JENKIN, C. R. & ROWLEY, D. (1959). *J. gen. Microbiol.* 21, 191–202

Toxic Proteins from *Vibrio cholerae* and Water Vibrios which are Lethal for Mice

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SUMMARY: A toxic protein has been isolated from Inaba and Ogawa strains of *Vibrio cholerae* and from water vibrios by dissolving the bacteria in 2·5M-urea and subsequent fractional precipitation with ammonium sulphate. This toxic protein accounted for the major portion of the toxicity of whole organism. Immunological and chemical data suggest that the toxic protein is the protein component of the Boivin antigen. The lipopolysaccharide endotoxins of the strains examined were relatively non-toxic as compared with the lipopolysaccharides from other Gram-negative bacteria, though in other biological and chemical properties they resembled the general class.

There is a marked tendency on the part of many research workers in the cholera field to assume that the manifestations of the disease are caused by a specific toxin which is liberated from the bacteria during growth. The proof of this hypothesis is difficult for two reasons. First, there is little precise information about the toxins of *Vibrio cholerae*, and much confusion exists as to the chemical nature and similarity of those substances which have been described. The second difficulty is that it has not been possible to produce a disease resembling cholera in experimental animals; therefore it is difficult to test toxins for their ability to reproduce the signs of the natural disease.

Boivin & Mesrobian (1935) isolated a toxic antigenic complex from *Vibrio cholerae* by extraction with cold trichloroacetic acid and characterized it as a 'glucolipid'. Similar Boivin antigens have been extracted from many Gram-negative bacteria and have been shown to possess the 'O'-antigenic specificity of the parent organisms. It is not clear in the case of *V. cholerae* how completely this glucolipid complex could account for the toxicity of whole organisms. Bernard & Gallut (1945) confirmed the existence of a toxic Boivin-type antigen and reported a second small-molecular toxin which was later purified to some extent by Gallut & Grabar (1945). Burrows (1944) claimed to have extracted a highly toxic phospholipid from *V. cholerae* by using hot ethanol. This material contained, on occasion, 500 mouse LD₅₀ doses/mg. and, moreover, caused an increase in permeability to water of the small intestine of rabbits, when tested *in vitro*. This effect on permeability was abolished by using intestines from animals immunized with *V. cholerae*. Freter (1956) described the isolation of two toxic fractions from *V. cholerae*, one acid-soluble and the other insoluble in HCl at pH 3·5. The acid-insoluble material had a mouse LD₅₀ of approximately 500–750 µg. and appeared to be a lipoprotein; the acid-soluble toxic material had a mouse toxicity of

50–100 μ g. and on analysis resembled the Boivin type-antigen (4–6 % N; 1.5–2 % P).

At least five potentially different toxins have therefore been reported in the literature. Their relationship to each other is obscure and their quantitative importance in accounting for the overall toxicity of *V. cholerae* is unknown, since none of these authors gave any exact data on the percentage recovery of toxicity in their fractions.

In the present work an attempt has been made to repeat these earlier investigations in a quantitative manner. A new method by which a potent toxic protein can be extracted in good yield from vibrios is described. The immunological relationships between these various toxins will be described later.

METHODS

Organisms. Strains Ogawa 5596, Inaba 4693 and Hikojima 7270 were obtained from the National Collection of Type Cultures (Colindale, London), whilst strains Ogawa 161, 162, 163 and water vibrios 53, 54 and 55 were kindly supplied by Dr K. Bhaskaran of the Central Drug Institute, Lucknow, India.

Maintenance of cultures. All cultures were freeze-dried in 10 % (v/v) serum + 5 % (w/v) glucose in nutrient broth. One freeze-dried culture was used to inoculate 6 nutrient agar slopes for routine use. After growth the agar slopes were kept at 4° and no slope was opened on more than ten occasions. The Inaba and Ogawa strains were tested periodically against homologous antisera.

Growth and harvesting of the organisms. Organisms were grown for 48 hr. at 37° on nutrient agar (pH 8.6) and harvested in saline in an ultraviolet irradiated cabinet. The harvested suspension was passed through glass wool to eliminate pieces of agar.

Toxicity tests. For these tests mice weighing 18–20 g. were injected intraperitoneally. They were observed for 72 hr. and then discarded. LD 50 doses were calculated by the method of Reed & Muench (1938). A minimum of 5 mice were used at each dose level and for the more important materials such as the toxic protein, the total numbers of mice tested at each level amounted to more than fifty (Table 8).

