INFECTION AND IMMUNITY, Dec. 1981, p. 835-843
0019-9567/81/120835-09$02.00/0

Vol. 34, No. 3

Binding of *Salmonella typhimurium* Lipopolysaccharides to Rat High-Density Lipoproteins

ROBERT S. MUNFORD,* CATHERINE L. HALL, AND JOHN M. DIETSCHY

Departments of Internal Medicine and Microbiology, University of Texas Health Science Center at Dallas, Dallas, Texas 75235

Received 20 April 1981/Accepted 10 August 1981

These studies were undertaken to investigate the binding of gram-negative bacterial lipopolysaccharides (LPS) to high-density lipoproteins (HDL) of rat plasma. Purified *Salmonella typhimurium* LPS, intrinsically labeled with [^{3}H]-galactose, bound rapidly in vitro to isolated rat HDL. Maximal binding of LPS to HDL occurred when LPS and HDL were incubated with lipoprotein-free plasma ($\rho > 1.21$ g/ml). Since LPS, when purified, form large aggregates, we tested the hypothesis that disaggregation of LPS enhances LPS-HDL binding. We found that calcium chloride (1 mM), an agent which prevents LPS disaggregation, inhibited binding of LPS to HDL by interfering with the modification of LPS by lipoprotein-free plasma. Conversely, sodium deoxycholate (0.15 g/dl), which disaggregates LPS, greatly increased binding of LPS to HDL in the absence of lipoprotein-free plasma. Analysis of labeled LPS by sodium deodecyl sulfate-polyacrylamide gel electrophoresis showed only minor differences in the sizes of LPS molecules before and after binding to HDL, suggesting that chemical modification of LPS is not required for binding. The results provide evidence that disaggregation increases the binding of LPS to HDL.

The cell wall lipopolysaccharides (LPS) or endotoxins of gram-negative bacteria, when injected into animals, cause fever, hypotension, coagulation abnormalities, and many other derangements (2). The striking similarity between these effects and the clinical manifestations of gram-negative bacteremia in humans has stimulated intensive investigation of LPS structure and biological activity. Although the mechanisms by which LPS are detoxified by the host have received less attention, interest in this subject has recently been stimulated by the finding that purified LPS bind in vitro and in vivo to plasma lipoproteins (5, 27). The available evidence indicates that lipoprotein-bound LPS are less potent than purified LPS for causing complement activation, neutropenia, and fever, whereas the two LPS forms may be equally effective in causing hypotension and coagulation abnormalities (27). LPS-lipoprotein binding may thus be an important step in the process which eventually leads to further detoxification and/or uptake of LPS by target tissues. In human, rabbit, and rat plasma, purified LPS appear to associate preferentially with high-density lipoproteins (HDL), although binding to other lipoproteins may also occur (5, 27).

The experiments described in this paper examine the role of lipoprotein-free plasma in promoting the binding of purified LPS and rat HDL. Our results are consistent with the hypothesis of Ulevitch et al. (27) that purified LPS are disaggregated by lipoprotein-free plasma to small subunits; disaggregated LPS then may bind to HDL.

MATERIALS AND METHODS

Materials. D-[1-^{3}H]galactose (14.2 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, Mass.). Sodium deoxycholate and ethylene diaminetetraacetate (EDTA) were from Sigma Chemical Co. (St. Louis, Mo.). Triethylamine was obtained from Eastman Chemical Co. (Rochester, N.Y.). Bacteriological media and reagents for polyacrylamide gel electrophoresis (PAGE) were as previously described (15).

LPS preparations. Intrinsically labeled LPS were prepared from *S. typhimurium* strain G-30, a mutant deficient in uridine diphosphate glucose 4-epimerase (E.C. 5.1.3.2). This strain does not utilize galactose as a carbon source and incorporates exogenous galactose almost exclusively into LPS (18). Cultures were grown in proteose peptone beef extract broth and labeled with [^{3}H]galactose as previously described (15). Cell envelopes were prepared from whole cells by French press disruption followed by ultracentrifugation (15). LPS were extracted from cell envelopes by the hot phenol-water method of Westphal and Jans (28), washed with ether to remove phenol and lipid, and treated enzymatically to remove contaminating nucleic acid and protein as described by Romeo et al. (19). Before the purification steps, nonradioactive LPS prepared from the same strain were added to serve as

carrier. Preparations were then lyophilized and stored at 4°C. For each experiment, weighed amounts of LPS were suspended in 0.9% NaCl and briefly sonicated in a water bath (Ultrasonic Cleaner; Mettler Electronics Corp., Anaheim, Calif.) to achieve a uniform suspension. Three preparations of [^{3}H]LPS were used; specific activities ranged from 1,000 to 16,000 cpm/μg. We noted no differences in the behavior of the different preparations in the assays to be described. In previous studies using this mutant of *S. typhimurium*, we found that most of the LPS molecules had O-chains with less than 8 repeat units (15).

Lipoprotein preparations. Female, Sprague-Dawley-derived rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were housed in colony cages in an isolated room with alternating 12-h periods of light and darkness. They were allowed free access to water and low-cholesterol rat chow (Wayne Laboratory Animal Diets; Allied Mills, Inc., Chicago, Ill.) for at least 2 weeks before being used in specific experiments. Blood was aspirated from the abdominal aorta and anticoagulated with EDTA (1 mg/ml). HDL were then harvested from the plasma between the densities of 1.095 and 1.21 g/ml; HDL were prepared in this narrow-density range to avoid contamination with low-density lipoproteins (11). The lipoproteins were washed and concentrated by centrifuging an additional time at ρ = 1.21 g/ml. These HDL preparations ran as a single band with α mobility on agarose gel electrophoresis (16). On 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels the HDL showed a major apoprotein band corresponding to apo AI and a minor, but distinct, band corresponding to apo E (10). HDL and lipoprotein-free plasma (ρ > 1.21 g/ml, "1.21 bottom") were dialyzed extensively against 0.9% NaCl at 4°C before use and were used within 48 h of preparation.

