Biological Activity, Lipoprotein-binding Behavior, and In Vivo Disposition of Extracted and Native Forms of *Salmonella typhimurium* Lipopolysaccharides

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ABSTRACT Although phenol-extracted gram-negative bacterial lipopolysaccharides (LPS) have been used to study the properties of endotoxins for many years, nothing is known about the behavior of native (unextracted) LPS in vivo. Accordingly, we have compared extracted and native forms of LPS with regard to their biological activity, their ability to bind to plasma high density lipoproteins (HDL), and their fate after intravenous injection into rats. The LPS of *Salmonella typhimurium* G-30 were labeled with [^{3}H]galactose, and whole bacteria, bacterial outer membranes, outer membrane fragments (harvested from the bacterial culture supernatant), and phenol extracts of the bacteria were prepared. After defining the LPS, phospholipid, and protein composition of these preparations, we compared the activity of the LPS in phenol extracts and membrane fragments in two assays. In both the Limulus lysate assay and the rabbit pyrogen test, the LPS in phenol extracts were slightly more potent than the LPS in membrane fragments. We next studied the ability of the LPS in each preparation to bind to rat lipoproteins in vitro, and each preparation was then injected intravenously into rats for measurements of LPS-HDL binding and tissue uptake in vivo. Two patterns of lipoprotein binding were observed. Less than 25% of the LPS in both outer membranes and whole bacteria bound to HDL in vitro. When the outer membranes and whole bacteria were injected into rats, their LPS again bound poorly to HDL and they were rapidly removed from plasma into the liver and spleen. In contrast, >50% of the LPS in both culture supernatant membrane fragments and phenol-water extracts bound to HDL in vitro. When these preparations were injected into rats, ~50% of the LPS in the membrane fragments and phenol-water extracts bound to HDL and remained in the plasma over the 10-min study period. Moreover, the LPS in these preparations accumulated in the ovary and the adrenal gland, two tissues that use HDL-cholesterol for hormone synthesis. Binding to HDL thus greatly influenced the plasma half-life and tissue uptake of both extracted and native LPS.

We conclude that extraction of *S. typhimurium* LPS with phenol does not significantly alter the biological activity or the lipoprotein binding behavior of the LPS and that the in vivo fates of phenol-extracted and membrane fragment LPS are essentially identical. The results thus provide important support for many previous studies that have used phenol-extracted LPS to mimic the activities of native LPS in vivo. However, the only native LPS that resembled the behavior of extracted LPS were the LPS that had been shed from the bacteria in fragments of membrane that had reduced amounts of protein and phospholipid. Removal of LPS from other outer membrane constituents, whether by chemical extraction or by a natural process of surface shedding, thus alters the behavior of the LPS; the most important feature of this alteration appears to be the ability of these LPS to bind readily to HDL.

INTRODUCTION

When purified gram-negative bacterial lipopolysaccharides (LPS)[1] are injected into animals they may

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[1] *Abbreviations used in this paper*: HDL, high density lipoprotein; LPS, lipopolysaccharide; PPBE, proteose peptone beef extract broth; TRI, thermal response index.

induce fever, neutropenia, coagulation abnormalities, complement activation, and hypotension. Similar alterations often occur in patients who have gram-negative bacteremia, and it has been widely assumed that these changes result from the presence of LPS, or of bacterial structures that contain LPS, in blood and other tissues. In fact, little is known about the LPS that are made by bacteria during clinical infections. Existing knowledge of the fate of LPS in vivo, for example, derives from studies of LPS that have been chemically extracted from bacterial cells, extensively purified, radiolabeled, and injected intravenously into animals (1). It is not known if such extracted LPS behave biologically as do the native, unextracted LPS.

The present studies were designed to define possible differences between phenol-extracted and native (unextracted) LPS. Because the binding of LPS to lipoproteins, particularly high density lipoproteins (HDL), is thought to influence the behavior of the LPS in vivo (2–5), most of these experimental comparisons dealt with the binding of extracted and native LPS to HDL. The LPS of *Salmonella typhimurium* G-30 were biosynthetically labeled with [^{3}H]galactose. Whole bacteria, bacterial outer membranes, outer membrane fragments (harvested from the culture supernatant), and phenol extracts of the bacteria were prepared, and the LPS, phospholipid, and protein compositions of the different preparations were compared. The biological activity of two of these preparations (phenol extracts and membrane fragments) was then studied quantitatively in two assay systems. Experiments were next performed in vitro to compare the binding of the different forms of LPS to HDL. Finally, we injected each of the preparations intravenously into rats and measured the binding of the LPS in each preparation to HDL in vivo and the uptake of the LPS by various tissues. The results indicate that the biological properties of phenol-extracted LPS may closely resemble those of native LPS, provided that the native LPS have been released from the bacterial outer membrane.

METHODS

Radioisotopes. The following radioisotopes were obtained from New England Nuclear, Boston, MA: D-[1-^{3}H]galactose (14.2 Ci/mmol), D-[1-^{14}C]galactose (56.5 mCi/mmol), [2-^{3}H]glycerol (9.5 Ci/mmol), L-[^{14}C]arginine (336 mCi/mmol), L-[^{14}C]leucine (339 mCi/mmol), and L-[^{14}C]tyrosine (499 mCi/mmol).

Bacteria, growth conditions. *S. typhimurium* G-30, a mutant that incorporates D-galactose almost exclusively into LPS (6), was provided by Dr. M. J. Osborn (University of Connecticut Health Science Center, Farmington, CT). Cells were grown at 37°C with agitation (150 rpm) in proteose peptone beef extract broth (PPBE) (7) that contained 0.05 mM nonradioactive D-galactose. At the mid-logarithmic phase of growth (OD_{540} = 0.4 [Coleman Jr. II spectrophotometer, Perkin-Elmer Corp., Oak Brook, IL, using 1.2 cm light path]), the bacterial cells were harvested by centrifugation, washed once with 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes) buffer, pH 7.4, and were either used immediately or stored at −70°C.

Labeling of LPS, protein, and phospholipids. LPS were labeled by growing the bacteria in the presence of [^{3}H]- or [^{14}C]galactose added to 0.05 mM nonradioactive D-galactose as described above and in reference 8. Approximately 40–60% of the radioactive galactose was incorporated into whole cells under the conditions used. [2-^{3}H]Glycerol is incorporated almost exclusively into phospholipids by *S. typhimurium* G-30 grown in PPBE (7), and we double-labeled cells by growing the bacteria in 80 ml of PPBE containing 1.5 mCi [^{3}H]glycerol and 50 μCi [^{14}C]galactose. 10% of the [^{3}H]glycerol was incorporated into whole cells. When outer membranes and supernatant membrane fragments were extracted with chloroform-methanol (9), >90% of the [^{3}H]glycerol and <3% of the [^{14}C]galactose were found in the chloroform phase. In other experiments, LPS were labeled with [^{3}H]galactose (100 μCi) and bacterial proteins were labeled by growing the cells in 80 ml of PPBE containing [^{14}C]arginine (50 μCi), [^{14}C]leucine (100 μCi), or [^{14}C]tyrosine (75 μCi); the amount of the radioactivity incorporated into the cells from these three amino acids equaled 52, 2, and 10%, respectively.

