INFECTION AND IMMUNITY, July 1975, p. 187-192
Vol. 12, No. 1
Copyright © 1975 American Society for Microbiology
Printed in U.S.A.

Partial Purification and Properties of Somatic Antigen Spontaneously Released from *Vibrio cholerae*

ROBERT M. PIKE* AND CLEO H. CHANDLER

Department of Microbiology, The University of Texas Southwestern Medical School, Dallas, Texas 75235

Received for publication 4 April 1975

The supernatant fluids from cultures of *Vibrio cholerae* grown for 40 h in a dialyzable medium were dialyzed, concentrated, and fractionated on agarose columns. The fractions containing most of the antigen which inhibited the vibriocidal activity of homologous antiserum were pooled, dialyzed, and concentrated to provide material with about 100 times the specific activity of the original culture supernatant. This material, containing 12 to 20% protein, 11 to 19% carbohydrate, and about 16% unbound lipid, had a mean lethal dose for mice of about 500 μg. This partially purified antigen absorbed all the vibriocidal antibody from homologous antiserum against live cultures and produced a single line of precipitation in gel diffusion tests with the same antiserum.

Significant amounts of heat-stable somatic antigen have been demonstrated in filtrates of broth cultures of *Vibrio cholerae* (6-8, 17, 20). Evidence that the antigen is present in the culture fluid primarily as a result of spontaneous release rather than as a consequence of bacterial lysis has recently been presented (17). The elaboration of relatively large amounts of antigen in a dialyzable culture medium offers an opportunity to separate the antigen from the other constituents of culture filtrates without the use of chemical extraction methods which have conventionally been employed for the preparation of bacterial endotoxins (13). This report describes some of the serological and chemical properties of antigenic material obtained from culture filtrates by procedures which resulted in a 100-fold increase in specific activity.

MATERIALS AND METHODS

Cultures. *V. cholerae* Ogawa strains NIH 41 and 17 (El Tor) and Inaba strains NIH $35A_3$ and V86 (El Tor) were used in this study. The sources of these strains, their maintenance, and the method of cultivation in Syncase broth (5) have previously been described (17).

Immune sera and immunoglobulin preparations. The preparation of hyperimmune rabbit sera against live organisms and the separation of immunoglobulin G- and immunoglobulin M-containing fractions were done as described previously (16).

Vibriocidal antibody titration and inhibition. The methods for determining the 50% vibriocidal dose of serum or of an immunoglobulin preparation and for determining the 50% vibriocidal antibody inhibiting dose of antigen were described in a previous publication (17). In this study, immunoglobulin G fractions of homologous antisera against live vibrios were used in the determination of the 50% vibriocidal antibody inhibiting dose of antigen.

Precipitin reaction. Gel double diffusion was done as previously described (17).

Immunoelectrophoresis. The gel consisted of 0.8% agarose in 0.1 M tris(hydroxymethyl)aminomethane buffer at pH 8.6. Electrophoresis was performed with LKB 6800A equipment (LKB Produkter AB, Stockholm, Sweden) at 250 V for 1 h.

Fractionation of culture supernatants. Cultures grown in Syncase broth for 40 h were processed as outlined in Fig. 1. Gel filtration on Bio-Gel agarose A-5m (Bio-Rad Laboratories, Richmond, Calif.) was performed in a column (2.5 by 90 mm) with an upward flow rate of 18 ml per h. Four-milliliter fractions were eluted with a buffer containing 0.0013 M ethylenedinitrilotetraacetic acid, 0.05 M tris(hydroxymethyl)aminomethane, 0.2 M sodium chloride, and 0.003 M sodium azide, adjusted to a pH 7.5 with hydrochloric acid. The optical density of the eluate was monitored at 280 nm with an Isco UA-2 ultraviolet analyzer (Instrumentation Specialities Co., Lincoln, Neb.).

Chemical analyses. Protein was determined according to Lowry et al. (12), using bovine serum albumin as a standard. Carbohydrate was estimated as glucose by the phenol-sulfuric acid method (4). The ratio of optical density at 260 nm to that at 280 nm was used as an indication of the nucleic acid content. Lipid, extractable without hydrolysis, was determined by chloroform-methanol extraction as described by Knox et al. (10).