In vitro assay for LPS-HDL binding. Measured amounts of HDL (in 1.0 ml), lipoprotein-free plasma (1.0 ml), and [^{3}H]LPS (0.1 ml) were agitated (100 oscillations per min) in polyallomer ultracentrifuge tubes in a 37°C water bath. After incubation for the desired time period, the tubes were quickly chilled, and the density was adjusted to 1.21 g/ml with a chilled, concentrated solution of KBr. The tubes were centrifuged for 24 h at 227,000 × *g* at 4°C. 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 samples were added to 10 ml of Aquassure (New England Nuclear Corp.) before counting. The appearance of [^{3}H]LPS in the "1.21 top" fraction (containing HDL) was used as an index of LPS-HDL binding.

In vitro assay for LPS disaggregation. The in vitro assay for LPS disaggregation was based on the observation that treatment of LPS with delipidated plasma causes a shift in the sedimentation profile of LPS on sucrose gradients (9, 27). Although the explanation for this change in sedimentation behavior is uncertain, it probably reflects a decrease in the size of LPS aggregates, since disaggregation lowers the sedimentation coefficient of LPS (6, 9). [^{3}H]LPS were incubated with lipoprotein-free plasma or other agents for 60 min at 37°C. Samples (0.2 ml) of the reaction mixture were layered onto 4.5-ml continuous sucrose gradients [from 5 to 25% prepared with 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, (pH 7.5) in 5.5-ml nitrocellulose ultracentrifuge tubes] and overlaid with saline. These were centrifuged for 4 h at 150,000 × *g* at 4°C. Fractions (0.5 ml) were collected from the bottom of each tube, diluted with 0.4 ml of distilled water, added to 5 ml of Aquasol (New England Nuclear Corp.), and counted for ^{3}H activity.

SDS-PAGE. Samples of LPS-HDL (reisolated after LPS-HDL binding in the presence of 1.21 bottom) and LPS were exhaustively dialyzed against 0.9% NaCl, extracted once with cold diethyl ether, and dried under N_2. Samples were then suspended in the digestion buffer of Fairbanks et al. (3) and analyzed by tube SDS-PAGE as previously described (15). Gel slices (2 mm) were soaked in 0.2 ml of a solution containing 0.1% SDS and 1 mM EDTA for 12 h at 60°C and cooled; 5 ml of Aquasol was added before counting.

Miscellaneous assays. Protein was measured by the method of Lowry et al. (12).

RESULTS

In vitro binding of LPS and HDL. When [^{3}H]LPS were incubated with whole rat plasma and the mixture was fractionated into different density ranges by repetitive ultracentrifugation, 85% of the radioactivity appeared in the density range 1.050 to 1.21 g/ml (the fraction containing predominantly HDL), in agreement with previous evidence that LPS preferentially bind to HDL in rat plasma (5). Subsequent experiments focused on the binding of LPS to isolated HDL. As shown in Table 1, binding of [^{3}H]LPS to HDL was most complete when both HDL and lipoprotein-free plasma (1.21 bottom) were present in the reaction mixture. No binding occurred when the mixtures were incubated at 0°C. As seen in Fig. 1A, binding reached maximal levels within 20 min of incubation at 37°C. Maximal binding occurred with as little as 0.5 mg of HDL protein (Fig. 1B) or 10 mg of 1.21 bottom protein (Fig. 1C), provided that the other non-LPS com-

TABLE 1. *In vitro binding of S. typhimurium LPS to rat HDL*[a]

Additions to incubation mixture	Incubation temp (°C)	% of [^{3}H]LPS in 1.21 top fraction
[^{3}H]LPS	37	0.82 ± 0.21
[^{3}H]LPS, HDL	37	18.6 ± 7.4
[^{3}H]LPS, 1.21 bottom	37	14.0 ± 8.9
[^{3}H]LPS, HDL, 1.21 bottom	37	87.9 ± 2.6
[^{3}H]LPS, HDL, 1.21 bottom	0	1.3

[a] Reaction mixtures contained 50 μg of [^{3}H]LPS (50,000 cpm), 1.0 mg of HDL protein, and 25 mg of 1.21 bottom protein. All incubations were for 60 min. Each value equals the mean ± 1 standard deviation of four determinations; the 0°C incubation was performed twice.

