

Purification and Characterization of a Hemolysin Produced by *Vibrio cholerae* Biotype El Tor: Another Toxic Substance Produced by Cholera Vibrios

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A thermolabile direct hemolysin from an El Tor cholera vibrio strain has been isolated and partially characterized as a simple protein of ca. 20,000 molecular weight. In addition to its hemolytic activity, the hemolysin is cytotoxic, cardiotoxic, and rapidly lethal. In these respects it resembles the thermostable direct hemolysin/cytotoxin/cardiotoxin/lethal toxin of *Vibrio parahaemolyticus* and certain other bacterial hemolysins, although there are other significant differences. Because identical diseases are produced by both hemolytic and nonhemolytic cholera vibrios, the El Tor hemolysin may be presumed to be pathogenetically irrelevant. These observations raise the question of "When is a toxic substance also a toxin?"

Historically, a subset of cholera vibrios, now known as *Vibrio cholerae* biotype El Tor, were differentiated from classical cholera vibrios, first isolated by Koch, on the basis of their ability to cause hemolysis of goat or sheep erythrocytes (RBC) in a tube test known as the Greig test (12, 20). Other properties have since been recognized to differentiate these two groups of cholera vibrios, which are believed to cause identical diseases (7, 45) by means of closely related or identical enterotoxins (22). *V. cholerae* biotype El Tor is the cause of the present pandemic of cholera. However, during the evolution of the present pandemic, most El Tor vibrio isolates have lost the capability of producing hemolysin, or express it only feebly (11). Although the clinical syndromes caused by the two groups of cholera vibrios, El Tor and classical, are believed to be identical, El Tor vibrios have been reported to produce additional toxic factors and to have exalted virulence in some models (6, 9, 10, 30, 35, 46). Although the hemolytic properties of cholera vibrios have been the subject of many investigations (8, 16, 18, 27, 28, 36, 47, 48), the precise nature and pathogenic role (if any) of the El Tor hemolysin remains unclear.

Our interest in this as yet unresolved problem was stimulated in part because of the attention being devoted to the hemolysin produced by an unrelated group of vibrios which also produce

diarrheal disease: namely, the thermostable direct hemolysin of Kanagawa phenomenon-positive *V. parahaemolyticus*. This hemolysin, in a series of studies (17, 23, 25, 39, 41, 51, 52), has been shown to be cytotoxic, lethal, and cardiotoxic; it is believed by many to play a role in the pathogenesis of enteritis caused by *V. parahaemolyticus*. The present paper describes partial purification, characterization, and some of the properties of the El Tor hemolysin.

MATERIALS AND METHODS

Bacterial strain. The markedly hemolytic *V. cholerae* strain used, 26-3, serotype Ogawa, biotype El Tor, was isolated by one of us (R.A.F.) from a sample of fluid from the small bowel of a purging cholera patient at San Lazaro Hospital in Manila, The Philippines, 1961, and was maintained by lyophilization since that time.

Assay of hemolysin. Hemolytic activity was assayed by two techniques. In the test tube method, 1 ml of 1% goat RBC in phosphate-buffered saline (0.02 M phosphate, pH 7.0) was added to 0.1 ml of serially diluted samples. The mixtures were incubated at 37°C for 1 h, and the optical density of the supernatant was determined at 540 nm. One hemolytic unit (HU) was defined as the smallest dose in 0.1 ml of sample which caused 50% hemolysis. Because the test tube method was rather laborious, we developed a simpler microplate method (modified from that of reference 52). Goat RBC (1%) were added to molten agarose (1%) in phosphate-buffered saline, and 3.5 ml of the suspension was pipetted onto a 3- by 7-cm plate (Immuno-plate, Hyland, Inc.). Sample solution (10 μ l) was added to wells 2 mm in diameter, and the plates were incubated at 37°C. There was a linear correlation between HU and diameter of the hemolytic zone at 1 h of incubation (Fig. 1). The zone sizes continued to in-

