in the face of mounting osmolar clearance indicates that $T^c_{H_2O}$ was inhibited from 40 to 70 percent of its expected value at that level of C_{osm}.

The demonstration of in vivo inhibition of both cortical and medullary adenosine triphosphatase at a time when net sodium reabsorption by the kidney was also depressed suggests that in both these regions the enzyme may participate in active sodium transport. The depression in $T^c_{H_2O}$ [in view of our demonstration that digoxin inhibits renal diluting capacity (*4*)] further indicates that the Na^+,K^+-adenosine triphosphatase plays a principal role in renal concentration and dilution. Since the bulk of sodium transport is believed to occur in the cortex (*8*), the finding of a significantly and reproducibly higher Na^+,K^+-adenosine triphosphatase activity in the medulla requires explanation. There are two possibilities: (i) the rate of sodium transport in this region is greater than in the cortex, and, therefore, there is a requirement for a more active transport enzyme and (ii) the medullary site may represent an area of the kidney which transports Na^+ against a concentration gradient significantly higher than any other area in the organ. Although it is impossible at present to distinguish between the alternatives, current data would support the latter as the most likely explanation. These data represent additional evidence that Na^+,K^+-adenosine triphosphatase is an important pharmacological receptor for cardiac glycosides (*7*).

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Separation of Type 2 Toxins of Vibrio cholerae

Abstract. *Choleragenic toxin was separated from vascular permeability factor by ion-exchange chromatography of supernatants of dialyzed peptone cultures of* Vibrio cholerae. *The choleragenic toxin eluted from columns of QAE-Sephadex with low-ionicity systems is free of permeability factor activity. Further elution of these columns with 0.5*M *NaCl removes both the permeability factor and residual choleragenic toxin. When this latter material is chromatographed on columns of carboxymethyl-Sephadex, the permeability factor toxin is eluted by 0.02*M *phosphate buffer and is free of choleragenic activity. Therefore, choleragenic and permeability factor activities of the type 2 cholera toxins are different and can be separated by these procedures.*

Cholera in man is considered an intoxication because the vibrios appear to be confined to the intestinal lumen and do not invade the tissues. It is now recognized that, in addition to the endotoxin, *Vibrio cholerae* produce exotoxins which can induce in animals an acute diarrheal disease resembling that of human beings. These choleragenic toxins are found in culture filtrates as well as in the stool of patients with acute cholera. According to the classification and interrelations of the several cholera toxins, reviewed by Burrows (*1*), those most likely to take part in the pathogenesis of cholera are designated type 2. These toxins may be demonstrated by their capacity to induce fluid accumulation in the small bowel of the infant rabbit (*2*) or in the ligated ileal loop of the adult (*3*), and by increased vascular permeability when injected intradermally in rabbits (*4*).

It is important to distinguish those toxins which truly play a role in pathogenesis and to devise effective methods for assessing their presence and immunogenic potency. Although the fluid-inducing toxins seem to be directly related to the disease process, the status of the vascular permeability factor (PF) is somewhat uncertain in its relation to choleragenic toxin and to the pathogenesis of cholera. Only a few investigators studied both choleragenic activity and PF in the same toxin preparation. Evans and Richardson (*5*) described the production, in liquid cultures, of toxin choleragenic for the infant rabbit and vascular permeability factor. In a semisynthetic medium which gave high yields of PF, their cultures also elaborated choleragenic toxin. They further purified these toxins by precipitation with dextran sulfate (*6*) and again observed high PF activity associated with choleragenic activity, although exact quantitation of the latter was not attempted.

By the use of chromatography on ion-exchange polymers, we have shown that the choleragenic and PF activities can be separated from one another and, therefore, do not appear to be two manifestations of the same toxin.

Choleragenic toxin was titrated in the ligated ileal loop of young adult rabbits by a modification of the methods developed in Burrows' laboratory (*7*). The toxin unit we have used is that amount which results in fluid accumulation of 1.0 ml per centimeter of intestinal loop. Although this system gives results approximating those of Burrows' group, it is somewhat less precise.

The vascular permeability factor was titrated by the methods of Craig (*4*). The test material was injected in 0.1 ml amounts intradermally in the rabbit, 18 hours later the rabbit was given an intravenous injection of Niagara sky-blue dye. The toxin unit is the bluing dose and is defined as that amount of toxin which yields a blue area, at the injection site, with a mean diameter of 0.7 cm.

Crude toxin was produced in dialyzed peptone medium with *Vibrio cholerae* 569B according to the procedure of Coleman *et al.* (*7*). The cultures were grown for 6 hours on a rotary shaker at 37°C, the cells were removed by centrifugation, and the supernatant was sterilized by membrane filtration. This peptone supernatant was concentrated by flash evaporation and exhaustively dialyzed to remove medium constituents. Final concentration was 50- to 100-fold over the original culture filtrate.

Table 1. Choleragenic and permeability factor activities eluted from ion-exchange columns. PS, peptone supernatant; FrC, fraction C.