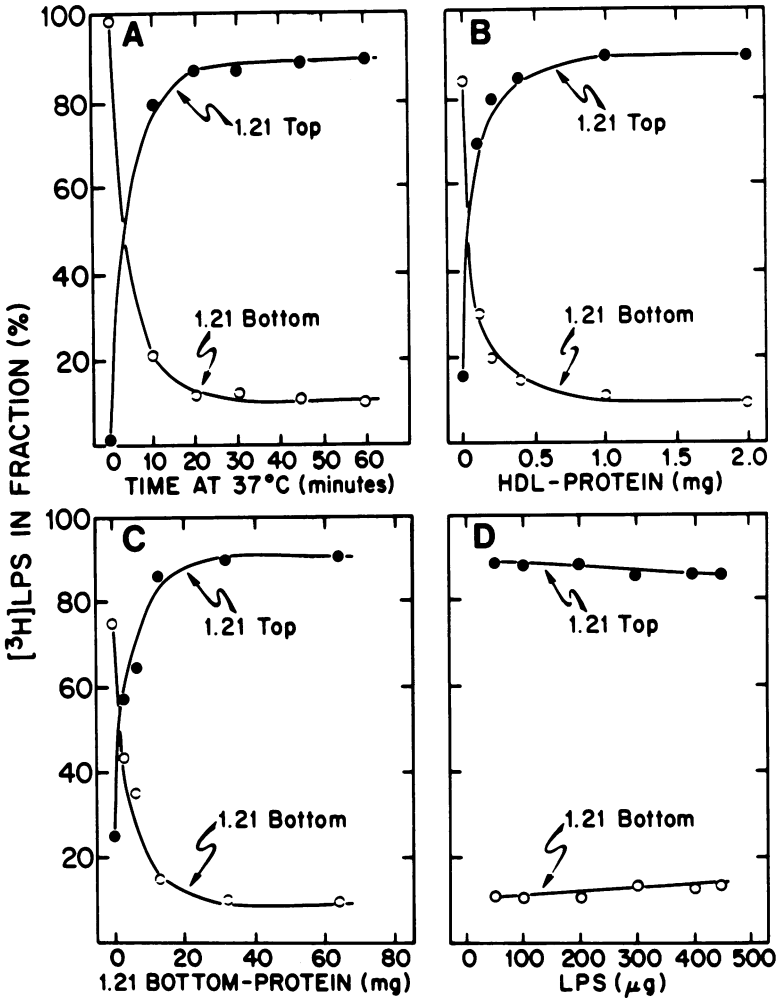

FIG. 1. *In vitro binding of S. typhimurium LPS to rat HDL. [^{3}H]LPS, HDL, and 1.21 bottom were incubated together at 37°C as described in the text. The reaction mixtures were then chilled, adjusted to 1.21 g/ml with concentrated KBr, and centrifuged. The tubes were cut, and the 1.21 top (containing HDL) and 1.21 bottom (lipoprotein-free) fractions were obtained. A, Time course of the in vitro reaction with the complete mixture (63 mg of 1.21 bottom protein, 2.0 mg of HDL protein, and 50 μg of [^{3}H]LPS). B, Quantitation of the requirement for HDL protein (other reaction components as in A). C, Effect of changing the amount of 1.21 bottom (other reaction components as in A). To determine the amount of LPS bound by the complete mixture (D), nonradioactive S. typhimurium G-30 LPS were added to 50 μg of [^{3}H]LPS (other components as in A). In B through D, incubation was for 60 min at 37°C.*

ponent of the reaction mixture was present in excess. We were unable to saturate 2.0 mg of HDL protein with up to 500 μg of LPS (Fig. 1D).

Inhibition of in vitro LPS-HDL binding by calcium chloride. We prepared HDL and 1.21 bottom in the presence of 10 mM EDTA and dialyzed them against 0.9% saline before use. The in vitro HDL-LPS binding reaction could be inhibited by adding calcium chloride to the reaction mixture before incubation of [^{3}H]LPS, HDL, and 1.21 bottom at 37°C (Fig. 2A). When LPS-HDL complexes were isolated by ultracentrifugation and then exposed to calcium chloride, in contrast, there was no effect on LPS-HDL binding (Fig. 2B). These data indicate that calcium chloride interferes with the overall binding reaction but does not disrupt preformed LPS-HDL complexes. In another experiment, LPS (50 μg) were incubated with 1.21 bottom (18 mg of protein) for 60 min at 37°C. When HDL (1.0 mg of protein) and calcium chloride (5 mM) were then added and incubation was

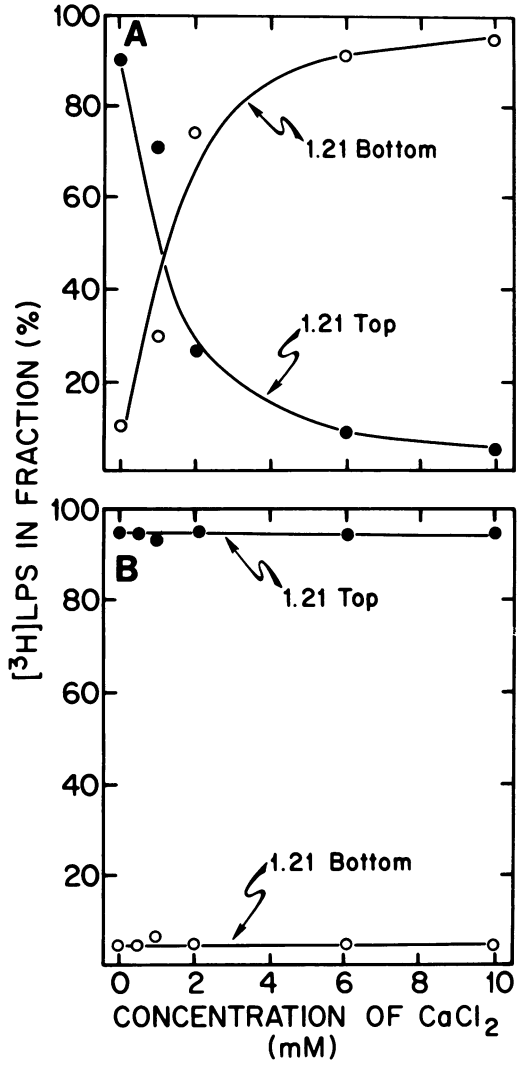

FIG. 2. *A, Inhibition of LPS-HDL binding reaction by calcium chloride added before incubation of [^{3}H]LPS (50 μg), 1.21 bottom (65 mg), and HDL (2.0 mg) for 60 min at 37°C; 50% inhibition of the binding reaction was obtained with approximately 1.5 mM $CaCl_2$. B, Effect of calcium chloride on LPS-HDL complexes. [^{3}H]LPS-HDL complexes were isolated from top fractions and dialyzed against 0.9% NaCl to remove the KBr. Samples were reincubated for 60 min at 37°C with different concentrations of calcium chloride and then centrifuged. Since most of the ^{3}H remained in the top fraction, calcium chloride did not disrupt prebound [^{3}H]LPS-HDL complexes.*

