INFECTION AND IMMUNITY, Oct. 1971, p. 362-367
Vol. 4, No. 4

Printed in U.S.A.

Effect of Alkali-Treated Lipopolysaccharide on Erythrocyte Membrane Stability

I. ČIŽNÁR[1] AND J. W. SHANDS, JR.

Department of Immunology and Medical Microbiology, University of Florida College of Medicine, Gainesville, Florida 32601

Received for publication 20 May 1971

The interaction of various lipopolysaccharides (LPS) with sheep erythrocytes was studied. When subjected to mild alkaline hydrolysis, the affinity of LPS for the red cell surface was greatly increased, as others have reported. In addition, excessive quantities of alkali-treated LPS (but not parent or heated products) were found to cause hemolysis of red cells. Experiments indicated that the hemolysis was caused by the LPS particles themselves and not by liberated free fatty acids.

The adsorption of gram-negative lipopolysaccharides (LPS) onto the surface of erythrocytes provides a convenient procedure for the detection and titering of antibodies made in response to a particular somatic antigen (8, 9). This affinity for cell membranes extends to other types of cells as well, including polymorphonuclear leukocytes (5), monocytes, platelets, and even L cells (Shands, *unpublished data*). The ability of LPS to associate with cell surfaces is a property which is apparently conferred by the lipid moiety of LPS, lipid A (6). The polysaccharide moiety of LPS, when freed of lipid by acid hydrolysis, does not possess this property (6). Interestingly, mild alkaline hydrolysis of LPS markedly enhances its affinity for cell surfaces (10). This treatment, which cleaves the ester-linked fatty acids, but not those that are amide-linked, reduces the size and alters the conformation of LPS particles (13; Shands, *submitted for publication*).

Our interest in the association of LPS with mammalian cells resides in the possibility that this represents the initial step in some of the biological effects elicited by LPS. The observations presented in this paper, which show that alkali-treated LPS produces membrane instabilities in sheep erythrocytes, may be pertinent to some of the activities of LPS.

MATERIALS AND METHODS

Bacteria. *Salmonella typhimurium* 7 was obtained from M. Herzberg (Univ. of Hawaii). *S. enteritidis* SR 11 was obtained from K. Saito (Keio Univ. Medical School, Tokyo). The bacteria were grown in glucose-mineral salts medium (1) supplemented with 0.1% Casamino Acids (Difco) at 37 C for 24 hr. Formalin was then added to 0.2% by volume, and after 2 hr the bacteria were harvested by centrifugation and washed. Ra and Re mutants of *S. typhimurium*, strains TV 119 and SL 1102, were obtained from B. Stocker (Stanford University, Palo Alto, Calif.) and were grown in Brain Heart Infusion medium (Difco).

Lipopolysaccharides. These were extracted from the bacteria by the phenol-water procedure (14). One sample of LPS was prepared from *S. typhimurium* 7 by the aqueous-ether procedure (11) by using unfixed bacteria. One phenol-water extract of *S. typhimurium* was obtained from H. Baer (Univ. of Florida). The absence of contaminating ribonucleic acid (RNA) was confirmed by ultraviolet (UV) absorption spectra. Biosynthetically labeled ^{14}C-LPS was prepared by a previously described procedure by using ^{14}C-glactose and a uridine diphosphate-gal-4 epimeraseless mutant of *S. typhimurium* (3). ^{51}Cr labeling of LPS was performed by the method described by Braude et al. (2).

Alkaline hydrolysis of LPS. LPS was solubilized in distilled water, and an equal volume of 0.5 N NaOH was added. After incubation at 37 C for 3 hr, the solution was neutralized with 0.25 N HCl. Those preparations which were ^{51}Cr-labeled were subsequently dialyzed for 3 days against distilled water and for 1 day against isotonic saline to remove free ^{51}Cr liberated by the procedure. Approximately one-third of the bound ^{51}Cr was released by treatment with alkali.

Sensitization of sheep erythrocytes. Fresh sheep erythrocytes were pelleted at 700 × *g* and washed three times with isotonic saline. The final suspension was made up to 1% in isotonic saline (2.8×10^8 cells/ml). Various amounts of LPS were added to 5-ml samples of the erythrocyte suspension. After 1 hr of incubation at 37 C, the cells were centrifuged at 700 × *g* and washed three times with saline to remove excess LPS. ^{51}Cr-labeled cells were counted in a gamma counter, model 530 (Baird Atomic). ^{14}C-

[1] Present address: Research Institute of Epidemiology and Microbiology, Sasinkova, Bratislava, Czechoslovakia.

labeled cells were lysed in 0.015 N NaCl. The stromata were pelleted at 30,000 × *g* for 1 hr and dissolved in 0.25 ml of Soluene (Packard Instrument Co.). After addition of 10 ml of scintillation fluid, the radioactivity was measured in a Packard liquid scintillation spectrometer.

