proc Natl Acad Sci USA 48:1831–1838, 1962
13. Ward PA, Hill JH: Detection of lipopolysaccharide (LPS): An improved method for isolation of the Limulus extract. Proc Soc Exp Biol Med 141:898–900, 1972
14. Chase MW, Williams CA: Chemical analysis. Methods in Immunology and Immunochemistry, Vol 2. Edited by CA Williams, MW Chase. New York, Academic Press, Inc., 1968, pp 307–308
15. Till G, Ward PA: Two distinct chemotactic factor inactivators in human serum. J Immunol 114:843–847, 1975
16. Galanos C, Rietschel ET, Lüderitz O, Westphal O: Interaction of lipopolysaccharides and lipid A with complement. Eur J Biochem 19:143–152, 1971
17. Braude AI, Douglas H, Davis CE: Treatment and prevention of intravascular coagulation with antiserum to endotoxin. J Infect Dis 128 (Suppl):157–164, 1973
18. Oroszlan SI, Mora PT: Dissociation and reconstitution of an endotoxin. Biochem Biophys Res Commun 12:345–349, 1963
19. Johnson KJ, Ward PA: Unpublished observations