continued for 60 min, LPS-HDL binding was inhibited only 37% (as opposed to 88% inhibition when the calcium was incubated with the complete mixture). The ability of calcium to inhibit the binding reaction thus was greatest when calcium was present during the interaction of LPS with 1.21 bottom. To examine this interaction further, we studied the influence of calcium on the sedimentation behavior of LPS in sucrose gradients.

Modification of LPS in vitro. The ability of 1.21 bottom to modify LPS in the absence of HDL was examined by using sucrose gradient ultracentrifugation. Incubation of [^{3}H]LPS with 1.21 bottom at 37°C caused a shift in the peak of radioactivity toward the top of the gradient (Fig. 3A). The addition of calcium chloride before incubation of LPS with 1.21 bottom inhibited this shift in peak radioactivity (Fig. 3B). The addition of calcium after incubation of LPS with 1.21 bottom, with further incubation at 37°C for 30 min, did not alter the profile of ^{3}H activity found after incubation with 1.21 bottom alone (data not shown). These data are consistent with the conclusion that calcium chloride interferes with the initial step in which LPS are modified by 1.21 bottom. After this modification has occurred, the addition of calcium has little effect on either the shift in sucrose gradient sedimentation profile or on the binding of LPS to HDL.

Substitutes for 1.21 bottom. We then sought to find reagents which might substitute for lipoprotein-free plasma in "activating" LPS for HDL binding (Table 2). Treatment of [^{3}H]LPS with 10 mM EDTA, with or without 10 mM Tris-hydrochloride (as described by Shands and Chun [23]), did not alter the sedimentation profile of LPS on sucrose gradients or allow in vitro binding of LPS to HDL. Triethylamine, which disperses LPS (6), had a minimal effect (Fig. 4). In contrast, sodium deoxycholate produced a striking shift in the sedimentation profile of [^{3}H]LPS on sucrose gradients (Fig. 4). and allowed binding of LPS to HDL in the absence of 1.21 bottom (Fig. 5). We observed two differences in the activation of LPS by deoxycholate and by 1.21 bottom. First, the LPS sucrose gradient sedimentation profiles generated by the two treatments were not identical (Fig. 3 and 4). Second, whereas deoxycholate-treated LPS retained its ability to bind to HDL after 21-fold dilution (to deoxycholate concentrations of less than 0.005 g/dl), similar dilution of LPS which had been treated with 1.21 bottom (so that 0.75 mg of 1.21 bottom protein was present with 1.0 mg of HDL protein) reduced HDL binding (Table 2). Deoxycholate disaggregates LPS to small subunits (probably to dimers or trimers of the unit structure [23]), and the data suggest that such disaggregation is sufficient to allow LPS-HDL binding. Although 1.21 bottom may also activate LPS by promoting their disaggregation, the differences noted above suggest that lipoprotein-free plasma and deoxy-

cholate alter LPS in somewhat different ways.

Effect of in vitro binding on LPS structure. We studied the effect of HDL binding on LPS structure by using SDS-PAGE, a technique which separates LPS molecules according to size (8, 15). Molecules with low polysaccharide/lipid A ratios migrate more rapidly in SDS-PAGE than do molecules with high ratios (8). In preliminary experiments, we found that [^{3}H]LPS-HDL gave an unexpected profile of ^{3}H activity in SDS-PAGE: there was a large ^{3}H peak located immediately below the top of the gel. This peak did not correspond to the position of the HDL apoproteins, which migrated normally (determined by Coomassie blue staining of the gels). Treatment of [^{3}H]LPS-HDL with diethyl ether eliminated this peak; after ether extraction, the radioactivity profiles on tube gels were similar for [^{3}H]LPS-HDL and [^{3}H]LPS (Fig. 6) and were essentially identical to previous analyses of [^{3}H]galactose-labeled *S. typhimurium* LPS (15). The only reproducible difference in the gel patterns was the appearance of approximately 5% of the counts in LPS-HDL preparations at the origin (top) of the gel.