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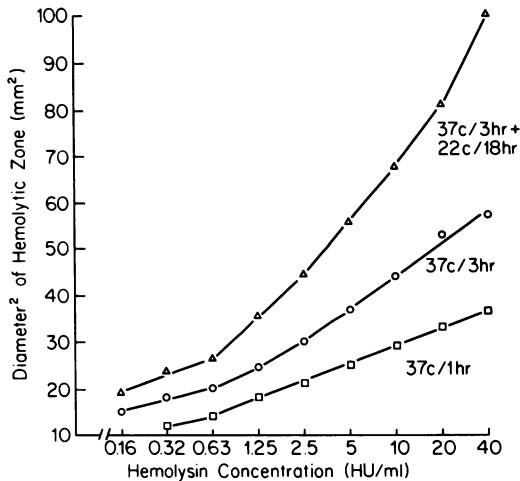


FIG. 1. Comparison of hemolytic activity by tube and microplate methods.

crease upon further incubation, and the dose-response curve assumed a curvilinear aspect.

Assay of mouse lethal toxicity. Lethal toxicity was assayed by intravenous inoculation of BDF₁ mice (20 to 25 g of body weight) with 0.2 or 0.25 ml of sample and measuring the survival time of the animals.

Assay of cytotoxicity. Cytotoxicity was determined with cultured Y-1 adrenal cells (37). Cell suspensions treated with toxin were mixed with an equal volume of 0.5% Trypan blue (Allied Chemical) solution, and the percentage of cells stained with Trypan blue was determined microscopically. The effect of preparations on cultured beating rat heart cells (33) was determined by direct microscopic observation.

Chemical determinations. Protein was determined by the Bradford technique (5), carbohydrate was determined with anthrone reagent (32), and phospholipid was determined as described by Yamashita and Numa (49). Polyacrylamide gel electrophoresis (PAGE) was as previously described (14).

Size approximation by gel filtration. The molecular size of the partially purified hemolysin was approximated by gel filtration in comparison with standard molecular size markers (aldolase, ovalbumin, chymotrypsinogen A, and ribonuclease) on both ACA 34 (LKB Instruments, Inc.) and Sephadex G75 (Pharmacia Fine Chemicals, Inc.) gels.

Preparation of antiserum. Antiserum against the hemolysin was prepared in rabbits inoculated intramuscularly with the antigen (ca. 10 HU) mixed with Freund incomplete adjuvant. The antigen for this purpose was partially purified through the Sephadex G75 step (see Results) and was then subjected to PAGE. After PAGE, the gel was cut into 0.3-mm slices and extracted with 0.2 ml tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetic acid-azide-NaCl (TEAN) buffer (14). Samples with hemolytic activity were then used as antigen.

RESULTS

Preliminary experiments revealed that brain-heart infusion broth (Difco Laboratories) and

heart infusion broth (Difco) were superior to syncase medium (13) and Trypticase soy broth (BBL Microbiology Systems) for hemolysin production. Table 1 compares hemolysin production in heart infusion and brain heart infusion broth at 30 and 37°C in both stationary and shaken cultures, with and without supplementation with 3% glycerol. In accordance with previous observations (4, 16, 38), glycerol stimulated the production of hemolytic activity. Other studies (results not shown) indicated that a concentration of 3% glycerol was nearly optimal. There was no clear correlation between the hemolytic activity and the final pH of the culture supernatant. Based on the results in Table 1, stationary cultures in heart infusion medium with glycerol at 37°C were adopted for routine use.