Phenol-water extraction of antigen. Bacteria, dried from acetone, were extracted as described by Staub (19). After exhaustive dialysis of the aqueous phase, 100 μg of deoxyribonuclease, 100 μg of ribonuclease, and 0.2 ml of 0.15 M magnesium sulfate were added for each milliliter of highly viscous extract. After incubation at 37 C for 30 min, the extract, which

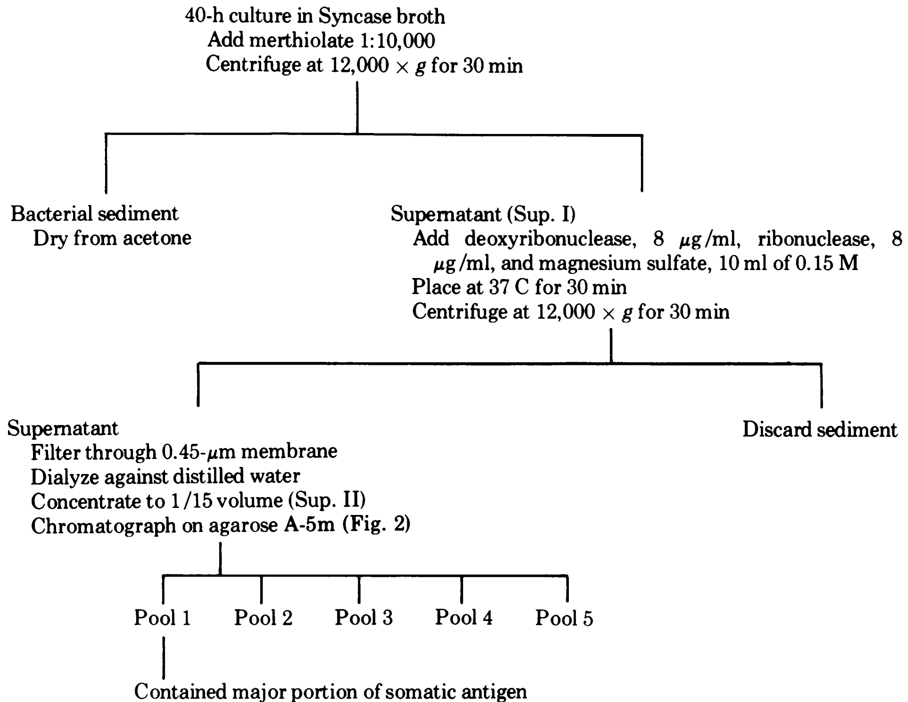

FIG. 1. *Isolation of fraction containing somatic antigen from V. cholerae broth culture.*

was no longer noticeably viscous, was centrifuged at 12,000 × g for 30 min, filtered through a 0.45-μm membrane, dialyzed again, and chromatographed on Bio-Gel A-5m. Fractions containing antigen detectable by gel diffusion were pooled, dialyzed against distilled water, and lyophilized.

Mouse toxicity. Female Swiss mice, 18 to 20 g (Texas Inbred Mice, Inc., Houston, Tex.) were inoculated intraperitoneally with graded doses of antigen preparation in 0.5 ml. Six mice received each dose in a series of fourfold dilutions. The amount of antigen killing half the mice in 48 h (mean lethal dose [LD_{50}]) was estimated by the method of Reed and Muench (18).

Absorption of antisera. One milliliter of antiserum diluted with 4 ml of saline was added to a tube containing 1 mg of lyophilized antigen. After 30 min at 37 C, the mixture was centrifuged at 34,000 × g for 30 min. A small portion of the supernatant was saved for testing and the remainder was added to a second milligram of antigen. The process was repeated until five absorptions were completed. The fifth absorption mixture was placed at 4 C overnight.

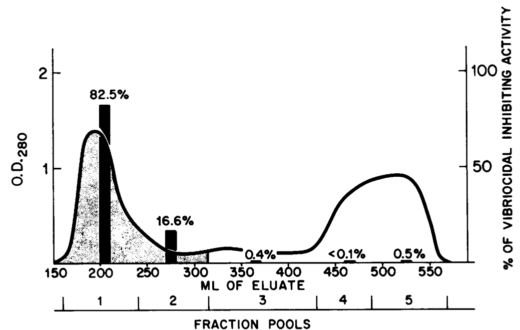

FIG. 2. *Elution of concentrated, enzyme-treated Ogawa 17 culture supernatant (Sup. II) from Bio-Gel A-5m showing optical density at 280 nm (OD_{280}) of eluate, solid line; location of somatic antigen by qualitative gel precipitation, shaded area; and % distribution of vibriocidal antibody inhibiting activity in fraction pools, columns.*