Centrifugation. Centrifugation at 1200–5000 *g* was obtained in an M.S.E. 'Magnum' refrigerated centrifuge; at 6000–20,000 *g* in a Servall SS 2 ultracentrifuge; at 20,000–78,000 *g* in a Spinco ultracentrifuge.

Ultrasonic disintegration. This was carried out in a 2 kW. Mullard ultrasonic disintegrator type E. 7590 A with a frequency output of 25 kc./sec. In all cases the apparatus was used with maximum power output at maximum efficiency, which was found by trial and error to occur when the stainless steel cup contained 30–40 ml. suspension of bacteria at a concentration of 30 mg. dry wt./ml. This operation was conducted at 0°.

Chemical analyses. Nitrogen estimations were by the macro-Kjeldahl method, with the apparatus of Markham (1942) for the distillation.

Phosphorus determinations were made by a modification of the Fiske & SubbaRow (1925) method. *Total carbohydrate* was determined as described

by Seifter, Dayton, Novic & Muntz (1950). *Protein* was determined by the Folin-Ciocalteu reagent as described by Kabat & Mayer (1948) with bovine albumin as a standard. *Lipid A* was extracted as described by Westphal & Lüderitz (1954). *Methylpentoses*, *hexoses* and *pentoses* were determined by the methods of Dische & Shettles (1948) and Dische, Shettles & Osnos (1949). *Nucleic acid* was estimated by measuring the absorption of a solution containing 250 μ g. fraction/ml. by the Beckman u.v.-spectrophotometer at 260 m μ ., comparing with a yeast nucleic acid standard.

Electrophoresis. The conjugated toxic protein toxin was examined for purity by: (a) starch-gel electrophoresis by the method described by Smithies (1955); (b) zone electrophoresis on Whatman no. 1 paper at 6 V./cm. in borate buffer (pH 7.3; ionic strength 0.05) and in veronal buffer (pH 8.6; ionic strength 0.075); (c) moving-boundary electrophoresis carried out on a 1% (w/v) solution of the toxic protein in a Perkin-Elmer modification of the Tiselius apparatus at pH 7.4 in a phosphate buffer containing $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (0.460 g./l.), $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (5.95 g./l.) and NaCl (8.78 g./l.).

Biological tests on the lipopolysaccharides. These materials were tested for the ability to produce the following effects: (a) non-specific immunity by the method of Rowley (1956); (b) Schwartzman effect as described by Schwartzman (1937); (c) pyrogenic response. The rectal temperatures of rabbits were taken during 2 hr. to ensure a constant zero reading. Three rabbits were each given 0.05 μ g. lipopolysaccharide intravenously in 1 ml. pyrogen-free saline. Temperatures were taken at intervals during 5–6 hr. after injection.

RESULTS

Alcohol extraction (after Burrows)

No toxic materials could be extracted by hot alcohol from the three *V. cholerae* serotypes, whilst the residue from these alcohol extractions was always non-toxic.

Hydrochloric acid extraction

It was sometimes found by following Freter's (1956) method of extraction that 40–50% of the toxicity of whole organisms could be extracted. However, this did not always occur; occasionally very little toxicity was extracted and on testing the residue it was found that considerable loss of toxicity had occurred during incubation with the hydrochloric acid. Even in these cases where toxicity had been extracted it was always lost during the subsequent purification steps, which involved solvent extraction and precipitation from hot ethanol.

Extraction with trichloroacetic acid

Preliminary experiments showed that the toxicity of *Vibrio cholerae* whole organisms decreased markedly when heated at 100° for 60 min. or by exposure to strong trichloroacetic acid solutions. For this reason the extraction was performed on vibrio suspensions (killed at 75° for 30 min.) by using 0.05 M-trichloroacetic acid, the whole extraction being conducted during ultrasonic disintegration for 60 min. at 0°. The mixture was centrifuged at 3500 g for