The hemolysin was purified by sequential steps involving precipitation with ammonium sulfate, diethylaminoethyl-cellulose (Schleicher and Schuell), ion-exchange chromatography, gel filtration on Sephadex G75, chromatography on hydroxyapatite (Sigma Chemical Co.), and, finally, gel filtration on ACA 34 (Table 2; Fig. 2). It will be noted that there were substantial losses in total activity and yield at each step during the purification, but the relative activity was increased ca. 140-fold. In PAGE (Fig. 3A), the final product was still not homogeneous. Faint bands, in addition to the major band associated with hemolytic activity (Fig. 3B), were still evident, as was some aggregated material which failed to penetrate the gel (Fig. 3A), but which still had some hemolytic activity (Fig. 3B1). The molecular size of the hemolysin was ca. 20,000, as determined by gel filtration on both Sephadex G75 and ACA 34. Antiserum raised against the hemolysin (see Materials and Methods) produced a single precipitin band, identifying the hemolysin (Fig. 3B). In PAGE also (Fig. 4), the antihemolysin serum produced a single precipitin arc, which corresponded in mobility with both crude ammonium sulfate-precipitated hemolysin and the material purified through the ACA 34 gel filtration step.

TABLE 1. Evaluation of culture conditions for hemolysin production by *V. cholerae* biotype El Tor strain 26-3

Medium ^a	Glycerol (3%)	HU/ml (pH) ^b			
		Shaking		Stationary	
		30°C	37°C	30°C	37°C
HI	-	0 (8.2)	0 (8.0)	2.6 (7.8)	2.8 (7.1)
	+	2.3 (6.1)	2.1 (5.3)	7.2 (6.7)	7.4 (7.0)
BHI	-	2.2 (7.9)	2.7 (8.1)	2.5 (7.1)	2.6 (7.0)
	+	4.5 (6.4)	4.4 (6.4)	2.2 (6.6)	4.0 (6.8)

^a HI, Heart infusion broth; BHI, brain heart infusion broth.

^b pH at the end of culture (20 h). Preinoculum pH was 7.3 in both media.

TABLE 2. Purification of *El Tor* hemolysin

Fraction	Total vol (ml)	Total protein (mg)	Total activity (HU)	Sp act (HU/mg)	Relative activity	Yield
Culture supernatant	50,000	9,600	468,000	48.7	1	100%
Ammonium sulfate (50%)	580	2,784	417,600	177.4	3.64	89.2
DEAE-cellulose ^a	40	256	136,000	531.2	10.91	29.1
Sephadex G75	5	21.6	31,680	1,173.3	24.09	6.8
Hydroxyapatite	2.1	3.4	12,000	3,767.6	77.36	2.5
ACA 34	1.0	0.31	2,088	6,735.5	138.31	0.5

^a DEAE, Diethylaminoethyl.