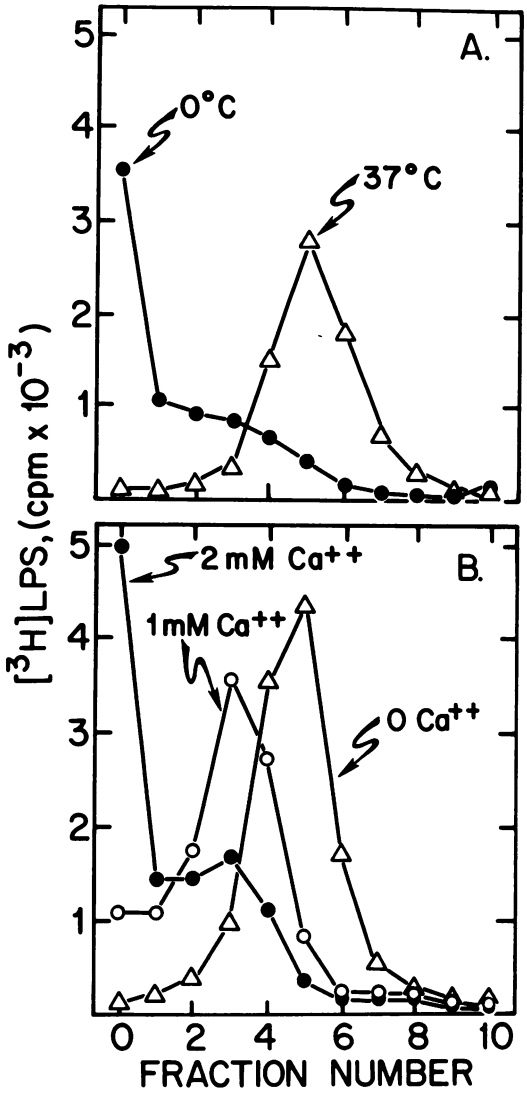

FIG. 3. *Sucrose gradient analysis of [^{3}H]LPS (50 μg) and 1.21 bottom (20 mg). A, Alteration in [^{3}H]-LPS sedimentation behavior that occurred after incubation at 37°C for 60 min. B, Inhibitory effect of calcium chloride on the shift in the sedimentation profile of [^{3}H]LPS. Samples of [^{3}H]LPS and 1.21 bottom, with and without calcium chloride, were incubated for 60 min at 37°C and centrifuged. When [^{3}H]LPS and 1.21 bottom were incubated for 60 min, calcium chloride (5 mM) was added, incubation was continued for 30 min, and centrifugation was performed, the ^{3}H profile in the gradient was similar to that shown for "0 Ca^{++}".*

TABLE 2. *Treatment of LPS to allow LPS-HDL binding in the absence of 1.21 bottom*[a]

LPS treatment	Sucrose gradient analysis: % of [^{3}H]-LPS in fractions 4-10	In vitro [^{3}H]-LPS-HDL binding: % of [^{3}H]LPS in 1.21 top fraction
1.21 bottom (20 mg/ml) 37°C	92	30
0°C	11	1.3
EDTA (10 mM)	28	13
Tris-EDTA (10 mM)	18	18
Triethylamine (0.5 ml/dl)	35	22
Sodium deoxycholate (0.15 g/dl)	94	89
None	18	18

[a] [^{3}H]LPS (approximately 50 μg, containing 50,000 cpm) were incubated with each agent in 0.9% NaCl at 37°C for 60 min. Samples were then layered onto sucrose gradients and centrifuged. Each experiment included [^{3}H]LPS which had been incubated at 0°C (negative control) and 37°C (positive control) in the presence of 1.21 bottom (20 mg/ml). Modification of LPS was indicated by a shift in ^{3}H counts per minute toward the top of the gradient (fractions 4 through 10 in Fig. 3). In separate experiments, each agent was incubated with [^{3}H]LPS (approximately 100,000 cpm in a total volume of 0.25 ml) for 60 min at 37°C; 0.1-ml aliquots were added to HDL (1.0 mg) in a total volume of 2.1 ml of 0.9% NaCl, and incubation was continued for 60 min at 37°C before adding KBr and centrifuging. In the latter experiments, the concentration of each agent was thus reduced 21-fold during incubation of LPS with HDL. The final amount of 1.21 bottom, after dilution, was 0.75 mg. Each result is the average of at least two determinations.

DISCUSSION

Purified gram-negative bacterial LPS are amphipathic molecules which form large, polydis-

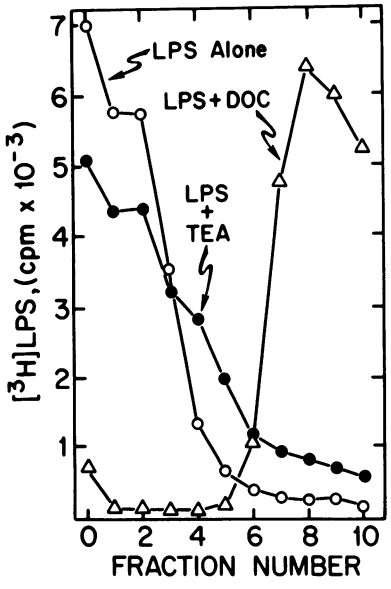

FIG. 4. *Effect of triethylamine and sodium deoxycholate on LPS sedimentation behavior. Triethylamine (0.5 ml/dl) or sodium deoxycholate (0.15 g/dl) was incubated with [^{3}H]LPS at 37°C for 60 min, and the mixtures were centrifuged on sucrose gradients. Triethylamine caused a slight change in the profile of ^{3}H activity (LPS + TEA), whereas deoxycholate produced a striking shift in the peak of ^{3}H toward the top of the gradient (LPS + DOC).*

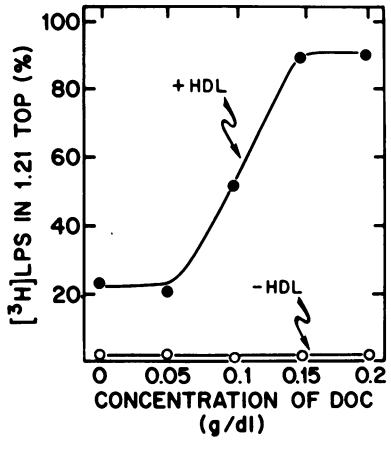

FIG. 5. *Binding of deoxycholate-treated LPS to HDL. [^{3}H]LPS (20 μg) were incubated for 60 min at 37°C in the presence of sodium deoxycholate in the concentrations shown. Samples (0.1 ml) were added to HDL (1.5 mg) or saline in a total volume of 2.1 ml. The mixtures were incubated at 37°C for 60 min, adjusted to a density of 1.21 g/ml with KBr, and centrifuged as described in the text. [^{3}H]LPS appeared in the 1.21 top fraction only when HDL was present in the reaction mixtures.*