Applied sample	Eluted fraction	Choleragenic activity			Permeability factor activity		
		Units*		Recovery (%)	Bluing doses†		Recovery (%)
		Starting	Recovered		Starting	Recovered	
		QAE-Sephadex A50					
PS		6400			50,000		
	A		1400	22		None‡	None
	B		None‡	None		None‡	None
	C		1900	30		8500	17
		CM-Sephadex A50					
FrC		1900			7000		
	D		None‡	None		1500	21

* Amount of toxin resulting in 1.0 ml fluid accumulation per centimeter intestinal loop. † Amount of toxin resulting in 0.7 cm diameter zone of permeability in rabbit skin. ‡ Represents undetectable amount of toxin at minimum dilution of 1 : 20.

The concentrates of the peptone supernatant, as well as the column fractions, were assayed for protein by the method of Lowry *et al.* (*8*), with crystalline bovine serum albumin as a standard. Carbohydrate was estimated by the anthrone test as glucose equivalents. Concentrated peptone supernatants usually contained 2000 to 3000 units of choleragenic toxin per milliliter and 20,000 to 30,000 bluing doses per milliliter of PF toxin.

These crude toxin preparations were chromatographed on Sephadex ion exchangers in cooled columns (Pharmacia Fine Chemicals). In the primary separation, the sample was applied to a column 2.5 by 16 cm of a strongly basic anion exchanger, QAE-Sephadex A50, equilibrated with dilute sodium chloride solution to the same ionicity as the peptone supernatant. This column was first eluted with the equilibrating resolvent and was followed by elution with 0.5*M* NaCl solution. The column eluates were monitored directly for ultraviolet absorption at 254 nm (ISCO ultraviolet analyzer; flow cell of 5 mm light path). Two distinct peaks (Fig. 1) were removed with the low-ionicity system. The first contained 7 percent of the protein applied to the column but had no detectable carbohydrate. This peak (fraction A) contained choleragenic but not PF activity and is analogous to fraction I of Coleman *et al.* (*7*). The following peak (fraction B) contained 6 percent of the original protein but had neither detectable toxins nor carbohydrates. When the 0.5*M* NaCl solution was passed through the column, a third ultraviolet peak was eluted (fraction C). This fraction contained 57 percent of the eluted protein and all of the anthrone-positive material. This peak, which is analogous to fraction II of Coleman *et al.* (*7*), had both choleragenic and PF activity.

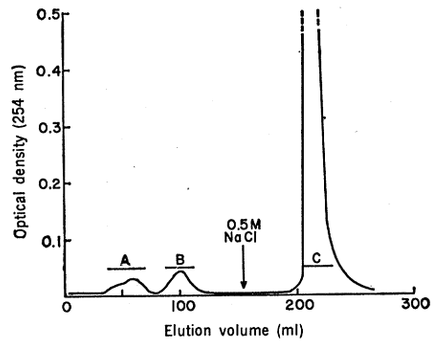

Fig. 1. Elution profile of peptone supernatant from QAE-Sephadex equilibrated and first eluted with 0.01*M* NaCl; the second elution was with 0.5*M* NaCl. Horizontal bars represent fractions tested.

In order to minimize loss due to the lability of the toxicities, all column components were eluted into portions of 0.05*M* phosphate buffer at *p*H 7.0. Earlier experiments had shown that both choleragenic and PF activities are stable at this *p*H for several days in the cold.

The fraction C from QAE-Sephadex was concentrated, dialyzed against 0.02*M* phosphate buffer at *p*H 7.0, and applied to a column of carboxymethyl-Sephadex (a weakly acidic cation exchanger) equilibrated in the same buffer. A single, sharp ultraviolet-absorbing peak (0.65 optical density units at 254 nm) is eluted with this buffer. This fraction contained 82 percent of the protein and 40 percent of the carbohydrate applied to this column. The fraction contained PF but showed only a trace of choleragenic activity. No additional ultraviolet-absorbing material could be eluted when the ionicity was increased to 0.5 with sodium chloride.

Table 1 shows the distribution of the choleragenic and PF activities among these column fractions. Approximately 52 percent of the choleragenic activity is recovered from the QAE-Sephadex in fractions A and C. Only about 17 percent of the PF activity is recovered. Of the choleragenic toxin applied to the column only 22 percent is present in fraction A and is free of detectable PF. When fraction C is chromatographed on carboxymethyl-Sephadex, all of the detectable choleragenic toxin is removed. Of the PF applied to this column 21 percent can be eluted.

When a weaker anion exchanger, diethylaminoethyl-Sephadex, was substituted for the QAE-Sephadex, a similar elution pattern was observed, except that the first and second peaks (fractions A and B) were not so distinct and were contaminated with a trace of PF activity. However, upon elution of the column with 0.5*M* NaCl, almost 90 percent of the recovered PF activity and only about 10 to 20 percent of the recovered choleragenic activity is found in the third peak (fraction C).

The loss of activity of both toxins in these column fractionation procedures is due to their lability and to the fact that only the middle portion of each peak was taken for assay. The data presented here indicate that choleragenic toxin and PF activity can be separated by ion-exchange chromatography, and that PF activity is not necessary for fluid accumulation in the bowel. Since fluid and ion movement is one of the outstanding aspects of cholera pathophysiology, the role of PF in the disease mechanism becomes uncertain. If this apparent lack of identity between choleragenic and PF toxins is confirmed by immunological studies, then studies on the potency of immunizing antigens for antitoxic immunity in man must take into consideration this diversity of toxins.

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