Stability of a Fluid Cholera Mucinase Preparation When Combined with a Commercially Prepared Cholera Vaccine. (22676)

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The demonstration by Burnet and Stone (1) that the mucinase of *Vibrio comma* causes desquamation of guinea pig intestinal epithelium suggested to these investigators that this enzyme may be involved in the pathogenesis of Asiatic cholera. Evidence to support this hypothesis is provided by the recent report of Lam, Mandle and Goodner(2) that the cholera mucinase increases the permeability of the mouse intestinal wall *in vivo* as well as *in vitro*. It is postulated that the mucinase, by causing desquamation of the intestinal epithelium, alters the permeability of the bowel wall to permit absorption of toxic substances and loss of fluids and electrolytes.

The usual cholera vaccine, which consists of non-viable vibrio cells, does not stimulate production of antimucinase when injected into animals(1,3). Vibrio culture filtrates containing active mucinase, on the other hand, are antigenic when administered parenterally to rabbits and guinea pigs, and result in the appearance of specific antibodies which inhibit the *in vitro* desquamation and depolymerization activities of the homologous mucinolytic enzyme(1,4). It would appear, then, that the addition of cholera mucinase to the vaccine would enhance the protective potency of this immunizing agent against an enteric cholera infection, provided the mucinase retains its antigenic properties in such a preparation.

Jensen(3) studied the stability of the cholera mucinase in culture filtrates and reported that the enzyme in this state is extremely sensitive to heat, storage and chemical preservatives. He demonstrated that *in vitro* enzyme activity and antigenicity are related, and that both properties can be preserved by lyophilization.

The studies reported herein were initiated to ascertain whether the application of a concentration and purification procedure would result in a stabilized fluid mucinase preparation, permitting the combination of the mucinase with the present cholera vaccine without affecting the *in vitro* activity and antigenicity of the enzyme, or the antigenicity of the somatic antigens.

Materials and methods. Culture: *V. comma* strain 20A78, the WRAIR designation for strain P93A obtained from Prof. K. Goodner, Jefferson Medical College, Philadelphia, Pa., was used for the preparation of mucinase by the method described below. *Cholera vaccine*. Lederle Cholera Vaccine, Lot #1976-67K (expiration date 21 Oct. 1955), was obtained in Nov. 1954 for use in these studies. The vaccine contained 8,000 million killed cholera vibrios (equal numbers of Inaba and Ogawa strains) per ml, preserved in 0.45% phenol. *Mucinase titrations*: *In vitro* mucinolytic activity was measured by the technic described elsewhere(5), which is based on the ability of the mucinase to depolymerize ovomucin so that the latter is no longer clotted by cetyl trimethyl ammonium bromide (CTAB). The ovomucin was prepared by the method of Gottschalk and Lind(6) and was standardized by the addition of borate-buffered calcium saline, pH 7.0,* to the maximum dilution containing sufficient dissolved ovomucin to form a characteristic fibrous clot on addition of one drop of 1% aqueous CTAB to 0.5 ml of the standard solution in 0.5 ml of buffered saline. The mucinase titers recorded are the reciprocals of the highest dilutions of the enzyme solutions which prevented the formation of the ovomucin-CTAB clot. *Antimucinase titrations*: The antimucinase activity of serum was measured by the constant antigen-varying antibody technic previously described (5). The titers recorded are the reciprocals of the highest serum dilutions which neutral-

* Borate-buffered calcium saline, pH 7.0: Per liter: 0.052 g $Na_2B_4O_7 \cdot 10H_2O$, 1.0 g $CaCl_2$ (anhydrous), 8.5 g NaCl, 1.203 g H_3BO_3.

ized the depolymerizing activity of 2 mucinase units, permitting the formation of the ovomucin-CTAB clot. *Agglutinin titrations*: Serum agglutinin titers were obtained by employing as antigen a saline suspension of agar-grown viable vibrios of the same strain from which the mucinase was prepared (20A78). Antigen-antiserum mixtures were incubated at 37°C for 2 hours followed by refrigeration overnight. The titers recorded are the reciprocals of the highest dilutions of the sera which yielded definite macroscopic agglutination.

Experimental results. Purification of Mucinase: In order to facilitate purification of the mucinase from culture filtrates an attempt was made to cultivate the cholera vibrio in a protein-free medium. Several synthetic media and media containing protein hydrolysates were tried, and although good yields of bacterial cells were obtained in many instances, little or no mucinolytic activity was demonstrated in the culture supernates. On the other hand, a dialysate of Brain-Heart Infusion Broth was found to support the growth of the organism and to yield satisfactory mucinase titers, and was consequently used as the culture medium.