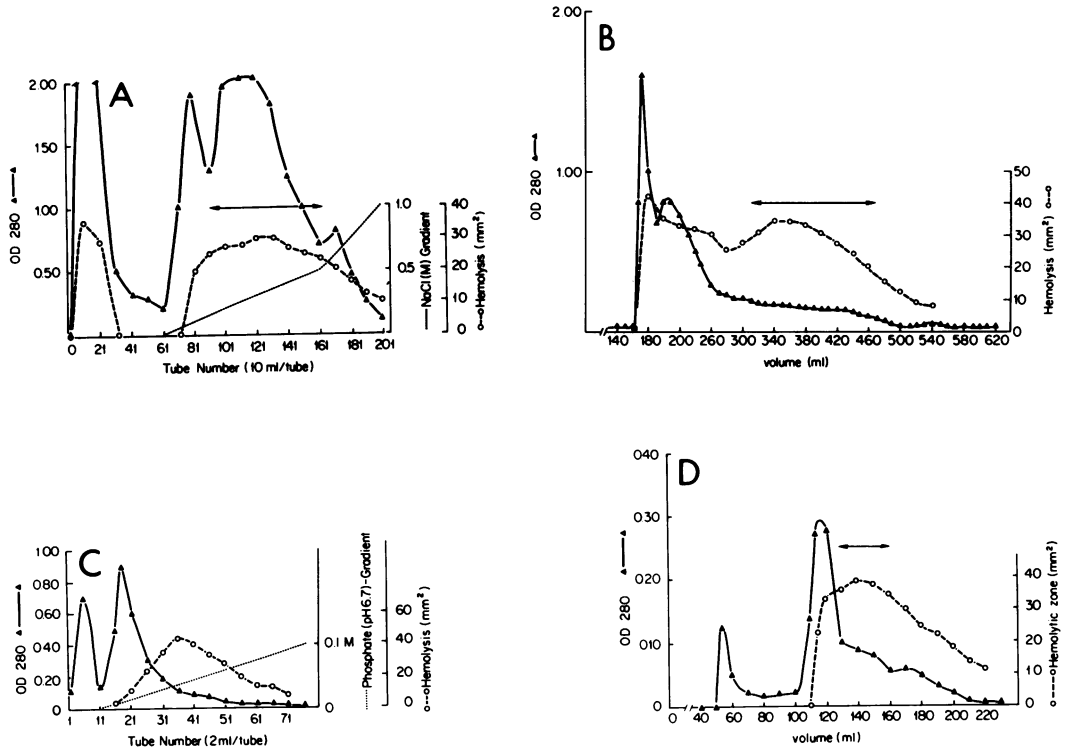


FIG. 2. Purification of *El Tor* hemolysin. (A) Diethylaminoethyl-cellulose ion-exchange column (2.4 by 100 cm, 0.01 M tris(hydroxymethyl)aminomethane buffer, pH 7.5) chromatography. (B) Gel filtration on Sephadex G75 (2.4 by 110 cm, TEAN buffer). (C) Chromatography on hydroxyapatite column (8-ml volume, washed with TEAN, gradient to 0.1 M phosphate, pH 6.7). (D) Chromatography on ACA 34 (1 by 95 cm, TEAN buffer). OD₂₈₀, Optical density at 280 nm.

Additional activities of this antiserum are depicted in Fig. 5, where it may be seen that the antiserum produced a single precipitin line against both crude and purified hemolysin, which corresponded with an inhibition of hemolytic activity when the same Ouchterlony plate was subsequently overlaid with goat RBC in agar. The serum did not react with concentrated crude culture supernatant from a classical biotype cholera vibrio, strain 569B, included as a control. Antiserum against the B subunit of cholera enterotoxin did not react with the hemolysin, but recognized cholera toxin antigen in the 569B preparation. This unrelated antigen-

antibody reaction had no effect on the hemolytic zone (Fig. 5B). Preimmune serum did not precipitate with the hemolysin.

Preparations containing the *El Tor* hemolysin possess additional biological activities. Both crude and purified hemolysin were rapidly lethal for mice and cytotoxic for cultured Y-1 adrenal cells (Table 3). When adjusted to the same hemolytic activity, the lethal and cytotoxic activities of the preparations at different stages in purification were essentially similar, suggesting that a single molecule was responsible. Additional evidence that the same entity was responsible for the three activities was provided by

neutralization studies (Table 4). The antiserum, which gave a single precipitin band with crude and purified hemolysin, neutralized the three activities at similar dilutions. Further, both the hemolytic and lethal activities were similarly

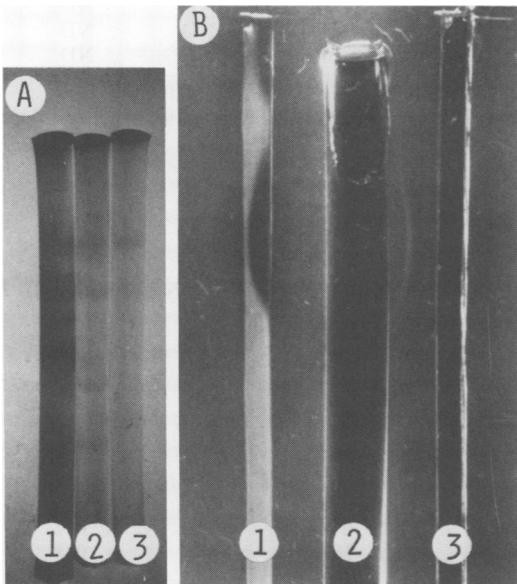


FIG. 3. PAGE of El Tor hemolysin. (A) (1) Hemolysin from ammonium sulfate precipitate. (2) Hemolysin from Sephadex G75. (3) Hemolysin from ACA 34. (1) Contains 1% goat RBC in agar and shows the hemolytic zone corresponding to the major band in (A3), as well as some hemolytic activity corresponding to the aggregate. (B) (2) The disc gel from (A3) was embedded in agarose. (3) Contains antihemolysin serum which produced a single precipitin arc corresponding to the major protein band (A3).