Preparation of labeled whole bacteria. Cells that had been grown in the presence of [^{3}H]galactose and 0.05 mM nonradioactive galactose were sedimented by centrifugation, resuspended in an equal volume of fresh PPBE that contained 1 mM nonradioactive galactose, and incubated at room temperature for 20 min to allow complete translocation of [^{3}H]LPS to the outer membrane (10). The cells were sedimented again, resuspended in cold 0.9% NaCl, and dialyzed against 0.9% NaCl at 4°C until they were used (usually 1–2 h). Viable bacteria were counted by performing 10-fold dilutions in sterile 0.9% NaCl and plating onto PPBE agar.

Preparation of outer membranes. Bacterial cells were disrupted by passage through a chilled French press and cell envelopes were isolated by centrifugation of the disrupted cellular material onto a cushion of 60% sucrose (8). Outer and inner membranes were then separated by centrifuging the cell envelopes on linear gradients of 30–55% sucrose. Fractions were collected from the bottom of each tube and assayed for radioactivity as previously described (8). Outer membranes were either kept at 4°C (no longer than 24 h) or stored at −70°C; before use in experiments they were dialyzed against 0.9% NaCl at 4°C to remove the sucrose and Hepes.

Preparation of culture supernatant membrane fragments. Membrane fragments were prepared from the filtered culture supernatant by ammonium sulfate precipitation as previously described (8). After dialysis of the precipitate against 0.9% NaCl at 4°C to remove the ammonium sulfate, the membrane fragments were kept at 4°C or −70°C until they were used.

Preparation of purified LPS. LPS were extracted from labeled cell envelopes or outer membranes (after removal of sucrose by dialysis against distilled water) at 68–70°C by the phenol-water method of Westphal and Jann (11). The subsequent purification steps have been described (8); the LPS were then lyophilized and stored at 4°C. The lyophilized LPS contained 20,000 cpm of ^{3}H/μg. When the properties of the lyophilized LPS were compared with those of the LPS in the other preparations, care was taken to assure that the cells used for all of the preparations were labeled identically, so that the specific activity of the LPS in each preparation would be the same. Before use in experiments,

the purified LPS were suspended in distilled water, sonicated gently in a water bath sonicator to achieve a uniform suspension, and dialyzed against 0.9% NaCl at 4°C. In some experiments, LPS that had been extracted with the phenol-water method were simply dialyzed against distilled water at 4°C overnight, with no further purification steps; the binding of these LPS to HDL was quantitatively identical to that of lyophilized LPS.

Preparation of rat lipoproteins. Female, Sprague-Dawley-derived rats were purchased from Charles River Breeding Laboratories, Inc., Wilmington, MA. They were housed and fed as previously described (5). Lipoproteins were isolated from fresh rat plasma using standard ultracentrifugation methods (5). HDL (ρ = 1.095–1.21 g/ml), HDL_2 (ρ = 1.095–1.125 g/ml), and HDL_3 (ρ = 1.125–1.21 g/ml) were dialyzed against 0.9% NaCl, passed through a 0.45-μm filter, and stored at 4°C. Preparations were used for experiments within 8 d. Purity of the HDL was established as previously described (5); when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12), preparations of HDL_2 and HDL_3 were enriched and depleted, respectively, in apolipoprotein E content. Lipoprotein-free plasma ("1.21 bottom") was prepared by extensive dialysis (0.9% NaCl, 4°C) of the constituents of plasma that sedimented to the bottom half of the tube when centrifuged at a density of 1.21 g/ml for 24 h at 227,000 *g* (13). Fresh serum was pooled from 10–20 rats and stored at 4°C until use.

In vitro assay for LPS-lipoprotein binding. This assay takes advantage of the difference in density between the preparations that contained LPS (ρ > 1.21 g/ml) and plasma lipoproteins (ρ < 1.21 g/ml); LPS that are bound to lipoproteins assume a density of <1.21 g/ml (13). In experiments designed to analyze the binding of LPS to the lipoproteins in whole serum, preparations containing LPS were adjusted so that each contained 45,000 cpm (equivalent to 2.25 μg of lyophilized LPS) in 0.1 ml of 0.9% NaCl. The preparation of whole bacteria contained 9.0×10^8 colony-forming units/ml. Each preparation (0.1 ml) was then added to 1.0 ml of fresh rat serum and the mixtures were agitated (100 oscillations/min) in a 37°C water bath for 60 min. Duplicate tubes were adjusted to densities of 1.006, 1.020, 1.055, 1.095, and 1.21 g/ml with ice-cold KBr, and the tubes were centrifuged for 22 h at 227,000 *g* at 4°C. After centrifugation the tubes were cut approximately two-thirds of the way from the bottom; top and bottom fractions were brought to 10 ml with distilled water and 1.0-ml aliquots were added to 10 ml of Aquassure (New England Nuclear) for counting. The percentage of labeled LPS that floated at each density was then calculated. Aliquots of each preparation were also incubated with serum in an ice bath for 60 min and centrifuged at a density of 1.21 g/ml. The percentage of [^{3}H]LPS in each preparation that floated at ρ = 1.21 after incubation at 0°C (<3% in each instance) was subtracted from the percentage of [^{3}H]LPS that floated at this density when the incubation was carried out at 37°C.

In separate experiments, we also studied the ability of the LPS in the different preparations to bind to isolated rat HDL. Purified HDL (1.4 mg HDL-protein in 1.0 ml), lipoprotein-free plasma (24.0 mg protein in 1.0 ml), and a preparation containing labeled LPS (35,000 cpm in 0.1 ml of 0.9% NaCl, equivalent to 1.75 μg of lyophilized LPS) were agitated in polyallomer ultracentrifuge tubes in a 37°C water bath for 60 min. The tubes were then quickly chilled and the density was adjusted to 1.21 g/ml with a cold, concentrated solution of KBr. The tubes were centrifuged and cut as described above. The [^{3}H]LPS that appeared in the top fraction were assumed to be bound to HDL.