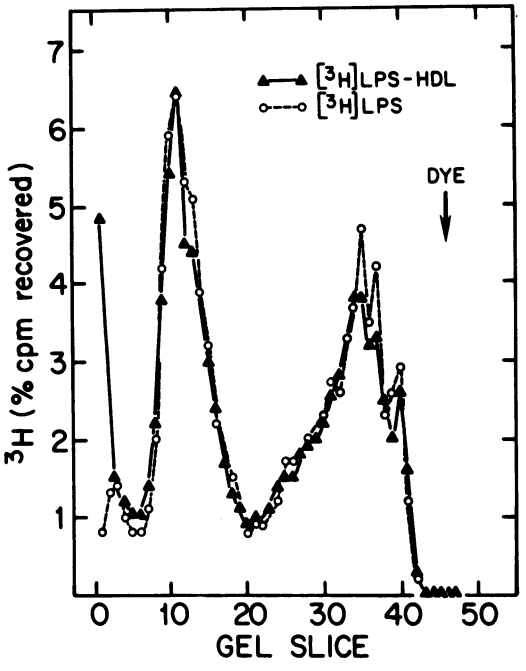

FIG. 6. *SDS-PAGE analysis of [^{3}H]LPS and [^{3}H]LPS-HDL. Samples of [^{3}H]LPS and [^{3}H]LPS-HDL were treated with ether and analyzed by SDS-PAGE. The arrow indicates the migration position of the pyronin Y dye marker. The results from companion gels, run simultaneously, are plotted. The ^{3}H profiles are similar, except that approximately 5% of the counts per minute in [^{3}H]LPS-HDL remained at the top of the gel.*

perse aggregates in aqueous suspension (6, 23). The mechanisms by which plasma decreases the toxicity of these molecules have been studied for over 25 years. Early observations indicated that plasma induced a change in the migration pattern of LPS in agar diffusion gels, consistent with the disaggregation of LPS into smaller units (1), and that divalent cations were important inhibitors of LPS detoxification (20). Later investigations by Rudbach and his colleagues found that LPS which had been treated with plasma or sodium deoxycholate were less pyrogenic in rabbits than native LPS, yet reextraction of LPS from plasma by pronase treatment and ethanol precipitation allowed essentially complete recovery of pyrogenicity (21, 22). These workers argued that the detoxification of LPS by plasma was primarily due to LPS disaggregation and not to an enzymatic modification of LPS structure. A different view, that LPS were chemically modified during exposure to plasma, was advanced by Skarnes (26), who also presented evidence that LPS bind to plasma lipoproteins (25). In more recent studies, Ulev-

itch and his co-workers have shown that LPS interact preferentially with HDL in rabbit and human plasma, and that lipoprotein-bound LPS appear to be less toxic than purified LPS, at least when tested in certain assays (27). They proposed that disaggregation of LPS probably precedes LPS-HDL interaction according to a two-step sequence: step 1, LPS + plasma factor(s) $\rightleftarrows$ (LPS) disaggregated; step 2, (LPS) disaggregated + HDL $\rightarrow$ (LPS) $\rho < 1.21$ g/ml.

The experimental data presented in this publication support this last hypothesis. We found that calcium chloride, which prevents disaggregation of LPS (6, 17), interferes with the overall LPS-HDL binding reaction by inhibiting the modification of LPS by lipoprotein-free plasma (step 1). Second, we have shown that deoxycholate, which disaggregates LPS, will augment LPS-HDL binding in the absence of 1.21 bottom. Both of these observations are consistent with the proposal that disaggregation of LPS enhances LPS-HDL binding.

Several agents (EDTA, Tris-EDTA, triethylamine) which did not modify the sedimentation profile of LPS on sucrose gradients also did not activate LPS for HDL binding. Since these agents reduce the size of LPS aggregates, their inability to promote LPS-HDL binding may seem to contradict the proposal outlined above. The most economical explanation for our findings is that these agents did not entirely disaggregate LPS, and that only small aggregates (such as those produced by deoxycholate) are able to bind to HDL. Unfortunately, there is very little evidence which bears directly on this hypothesis. Although triethylamine is widely used to disperse LPS preparations in aqueous suspension, for example, the actual size of the resulting aggregates is not known. Galanos and Luderitz found that triethylamine did not alter the sedimentation coefficient of *Salmonella abortus equi* LPS unless the LPS were first electrodialyzed to remove cations (6); electrodialysis followed by conversion of the LPS to their triethylamine salts lowered the sedimentation coefficient from 83 to 9.3, whereas triethylamine treatment alone was ineffective. These findings suggest that triethylamine treatment, such as that used in our experiments, probably does not greatly reduce the size of LPS aggregates. EDTA and Tris-EDTA remove cations which are present in LPS preparations; this increases the net negative charge of the LPS molecules and may contribute to dispersion of LPS by charge-charge repulsion (23). Olins and Warner found that Tris-EDTA treatment lowered the sedimentation coefficient of *Azotobacter vinelandii* LPS from 45 to 5.7, giving an estimated subunit molecular weight of 134,000; treatment of the dissociated LPS with Tris-calcium buffer raised the molecular weight of the aggregates to 873,000 (17). Combined treatment of the LPS with Tris-EDTA and SDS lowered the aggregate size to an estimated 65,600 daltons (17). This size was consistent with an association of six or seven molecules to form the aggregate. Similar estimates using triethylamine or EDTA treatment of *S. typhimurium* LPS have not been described, and the results obtained with different LPS preparations are not strictly comparable. Nevertheless, the available evidence suggests that deoxycholate may reduce *S. typhimurium* LPS to aggregates with molecular weights of 15,600 (for smooth LPS), consistent with the existence of LPS in a dimeric form (23). Moreover, the deoxycholate concentration which allowed maximal LPS-HDL binding in our study (0.15 g/dl) was associated with near-maximal reduction in the sedimentation coefficient of *S. typhimurium* LPS (23). The available data thus appear consistent with the proposal that dissociation of LPS molecules to small aggregates greatly increases the binding of the LPS to HDL. It is possible that other factors may also be important: the detergent properties of deoxycholate itself may be critical, for example, or there may be charge interactions between LPS and HDL which influence binding. It should also be noted that the factor(s) in 1.21 bottom which prepare LPS for HDL binding are unknown. We found differences in our assays between the results of LPS treatment with 1.21 bottom and deoxycholate; these differences and the observation that delipidated serum modifies LPS to enhance lipoprotein binding (27) suggest that bile salts are probably not the factor in 1.21 bottom which disaggregates LPS.

