INFECTION AND IMMUNITY, Aug. 1971, p. 167-172

Vol. 4, No. 2
Printed in U.S.A.

NOTES

Evidence for a Bilayer Structure in Gram-Negative Lipopolysaccharide: Relationship to Toxicity

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Received for publication 4 May 1971

Additional evidence for a bilayer structure in isolated gram-negative lipopolysaccharide (LPS) is demonstrated. Both heating in an electron beam and cleavage of ester-bound fatty acids by alkali were found to split the bilayer structure of LPS into apparent monolayers. The altered structure of LPS resulting from alkaline hydrolysis may explain some of the altered biological activities possessed by the hydrolyzed product.

Evidence was published previously indicating that lipopolysaccharide (LPS), as isolated by various procedures, exists as a bilayer in aqueous solutions, and a model for its structure was proposed (9, 10). In this model, the LPS particle was pictured as two monolayers of polysaccharide and covalently bound lipid arranged in such a way that the two were bound together by their hydrophobic lipid moieties, whereas the polysaccharides made up the hydrophilic, exposed surfaces of the particle. The evidence for the bilayer structure arose from the interaction of LPS with deoxycholate, diethyl ether, or pyridine which appeared to result in the splitting of some bilayers into monolayers and the interaction with Tween 80 which appeared to push some of the monolayers apart (9, 10). In this paper, it is shown that LPS bilayers may also be separated by heating in an electron beam and by cleavage of the ester-linked fatty acids by alkali.

Figure 1 shows a phenol-water extract of wild-type *Salmonella typhimurium* stained with uranyl acetate. This preparation was heated in an electron beam which caused some focal instability in the support film. It is apparent that the parallel dense lines of the LPS have separated in several areas (arrows) to such an extent that the fatty acids of the two sides of the ribbons could not conceivably be in contact with one another.

Figure 2 shows a similar LPS preparation treated with 0.05 N NaOH at 37 C for 3 hr. The preparation was stained by saturation with uranyl acetate followed by dialysis. Treatment with this concentration of alkali dispersed the LPS but did not disturb the basic architecture, nor was the toxicity of the preparation, as measured by lethality in BCG-infected mice, decreased by this treatment ($LD_{50} < 1.0$ μg). In the preparation, one can see the "bilayers," the discs, and the "ribbon-like" structures typical of this LPS. Figures 3 and 4 show the appearance of LPS after hydrolysis in 0.25 N NaOH for 3 hr at 37 C. This treatment caused extensive alteration of the basic architecture of the LPS and a loss of toxicity as measured by lethality in BCG-infected mice. In this preparation, few if any bilayer-like structures can be seen, and, in some areas (arrows), one could interpret the structures seen as aggregates of monolayers. Treatment with alkaline hydroxylamine (7) had a similar effect (Fig. 5). Again, although some very small ribbons are present, definitive bilayers cannot be found, and many of the stained particles could be interpreted as monolayers (arrows). In this preparation also toxicity for BCG mice was substantially reduced ($LD_{50} = 16.0$ μg, a greater than 16-fold reduction in toxicity).

These data taken with those published previously indicate that LPS is a bilayer composed of monolayers of covalently linked lipid and carbohydrate. Since manipulations that tend to break hydrophobic forces or diminish them, i.e., lowering of the dielectric constant of the medium or removal of ester-bound fatty acids, cause the bilayer to fall apart, this suggests that hydrophobic forces are involved in holding the two halves of the particle together.