60 min. to recover undisrupted bacteria and other material, the supernatant fluid from this was centrifuged for 60 min. at 16,000 *g* and the resulting supernatant fluid from this spun at 78,000 *g* for 2 hr. The latter centrifugation sedimented the Boivin-type macromolecular complex. All these fractions were dialysed and the dry weight measured, before testing for toxicity in mice. The results of a typical experiment with the *V. cholerae* strain Ogawa 5596 (Table 1) show that *c.* 16 % of the original toxicity was recovered in the Boivin antigen with a toxicity of 5 mouse LD 50/mg. Similar extraction experiments with the Inaba 4693 and Hikojima 7270 strains did not yield any material which would sediment at 78,000 *g* even when 0.5M- or 0.1M-trichloroacetic acid was used for extraction. Analysis of the toxic complex obtained from the Ogawa strain 5596 showed it to contain 35 % protein. The Boivin antigen extracted from *V. cholerae*, Ogawa strain 5596, was relatively heat labile; heating at 100° for 60 min. led to considerable loss in toxicity (Table 2). The heat lability of this fraction is surprising since endotoxic complexes from other Gram-negative bacteria are remarkably heat stable.

Table 1. *Extraction of Vibrio cholerae, Ogawa strain 5596, with 0.05M-trichloroacetic acid in the ultrasonic apparatus for 60 min.*

	Weight (g.)	Recovery	
		% by weight	% of initial toxicity
Starting material	3.45	—	—
Residue from extraction	2.13	62	35
Deposit 16,000 <i>g</i>	0.36	10.4	39
Deposit 78,000 <i>g</i>	0.05	1.4	16
Total recovery	—	73.8	90

Table 2. *Stability of the Boivin-type toxin from Vibrio cholerae, Ogawa strain 5596, at 100°*

Injected dose of Boivin toxin (μg.)	Time (min.) of heating at 100°		
	0	30	60
	No. mice dead/no. mice injected		
500	7/8	6/9	3/9
250	5/9	4/9	0/9

Extraction with phenol/water

Suspensions of the three serotypes of *Vibrio cholerae* were killed by heating at 100° for 10 min. and were extracted at a concentration of 20 mg. dry wt. organisms/ml. water layer by the phenol/water method of Westphal, Lüderitz & Bister (1952). The data from typical extractions on these serotypes are collected in Table 3. The weights of the various fractions are those of the residual non-dialysable material, since the phenol had been removed by prolonged dialysis. The final yields of purified lipopolysaccharides varied between 0.5 and 2.0 % of the original dry weight, which is similar to the

amounts obtained from other Gram-negative organisms. However, both the toxicity and the recovery of toxicity were extremely low in all cases by comparison with a typical extraction of *Escherichia coli* (Table 3).

Chemical analysis of the lipopolysaccharides. Deposits at 78,000 g, which by their previous extraction history were presumptive lipopolysaccharides, from various vibrio strains were analysed for nitrogen, phosphorus, sugar and Lipid A content (Table 4).

Biological properties. Although the toxicities of lipopolysaccharides from the vibrio strains were low by comparison with those from most other Gram-negative organisms, further tests showed that these compounds possessed biological properties typical of the general class.

Table 3. *Extraction of the three serotypes of Vibrio cholerae and Escherichia coli 2380 with 45 % (w/v) phenol in water*

	Inaba 4693	Ogawa 5596	Hikojima 7270	<i>E. coli</i> 2380
Wt. of starting material (g.)	15.7	3.52	10.5	4.6
No. LD 50 doses	6240	1408	1750	1840
Wt. in water layer (g.)	5.14	0.40	3.1	1.2
Recovery of toxicity (%)	16.4	—	—	—
Wt. in phenol layer (g.)	3.12	0.57	1.8	0.8
Recovery of toxicity (%)	< 5	< 2.7	< 7	—
Wt. in residue (g.)	4.7	1.9	2.3	1.4
Recovery of toxicity (%)	7.5	9	7	15
Wt. of lipopolysaccharide (g.)	0.39	0.02	0.18	0.11
Recovery of toxicity (%)	11	0.7	5	74

Stimulation of non-specific immunity

Groups of mice were given intravenously, in 0.2 ml. of saline, 50 µg. or 10 µg. lipopolysaccharide from *Vibrio cholerae* Ogawa, Inaba and Hikojima strains. The control mice were given 0.2 ml. of saline only, by the same route. After 48 hr. the mice were challenged intraperitoneally with a virulent strain of *Escherichia coli* 145 (10^5 organisms in 0.2 ml. of 1.75 % (w/v) solution of hog gastric mucin). The results and a comparison made with a group of mice given 10 µg. lipopolysaccharide from *E. coli* 2380 are given in Table 5. In all cases lipopolysaccharide derived from the three serotypes of *V. cholerae* stimulated non-specific immunity to an extent approaching that caused by *E. coli* 2380.