Immunoprecipitation methods. IgG antibodies to LPS were prepared from the serum of rabbits that had been immunized with heat-killed cells of *S. typhimurium* (14). The IgG were adsorbed to Cowan strain staphylococci (prepared by the method of Kessler [15] and kindly provided by Dr. L. Eidels), and then used for LPS immunoprecipitation. Measured volumes of coated staphylococci (100 μl of a 10% suspension) were incubated overnight at 4°C with samples of plasma that contained labeled LPS (1 ml). The staphylococci were sedimented by centrifugation, washed three times with phosphate-buffered saline (0.01 M potassium phosphate, 0.9% NaCl, 0.02% sodium azide), and suspended in 100 μl of 1.0% sodium dodecyl sulfate. Aliquots (20 μl) were added to 10 ml Aquasol (New England Nuclear) for counting. Similar results were obtained if the supernatants of the immunoprecipitation mixtures were counted, and the amount of radioactivity in the precipitate was determined by difference. To control for nonspecific binding to the staphylococci, samples were also adsorbed with staphylococci that had been coated with IgG from a rabbit antiserum to *Escherichia coli* 055 (14). In some experiments, specificity was further demonstrated by swamping out the adsorption of the [^{3}H]LPS by adding a large mass of nonradioactive LPS purified from *S. typhimurium* G-30. Each adsorption was performed in duplicate.

In vivo experiments. Preparations that contained LPS were injected via a tail vein catheter into fully conscious rats. Approximately 2 million cpm of ^{3}H in each preparation (500,000 cpm of the [^{3}H]LPS in culture supernatant membrane fragments) were injected into each animal. After intervals of 40 s and 10 min, rats were anesthetized with diethyl ether and blood was withdrawn from the abdominal aorta into syringes that contained EDTA (1 mg/ml). The blood was promptly chilled and centrifuged (600 *g*, 15 min, 4°C) to separate the cellular components from plasma. Aliquots of plasma (100 μl) were added to 10 ml Aquassure for counting. The amount of ^{3}H injected into each animal was divided by the plasma volume of the rat (calculated as 4.04 ml/100 g body weight [16]) to obtain the theoretical plasma concentration (cpm/ml) at time zero. The concentration of radioactivity that remained in the plasma at 40 and 10 min was then expressed as the percentage of the time zero concentration, i.e., the inoculum. Plasma (1.0 ml) was also added to 0.9% NaCl (1.0 ml) and the mixture was adjusted to a density of 1.21 g/ml with a cold solution of KBr. The percentage of plasma [^{3}H]LPS that floated at ρ = 1.21 g/ml was multiplied by the percentage of the time zero concentration that remained in plasma; this product gave the percentage of the inoculum that appeared in the ρ < 1.21-g/ml fraction (i.e., which was lipoprotein-bound) at each time point. Aliquots of plasma were also centrifuged at specific densities to locate the LPS within the lipoprotein-containing fraction. To determine the distribution of labeled LPS in the tissues of rats, we removed and counted six organs: adrenal gland, ovary, liver, spleen, kidney, heart. Digestion of the tissues before counting was performed as previously described (5). To compare the uptake of the different LPS preparations by specific tissues we expressed the results as the counts per minute of ^{3}H per gram of tissue; the data are normalized to a constant inoculum of 2.0×10^6 cpm.

Biological activity assays. The Limulus lysate test was performed as described previously (17). Samples were diluted in pyrogen-free water (Travenol Laboratories Inc., Deerfield, IL). Purified *S. typhiurium* LPS (List Laboratories, Campbell, CA) were used as the standard. This assay measures the activation of the Limulus clotting system by lipid A. To test the ability of the LPS preparations to cause

fever, 0.1-ml samples were injected via the marginal ear vein into six male New Zealand White rabbits; rectal temperatures were monitored at 10-min intervals, and the integrated thermal response index (TRI) was calculated by a standard technique (18). Results are expressed as the TRI (the product of the temperature above base line [°C] and time[degree-hours]).

Miscellaneous techniques. Phospholipids were extracted by the method of Bligh and Dyer, as modified by Ames (9). Protein was measured by the method of Lowry (19).

Radioactivity detection. Samples containing ^{3}H or ^{14}C were counted in a liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL). Results in samples that contained both isotopes were corrected for background and channel spillover. Quench correction was performed by an external standardization method.

RESULTS

Comparison of outer membranes, culture supernatant membrane fragments, and purified LPS. The protein, phospholipid, and LPS compositions of *S. typhimurium* outer membranes have been well characterized (20). These bacteria are also known to shed membrane fragments during growth in vitro (21), but the composition of these fragments has received little investigation. Our working hypothesis was that the behavior of the LPS in the different preparations would be influenced by the association of the LPS with other bacterial membrane components. Accordingly, we began by comparing the composition of outer membranes, culture supernatant membrane fragments, and phenol-water extracts from the same broth cultures of *S. typhimurium*.

LPS and phospholipid composition. Cell envelopes and supernatant membrane fragments were prepared from cells that had been labeled by growing them in the presence of [^{3}H]glycerol and [^{14}C]galactose. These preparations were then centrifuged on 30–55% sucrose gradients. As shown in Fig. 1A, outer membranes reached an apparent buoyant density of 1.24 g/ml and were enriched in [^{14}C]galactose. In contrast, the inner membranes had a density of 1.17 g/ml and showed little labeling with [^{14}C]galactose. These results are similar to those previously reported for sucrose gradient ultracentrifugation of inner and outer membranes from *S. typhimurium* G-30 (7). The results from a companion sucrose gradient that was loaded with membrane fragments obtained from the supernatant fraction of the culture medium are shown in Fig. 1B. The [^{14}C]galactose appeared in a band with an apparent buoyant density of 1.18 g/ml; this material contained less [^{3}H]glycerol, relative to [^{14}C]galactose, than did the outer membranes shown in Fig. 1A.