It is of interest that treatment with alkali of

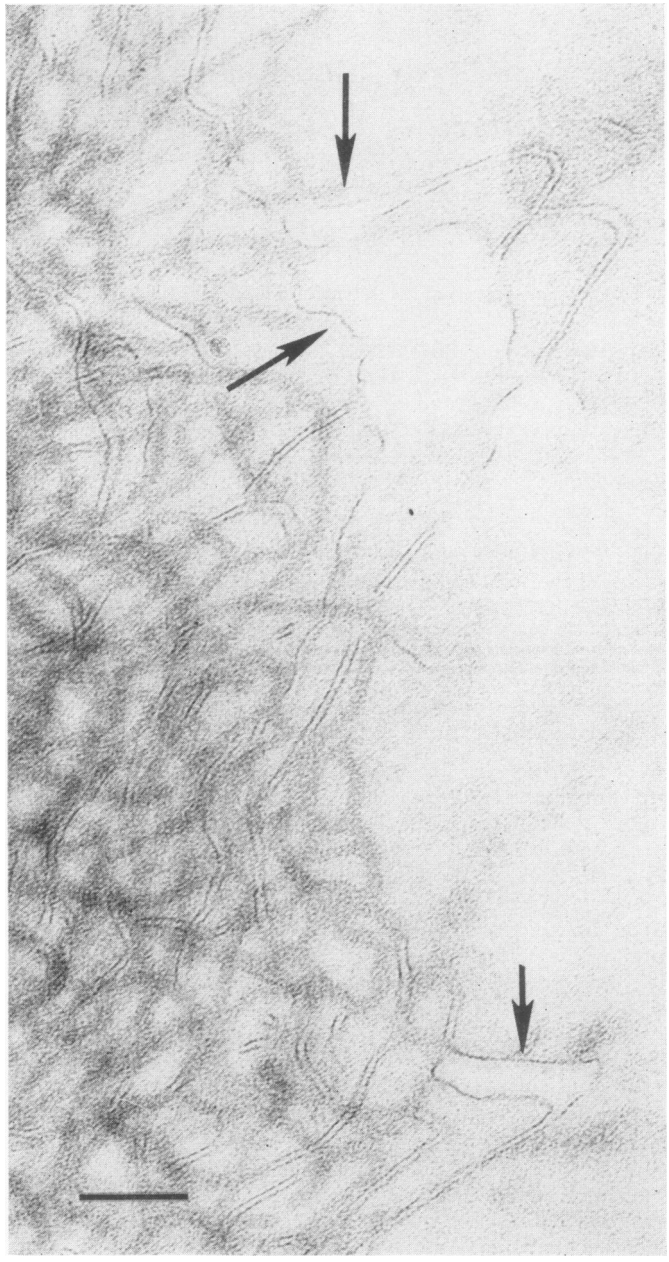


FIG. 1. *Wild-strain lipopolysaccharide from Salmonella typhimurium stained with uranyl acetate.* × *140,000. Bar = 100 nm.*