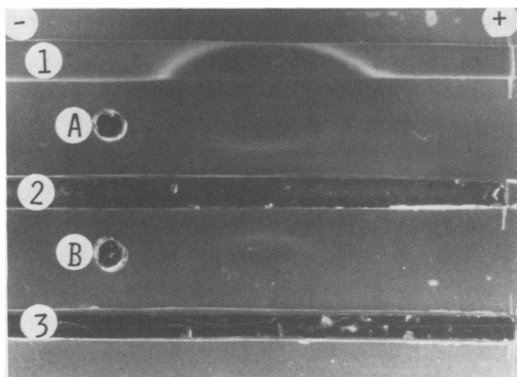


FIG. 4. Immunoelectrophoresis of hemolysin (ca. 2 HU) in 1% agarose (4.5 by 9.5 cm, 4 mm depth; 10 mA for 18 h at 4°C). (A) Ammonium sulfate-precipitated hemolysin. (B) ACA 34-purified hemolysin; (1) agar containing goat erythrocytes, 1%; (2) antihemolysin serum; (3) normal (preimmune) serum.

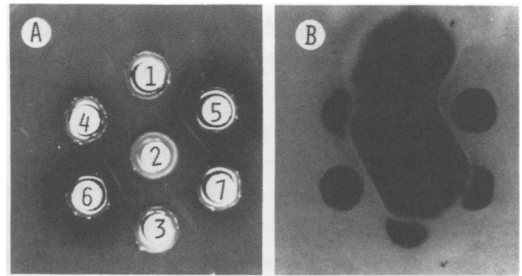


FIG. 5. Ouchterlony-type immunodiffusion reactions. (A) Wells contain: (1) ACA 34-purified hemolysin; (2) crude hemolysin (ammonium sulfate precipitated); (3) concentrated *V. cholerae* 569B culture supernatant; (4) normal serum; (5) and (6) anti-hemolysin serum; (7) goat "anti-cholera B" serum. (B) The same plate has been overlaid with agar containing goat RBC.

heat-labile (Table 5). Treatment at 55°C for 15 min destroyed both activities almost completely in both crude and purified preparations. In other studies (data not shown) the optimum pH for hemolytic activity was found to be near neutrality (little activity at pH 5 and 9), and the activity was unaffected by addition of divalent cations (Mg, Ca, and Mn at 5 mM).

In addition to its cytotoxicity for cultured Y-1 cells, the hemolysin also showed marked cytotoxic effects on cultured beating rat heart cells (33). About 1 min after the addition of crude and purified hemolysin preparations, at 10 HU/ml, the beating of the heart cells was observed to stop, and the cells degenerated ca. 3 min after the hemolysin challenge.

Preliminary studies reveal (Fig. 6) that the hemolytic activity was inhibited by mixed gangliosides (type III, Sigma). The addition of purified cholera toxin to the ganglioside mixture had no effect on this inhibition, suggesting that a factor other than the G_{M1} ganglioside was responsible. This El Tor hemolysin may be regarded as a "direct" hemolysin (40) as the addition of lecithin (L- α -phosphatidyl choline from egg yolk [Sigma], 500 μ g/ml) did not enhance its activity (data not shown).

Finally, studies with RBC from different species revealed (Table 6) that there is a spectrum of sensitivity to the hemolysin: the activity was higher with rabbit, goat, mouse, and sheep, and lower with chicken, horse, and human RBC. The spectrum differs from that of *V. parahaemolyticus* hemolysin (50).