LPS and protein composition. In another experiment, cells were labeled with [^{14}C]leucine and [^{3}H]galactose, and the cell envelopes and supernatant fragments were centrifuged on sucrose gradients. The

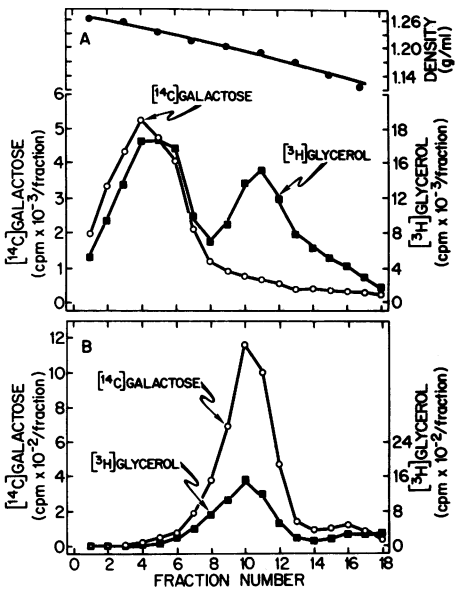

FIGURE 1 Sucrose gradient analysis of cell envelopes and membrane fragments from the culture supernatant. The density of sucrose in the fractions in both gradients is displayed at the top of A. A. Separation of outer and inner membranes from cell envelopes of *S. typhimurium* G-30 labeled with [^{3}H]glycerol and [^{14}C]galactose as described in Methods. Outer membranes (left peak) had an apparent bouyant density of 1.24 g/ml and contained ~85% of the [^{14}C]galactose (LPS) in the cell envelope. B. Profile of radioactivity found for the membrane fragments harvested from the supernatant fluid of the same culture, centrifuged on a companion gradient. The [^{14}C]galactose peak occurred at a density of 1.18 g/ml and was accompanied by a peak of [^{3}H]glycerol. When compared with the outer membranes in A, the supernatant fragments in B appear depleted in glycerol-labeled phospholipid, relative to their LPS content.

results shown in Fig. 2A indicate that outer membranes (ρ = 1.25 g/ml) contained almost all of the [^{3}H]galactose. [^{14}C]Leucine, in contrast, was incorporated into the proteins of both inner and outer membranes. As shown in Fig. 2B, supernatant fragments from the same culture banded with an apparent density of 1.19 g/ml and appeared to contain little [^{14}C]leucine-labeled protein (relative to the outer membranes in Fig. 2A). The [^{14}C]leucine at the top of the gradient in Fig. 2B probably represents proteins that were released into the culture media by growing cells. Similar results were found with cells that had been labeled with [^{3}H]galactose and [^{14}C]arginine (data not shown).

These experiments suggested that the membrane fragments harvested from the culture supernatant contained less phospholipid and protein, relative to their LPS content, than the outer membranes isolated from bacteria in the same cultures. Direct comparisons of the amounts of both [^{3}H]glycerol-labeled phospholipid

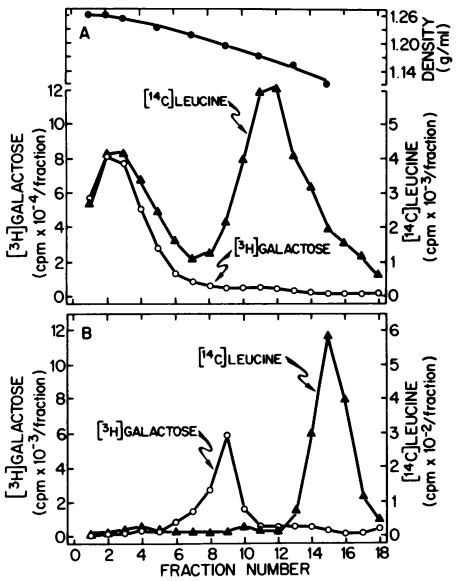

FIGURE 2 Sucrose gradient analysis of cell envelopes and membrane fragments from the culture supernatant. A. Separation of outer membranes (left peak) and inner membranes (right peak) from cell envelopes that had been labeled with [^{3}H]galactose and [^{14}C]leucine. B. Profile of radioactivity found with culture supernatant membrane fragments from the same culture, centrifuged on a companion gradient. When compared with the outer membranes in A, the supernatant fragments in B appear to have a lower apparent bouyant density and, relative to their LPS content, to be markedly depleted in protein.

and [^{14}C]-LPS in outer membranes and membrane fragments indicated that the LPS in the membrane fragments were associated with only 54.6±11.0 (SD of three determinations) % of the phospholipid that accompanied the LPS in outer membranes. Similarly, comparison of the amounts of [^{14}C]-amino acids (leucine, arginine, or tyrosine) and [^{3}H]-LPS in outer membranes and membrane fragments showed that the LPS in the membrane fragments were accompanied by only 10.0±1.1 (SD of three experiments using different amino acid labels) % of the labeled protein that was present with LPS in the outer membranes. As expected, LPS that had been extracted from outer membranes with hot phenol-water contained even less phospholipid and protein; relative to their LPS content, phenol-water extracts contained <1.0% of the [^{3}H]glycerol and [^{14}C]-amino acids that were present in outer membranes (averages of two determinations). When centrifuged on sucrose gradients as described above, the extracted LPS assumed an apparent density of 1.23–1.25 g/ml.

That there is a disproportionate loss of protein (90%) compared to phospholipid (50%) in the membrane fragments is consistent with the observation that the membrane fragments have a lower apparent buoyant density than outer membranes on sucrose gradients (Figs. 1 and 2); similar densities have been reported for reconstituted complexes of LPS and phospholipids (22). LPS that had been extracted from the outer membrane with hot phenol-water, in contrast, contained very low amounts of both phospholipid and protein and thus had the higher apparent buoyant density characteristic of purified LPS.

Biological activity. Two assays were used to compare the biological activity of the LPS in membrane fragments and phenol extracts. For these studies, membrane fragments and phenol-extracted LPS were prepared from the same culture of labeled cells. Each preparation was reisolated from a sucrose gradient before the experiment. The samples of phenol-extracted LPS and membrane fragments were adjusted to equivalent concentrations of LPS according to their ^{3}H content. When assayed in the Limulus lysate test, preparations of phenol-extracted and membrane fragment LPS had 28±10 (SD of three determinations) and 16±8 ng LPS, respectively, per 1,000 cpm of [^{3}H]galactose. Adsorption of the two preparations with staphylococci that had been coated with antibodies to *S. typhimurium* removed over 95% of their ^{3}H and reduced their activity in the Limulus assay by 10-fold or greater; adsorption with staphylococci that were coated with *E. coli* antibodies did not decrease either the ^{3}H content of the preparations or their Limulus activity. LPS-HDL complexes (derived from either phenol-extracted or native LPS) had reduced activity in the Limulus test, but the ability of unbound HDL to inhibit the activity of LPS in the assay confounded the interpretation of this result.

The two preparations were also diluted in pyrogen-free saline and injected into groups of six rabbits as described in Methods (Fig. 3). The TRI for rabbits that received 1,000 cpm of [^{3}H]LPS in both phenol extracts and membrane fragments was 2.66±1.29 (SD) and 1.51±0.62, respectively. Other groups of rabbits were injected with 3,000 cpm of one of the preparations; rabbits that received phenol-extracted LPS had a mean TRI of 3.09±1.19, whereas those that were given membrane fragments responded with a TRI of 3.0±1.57. Adsorption of the preparations with staphylococci that were coated with antibodies to *S. typhimurium* removed their pyrogenic activity (Fig. 3). Binding of phenol-extracted and membrane fragment LPS to HDL also abolished the pyrogenicity of the LPS (Fig. 3), in agreement with a previous report indicating that complexes of extracted LPS and HDL had reduced pyrogenicity (3).