RESULTS

Separation of somatic antigen fractions from culture supernatants. Supernatant II (Fig. 1) contained all the nondialyzable constituents of the enzyme-treated culture supernatant fluids. When this material from strain 17 was chromatographed on agarose, the results shown in Fig. 2 were obtained. The other three strains gave similar elution patterns. The concentrated supernatants from the two El Tor strains were nearly colorless whereas those from classical strains were brownish. The brown pigment appeared in pool 5. Table 1 summarizes the analysis of the agarose pools from strain V86 as compared to the untreated culture supernatant (Sup. I) and the enzyme-treated, dialyzed, concentrated supernatant (Sup. II). Pool 1 represented less than 1% of the total

solids in the untreated supernatant but contained the major portion of the antigenic activity. The specific activity of pool 1 was about 100 times that of the original supernatant as measured both by inhibition of vibriocidal action and by precipitation with antiserum. Similar results were obtained with the other three strains.

Properties of the partially purified somatic antigen. Table 2 summarizes the results of analysis of pool 1 antigens from all four strains. The data reveal no consistent differences between Inaba and Ogawa strains. Although the ratios of the optical density at 260 nm to the optical density at 280 nm were all reduced as compared to Sup. I, the values greater than 1 for three preparations suggest that the nucleic acids were not entirely removed. Without the addition of the enzymes, particularly deoxyribonuclease, some of the preparations would have been too viscous to process. The toxicity of pool 1 material for mice was within the range reported for somatic antigens extracted from other gram-negative bacteria (15).

The amount of pool 1 antigen obtained from 600 ml of culture supernatant was almost as great as the amount of antigenic material extracted from the bacteria by the phenol-water method (Table 2). The serological activity of the two preparations was also comparable except that less phenol-extracted antigen seemed to be required for precipitation. Phenol-extracted antigens, however, were lower in protein and higher in carbohydrate content. Lyophilized phenol antigens were more difficult to suspend in saline, which may have accounted for their greater LD_{50}.

In gel diffusion, pool 1 antigens showed a single line of precipitation (Fig. 3). Ogawa and Inaba antigens reacted with heterologous anti-

TABLE 1. *Analysis and serological activity of V. cholerae, strain V86, culture supernatant, and chromatographic fractions of supernatant*

Prepn	Total dry wt (mg)	OD_{260}/OD_{280}[a]	Protein (%)	Carbohydrate (%)	VAI_{50}[b] (μg)	Precipitating titer (μg/ml)
Sup. I[c]	10,539	1.8			49.3[d]	2,460
Sup. II[e]	503	1.3	6.3	4.0	3.5	115
Agarose A-5m						
Pool 1	86	1.1	13.8	12.2	0.5	30
Pool 2	47	1.2	1.7	2.0	6.2	160
Pool 3	127	1.1	0.7	0.2	283	1,980
Pool 4	101	1.2	2.0	0.2	995	NR[f]
Pool 5	93	1.2	5.8	0.2	119	NR

[a] OD, Optical density at 260 and 280 nm.
[b] Amount of antigen required to give 50% inhibition of 10 mean vibriocidal doses of homologous immunoglobulin G.
[c] Crude culture supernatant.
[d] Determined on a sample of Sup. I after dialysis to remove merthiolate but expressed in terms of dry weight before dialysis.
[e] After treatment of Sup. I with deoxyribonuclease and ribonuclease, filtration, dialysis, and concentration.
[f] NR, no reaction.

TABLE 2. *Properties of pool 1 antigens compared with antigens prepared from V. cholerae by the phenol-water method*

Antigen	Strain	Dry wt from 600 ml of culture (mg)	OD_{260}/OD_{280}[a]	Protein (%)	Carbohydrate (%)	VAI_{50}[b] (μg)	Precipitating titer (μg/ml)	LD_{50} (μg)
Pool 1	NIH 41	78	1.3	15.7	19.0	0.6	34	234
	17	88	0.9	12.5	11.5	1.0	30	750
	NIH $35A_3$	58	1.6	20.0	17.7	1.1	47	660
	V86	86	1.1	13.8	12.2	0.5	30	300
Phenol	NIH 41	121	0.9	4.0	20.0	0.5	7	3,000
	NIH $35A_3$	77	1.3	3.6	30.3	0.3	12	4,200

[a] OD, Optical density at 260 and 280 nm.
[b] See footnote b, Table 1.