Stability of sheep erythrocytes. The stability of erythrocytes was determined as follows. Five-milliliter samples of a 1% suspension of cells were incubated at 37 C for 1 hr with various quantities of LPS. After 1 hr, the cells were pelleted at 700 × *g* and resuspended to a 1% concentration in isotonic saline. The cells were kept at 4 C overnight, and the amount of hemoglobin released into the supernatant fluid was measured by optical density at 412 nm. The per cent hemolysis was based on a comparison with a sample of red blood cells hemolyzed with distilled water.

Isotopes. ^{51}Cr was purchased as sodium chromate (specific activity 185 mCi/mg) from New England Nuclear Corp. The scintillation fluid used consisted of 6.5 g of 2,5-diphenyloxazole, 130 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene, and 104 g of naphthalene in a mixture of 500 ml of toluene, 500 ml of dioxane, and 300 ml of methanol.

RESULTS

Table 1 shows quantitative aspects of the association of intrinsically ^{14}C-labeled LPS with erythrocytes. In this experiment, alkali-treated LPS and LPS heated at 100 C for 1 hr were compared with the parent product. It is apparent that heating the product, which reportedly increases the affinity of LPS for cells (10), had little effect, whereas the alkali treatment increased the binding to erythrocytes by 20-fold. Also, saturation of the system by using parent or heated LPS seemed to occur at a concentration of 80 μg/ml, and a further increase in the amount of LPS added caused no further increase in the amount bound to cells. Unfortunately, the data in this experiment were insufficient to detect a saturation dose with the alkali-treated LPS.

In subsequent experiments, we observed that, when one increased the LPS levels above those listed in Table 1, hemolysis of the erythrocytes often resulted, but only with the use of alkali-treated LPS, never with the heated or parent product. Figure 1 shows the extent of hemolysis versus time when using an alkali-treated, phenol-water extract of *S. enteritidis* at three concentrations: 100, 300, and 500 μg per ml. These data show that the phenomenon is dose-dependent and also that it is not a rapid one but requires 15 to 24 hr for completion. Figure 2 shows the effect of different temperatures on the reaction. In each case, sensitization of the erythrocytes was performed at 37 C, and, subsequently, the cells were kept at either 4, 22, or 37 C. Hemolysis occurred at each temperature and seemed to be greater at the higher temperatures. Any interpretation of this latter phenomenon, however, is complicated by the probability that the different incubations led to variations in the relative concentrations of oxyhemoglobin, methemoglobin, etc., which would be reflected in changes in the optical density. In all subsequent experiments, 4 C was the temperature chosen for convenience.

Different LPS preparations were assayed to see whether the hemolytic effect was a general property of alkali-treated LPS. Figure 3 shows results with four different preparations: (A) an aqueous-ether extract of *S. typhimurium*, (B) a phenol-water extract of *S. typhimurium* obtained from H. Baer, (Univ. of Florida), (C) a phenol-water extract of *S. enteritidis*, and (D) a phenol-water extract of *S. typhimurium*. It is evident that some hemolytic activity was possessed by each product, but the potency was variable. B had consistently low hemolytic activity, whereas C and D had consistently high activity. The activities of LPS from various mutant strains of *S. typhimurium* are compared to those of two wild strains of *Salmonella* (Table 2). Both the Ra and Re chemotype LPS when treated with alkali were hemolytic but possessed less activity than the complete LPS.

TABLE 1. *Quantitative aspects of attachment of lipopolysaccharide (LPS) to erythrocytes*

Amt LPS used to sensitize RBC[a]		Amt LPS attached to stroma from 2.8 × 10^8 RBC					
		NaOH-LPS[b]		Δ-LPS[c]		Parent LPS	
Counts/min	μg	Counts/min	μg	Counts/min	μg	Counts/min	μg
15,000	20	450	0.56	41	0.05	21	0.03
31,200	40	1,766	2.3	140	0.18	90	0.11
62,400	80			284	0.36	180	0.22
93,600	120	3,172	4.1	256	0.35	181	0.22

[a] Amount of LPS added per milliliter containing 2.8 × 10^8 red blood cells (RBC).
[b] LPS treated with NaOH as in text.
[c] LPS heated at 100 C for 1 hr.