Binding of LPS to rat plasma lipoproteins in vitro. The LPS in labeled phenol-water extracts, culture supernatant membrane fragments, outer membranes,

and whole bacteria were compared for their ability to bind to rat lipoproteins in vitro. Aliquots were incubated with fresh rat serum for 60 min at 37°C and then centrifuged at different densities to determine the percentage of labeled LPS that bound to different lipoprotein classes. As shown in Fig. 4, the LPS floated predominantly in the density range (ρ = 1.095–1.21 g/ml) that contains HDL (5). In contrast, insignificant amounts of labeled LPS were found in the fractions that contained low density lipoproteins (ρ = 1.020–1.055 g/ml) and very low density lipoproteins (ρ < 1.006 g/ml). The density range ρ = 1.055–1.095 g/ml contains both LDL and HDL in the rat (5), and the labeled LPS that floated in this range were presumably bound to HDL. Fig. 4 also demonstrates important differences in the ability of LPS in the different preparations to bind to lipoproteins: 50% or more of the LPS in phenol-water extracts (A) and culture supernatant membrane fragments (B) floated in the HDL fraction, whereas <10% of the LPS in outer membranes (C) and whole bacteria (D) appeared in this fraction. Because the number of viable bacteria decreased by ~20-fold during the incubation period, killing of the bacteria was obviously not sufficient to allow large amounts of their LPS to bind to HDL. It should be emphasized that each of the preparations

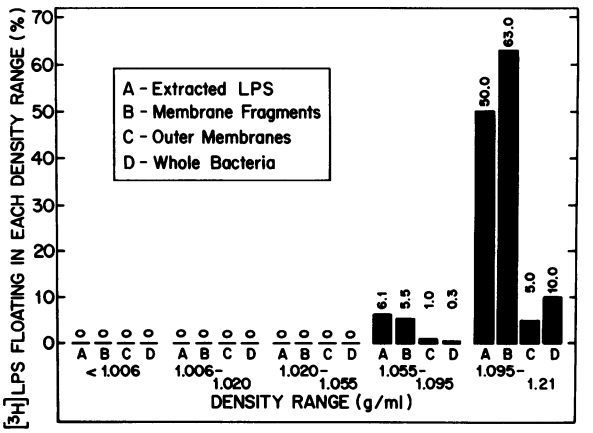

FIGURE 4 Binding of [^{3}H]LPS to rat lipoproteins in vitro. Samples of each LPS preparation (0.1 ml) were added to 1.0 ml of rat serum and incubated at 37°C for 60 min. The serum was then separated into different density classes as described in Methods. The [^{3}H]LPS floated in the density range that contained HDL (1.095–1.21 g/ml); no [^{3}H]LPS were found in the density ranges that contained only very low density lipoproteins (ρ < 1.006 g/ml) or low density lipoproteins (1.020–1.055 g/ml). The range 1.055–1.095 g/ml contains both HDL and LDL. Binding of LPS to HDL was greatest when phenol-water extracts (A) or culture supernatant membrane fragments (B) were used.

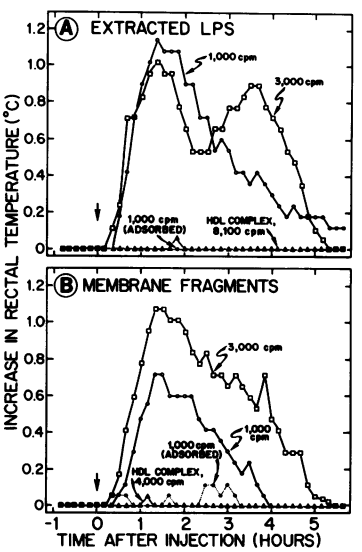

FIGURE 3 Fever responses in rabbits. A. Temperature responses of groups of rabbits that were injected with phenol-extracted LPS. B. Responses of rabbits that were injected with membrane fragment LPS. Each point represents the mean of five or six rabbits; SE did not exceed 0.08°C. Adsorption of the LPS preparations with antibodies to *S. typhimurium* abolished the febrile responses, as did complexing the LPS with HDL. When assayed in the Limulus lyate test, 1,000 cpm of phenol-extracted and membrane fragment LPS had 28 and 16 ng of LPS activity, respectively.

of LPS, when centrifuged under identical conditions after incubation with serum in an ice bath, had a density >1.21 g/ml and so was found in the bottom of the centrifuge tubes. The difference in apparent density of the culture supernatant membrane fragments, when determined on sucrose gradients and in this assay, is probably attributable to differences in the sedimentation characteristics of the two gradient media.

In another experiment we incubated labeled phenol-water extracts, culture supernatant membrane fragments, outer membranes, and whole bacteria with isolated rat HDL and 1.21 bottom. As shown in Fig. 5, very little LPS floated at a density of 1.21 g/ml after incubation with either HDL or 1.21 bottom. When both HDL and 1.21 bottom were present, in contrast, ~50% of the LPS in culture supernatant membrane fragments and 90% of the LPS in phenol-water extracts floated at this density, indicating binding to HDL (13). The ability of 1.21 bottom (nonlipoprotein constituents of plasma) to promote LPS-HDL binding has previously been noted (3, 13); it is thought that some component(s) of 1.21 bottom disaggregate LPS to a size that can then interact with HDL. The results of this experiment indicate that 1.21 bottom is also required for the LPS in culture supernatant membrane fragments to bind to HDL, suggesting that disruption of the membrane fragments may precede LPS-HDL binding. In agreement with the experimental data pre-

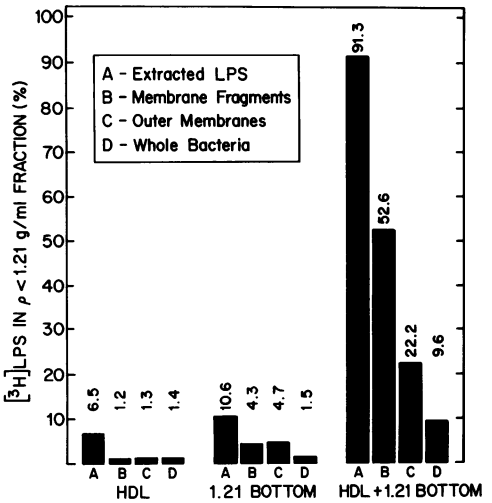

FIGURE 5 Binding of [^{3}H]LPS to rat HDL in vitro. Samples of each LPS preparation were added to mixtures that contained isolated HDL, 1.21 bottom, or both HDL and 1.21 bottom. After incubation at 37°C for 60 min the mixtures were brought to a density of 1.21 g/ml with cold KBr and centrifuged as described in Methods. Binding of [^{3}H]LPS to HDL was greatest when 1.21 bottom was present in the reaction mixture, and when phenol-water extracts (A) or culture supernatant membrane fragments (B) were used. The [^{3}H]LPS in outer membranes (C) and whole bacteria (D) underwent significantly less binding to HDL under the conditions of the experiment.