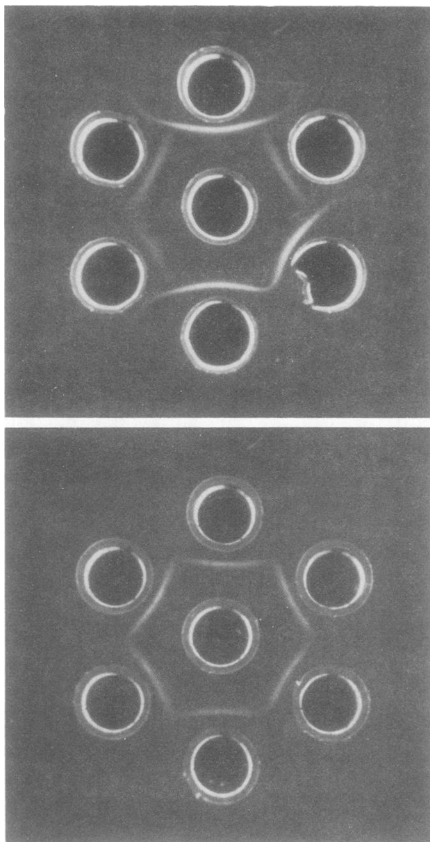

FIG. 3. *Reaction of pool 1 antigens with homologous and heterologous antisera. Top, center well, anti-Ogawa serum. Bottom, center well, anti-Inaba serum. Clockwise from the top (both plates), NIH 41, NIH 35A$_3$, 17, NIH 41, V86, NIH 35A$_3$. All antigens at a concentration of 1 mg/ml.*

sera showing reactions of partial identity. On immunoelectrophoresis (Fig. 4), the pool 1 antigens appeared to be relatively homogeneous moving slowly towards the anode. The reaction between Inaba antigen and anti-Ogawa serum was relatively weak as it was in double diffusion.

For the determination of unbound lipid a second batch of pool 1 antigen was prepared from strain NIH 41. The chloroform-soluble portion of the combined chloroform-methanol extracts amounted to 16% of pool 1 material and had little if any serological activity (Table 3). Removal of the methanol-chloroform-soluble portion increased the specific activity measured by precipitation and by inhibition of vibriocidal antibody, but the insoluble material was more difficult to suspend in water than the lyophilized pool 1 antigen.

Absorption of vibriocidal antibody. Although small amounts of pool 1 antigens could inhibit 10 mean vibriocidal doses of immunoglobulin G antibody, it remained to be determined whether or not the antigen could absorb all the vibriocidal antibody from whole serum. The results in Table 4 show that both Inaba and Ogawa antigen removed all detectable vibriocidal antibody from homologous antiserum. In parallel absorptions with phenol-extracted antigens, a small residue of vibriocidal antibody remained even after five absorptions.

DISCUSSION

Although a significant increase in the specific activity was achieved by the mild methods used, the pool 1 material is certainly not pure somatic antigen. The composition of the material, however, is similar to that of antigens isolated from the culture supernatants of other gram-negative bacteria. The material derived from rough *Escherichia coli* under lysine limiting conditions by Knox et al. (10) contained 11% protein, 7% glucose, and 26% lipid extractable without hydrolysis. The "free endotoxin" obtained from *E. coli* by Crutchley et al. (3) had a mouse LD_{50} of 241 μg. *V. cholerae* antigens prepared by methods entirely comparable to ours have not been reported but the highly purified mouse-protective antigen described by Watanabe and Verwey (20) had a lower protein (1.5% total nitrogen), a higher carbohydrate (60% glucose), and a mouse LD_{100} of 1,250 μg. The lipopolysaccharide extracted from *V. cholerae* by Neoh and Rowley (14) by the phenol-water method contained 10.4% protein and 8.6% carbohydrate.

Absorption experiments indicated that the pool 1 antigen could absorb all the vibriocidal antibody induced by immunization with live organisms whereas absorption with phenol-water-extracted antigen left a small portion of vibriocidal antibody in the serum. This coin-

TABLE 3. *Serological activity of fractions obtained from strain NIH 41 pool 1 antigen by treatment with methanol and chloroform*

Prepn	Amt (mg)	Precipitating titer (μg/ml)	VAI_{50}[a] (μg)
Pool 1 antigen	53.4	25	0.66
Methanol-chloroform insoluble	34.0	13	0.44
Chloroform soluble	8.8	> 6,800	> 1,000

[a] See footnote b, Table 1.