An important question posed at this point was whether the hemolytic activity was a property of the LPS particle or whether the alkaline hydrolysis liberated some low-molecular-weight hemolysin. For instance, free fatty acids in sufficient concentration are hemolytic, and these could be responsible for the effect observed. Several experiments were performed to test this possibility. First, after alkaline hydrolysis, dialysis against water or isotonic phosphate-buffered saline (*p*H 7.4) did not decrease the hemolytic activity. Second, repeated extraction with chloroform and petroleum ether did not alter the hemolytic activity, and third, when fractionated on Sephadex G-200, both the hemolytic and antigenic activity were recovered in the void volume. These data indicate that free fatty acids were not responsible for the hemolytic activity nor was some unknown low-molecular-weight substance responsible for

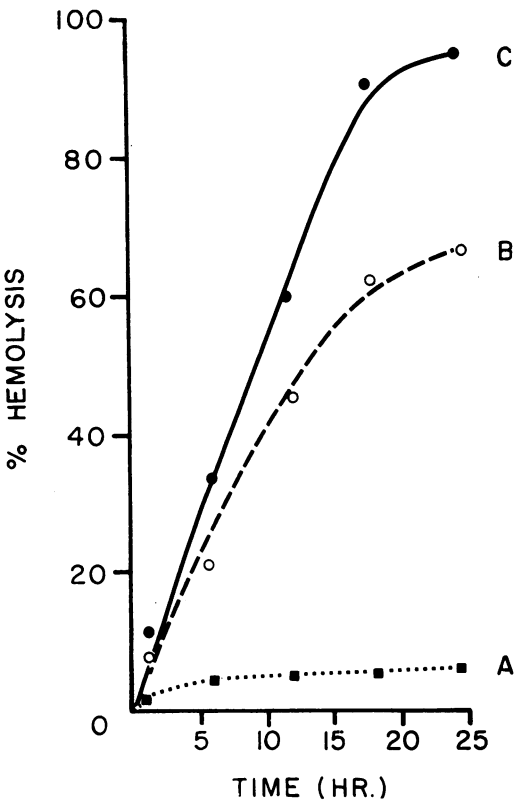

FIG. 1. *Extent of hemolysis versus time. A, B, and C cells were sensitized with 100 μg, 300 mg, and 500 μg of LPS per ml, respectively.*

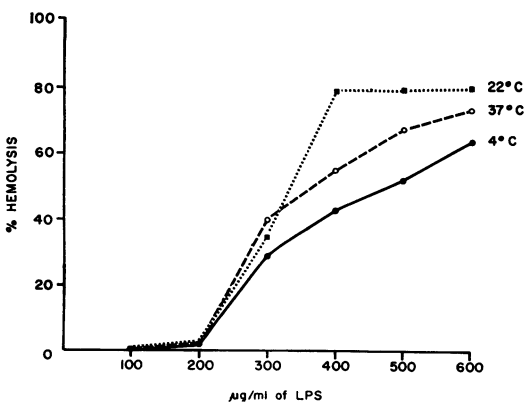

FIG. 2. *Effect of temperature and concentration of alkali-treated LPS on the extent of hemolysis.*

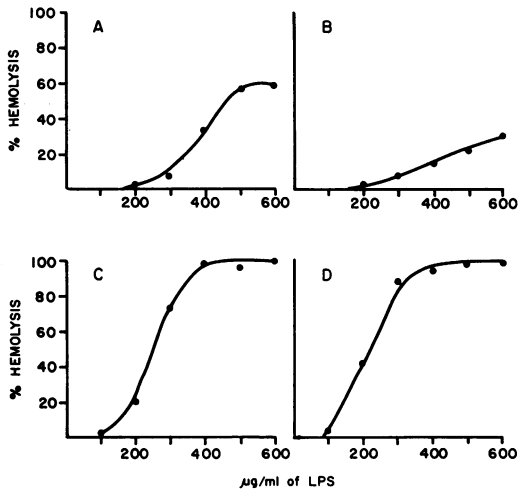

FIG. 3. *Hemolytic activity of four different preparations of alkali-treated LPS: (A) an aqueous-ether extract of S. typhimurium, (B) a phenol-water extract of S. typhimurium obtained from H. Baer, (C) a phenol-water extract of S. enteritidis, and (D) a phenol-water extract of S. typhimurium.*

TABLE 2. *Hemolytic activity of different types of lipopolysaccharides (LPS)*

LPS from[a]	Structure of LPS	Per cent hemolysis
Salmonella enteritidis	Complete	98
S. typhimurium 7	Complete	94
S. typhimurium TV119	No O side chains (Ra)	43
S. typhimurium SL1102	KDO only polysaccharide (Re)	60

[a] For each preparation, 400 μg of alkali-treated LPS was added per milliliter containing 2.8×10^8 red blood cells.