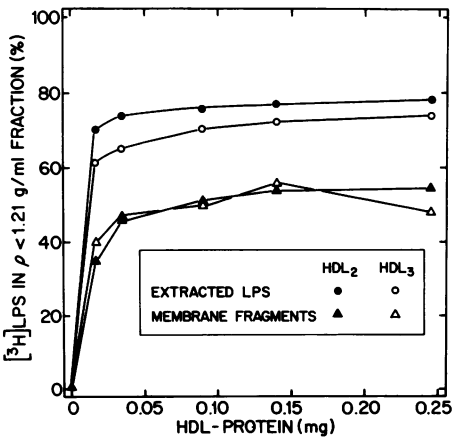

FIGURE 6 Binding of [^{3}H]LPS to subpopulations of rat HDL. The [^{3}H]LPS in phenol extracts (56,000 cpm) and membrane fragments (57,000 cpm) were incubated in vitro with 30 mg of 1.21 bottom-protein and increasing amounts of HDL_2 or HDL_3. After incubation for 60 min at 37°C, LPS-HDL complexes and unbound LPS were separated by centrifugation as described in Methods. The LPS in phenol extracts bound equally well to HDL_2 and HDL_3; similarly, the [^{3}H]LPS in membrane fragments bound equally well to the two HDL fractions.

sented in Fig. 4, only small amounts of the LPS in whole bacteria (D) and outer membranes (C) bound to HDL in these experiments using reconstituted mixtures of HDL and 1.21 bottom.

In a final in vitro experiment, we compared the binding of the LPS in both phenol extracts and membrane fragments to purified rat HDL_2 and HDL_3. The results shown in Fig. 6 indicate that each form of LPS had essentially equivalent binding to the two HDL subfractions. HDL_2 and HDL_3 differ principally in their content of apolipoprotein E; the results thus suggest that this apolipoprotein does not greatly influence the binding of LPS to HDL.

Binding of LPS to rat HDL in vivo. To test the ability of LPS in phenol-water extracts, membrane fragments, outer membranes, and whole bacteria to bind to rat HDL in vivo, we injected these preparations intravenously into groups of four to seven rats and withdrew blood for analysis at both 40 s and 10 min after injection. As a control, LPS-HDL complexes that had been prepared in vitro (5) were also injected into a group of rats. To show that the label that remained in plasma was in LPS, plasma samples were adsorbed with staphylococci that had been coated with IgG antibodies to *S. typhimurium* LPS; regardless of the preparation of [^{3}H]LPS injected, >85% of the counts per minute in the plasma were precipitable with this technique (Table I).

The results of this experiment are shown in Fig. 7. As we have previously found (5), [^{3}H]LPS-HDL com-

TABLE I
Immunoprecipitation of [^{3}H]LPS from Rat Plasma

[^{3}H]LPS preparation	^{3}H precipitated		
	Staph-homologous IgG		
	No LPS added	With LPS added	Staph-heterologous IgG
	%		
LPS-HDL complexes	96.1	6.6	3.9
Phenol-water extract	97.4	NT	6.8
Membrane fragments	85.4	13.2	NT
Outer membranes	99.0	8.1	13.3
Whole bacteria	88.0	NT	11.2

NT, not tested.

Samples of rat plasma, obtained 10 min after intravenous injection of one of the preparations listed, were tested as described in Methods. Aliquots of plasma from two rats in each group were diluted 1:10 in phosphate-buffered saline; the diluted plasma contained ~1,000–2,000 cpm ^{3}H/ml. Immunoprecipitation was performed using staphylococci coated with IgG antibodies to homologous LPS (*S. typhimurium*) or to heterologous LPS (*E. coli* 055). Immunoprecipitations with homologous LPS were also carried out in the presence of a large amount (50 μg) of unlabeled phenol-extracted LPS from *S. typhimurium*. Results are the average of four determinations.

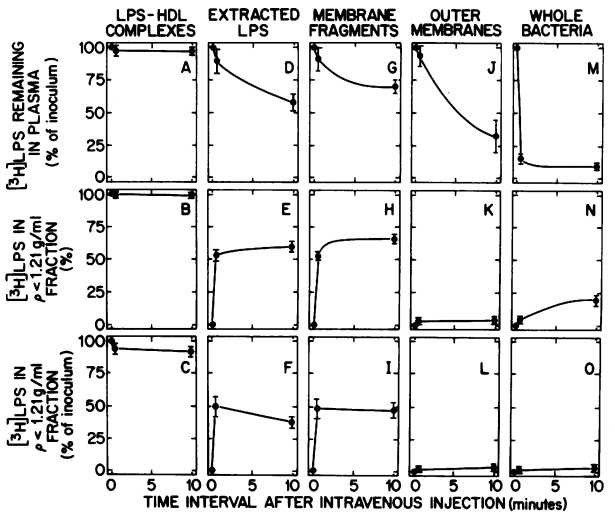

FIGURE 7 Binding of [^{3}H]LPS to rat lipoproteins in vivo. Samples of each preparation were injected into conscious rats and plasma was obtained 40 s and 10 min later. The figure shows three panels for each preparation, and the data points represent means±1 SD for four to seven animals. The top row of panels describes the time course of disappearance of each preparation of [^{3}H]LPS from the plasma and is expressed as the percentage of the injected material that remained in the plasma compartment. The middle row of panels shows the percentage of the plasma [^{3}H]LPS that floated at a density of 1.21 g/ml (i.e., was bound to lipoproteins) at each time point. The bottom row of panels gives the percentage of the injected material that was bound to plasma lipoproteins at each time point.