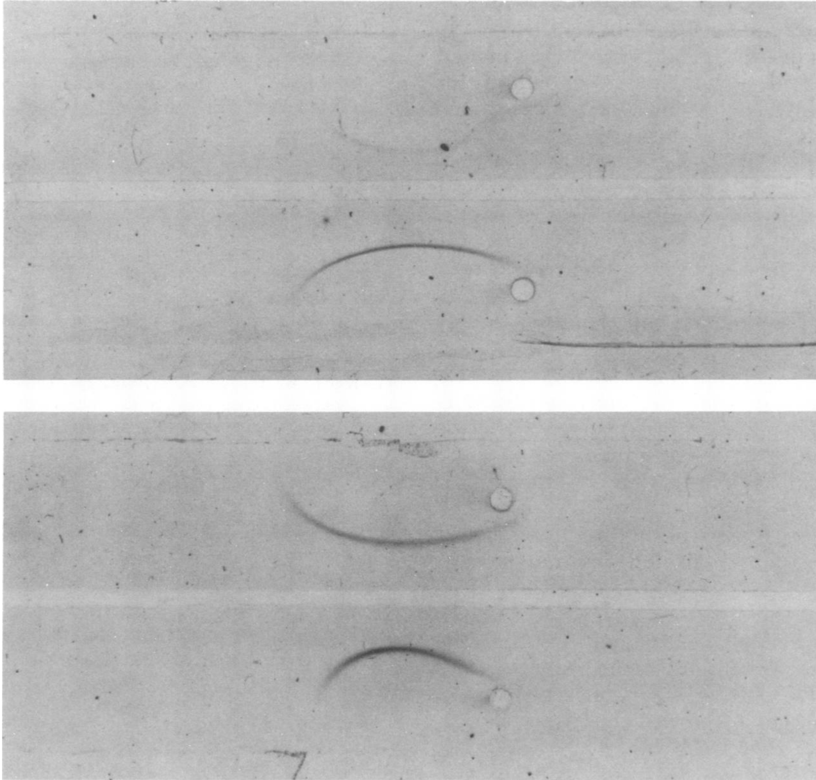

FIG. 4. *Immunoelectrophoresis of pool 1 antigens. Top and bottom wells, NIH 35A_3 antigen. Middle wells, NIH 41 antigen. Upper trough, anti-Ogawa serum. Lower trough, anti-Inaba serum. Anode is to the left.*

TABLE 4. *Absorption of anti-Ogawa and anti-Inaba sera with pool 1 and phenol antigens prepared from homologous serotypes*

Antiserum	Antigen used for absorption	No. of absorptions[a]	VD_{50}[b] (dilution)	Original titer remaining (%)
Anti-live NIH 41 (Ogawa)[c]	NIH 41 pool 1	0	4,550,000	
		1	50,000	1.10
		2	7,100	0.15
		3	1,600	0.03
		4	220	0.005
		5	<20	<0.0004
	NIH 41 phenol	5	66,000	1.45
Anti-live V86 (Inaba)[d]	V86 pool 1	0	1,580,000	
		1	435,000	27.61
		2	9,100	0.58
		3	1,470	0.09
		4	400	0.025
		5	<20	<0.001
	V86 phenol	5	34,500	2.18

[a] 1 ml of serum absorbed with 1 mg of antigen.
[b] Reciprocal of dilution of antiserum required to kill 50% of standard dose of vibrios.
[c] Tested with NIH 41.
[d] Tested with V86.

cides with the observation of Neoh and Rowley (14), who were unable to remove all the vibriocidal antibody by absorption with phenol-treated antigens, although Watanabe and Verwey (20) reported only about 0.1% of the original titer remaining after absorption with their antigen.

The manner in which somatic antigen is released into the culture fluid is a matter of some speculation. Our previous report (17) seemed to confirm the contention of Crutchley et al. (2) that cell lysis is not a prerequisite to antigen release from *E. coli*. Knox et al. (11) showed blebs of cell wall material on the surface of *E. coli* in electron photomicrographs from which they suggested the extracellular lipopolysaccharide originates. Similar saclike structures formed by bulging out and pinching off of cell wall material were seen on *V. cholerae* by Chatterjee and Das (1). Although differing in some respects from the above, extracellular material was observed during growth of *V. cholerae* by Kennedy and Richardson (9). It seems likely, therefore, whatever the true function of cell wall extrusions may be, that they account for extracellular somatic antigen in the absence of lysis although, under certain conditions and stages of growth, cell lysis can also result in antigen release.