It should be noted that approximately 18% of the LPS in our preparations could bind to HDL without treatment of the LPS with 1.21 bottom or deoxycholate. Since preparations of LPS include a heterogeneous assortment of molecular and aggregate sizes, it is possible that this finding reflects the binding to HDL of the smaller aggregates in the total population of LPS. Alternatively, HDL may bind unmodified LPS directly. Since LPS-HDL binding could be increased almost fivefold by treatment of the LPS with 1.21 bottom or deoxycholate, however, direct LPS-HDL binding appears to be quantitatively less important than the binding which occurs after the LPS have been disaggregated.

Although the biological properties of disaggregated LPS have not been studied in detail, there is evidence that low-molecular-weight LPS aggregates are less effective activators of serum

complement than are high-molecular-weight LPS preparations (7), and that deoxycholate-treated LPS are less pyrogenic than untreated LPS in rabbits (21). It thus appears that disaggregation may be important for the detoxification of purified LPS in vivo, although the relative contributions of disaggregation and lipoprotein-binding to this detoxification have not been clearly differentiated. The role of calcium in modulating LPS disaggregation (and therefore the binding of LPS to lipoproteins) in vivo is also uncertain. We found that LPS bind rapidly to HDL in vivo in rats which presumably had normal plasma calcium levels (R. S. Munford, J. M. Andersen, and J. M. Dietschy, J. Clin. Invest., in press). On the other hand, Skarnes and Moreau have presented evidence that LPS injection causes a transient lowering of serum ionized calcium levels in rabbits, and that rabbits made tolerant to LPS exhibit an even more striking reduction in serum ionized calcium after LPS injection (14, 26). These findings raise the possibility that hypocalcemia may be a host response to LPS injection, and our data would suggest that such hypocalcemia might facilitate the disaggregation of LPS and, thus, the binding of LPS to lipoproteins in vivo.

We used the decrease in density of LPS (to $\rho < 1.21$ g/ml) as an index of LPS-HDL binding. The validity of this assay depends upon the accuracy of the assumption that such a density change only occurs when LPS bind to lipoproteins. Several lines of evidence suggest that this assumption is correct. First, unreacted LPS consistently had a density >1.21 g/ml in our assays, in agreement with previous determinations of the density of LPS preparations (27). Even LPS which had been treated with 1.21 bottom or deoxycholate did not float at a density of 1.21 g/ml unless the LPS were first reacted with HDL. Second, calcium chloride did not disrupt LPS-HDL complexes. This experiment involved repetitive ultracentrifugation of the LPS-HDL complexes, indicating that they were also stable to prolonged treatment with KBr and centrifugation. Third, SDS-PAGE analysis provided evidence against a major molecular modification of the LPS after interaction with 1.21 bottom that would by itself account for the observed change in density. Fourth, the LPS-HDL complexes were not dissociated in vivo in the rat, as indicated by the binding of LPS-HDL to the same tissues which bind HDL and by the influence of hormonal manipulations which alter HDL binding in vivo (Munford et al., in press). Thus, for all of these reasons we feel that the simple flotation assay is a reasonable in vitro test for LPS-lipoprotein binding.

Previous studies on the LPS-HDL interaction have utilized extrinsically labeled LPS (13, 27). Although iodination does not appear to modify the biological activity of the LPS preparations used, the molecular location of the radioactive label in these preparations is unknown. For our experiments we biosynthetically labeled LPS with [^{3}H]galactose, which is found in the O-antigen and R-core regions of *S. typhimurium* LPS. LPS and LPS-HDL showed essentially identical profiles of ^{3}H activity in companion gels (Fig. 6), indicating that minimal modification of the lengths of the polysaccharide chains occurred during the HDL binding process. This observation agrees with that of Mathison and Ulevitch, who used [^{125}I]LPS from *E. coli* O111 and *S. minnesota* R595 (13). A small percentage of ^{3}H did not enter the gels when samples of LPS-HDL were analyzed by SDS-PAGE; these counts may represent lipid-free polysaccharides (15), and we are currently investigating the possibility that there is enzymatic deacylation of lipid A during the LPS-HDL binding process. This reaction, if present, is quantitatively minor, however.

These in vitro experiments provide important background for studies on the in vivo behavior of LPS and LPS-HDL in rats. HDL is the major lipoprotein class in rats; Freudenberg and coworkers have found that injection of large amounts of LPS increase HDL blood levels in rats and actually alters transiently the electrophoretic migration of HDL in agarose gels (5). Rat serum appears to be more effective than serum from many other species in detoxifying LPS (24), and rats are quite resistant to in vivo LPS toxicity (4). These observations suggest that investigation of the interactions between HDL and LPS in this species may shed light on the mechanisms of HDL turnover and LPS resistance.