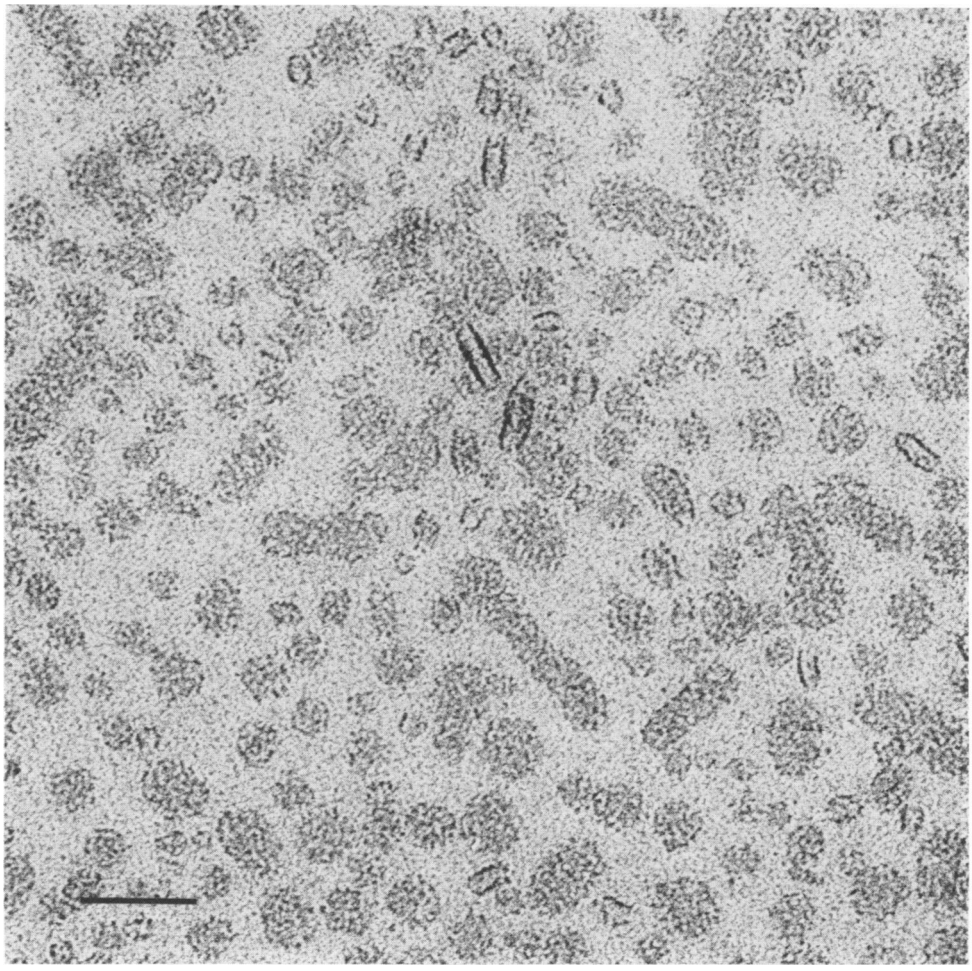
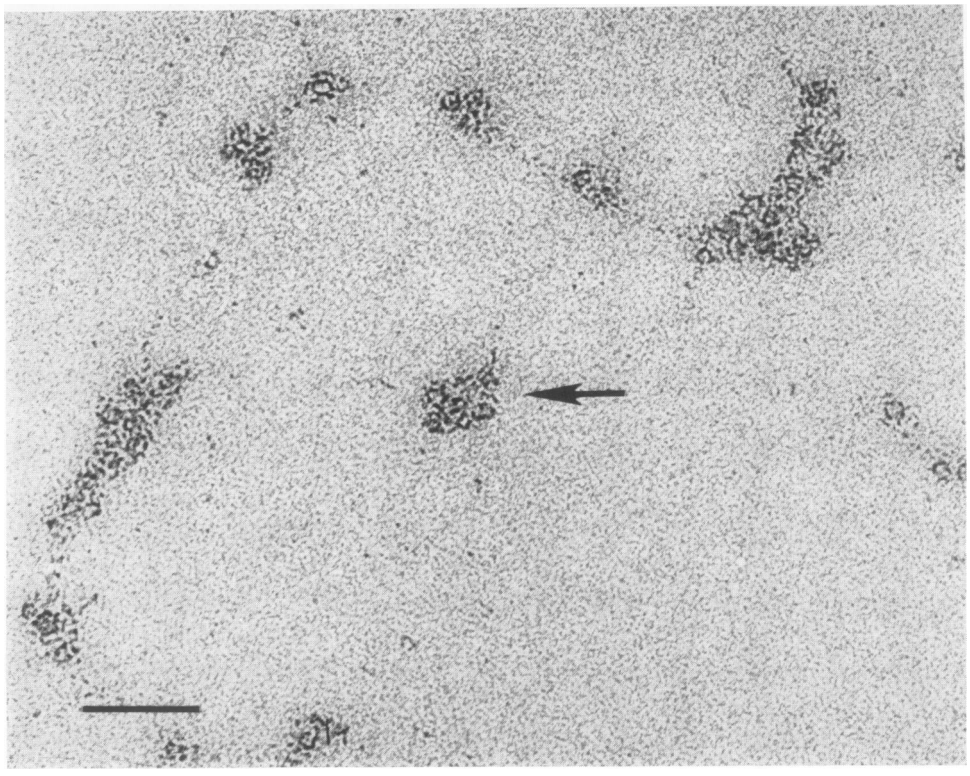


FIG. 2. *Wild-strain lipopolysaccharide from Salmonella typhimurium heated at 37 C for 3 hr in 0.05 N NaOH, neutralized, and stained with uranyl acetate. × 320,000. Bar = 50 nm.*