TABLE 3. *Attachment of lipopolysaccharide (LPS) to erythrocytes*

Prepn B		Prepn C	
Amt LPS used to sensitize RBC[a] (μg)	Amt LPS attached to 4.8×10^8 RBC (μg)	Amt LPS used to sensitize RBC[b] (μg)	Amt LPS attached to 2.8×10^8 RBC (μg)
70	1.4	30	1.5
140	1.5	60	2.8
210	2.0	120	5.2
280	2.6	180	9.3
350	3.0	240	9.8
		300	11.2
		360	12.8

[a] Amount of alkali-treated LPS added per milliliter containing 4.8×10^8 red blood cells (RBC).
[b] Amount of alkali-treated LPS added per milliliter containing 2.8×10^8 RBC.

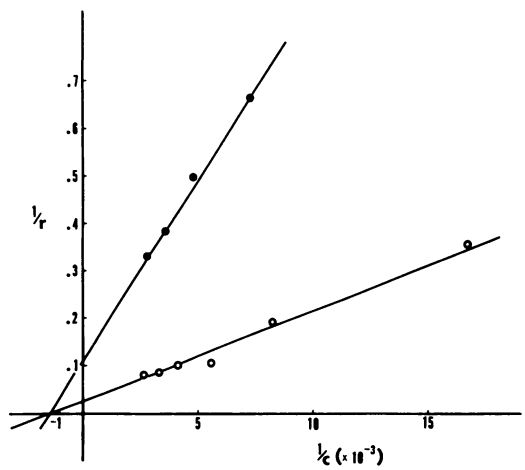

FIG. 4. *A double-reciprocal plot of amount of alkali-treated LPS bound versus concentration, c = micrograms of LPS per milliliter; r = micrograms of LPS bound per 2.8×10^8 red blood cells (preparation C) or by 4.8×10^8 red blood cells (preparation B). Closed circles, preparation C; open circles, preparation B.*

the activity. It appeared that hemolysis was caused by the LPS particle itself.

An effort was made to explain the difference in the hemolytic activities of two of the LPS preparations. Preparation B, with low hemolytic activity and preparation C, with high activity, both labeled with ^{51}Cr, were compared in their ability to bind to the surfaces of sheep erythrocytes. Table 3 shows the results after sodium hydroxide treatment. When compared to the values for preparation C, the extent of binding of B was roughly one fourth to one eighth of that possessed by C when corrections were made for the number of cells. A double reciprocal plot of these data is depicted in Fig. 4. By extrapolation, the amount of preparation C bound at infinite concentration was 40 μg per 2.8×10^8 cells. Assuming a molecular weight of 200,000 for the alkali-treated product, this is equivalent to approximately 4×10^5 particles per cell. In contrast, only 9 μg of preparation B would be bound by 4.8×10^8 cells under the same conditions. Preparation B was, therefore, approximately 7.5-fold less active in binding. This difference is compatible with the differences in hemolytic activity. Of interest is the observation that the K (equilibrium) for both preparations was ca. 7×10^2. The fact that this constant was the same for both preparations indicates that the interacting particles of both preparations were behaving in a similar fashion and is evidence against a qualitative difference.

Efforts to modulate the hemolytic effect of LPS included the addition of glucose and the addition of hydrocortisone. Since the red cells were incubated overnight in saline, it was thought that perhaps some energy substrate would allow the cells to maintain their integrity although the metabolism should be almost negligible at 4 C. The effect of glucose addition is shown in Fig. 5. Increasing quantities of glucose did inhibit hemolysis, but good protection was provided only at concentrations well above that needed for energy requirements. Since cortisone is thought to stabilize membranes and was found to stabilize phospholipase C-treated sheep erythrocytes (4),