LITERATURE CITED

1. Chatterjee, S. N., and J. Das. 1967. Electron microscopic observations on the excretion of cell-wall material by *Vibrio cholerae*. J. Gen. Microbiol. **49**:1-11.
2. Crutchley, M. J., D. G. Marsh, and J. Cameron. 1967. Free endotoxin. Nature (London) **214**:1052.
3. Crutchley, M. J., D. G. Marsh, and J. Cameron. 1968. Biologic studies on free endotoxin and a non-toxic material from culture supernatant fluids of *Escherichia coli* 078K80. J. Gen. Microbiol. **50**:413-420.
4. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350-356.
5. Finkelstein, R. A., P. Atthasampunna, M. Chulasamaya, and P. Charunmethee. 1966. Pathogenesis of experimental cholera: biologic activities of procholeragen A. J. Immunol. **96**:440-449.
6. Finkelstein, R. A., H. T. Norris, and N. K. Dutta. 1964. Pathogenesis of experimental cholera in infant rabbits. 1. Observations on the intraintestinal infection and experimental cholera produced with cell-free products. J. Infect. Dis. **114**:203-216.
7. Holmgren, J., I. Lönroth, and Ö. Ouchterlony. 1971. Immunochemical studies of two cholera toxin-containing standard culture filtrate preparations of *Vibrio cholerae*. Infect. Immun. **3**:747-755.
8. Kaur, J., and J. B. Shrivastav. 1964. Immunochemical studies in vibrio polysaccharides. Indian J. Med. Res. **52**:809-816.
9. Kennedy, J. R., and S. H. Richardson. 1969. Fine structure of *Vibrio cholerae* during toxin production. J. Bacteriol. **100**:1393-1401.
10. Knox, K. W., J. Cullen, and E. Work. 1967. An extracellular lipopolysaccharide-phospholipid-protein complex produced by *Escherichia coli* grown under lysine-limiting conditions. Biochem. J. **103**:192-201.
11. Knox, K. W., M. Vesk, and E. Work. 1966. Relation between excreted lipopolysaccharide complexes and surface structures of a lysine-limited culture of *Escherichia coli*. J. Bacteriol. **92**:1206-1217.
12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**:265-275.
13. Milner, K. C., J. A. Rudbach, and E. Ribi. 1971. General characteristics, p. 1-65. *In* G. Weinbaum, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. IV. Academic Press Inc., New York.
14. Neoh, S. H., and D. Rowley. 1970. The antigens of *Vibrio cholerae* involved in the vibriocidal action of antibody and complement. J. Infect. Dis. **121**:505-513.
15. Nowotny, A. M., S. Thomas, O. S. Duron, and A. Nowotny. 1963. Relation of structure to function in bacterial 0 antigens. I. Isolation methods. J. Bacteriol. **85**:418-426.
16. Pike, R. M., and C. H. Chandler. 1971. Serological properties of γG and γM antibodies to the somatic antigen of *Vibrio cholerae* during the course of immunization of rabbits. Infect. Immun. **3**:803-809.
17. Pike, R. M., and C. H. Chandler. 1974. The spontaneous release of somatic antigen from *Vibrio cholerae*. J. Gen. Microbiol. **81**:59-67.
18. Reed, L. J., and H. Muench. 1938. A simple method of estimating fity per cent endpoints. Am. J. Hyg. **27**:493-497.
19. Staub, A. M. 1967. Preparation of cell wall antigens from gram-negative bacteria, p. 28-34. *In* C. A. Williams and M. W. Chase (ed.), Methods in immunology and immunochemistry, vol. I. Academic Press Inc., New York.
20. Watanabe, Y., and W. F. Verwey. 1965. The preparation and properties of a purified mouse-protective lipopolysaccharide from the Ogawa subtype of the El Tor variety of *Vibrio cholerae*, p. 253-259. *In* O. A. Bushnell and C. S. Brookhyser (ed.), Proc. Cholera Res. Symp. Honolulu. U. S. Department of Health, Education and Welfare, Washington, D.C.