ACKNOWLEDGMENTS

These studied were supported by Public Health Service research grants AM-19329 and HL-09610 from the National Institutes of Health. R.S.M. is the recipient of Public Health Service young investigator research grant A1-14942 from the National Institutes of Health.

We thank Leon Eioels for reviewing the manuscript and gratefully acknowledge the help of Dorothy Dunham in preparing the manuscript.

LITERATURE CITED

1. **Cluff, L. E.** 1956. A study of the effect of serum on the immunological reaction of a bacterial endotoxin. J. Exp. Med. **103**:439–452.
2. **Elin, R. J., and S. M. Wolff.** 1976. Biology of endotoxins. Annu. Rev. Med. **27**:127–141.
3. **Fairbanks, G. T., T. L. Steck, and D. F. H. Wallach.** 1971. Electrophoretic analysis of the major polypeptides

of the human erythrocyte membrane. Biochemistry **10**: 2606–2617.

4. **Filkins, J. P.** 1970. Bioassay of endotoxin inactivation in the lead-sensitized rat. Proc. Soc. Exp. Biol. Med. **134**: 610–612.
5. **Freudenberg, M. A., T. C. Bog-Hansen, U. Back, and C. Galanos.** 1980. Interaction of lipopolysaccharides with plasma high-density lipoprotein in rats. Infect. Immun. **28**:373–380.
6. **Galanos, C., and O. Luderitz.** 1975. Electrodialysis of lipopolysaccharides and their conversion to uniform salt forms. Eur. J. Biochem. **54**:603–610.
7. **Galanos, C., and O. Luderitz.** 1976. The role of the physical state of lipopolysaccharides in the interaction with complement. Eur. J. Biochem. **65**:403–408.
8. **Jan, B., K. Reske, and K. Jann.** 1975. Heterogeneity of lipopolysaccharides. Analysis of polyacrylamide chain lengths by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Eur. J. Biochem. **60**:239–246.
9. **Johnson, K. J., P. A. Ward, S. Goralnick, and M. J. Osborn.** 1977. Isolation from human serum of an inactivator of bacterial lipopolysaccharide. Am. J. Pathol. **88**:559–574.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
11. **Lasser, N. L., P. S. Roheim, D. Edelstein, and H. A. Eder.** 1973. Serum lipoproteins of normal and cholesterol-fed rats. J. Lipid Res. **14**:1–8.
12. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
13. **Mathison, J. C., and R. J. Ulevitch.** 1979. The clearance, tissue distribution, and cellular localization of intravenously injected lipopolysaccharide in rabbits. J. Immunol. **123**:2133–2143.
14. **Moreau, S. C., and R. C. Skarnes.** 1973. Host resistance to bacterial endotoxemia: mechanisms in endotoxin-tolerant animals. J. Infect. Dis. **128**:S122–S133.
15. **Munford, R. S., C. L. Hall, and P. D. Rick.** 1980. Size heterogeneity of *Salmonella typhimurium* lipopolysaccharides in outer membranes and culture supernatant membrane fragments. J. Bacteriol. **144**:630–640.
16. **Noble, R. P.** 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. J. Lipid Res. **9**:693–700.
17. **Olins, A. L., and R. C. Warner.** 1967. Physicochemical studies on a lipopolysaccharide from the cell wall of *Azotobacter vinelandii*. J. Biol. Chem. **242**:4994–5001.
18. **Osborn, M. J., S. M. Rosen, L. Rothfield, and B. L. Horecker.** 1962. Biosynthesis of bacterial lipopolysaccharide. I. Enzymatic incorporation of galactose in a mutant strain of Salmonella. Proc. Natl. Acad. Sci. U.S.A. **48**:1831–1838.
19. **Romeo, D., A. Girard, and L. Rothfield.** 1970. Reassembly of a functional membrane system in a monomolecular film. J. Mol. Biol. **53**:475–490.
20. **Rosen, F. S., R. C. Skarnes, M. Landy, and M. J. Shear.** 1958. Inactivation of endotoxin by a humoral component. III. Role of divalent cation and a dialyzable component. J. Exp. Med. **108**:701–711.
21. **Rudbach, J., and A. G. Johnson.** 1966. Alteration and restoration of endotoxin activity after complexing with plasma proteins. J. Bacteriol. **92**:892–898.
22. **Rudbach, J. A., R. L. Anacker, W. T. Haskins, A. G. Johnson, K. D. Milner, and E. Ribi.** 1966. Physical aspects of reversible inactivation of endotoxin. Ann. N.Y. Acad. Sci. **133**:629–643.
23. **Shands, J. W., and P. W. Chun.** 1980. The dispersion of gram-negative lipopolysaccharide by deoxycholate. J. Biol. Chem. **255**:1221–1226.
24. **Skarnes, R. C., F. S. Rosen, M. J. Shear, and M. Landy.** 1958. Inactivation of endotoxin by a humoral component. J. Exp. Med. **108**:685–699.
25. **Skarnes, R. C.** 1968. In vivo interaction of endotoxin with a plasma lipoprotein having esterase activity. J. Bacteriol. **95**:2031–2034.
26. **Skarnes, R. C.** 1970. Host defense against bacterial endotoxemia: mechanism in normal animals. J. Exp. Med. **132**:300–316.
27. **Ulevitch, R. J., A. R. Johnson, and D. B. Weinstein.** 1979. New function for high density lipoproteins. J. Clin. Invest. **64**:1516–1524.
28. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides. Methods Carbohydr. Chem. **5**:83–91.