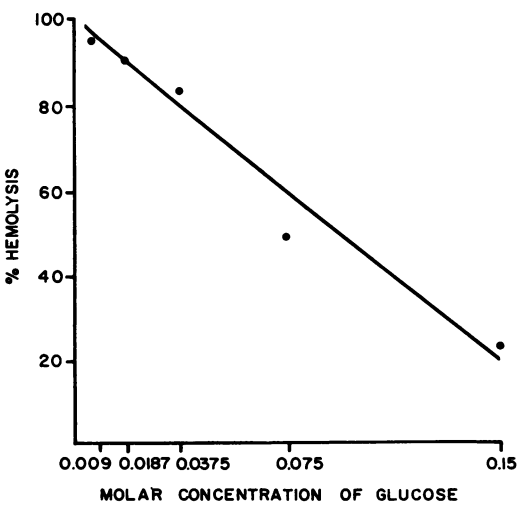

FIG. 5. *Effect of increasing glucose concentrations upon hemolysis of sheep erythrocytes by alkali-treated LPS from S. enteritidis (preparation C). The concentration of LPS used was 2,500 μg per ml.*

TABLE 4. *Effect of cortisone on lytic activity of lipopolysaccharide (LPS)*

Amt LPS used to sensitize RBC[a] (μg)	Per cent hemolysis	
	Normal RBC	HSS[b]-treated RBC
100	8	10
200	42	56
300	73	75
400	89	95
500	92	97

[a] Amount of preparation C added to 2.8×10^8 red blood cells (RBC) in 1 ml.
[b] HSS, hydrocortisone.

hydrocortisone (Solucortef, Upjohn) was added to the system at a concentration of 1 μg per ml before the addition of LPS and during the incubation for 1 hr with increasing quantities of LPS. The cells were then pelleted, resuspended in saline, and held at 4 C for 24 hr. The results shown in Table 4 show that the cortisone exerted no protective effect.

DISCUSSION

It has been known for some time that mild alkaline hydrolysis of gram-negative LPS results in an increased affinity of this material for the surface of erythrocytes (10). The quantitative aspects of this association, first described by Lüderitz et al. (7), were confirmed in these experiments. Alkali treatment of LPS resulted in a 20-fold increase in its ability to sensitize erythrocytes, whereas boiling led to a twofold or less increase. In terms of the number of particles attached to erythrocytes, our data are also in agreement with those of Lüderitz et al. (7). Although in our experiments a saturating quantity of LPS was not used, the most active preparation (Table 3, preparation C) sensitized each erythrocyte with an average of approximately 10^5 particles (assuming a 200,000 molecular weight), and this is the maximum figure quoted by Lüderitz et al. (7). Furthermore, it appears that the extent of sensitization of erythrocytes can be adequately measured by ^{51}Cr-labeled LPS since the results with ^{51}Cr label and ^{14}C intrinsic label were similar (Table 1, Table 3, preparation C).

When added in sufficient amounts to fresh sheep erythrocytes, alkali-treated LPS was found to possess hemolytic activity. Microscopic observation of the cells soon after sensitization revealed an alteration of cell morphology from a biconcave disc to a sphere, and when these cells were held in saline hemolysis occurred. Although there was variation in activity, all of the LPS preparations tested possessed some hemolytic activity, including those prepared from smooth strains and those from two rough mutants. The data indicated that the LPS particles per se were causing the hemolysis and not some unknown low-molecular-weight material or free fatty acids liberated by hydrolysis.

Although it is certainly not a proven fact, the data also suggest that hemolysis is related to the number of LPS particles which attach to the erythrocyte surface. Parent and boiled LPS preparations possessed little or no hemolytic activity and possessed very limited ability to adhere to the erythrocyte surface (Table 1). Also, the one alkali-treated product which was poorly hemolytic (preparation B) possessed one-fourth to one-eighth the sensitizing activity of the highly hemolytic preparation C. In part the low activity of preparation B might be explained by its high content of haptenic, low-molecular-weight material. One-third of the preparation failed to sediment at $100,000 \times g$ for 3.5 hr. In the analytical ultracentrifuge, this material had a S_{20w} of 2.5. Such haptenic material was not found in the other preparations.

Alkali-treated LPS, therefore, behaves in a fashion similar to *O*-stearoyl polysaccharides. Hämmerling and Westphal (6) found that the coupling of long-chain fatty acids to polysaccharides allowed their attachment to erythrocytes for passive hemagglutination. When used in excessive amounts, these *O*-steroyl derivatives deformed and lysed red blood cells.