Schwartzman effect. This phenomenon was described by Schwartzman in 1937. When endotoxin is injected into the skin of a rabbit and 24 hr. later a further amount is given intravenously, a haemorrhagic necrosis rapidly develops at the site of the first injection. In a typical experiment the abdomen of a rabbit was shaved and four squares drawn on the prepared area of skin. Into two of these 5 µg. Ogawa lipopolysaccharide in 0.2 ml. saline were injected subcutaneously, whilst the other two sites were injected with 0.2 ml. saline only. After 24 hr. the rabbit was given 20 µg. of the same lipopolysaccharide in 0.2 ml. intravenously, and the prepared skin squares examined after 18 hr. Haemorrhagic necrosis developed only at those sites previously injected with lipopolysaccharide.

Table 4. Collected chemical and biological data on the lipopolysaccharides from different vibrios

Organism	Yield of lipopoly-saccharide (%)	Mouse LD ₅₀ /kg. (mg.)	Recovery of toxicity as lipopoly-saccharide (%)	Nitrogen (%)	Phosphorus (%)	Lipid A (%)	Presence of		
							Methyl-pentose	Hexose	Pentose
<i>Vibrio cholerae</i>									
Ogawa 5596	0.67	50	2.5	2.1	2.0	54	+	+	+
Ogawa 161	2.07	25	16	nd	nd	50	nd	nd	nd
Ogawa 162	2.4	15	21	2.2	2.3	60	nd	nd	nd
Inaba 4693	1.83	37	14	2.4	1.9	22	+	+	+
Hikojima 7270	1.85	100	4.4	2.4	2.4	84	+	+	+
Water vibrio 53	2.35	25	16	2.4	2.0	65	+	+	+
Water vibrio 54	3.33	25	16	2.5	2.2	25	+	+	+
Water vibrio 55	2.39	25	16	2.25	2.1	86	+	+	+
<i>Escherichia coli</i> 2380	2.25	2.5	50	2.1	1.8	12	+	+	-

nd = analysis not done.

Pyrogenicity. The samples of Inaba, Ogawa & Hikojima lipopolysaccharides all proved to be highly pyrogenic in rabbits; 0.05 μ g. of any of them produced a 2° F. rise in temperature which was comparable to the temperature rise obtained with a similar amount of lipopolysaccharide from *Escherichia coli* 2380.

Table 5. *Stimulation of non-specific immunity by lipopolysaccharides*

The mice were challenged after 48 hr. with *Escherichia coli* 145 (10^5 organisms + mucin).

Lipopolysaccharide	Intravenous dose (μ g.)	Treated mice. No. deaths/no. injected
<i>V. cholerae</i> , Inaba 4693	50	1/8
	10	5/8
<i>V. cholerae</i> , Ogawa 5596	50	3/8
	10	4/8
<i>V. cholerae</i> , Hikojima 7270	50	2/8
	10	3/8
<i>Escherichia coli</i> , 2380	10	1/8

The control groups received 0.2 ml. saline and were challenged with *E. coli* with the treated groups, after 48 hr. All the mice died in each set of controls.

Table 6. *Antigenic specificity of lipopolysaccharides from Vibrio cholerae, Ogawa and Inaba strains*

Maximum dilution to produce agglutination or precipitation

	Ogawa 'O' antiserum	Inaba 'O' antiserum
Ogawa organisms (2 hr. at 100°)	1/4000	1/500
Ogawa lipopolysaccharide	1/256	1/16
Inaba organisms (2 hr. at 100°)	1/250	1/4000
Inaba lipopolysaccharide	1/8	1/256

Antigenic specificity. Lipopolysaccharide from Ogawa and Inaba strains of *Vibrio cholerae* were titrated against homologous and heterologous antisera which had been produced by injecting rabbits with a suspension of whole organisms heated for 2 hr. at 100°. Both samples of lipopolysaccharide were used in the precipitin tests at a concentration of 500 μ g./ml. The results (Table 6) showed that the lipopolysaccharides were specific for the parent organisms from which they had been isolated.

Urea and ammonium sulphate fractionation

Urea possesses the property of making proteins soluble and has been successfully used in the isolation of polysaccharides from *Mycobacterium tuberculosis* (Stacy, Kent & Nassau, 1951) and endotoxin complexes from *Salmonella typhi* (Walker, 1940). In all the following extractions living bacterial cultures were used. Preliminary tests showed that the bacteria were rapidly dissolved by the urea solution during disintegration in the ultrasonic apparatus at a constant low temperature. Dialysis of the opalescent solution deposited approximately 50% of the starting material, which weight for weight had a

similar toxicity to that of the suspension of whole organisms. This deposit could be recycled.