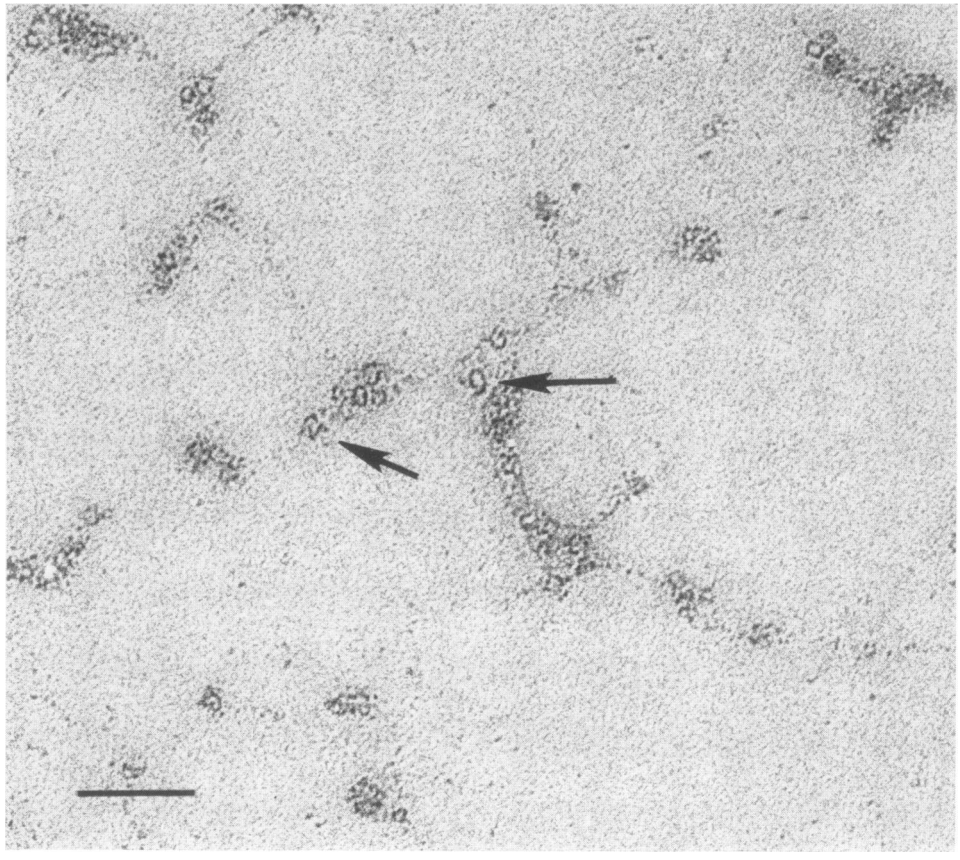


FIG. 3 and 4. *Wild-strain lipopolysaccharide from Salmonella typhimurium heated at 37 C for 3 hr in 0.25 N NaOH, neutralized, and stained with uranyl acetate. The excess uranyl acetate was removed by dialysis.* × *320,000. Bar = 50 nm.*