The type of injury caused by LPS which leads to hemolysis is not known. The addition of exogenous glucose protected the red cells only in concentrations well above those needed for a source of energy and may reflect osmotic stabilization. This point needs further investigation. Of interest is a report by Tenney and Rafter (12) demonstrating an endotoxin-mediated inhibition of leukocyte adenosine triphosphatase, and it may be that endotoxin also affects the sodium and potassium ion pump in the red cell. An analysis of ion fluxes would also allow a more subtle injury to be detected, and with this technique it is possible that alterations induced by heated and parent LPS will be found.

Does the membrane effect we have observed have anything to do with the toxicity of LPS? At the moment it appears unlikely since the amount of LPS required for this effect is far greater than would occur in vivo, nor have we been able to detect hemolysis by unaltered LPS. Therefore, unless we can find a cell system with much greater sensitivity to LPS, what we have observed may simply represent a laboratory artifact. The results, however, do indicate that the interaction of LPS with the erythrocyte membrane is more

than just a surface adsorption. The fact that membrane instabilities are produced indicates that the attachment of LPS causes membrane disorganization. What forces are involved in the production of this disorganization are not known, but they very likely involve hydrophobic interactions with intermixing of LPS fatty acids with membrane lipids. The participation of charge-charge interactions cannot be dismissed, however. Gimber and Rafter (5) have proposed a charge-charge interaction between LPS and the leukocyte surface, and such interactions may play some role in the interaction between LPS and erythrocytes.

LITERATURE CITED

1. Anderson, E. H. 1946. Growth requirements of virus-resistant mutants of *Escherichia coli* strain "B". Proc. Nat. Acad. Sci. U.S.A. **32**:120–128.
2. Braude, A. I., F. S. Carey, D. Sutherland, and M. Zalesky. 1955. Studies with radioactive endotoxin. I. The use of ^{51}Cr to label endotoxin from *E. coli*. J. Lab. Clin. Invest. **34**:850–857.
3. Čižnár, I., and J. W. Shands, Jr. 1970. Effect of alkali on the immunological reactivity of lipopolysaccharide from *Salmonella typhimurium*. Infec. Immun. **2**:549–555.
4. Eaglstein, W. H., H. S. Reasot, and S. L. Hsia. 1969. Membrane stabilization: effect of hydrocortisone sodium succinate on phospholipase C treated sheep erythrocytes. Proc. Soc. Exp. Biol. Med. **132**:919–923.
5. Gimber, P. E., and G. W. Rafter. 1969. The interaction of *Escherichia coli* endotoxin with leukocytes. Arch. Biochem. Biophys. **135**:14–20.
6. Hämmerling, U., and O. Westphal. 1967. Synthesis and use of *O*-stearoyl polysaccharides in passive hemagglutination and hemolysis. Eur. J. Biochem. **1**:46–50.
7. Lüderitz, O., O. Westphal, K. Sievers, E. Kröger, E. Neter, and O. H. Braun. 1958. Uber die fixation von P^{32}-markiertem lipopolysaccharide (endotoxin) aus *Escherichia coli* an menschlichen erythrocyten. Biochem. Z. **330**:34–36.
8. Neter, E. 1956. Bacterial hemagglutination and hemolysis. Bacteriol. Rev. **20**:166–188.
9. Neter, E., O. Westphal, O. Lüderitz, and E. A. Gorzynski. 1956. The bacterial hemagglutination test for the demonstration of antibodies to *Enterobacteriaceae*. Ann. N. Y. Acad. Sci. **66**:141–156.
10. Neter, E., O. Westphal, O. Lüderitz, A. Gorzynski, and E. Eichenberger. 1956. Studies of enterobacterial lipopolysaccharide. Effects of heat and chemicals on erythrocyte-modifying, antigenic, toxic and pyrogenic properties. J. Immunol. **76**:377–385.
11. Ribi, E., K. C. Milner, and T. D. Perrine. 1959. Endotoxic and antigenic fractions from the cell wall of *Salmonella enteritidis*. Methods for separation and some biologic activities. J. Immunol. **82**:75–84.
12. Tenney, S. R., and G. W. Rafter. 1968. Leukocyte adenosine triphosphatases and the effect of endotoxin on their activity. Arch. Biochem. Biophys. **126**:53–58.
13. Tripodi, D., and A. Nowotny. 1966. Relation of structure to function in bacterial O-antigens. V. Nature of active sites in endotoxic lipopolysaccharides of *Serratia marcescens*. Ann. N. Y. Acad. Sci. **133**:604–621.
14. Westphal, O., O. Lüderitz, and F. Bister. 1952. Uber die Extraction von Bacterien mit Phenol/Wasser. Z. Naturforsch. **7b**:148–155.