DISCUSSION

Bacterial hemolysins have long been fascinating to those interested in microbial pathogenesis. Easily assayed in the laboratory, hemolysins

TABLE 3. Comparison of hemolytic, lethal, and cytotoxic activities of *El Tor* hemolysin

Prepn	Hemolytic activity ^a (HU)	Lethal activity ^b (h)	Cytotoxic activity ^c (%)	Protein (μ g/ml)	Sugar (μ g/ml)	Lipid (nM/0.3 ml)
Culture supernatant	10	1.85	10-32-53	173	32	NT ^d
Ammonium sulfate (50%)	10	1.28	30-71-94	50.9	9.2	19.3
Sephadex G75	10	1.31	41-65-87	9.6	ND ^e	ND
ACA 34	10	1.08	38-70-92	2.7	ND	ND
Buffer (TEAN) control	0	24	14-17-20	0	ND	ND

^a Hemolytic activity was measured by the plate method. Each sample was adjusted to 10 HU/ml.

^b Lethal activity was measured as the mean survival time after intravenous inoculation of 0.2 ml containing 2.0 HU. There were four mice per sample.

^c Percentage of Y-1 adrenal cells stained with trypan blue 10, 30, and 60 min after toxin-challenge.

^d NT, Not tested.

^e ND, Not detectable.

TABLE 4. Neutralization of hemolytic, lethal, and cytotoxic activities by immune and normal serum^a

Serum or buffer	Hemolytic activity (HU)	Lethal activity ^b	Cytotoxic activity ^c
Immune serum			
1:2	0	0/4	7
1:10	2.8	1/4	54
1:50	7.6	4/4	83
Preimmune serum			
1:2	7.2	4/4	71
Buffer			
1:1	10	4/4	84

^a Sera were diluted as indicated; then 1.0 ml of each dilution was mixed with 1.0 ml of crude hemolysin preparation (20 HU). After 15 min of incubation at 37°C, biological activity was determined.

^b Number of dead mice at 4 h/total number challenged.

^c Percentage of Y-1 cells stained with trypan blue at 30 min of incubation with each preparation (buffer alone gave 6%).

have often been assumed to have significance in pathogenesis, even though the parent organisms cause no hemolytic manifestations. Although many investigators are aware that microbial products which are hemolytic in vitro do not necessarily function that way in vivo, the assumption that the hemolysin plays a role in pathogenesis is too frequently made. It is possible that the much studied thermostable direct hemolysin of *V. parahaemolyticus* is such a case. Despite intensive studies of this product, it has yet to be clearly implicated as playing an essential role in the disease process. The cardinal manifestation of "parahemolytic disease" is diarrhea, but the hemolysin/cytotoxin/cardiotoxin/lethal toxin does not evoke fluid movement in intestinal models (or does so only at extremely high doses [51]), and the disease is

TABLE 5. Heat stability of hemolysin and lethal toxicity

Prepn	Treatment	Hemolytic activity ^a (%)	Lethal activity ^b
Crude ^c	55°C		
	5 min	12.7	4/4
	15 min	4.1	1/4
	30 min	0	0/4
	100°C		
	5 min	0	0/4
Purified ^d	15 min	0	0/4
	30 min	0	0/4
	None	100	4/4
	55°C		
	5 min	3.5	2/4
	15 min	1.0	0/4
	30 min	0	0/4
	100°C		
	5 min	0	0/4
	15 min	0	0/4
	None	100	4/4

^a Percentage of original hemolytic activity remaining.

^b Lethal activity determined after intravenous injection of 0.25 ml of each preparation. Number of dead mice/total.

^c Crude ammonium sulfate precipitated hemolysin was used at a concentration of 20 HU/ml in TEAN.