Double strength Brain-Heart Infusion Broth (Difco) was dialyzed against an equal volume of distilled water at 4°C. After 24 hours the dialysate was collected and was replaced with another volume of water. This process was repeated again, after which the 3 resulting dialysate solutions were combined to constitute the medium for the growth of the vibrios. Nine liter bottles containing 2 liters of dialysate medium were seeded with 50 ml of a 6 hour vibrio culture (in the same medium) and were incubated at 37°C for 17 hours on a roller apparatus which constantly rotated the bottles (tilted at an angle of 30 degrees) at 40 revolutions per minute throughout the incubation period, providing maximum aeration without excessive foaming.

The bulk of the bacterial cells was removed by centrifugation and the remainder by filtration through a large Berkefeld filter of N (normal) porosity. The filtrate was then half-saturated with solid $(NH_4)_2SO_4$ and was placed in the cold room overnight. The resulting sediment was dissolved in borate-buffered calcium saline and was dialyzed against the buffered saline until the dialysate no longer precipitated with a saturated barium chloride solution. Sterilization of the product was accomplished by filtration through a Selas filter (02 porosity).

The final solution contained mucinase, other non-dialyzable ammonium sulfate precipitated extracellular products of vibrio metabolism, and cellular substances (somatic antigens, etc.) liberated through lysis of the cells. The material used in this study was prepared in May 1953 from culture filtrates of *V. comma* strain 20A78. The original culture filtrate contained 1.48 mg of nitrogen per ml and had a mucinolytic activity of 64 units per ml, while the final product contained 0.14 mg of nitrogen and 2048 mucinase units per ml (Table I). Comparable results have since been obtained with other preparations from the same strain, as well as with preparations from 4 other *V. comma* strains, including a recent isolate from a human cholera case.

Stability of in vitro enzyme activity: The partially purified mucinase in borate buffered calcium saline, prepared as described above, proved to be extremely stable when stored in the fluid state at 4°C, retaining its full *in vitro* enzymatic activity for at least 2½ years (column I, Table II). In Nov. 1954, after 1½ years storage, this mucinase solution was mixed with stock commercially prepared cholera vaccine in 4 different proportions. These mixtures, in rubber stoppered vaccine bottles, were stored at 4°C for an additional year, with periodic assays for *in vitro* mucinase activity. No significant variation in titer, be-

TABLE I. Comparison of Vibrio Culture Filtrate and Partially Purified Mucinase Solution.

Preparation	Vol, ml	Mucinase titer/ml	Mg N/ml	Mucinase units/mg N	Yield
Culture filtrate	7800	64	1.48	43.2	—
Purified mucinase	110	2048	.14	14629	45%

TABLE II. Mucinase Titers of Mucinase-Vaccine Mixtures.

	Mixture* No. 1	2	3	4	5
Mucinase solut'n†	20 ml	15 ml	10 ml	5 ml	1 ml
Cholera vaccine‡	0	5 "	10 "	15 "	19 "
Date tested	Mucinase titers				
5/18/53	2048	—	—	—	—
12/ 1/54	2048	1024	512	256	128
1/ 3/55	2048	1024	512	512	128
2/ 7/55	2048	2048	1024	512	128
3/21/55	2048	1024	1024	512	64
5/27/55	4096	2048	2048	1024	256
7/ 1/55	2048	1024	512	256	64
8/ 1/55	2048	1024	1024	512	64
9/30/55	2048	1024	1024	512	128
12/ 5/55	4096	2048	2048	1024	128

* Mixtures 2 through 5 prepared 11/24/54.
† Mucinase solution prepared in May 1953 from culture filtrate of *V. comma* strain 20A78.
‡ Lederle Cholera Vaccine, Lot No. 1976-67K. Mucinase titer = <4.

yond that inherent in a technic involving 2-fold serial dilutions of the enzyme solution and different preparations of the substrate, was noted with any of the mixtures during this period (Table II). *Stability of antigenic properties of the mucinase and of the somatic antigens*: Subsequent to storage at 4°C for one year, 3.25 ml of the mixture containing mucinase and vaccine in equal volumes was injected intravenously into 3 rabbits in 8 graded doses (ranging from 0.05 ml to 2.0 ml) at 3-4 day intervals. Simultaneously a second group of 3 rabbits received intravenous injections of 1.625 ml of the mucinase preparation alone and a third group was given 1.625 ml of the cholera vaccine alone, in graded doses ranging from 0.025 ml to 1.0 ml. The rabbits were bled 10 days following the final injection and the sera were assayed for agglutinin and antimucinase activity.