TABLE II
Density Distribution of Lipoprotein-bound [^{3}H]LPS in Rat Plasma

	Plasma [^{3}H]LPS in each density range (% total fraction with ρ < 1.21 g/ml)	
	Preparation injected	
Density range	Extracted LPS	Membrane fragments
<1.020	1.3	2.2
1.020–1.055	1.0	0.8
1.055–1.095	8.5	8.6
1.095–1.125	20.7	21.9
1.125–1.21	68.2	66.1
<1.21	100.0	100.0

Extracted LPS (500,000 cpm) or membrane fragments (300,000 cpm) were injected intravenously into rats. 10 min after injection the rats were killed and the plasma from three rats in each group was pooled. Duplicate aliquots (0.5 ml) were adjusted to the indicated densities and centrifuged as described in Methods. 59% of the extracted LPS and 45% of the membrane fragment LPS floated in the ρ < 1.21 g/ml fraction. For both phenol extracts and membrane fragments, the [^{3}H]LPS that was bound to lipoproteins floated primarily in the HDL fraction (1.095–1.21 g/ml). The distribution of the [^{3}H]LPS in the two preparations between HDL_2 (ρ = 1.095–1.125 g/ml) and HDL_3 (ρ = 1.125–1.21 g/ml) was also quite similar.

plexes that had been formed in vitro became distributed in the plasma volume and did not disappear rapidly from plasma over the time interval from 40 s to 10 min (A). The complexes were not dissociated in vivo, as the [^{3}H]LPS continued to float at a density of 1.21 g/ml (B); the percentage of the injected [^{3}H]LPS that remained in the HDL fraction at each time point (C) thus closely paralleled the amount that remained in plasma (A). The LPS in phenol-water extracts bound rapidly to HDL (E) but the total amount of LPS remaining in plasma decreased by 40 s (D), reflecting primarily the rapid tissue uptake of the LPS that did not become associated with HDL. The subsequent fall in both total LPS (D) and LPS-HDL (F) in plasma was much more gradual. Similarly, a large fraction of the LPS in culture supernatant membrane fragments bound rapidly to HDL (H) and resembled LPS-HDL complexes and extracted LPS in their subsequent slow rate of disappearance from plasma (I). In contrast, the [^{3}H]LPS in outer membranes (J–L) and whole bacteria (M–O) disappeared more rapidly from plasma and transferred considerably less LPS to lipoproteins during the 10-min period; only 1–3% of the injected LPS in these preparations was found in the lipoprotein fraction at the end of the study period (L and O).

To identify more precisely the location of the LPS within the lipoprotein fraction, aliquots of plasma from the 10-min time point were pooled, adjusted to different densities, and centrifuged as described in Methods. As is shown in Table II, almost all of the LPS that floated were found in the ρ = 1.095–1.21-g/ml fraction. Similar results were found for injected phenol extracts and membrane fragments.

To compare the uptake of [^{3}H]LPS in the five preparations by various organs, we calculated the counts per minute that appeared in 1 g of each tissue. Significant differences in the tissue binding (or uptake) of the different preparations were found in the adrenal gland, ovary, and spleen. The uptake of [^{3}H]LPS by the adrenal gland and ovary was greatest when large amounts of LPS were bound to HDL in plasma (LPS-HDL complexes [Fig. 8X], phenol-water extracts [A], and culture supernatant membrane fragments [B]) (Fig. 8, a and b). In contrast, uptake of [^{3}H]LPS by the spleen (Fig. 8, d) was greatest when the [^{3}H]LPS were injected in outer membranes (C) or whole bacteria (D), which showed little binding of LPS to HDL in vivo. There were no large differences in the uptake of [^{3}H]LPS in the different preparations by the kidney or

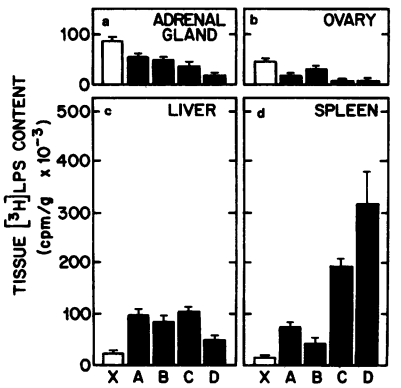

FIGURE 8 Uptake of [^{3}H]LPS by rat tissues. The content of [^{3}H]LPS in the various tissues was determined 10 min after injection of the different preparations as described in Methods. Values were normalized to a constant dose of administered material containing 2.0×10^6 cpm (the equivalent of 100 μg of lyophilized purified LPS). Each bar represents the mean±1 SEM for the results obtained in four to seven animals. [^{3}H]LPS-HDL complexes that had been re-isolated following in vitro binding (X) were compared with phenol-water extracts (A), membrane fragments (B), outer membranes (C), and whole bacteria (D). Uptake by the adrenal gland (a) and ovary (b) was greatest for the preparations in which the LPS were bound to HDL in plasma (LPS-HDL complexes, extracted LPS, and membrane fragments), whereas uptake by the spleen (d) was greatest for outer membranes and whole bacteria, the preparations whose LPS did not bind to HDL in vivo.

heart (there were <12,000 cpm/g in either tissue, regardless of the preparation injected).

DISCUSSION

LPS are amphipathic molecules that, when extracted from bacteria, form large aggregates in aqueous suspension (23). The size of these aggregates appears to determine certain biological activities of the LPS, particularly their ability to activate complement (24). Other evidence also suggests that the size of LPS aggregates influences both the binding of LPS to lipoproteins and the uptake of LPS by various tissues of the body. Ulevitch and his colleagues recently showed that LPS that were injected intravenously into rabbits could bind to plasma HDL, and they presented evidence that disaggregation of the LPS by nonlipoprotein constituents of plasma enhanced LPS-HDL binding (3). Our in vitro studies supported this hypothesis by showing that calcium inhibits the binding of *S. typhimurium* LPS to HDL by interfering with the modification (disaggregation) of LPS by lipoprotein-free plasma; we also found that deoxycholate, which disaggregates LPS, promoted LPS-HDL binding in the absence of other plasma constituents (13). The size of LPS aggregates, by influencing the binding of LPS to HDL, also has a role in determining the sites of uptake of LPS from the blood into tissues. On the basis of their studies in the rabbit, Mathison and Ulevitch concluded that LPS aggregates are rapidly cleared from the plasma by uptake into tissue phagocytes, principally hepatic Kupffer cells, whereas LPS-HDL complexes are removed from plasma much more slowly by uptake into tissues such as the adrenal gland, liver, and spleen (1, 3). Subsequent experiments in our laboratory showed that LPS-HDL complexes are taken up by the same tissues that bind or take up HDL, apparently by the same process that accounts for HDL metabolism (5). It seems reasonable to conclude from the available evidence that the physical state of injected LPS may be an important determinant of the rate of LPS-HDL binding in vivo and, therefore, of the sites of tissue uptake of the LPS.