^d Purified preparation from ACA 34 was used at a concentration of 10 HU/ml in TEAN.

rarely, if ever, directly lethal (2), although abnormalities in electrocardiograms have been reported (26). A separate cholera enterotoxin-like factor has been described (24) which could be responsible for the diarrhea. We initiated this study in part because of the feeling of one of us (R.A.F.) that the thermostable direct hemolysin of *V. parahaemolyticus* may in fact be irrelevant to "parahemolytic disease."

Both hemolytic *El Tor* and nonhemolytic classical biotype cholera vibrios cause cholera by means of the same enterotoxin, although *El Tor* vibrios seem to produce less of it (44), and

infection with El Tor vibrios is less likely to result in disease than is infection with the classical biotype (3). Cardiovascular abnormalities observed in cholera patients have been attributed to dehydration, acidosis, and electrolyte imbalance (34). If the hemolysin from El Tor vibrios was cytotoxic, lethal, and cardiotoxic, then, by analogy, this would provide evidence for the irrelevance of the *V. parahaemolyticus* hemolysin. The present results indicate that the hemolysin produced by a strain of *V. cholerae* of the El Tor biotype is also cytotoxic, lethal, and cardiotoxic. In these respects, it behaves like the heat-stable direct hemolysin from *V. parahaemolyticus*, streptolysin O, listerialysin, and others (21, 29, 42). However, as it differs from the *V. parahaemolyticus* hemolysin in a number of respects, e.g., heat lability, spectrum of activity, and physicochemical properties (15, 31), the analogy is therefore not completely sound. In both instances, evidence has been presented that

the hemolysin is produced in patients, in that specific serological responses have been observed (1, 15).

We are left then, in the case of the El Tor hemolysin, with a potential potent toxicity, present during the disease in humans, for which there is no known manifestation. Although from the evidence we do not consider it likely, we cannot completely exclude the possibility that the El Tor hemolysin/cytotoxin/lethal cardiotoxin may play some cryptic role in the pathogenesis of disease caused by hemolytic cholera vibrios of the El Tor biotype. In this connection, it should be recalled that the first patients from whom El Tor vibrios were isolated had dysentery (19). If in fact the factor has no role, this raises other questions: "Why not?" and "When is a toxic substance also a toxin?" Further studies on the mechanism of action of this interesting hemolysin may help resolve the enigmas which it presents.

Finally, in contrast to earlier studies (47, 48), which concluded that the El Tor hemolysin, purified from a different strain by more primitive procedures, contained 95% lipid, the present observations indicate that it is a simple protein of approximately 20,000 molecular weight (determined by gel filtration).

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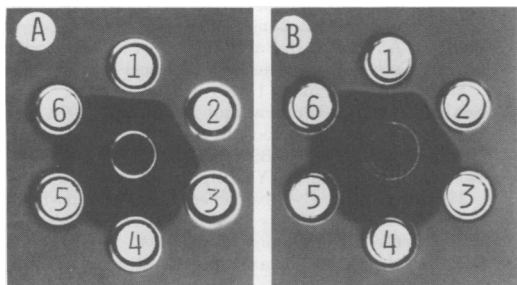


FIG. 6. Inhibition of hemolytic activity by mixed gangliosides (Sigma, type III) in agar containing goat RBC (1%) incubated at room temperature for 20 h. (A) Center well, crude hemolysin. (1) 50 µg of gangliosides; (2) 10 µg of gangliosides; (3) 2 µg of gangliosides; (4) 0.4 µg of gangliosides; (5) 0.08 µg of gangliosides; (6) phosphate-buffered saline control. (B) Same as (A), except that in well (1), 10 µg of cholera toxin have been mixed with 10 µg of gangliosides. This amount of cholera toxin should be sufficient to combine with (and block) the G_{M1} ganglioside in the mixed gangliosides (43).

TABLE 6. Hemolytic activities^a of El Tor hemolysin on erythrocytes from various species

Source of RBC	Reciprocal titer of 50% hemolytic dose
Rabbit	512
Goat	460
Mouse	460
Sheep	418
Chicken	256
Horse	256
Human B	225

^a Determined by tube test with (NH₄)₂SO₄ precipitated hemolysin.

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