By ammonium sulphate fractionation of the dialysed supernatant fluid, toxic fractions of high protein content were precipitated. The sharpness of separation was greatly improved by fractionation in the presence of 2.5 M-urea. It was important throughout the extraction and fractionation procedures to control carefully the temperature of the solutions; all extractions, precipitations and centrifugations were carried out at 0°. The toxic fractions were found to be more stable in the presence of phosphate ion; resuspension in saline or distilled water led eventually to loss in toxicity.

Fig. 1. Extraction of a protein toxin from *Vibrio cholerae*

The final extraction method was as follows. To a suspension of living vibrios in saline at a concentration of 30 mg. dry wt./ml., solid urea was added 2.5 M. The suspension was agitated in the ultrasonic apparatus at 0° for 5 min., during which time the turbidity greatly decreased. The opalescent suspension was dialysed against running tap water at 10° for 18 hr. and the contents of the dialysing bag centrifuged at 5000 g. Solid urea was again added to the clear amber-coloured supernatant fluid to 2.5 M and this solution was pre-

cipitated at 0° successively with neutral saturated ammonium sulphate at 1·9M, 2·8M and 3·8M saturation (Fig. 1). The precipitates at 2·8M and 3·8M saturation were highly toxic and were combined for further purification. The combined precipitates were dissolved in 0·0066M phosphate (pH 7·3) to give a colourless opalescent solution which was reprecipitated with neutral saturated ammonium sulphate to 2·8M. The resulting precipitate was again dissolved in 0·0066M-phosphate (pH 7·3) and centrifuged at 0° in the Spinco first at 20,000 *g* for 1 hr. and finally at 78,000 *g* for 2 hr. The deposit obtained at 78,000 *g* represented the purified toxic protein which contained 10–20 mouse LD 50 doses/mg. and accounted for between 10 and 45 % of the original toxicity, depending on the strain extracted. The results of a typical extraction of *Vibrio cholerae*, Ogawa strain 5596, are given in Table 7.

Table 7. *Combined results of four separate extractions of toxic protein from Vibrio cholerae, Ogawa strain 5596*

	Starting material	Deposit at 78,000 <i>g</i>
Weight (g.)	43·7	0·570
Weight recovery (%)	—	1·3
LD 50 (average)	2·2 mg.	75 µg.
Limits	1–3 mg.	50–100 µg.
Recovery of toxicity (%)	—	25
Protein (%)	—	100
Phosphorus (average %)	—	1·5
Limits (%)	—	1·3–1·7
Nitrogen (average %)	—	13·8
Limits (%)	—	13·6–14·0

Different strains of the same serotype, different serotypes or even water vibrios all yielded similar toxic protein fractions by this method. The results of some of these extractions are collected in Table 8.

The toxic protein obtained from Ogawa strain 5596 was examined for purity by starch-gel, zone and moving-boundary electrophoresis. By the latter two methods the material moved as one component which suggested a high degree of purity. However, no movement was detected in starch gel, which suggests that the toxic protein is of high molecular weight and is not contaminated with protein of smaller molecular size.

Chemical properties of the toxic protein

The toxicity of the protein was completely destroyed by heating at 100° for 10 min. The toxic proteins from the Ogawa and Inaba strains were less labile at 60°, about 50 % of the toxicity still remaining after heating for 60 min. This is somewhat surprising in view of the lability of the toxic material to changes in pH and temperature during its extractions, though like many other proteins its stability to physical changes may increase with degree of purification. Incubation of the toxic protein with proteolytic enzymes such as

trypsin, papain and pepsin, in an effort to break it down into smaller toxic units, led only to complete loss of toxicity. About 50% of the toxicity was lost as a result of freeze-drying the toxic protein. The toxicity was best retained by suspending the protein toxin in 0.0066M-phosphate (pH 7.3) and keeping the solution at 2°.