Unfortunately, it has been uncertain that chemically extracted LPS actually mimic the properties of native LPS in vivo. It seems improbable, for example, that the bacteria release LPS from their outer membranes as large aggregates that contain only LPS. It is more likely that LPS are shed from the bacteria in vivo as constituents of bacterial membrane fragments; the presence of other membrane components complexed to LPS might either hinder or enhance the interactions of LPS with various host molecules. Alternatively, disruption of bacterial membranes by plasma or tissues might release LPS from these other membrane components, making "free" LPS available. It is also possible that extraction of bacterial membranes with phenol-water or phenol-chloroform-petroleum ether (25) alters the physical state of the LPS (by removing the lipid moieties from the outer membrane, for example) so that extracted LPS have properties that the native LPS do not. The present studies were designed to examine these possibilities by comparing the behavior of native (unextracted) and purified forms of LPS in vitro and in vivo.

For these experiments we used *S. typhimurium* G-30, a well-characterized mutant that incorporates exogenous galactose almost exclusively into LPS. By using a mutant that could be intrinsically labeled, we were able to prepare LPS in different forms that had essentially identical specific activities; the amount of LPS in the preparations could thus be equalized for the experiments by adjusting the radioactivity. The first set of studies defined important chemical differences between the bacterial outer membranes, membrane fragments, and phenol extracts. In general agreement with a previous study of the membrane fragments released by chloramphenicol-treated cells (21), we found that the *S. typhimurium* membrane fragments differed significantly from the outer membranes isolated from cells in the same cultures, as the

LPS in the fragments were accompanied by less protein (10%) and phospholipid (50%). As expected, the LPS in phenol extracts were accompanied by essentially no outer membrane phospholipid or protein.

Examination of the biological activity of the LPS in phenol extracts and membrane fragments showed only small quantitative differences between the two preparations. These experiments used two assays, the rabbit pyrogen test and the Limulus lysate assay, which have previously been found to correlate well with each other (26, 27) and with the results of other tests of endotoxic activity, including mitogenicity (27), activation of mononuclear cell tissue factor (28), and chick embryo lethality (29). In each assay, the membrane fragments were slightly less active than the phenol extracts. Similar comparisons of the endotoxic activities of phenol extracts and membrane fragments (30, 31) or cell walls (29) of other gram-negative bacilli have given a variety of results. Our studies differed from the previously reported experiments in that (*a*) we compared the preparations based on their content of radiolabeled LPS, instead of measurements of total weight, and (*b*) we used immunoadsorption to rule out exogenous contamination. Our data do not exclude the possibility that the activity of the preparations is modified by the presence of non-LPS membrane component(s), however. It seems possible, for example, that the activity of the LPS in membrane fragments may be reduced because their lipid A is inserted into a phospholipid-containing micelle.

The importance of the chemical differences in the various preparations became apparent when the lipoprotein-binding behavior of the LPS in each preparation was examined. The LPS in those preparations that had reduced amounts of other outer membrane constituents (membrane fragments, phenol-extracts) underwent greater binding to HDL than the LPS in outer membranes or whole bacteria. It thus seems likely that removing the LPS from some outer membrane component(s) facilitates binding of the LPS to lipoproteins. The LPS in the membrane fragments and phenol extracts were unable to bind directly to HDL in the absence of 1.21 bottom (Fig. 5), so it appears that the important difference between these preparations and the outer membranes is the greater susceptibility of the former to disruption or disaggregation by nonlipoprotein plasma constituent(s). Although the putative factors that disrupt bacterial membranes and disaggregate purified LPS are unknown, it seems reasonable to conclude from the available data that both of these forms of LPS must undergo some modification by the nonlipoprotein components of plasma before binding to HDL can occur efficiently. The mechanism by which the LPS bind to HDL is not understood, although apolipoprotein Al has been identified in LPS-HDL complexes isolated from rabbit plasma (4); the results shown in Fig. 6 indicate that apolipoprotein E does not appear to influence the binding of either phenol-extracted or native LPS to HDL.

The final set of studies examined the behavior of the LPS in each of the preparations after intravenous injection into the rat. Although we did not monitor the physiological responses of the rats to the various preparations, the rat is quite resistant to the toxicity of LPS; the doses that we used ($\sim$50 μg/100 g body wt) were below those that have been used to induce death (32) or even modest hypotension (33). The results indicated that the fate of both extracted and native LPS in vivo is strongly influenced by the binding of the LPS to HDL. In keeping with previous studies, LPS-HDL binding prolonged the half-life of the LPS in plasma and was accompanied by uptake of the LPS into the adrenal gland and ovary (3, 5). Because LPS-HDL complexes are removed slowly from the plasma and accumulate in the endocrine tissues and the liver over several hours (5), the uptake of the LPS in membrane fragments and phenol extracts into these tissues was actually underestimated in the present studies that used a 10-min period of observation. The outer membranes and bacteria were removed more rapidly from the plasma during the 10-min study period, on the other hand, and minimal additional accumulation of these LPS in the tissues occurs over a 4-h period (unpublished results).

These studies have shown that the biological activities, lipoprotein binding behavior, and in vivo fate of the LPS in both phenol extracts and membrane fragments of *S. typhimurium* G-30 are quite similar. This is strong quantitative evidence that phenol extraction does not alter the biological properties of LPS; the findings thus lend legitimacy to studies that use phenol-extracted LPS to mimic the activities of native LPS. It should be noted, however, that the mechanisms for the disaggregation of purified LPS and the removal of LPS from membrane fragments, before HDL binding and other interactions, may be quite different. Moreover, these findings should probably not be generalized to other bacterial endotoxins in the absence of specific experimental data.

At the present time nothing is known about the physical state of LPS in the various tissues of the body during infection. Using the Limulus lysate assay, LPS have been found in the cerebrospinal fluids of patients with gram-negative bacterial meningitis, yet a clear distinction between free and bacteria-bound LPS has not been made in these studies (34, 35). Moreover, as in other work using this assay to detect endotoxins in body fluids, it is not certain whether the positive tests were generated by LPS or by other factors, such as serine proteases, which can initiate clotting of the Lim-

ulus coagulogen (36). Knowledge of the state of LPS in plasma and other tissues has also been limited by the absence of sufficiently sensitive and specific assays for these molecules. Our results indicate that, at least during a short period of observation, large amounts of LPS are not released from this strain of *S. typhimurium* in vivo. The bacteria were cleared rapidly from the plasma (Fig. 7M) and were taken up by the liver and spleen (Fig. 8). It is conceivable that over a longer time period tissue phagocytes might release LPS from the bacteria back into the blood (37), or that low-grade bacteremia might ensue, allowing more prolonged contact between bacteria and plasma (38). These possible mechanisms for the release of LPS from bacteria in vivo are under investigation. Their potential importance is underscored by the results of the present experiments, which indicate that the extent to which LPS are released from bacterial membranes should determine the degree to which LPS-HDL binding occurs in vivo.

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