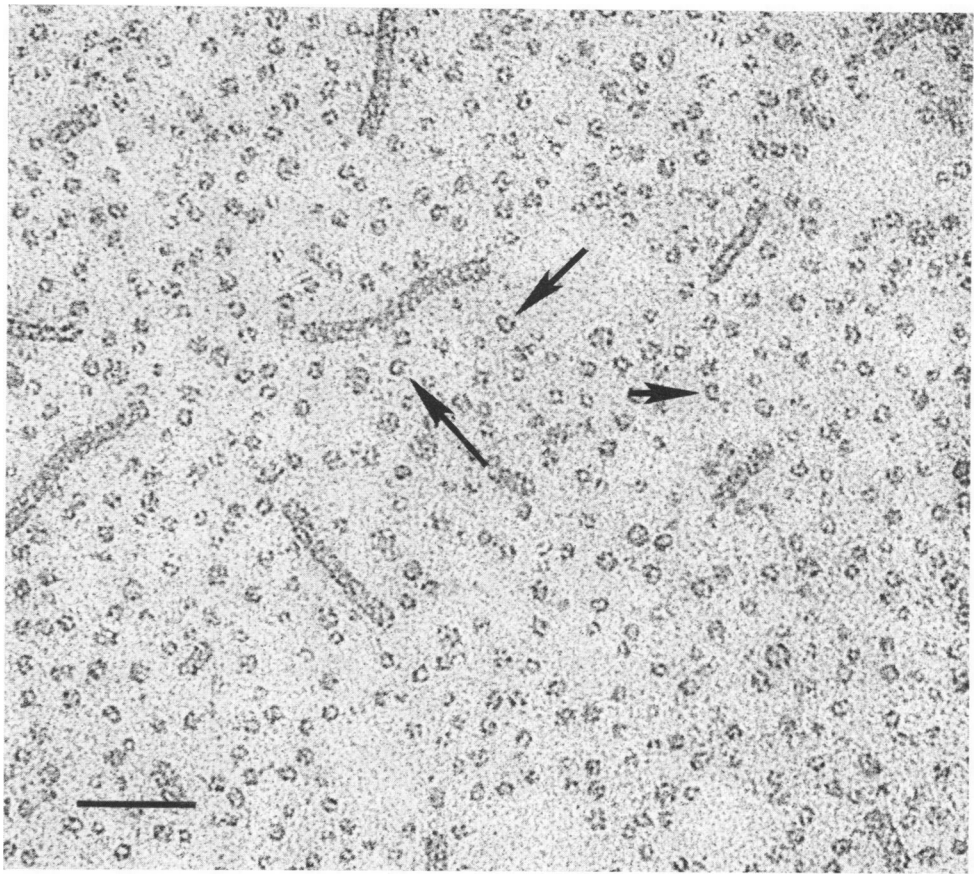


FIG. 5. *Wild-strain lipopolysaccharide from Salmonella typhimurium treated for 30 min at 65 C in alkaline hydroxylamine by the method of Niwa et al. (7) and stained with uranyl acetate. The excess uranyl acetate was removed by dialysis. × 320,000. Bar = 50 nm.*

sufficient degree to cause loss of toxicity also causes changes in the physical structure of LPS. Tripodi and Nowotny (14) used light-scattering techniques to show that the inactivation of *Serratia marcescens* LPS by alkaline hydrolysis was accompanied by an increase in dissymmetry and therefore a conformational change in the particles. Our morphological data are consistent with this observation.

One might speculate as to whether such a change in physical structure is related to the loss of toxicity and, if so, why? Neter et al. (6) have previously reported the modification of biological activity of LPS resulting from alkaline hydrolysis. Such treatment, on one hand, increased the capacity of LPS to bind to erythrocytes and, on the other, diminished its toxicity and pyrogenicity. We have made similar observations on the toxicity of 0.25 N NaOH-treated LPS and hydroxylamine-treated LPS (Ciznar and Shands, *unpublished data*). There are reasonable explanations for the increased capacity to bind to cells First, the LPS particles are much reduced in size by this treatment and, therefore, would be much less susceptible to removal from cell surfaces by shear forces. Secondly, in the current model of LPS structure, the particles consist of bilayers in which the hydrophobic fatty acids are, more or less, covered by the hydrophilic polysaccharide. The attachment of LPS to membranes is dependent on fatty acids and may involve the penetration of LPS fatty acids into membrane lipids (2). Assuming this to be true then the bilayer LPS particle with its "buried" amide and ester-linked fatty acids may present fewer reactive groups than the alkali-treated product, a monolayer, which still contains some amide-linked fatty acids (~3% by weight in the product used in these experiments).

Why this material is nontoxic is not clear, particularly since data are being accumulated suggesting that the toxicity of LPS is, in part,

due to its interaction with membranes (1, 3, 4, 13). A possible explanation for this paradox of increased membrane affinity and decreased toxicity involves the participation of host detoxification systems. It is well known that various components of serum bind to and diminish the toxicity of LPS (11, 12). Also serum inhibits in vitro sensitization of cells by LPS (5, 8). If the lipid of LPS is the important reactive moiety of the particle, then either complete removal of this moiety or covering it by serum components should reduce toxicity. In either case, the alkali-treated product with its accessible and diminished quantity of lipid should be much more susceptible to these systems than the bilayer with its relatively inaccessible lipid.

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