Table 8. *Accumulated data on the protein toxin extracted from various vibrios with 2.5M-urea*

	Ogawa 162	Ogawa 163	Water vibrio 55	Water vibrio 54	Inaba 4693
Weight of starting material (g.)	4.0	3.0	2.8	3.4	7.2
No. LD 50 doses	2000	1200	920	1100	3000
% weight recovery in deposit at 78,000 g (%)	1.0	0.66	2.85	1.17	0.33
LD 50 dose (μ g.)	100	80	200	100	80
Recovery of toxicity in protein (%)	22.5	20.8	43.3	36.3	10.3
Total recovery of toxicity in all fractions (%)	70	83.3	86.6	82	60
Protein in toxin (%)	100	100	100	100	100
Carbohydrate in protein (%)	3	1.2	1.7	1.2	nd

nd = analysis not done.

Recovery of toxic protein from Boivin antigen

Previous experiments had shown that the toxicity of the Boivin antigen was heat-labile and acid-labile. It seemed possible therefore that the toxic protein isolated might in fact be the protein component responsible for the toxicity of this macromolecular complex.

Boivin antigen (90 mg.) obtained by trichloroacetic acid extraction (containing 38% protein; mouse LD 50, 200 μ g.) was taken up in 2.5M-urea and fractionated with ammonium sulphate as previously described. The precipitate obtained at 3.8M weighed 32 mg. and contained 10 mouse LD 50 doses/mg. Analysis of the precipitate showed it to contain 92% protein and 8% carbohydrate.

These results show that a toxic protein may be isolated from the toxic Boivin antigen. From immunological data the protein toxin isolated from the whole organisms and that isolated from the Boivin antigen appear to be identical (Jenkin, unpublished).

DISCUSSION

Three toxic fractions have been prepared from *Vibrio cholerae* by different chemical methods. The protein toxin isolated by urea and ammonium sulphate fractionation appears to account for the major portion of the toxicity of whole organisms. This protein toxin occurs in the strains of *V. cholerae* and in the water vibrios tested. It is desirable to examine any relationship that might exist between the present protein toxin and those toxins of *V. cholerae* isolated previously, particularly in the work of Gallut & Grabar (1945) and of Freter

(1956). Gallut & Graber described a dialysable polypeptide toxin which was present in greatest amount when autolysis of a heavy suspension of *V. cholerae* proceeded for 72 hr. When the autolysis was stopped after 4 hr. very little, if any, of this toxin was found. The toxicity of the supernatant fluids due to the high molecular 'gluco-lipidique' Boivin complex was greatest after 4 hr. autolysis, and subsequently decreased when lysis continued for longer than this period, with the appearance of the small molecular toxin. With the progressive loss in toxicity they noted that the nitrogen content of 'gluco-lipidique' complex also diminished, and it was suggested that the polypeptide toxin was split from this large polymolecular complex by action of proteolytic enzymes. The suggested derivation of the polypeptide toxin from the Boivin antigen, taken in conjunction with the finding that the protein toxin isolated from *V. cholerae* in this work is part of this antigen, makes it seem likely that the polypeptide toxin of Gallut & Grabar and the toxic protein *V. cholerae* are similar. The only apparent reason to call the material polypeptide, was its ability to pass through a membrane of such pore size as to allow the passage of haemoglobin.

The acid-insoluble toxin isolated by Freter (1956) is similar in many ways to the toxic protein we isolated. The low toxicity of Freter's protein may have been due to the method of isolation and purification, which involved solvent extraction and precipitation from hot ethanol. All these procedures would certainly lead to loss in toxicity of the protein toxin isolated in this paper. When repeating Freter's method of acid extraction any extracted toxicity was always lost during the subsequent purification steps. It may be suggested that the toxic proteins of Gallut & Grabar and of Freter are similar to the toxic protein we isolated by urea and ammonium sulphate treatment, but that the latter method is far gentler and retains most of the toxicity of this fraction.

It must be emphasized that in the present work only those fractions of *Vibrio cholerae* responsible for the lethal effect of whole organisms injected into mice have been considered. Since death from any of these toxic fractions is rarely, if ever, accompanied by diarrhoea, it is by no means probable that these toxins cause the gross change in intestinal permeability which seems to be the most important clinical feature of cholera.

We wish to thank Dr T. Brogan and Miss B. Haywood for technical advice on electrophoresis and Mr W. Ali for the chemical data on *Escherichia coli* 2380 lipopolysaccharide. One of us (C.R.J.) is grateful to the Wright-Fleming Institute for the award of a Fleming (Pfizer) Fellowship during the period of this work, which forms part of a Ph.D. thesis submitted to the University of London.

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(Received 10 February 1959)