The antimucinase titers of the sera of rabbits immunized with the mucinase-vaccine mixture fell in the same range as those resulting from immunization with the mucinase solution alone (Table III). Thus there appears to be no decrease in the antigenic stability of mucinase as a result of its mixture with cholera vaccine, even after such a mixture has been stored for a year at 4°C. On the other hand, the appearance of agglutinins in the sera of these rabbits provides evidence that the presence of active mucinase in the vaccine does not destroy the antibody-stimulating properties of the vibrio somatic antigens.

The presence of agglutinins in the sera of the rabbits immunized with the mucinase solution alone is undoubtedly due to the precipitation of somatic antigens from the culture filtrate along with the mucinolytic enzyme during the preparation of the solution. Absorption of these sera with washed vibrios reduces the agglutinin titer without affecting the antimucinase activity(7). This, together with the observation that the cholera vaccine (containing non-viable cells) fails to stimulate the production of antimucinase, provides evidence that the mucinase and the somatic antigens of *V. comma* are not immunologically related, thus confirming the findings of Burnet and Stone(1) and Jensen(3).

Discussion. The role of the cholera mucinase in the pathogenesis of Asiatic cholera has not as yet been definitely established by direct evidence. The studies of Burnet and Stone(1) and those of Lam, Mandle and Goodner(2) suggest that this enzyme can induce intestinal damage such as may resemble the effects of cholera in man. However, the report by Singh and Ahuja(8) of mucinolytic activity in culture filtrates of vibrios isolated from non-cholera sources suggests that this enzyme may at best be only one of several

TABLE III. Antimucinase and Agglutinin Titers of Sera of Immunized Rabbits.

Immunizing antigens	Rabbit No.	Anti-mucinase titer	Agglutinin titer
None (pre-immunization sera)	1, 2	<20	<20
	4, 5	<20	<20
	7, 8	<20	<20
Mucinase solution*	1	640	1280
	2	320	320
	3	640	1280
Mucinase solution + cholera vaccine† (1:1)	4	320	640
	5	320	1280
	6	640	1280
Cholera vaccine	7	<20	1280
	8	<20	1280
	9	<20	1280

* Mucinase solution prepared from culture filtrate of *V. comma* strain 20A78.
† Lederle Cholera Vaccine, Lot No. 1976-67K.

factors involved in the disease process. If this proves to be the case, a truly effective cholera vaccine would have to contain a number of antigenic components. Jensen(3) has pointed out that the cholera mucinase must be maintained in its native state if it is to retain its antigenicity. Since the enzyme in the vibrio culture filtrate was extremely unstable, he suggested lyophilization as a method of preserving the *in vitro* activity and antigenicity of this material. The results of the present study, however, indicate that it is possible to obtain a stable mucinase solution, which can be combined with the present fluid cholera vaccine without altering the antibody-stimulating properties of the enzyme or of the somatic antigens.

No experimental data are available at this time to provide an explanation for the stability of the mucinolytic enzyme in the purified preparation as contrasted with the extreme lability of this enzyme in the culture filtrates. The purification procedure may have removed or inactivated some substance, perhaps another enzyme, which is responsible for the rapid deterioration of the mucinase. The possibility that one of the ingredients of the buffered calcium saline solution, employed as the solvent for the purified material, is protective must also be considered since Burnet and Stone found that the enzyme in culture filtrates diluted in buffered calcium saline is less readily inactivated by heat than in undiluted filtrates. Additional studies are planned to elucidate this point.

The partially purified mucinase solution has, in addition to its stability, other features which enhance its value as an immunizing antigen. The use of a dialyzed medium, which is not precipitated by ammonium sulfate, in the preparation of this material has eliminated high molecular weight medium constituents which might cause undesirable reactions when injected into animals or humans, and thus eliminates the necessity of developing chemical fractionation procedures for the removal of these substances. Furthermore, the preparation contains, in addition to the mucinase, other products of vibrio metabolism, some or all of which may be desirable components of an immunizing agent for Asiatic cholera.

Summary. 1. A method is described for the preparation from cholera culture filtrates of a mucinase solution which retains its *in vitro* mucinolytic activity for periods in excess of 2 years. 2. This preparation has been combined with a commercially prepared fluid cholera vaccine without affecting the *in vitro* activity of the enzyme, the antigenicity of the mucinase, or the antigenicity of the vibrio somatic substances. 3. The advantages of employing such a preparation as an immunizing agent for Asiatic cholera are discussed.

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