Affinity Chromatography and Structural Analysis of *Vibrio cholerae* Enterotoxin–Ganglioside Agarose and the Biological Effects of Ganglioside-Containing Soluble Polymers†

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ABSTRACT: Columns of agarose derivatives containing covalently attached gangliosides quantitatively adsorb the ^{125}I-labeled cholera toxin of chromatographed samples. The most effective derivatives are those in which the gangliosides are attached to macromolecules (albumin, poly(L-lysine-DL-alanine) copolymers) which are covalently linked to agarose. Ganglioside adsorbents (1-ml columns) containing such macromolecular "arms" can effectively adsorb cholera toxin even after the adsorbent is diluted 200- to 600-fold with unsubstituted agarose. Selective adsorption is blocked if the toxin is incubated with free gangliosides before chromatography. Quantitative elution is achieved with buffers containing 5–7 M guanidine·HCl. The biological activity of purified samples of cholera toxin is completely removed by chromatography on small ganglioside–agarose affinity columns, and this activity can be quantitatively recovered upon elution with guanidine·HCl. Small (5-ml) affinity columns can remove virtually all (more than 99%) of the cholera toxin activity and ^{125}I-labeled toxin present as a tracer in preparations of filtrates of *Vibrio cholerae* derived from about 4 l. of crude culture medium. Ganglioside–agarose beads can block the lipolytic effects of cholera toxin on isolated fat cells. Soluble ganglioside polymers, prepared by covalently attaching the glycolipids to branched copolymers of lysine and alanine, can prevent the binding of ^{125}I-labeled toxin to liver membranes as well as block completely the lipolytic activity of cholera toxin on fat cells. These polymeric ganglioside derivatives may prove useful in the management of the manifestations of clinical cholera. Studies with sodium dodecyl sulfate disc gel electrophoresis indicate that cholera toxin is composed of two major subunits having molecular weights of about 66,000 and 36,000. Reduction and alkylation convert the larger subunit into components having a molecular weight of about 8000, and the smaller subunit is converted to two components having molecular weights of about 27,000 and 8000; the role of disulfide bonds in maintaining or stabilizing the oligomeric structure of the two major subunits is uncertain. The larger subunit (mol wt 66,000) appears to be very similar to or identical with choleragenoid, a toxin derivative which is antigenically very similar to toxin, which is biologically inactive, and which competitively inhibits the binding and biological activity of cholera toxin. The smaller subunit (mol wt 36,000) does not appear to bind to cells. It is proposed that the ability of cholera toxin to bind specifically to cells is governed solely by the larger subunit, but that the ability to elicit a specific biological response resides in the smaller subunit. Cholera toxin thus consists of one subunit which acts to deliver to the cell membrane, in a highly specific way, another molecule (subunit) which in turn is capable of inducing subsequent changes which lead to the biological response.

It has recently been demonstrated that the enterotoxin from *Vibrio cholerae*, which is responsible for the gastrointestinal manifestations of clinical cholera, binds very strongly to brain gangliosides and less strongly to certain glycoproteins such as fetuin and thyroglobulin (Cuatrecasas, 1973a,b). Gangliosides block the biological effects of cholera toxin on isolated fat cells (Cuatrecasas, 1973a,b; van Heyningen *et al.*, 1971) and on the small intestine (van Heyningen *et al.*, 1971; Pierce, 1973; Holmgren *et al.*, 1973), and they prevent the binding of ^{125}I-labeled cholera toxin to specific receptors on the cell membranes of various tissues, such as adipose tissue, liver, erythrocytes, and intestinal epithelial cells (Cuatrecasas, 1973a,b). There is considerable evidence indicating that gangliosides, and specifically G_{M1} gangliosides, are the natural membrane receptors with which cholera toxin specifically interacts to elicit its biological effects in tissues (Cuatrecasas, 1973a,b).

The present studies describe the preparation and use of insoluble agarose derivatives containing covalently linked gangliosides for the extraction and purification of cholera toxin by affinity chromatography (Cuatrecasas *et al.*, 1968; Cuatrecasas, 1972). The potential utility of such insoluble biospecific adsorbents, as well as of soluble polymers which contain covalently attached gangliosides, in the therapeutic approach to clinical cholera is explored by demonstrating that such derivatives effectively block the binding and the metabolic effects of cholera toxin in isolated adipocytes.

Experimental Section

Materials. The crude culture filtrate cholera toxin from *Vibrio cholerae* was lot 002 (Wyeth), kindly provided by Dr. R. Northrup of the SEATO Cholera Research Program, NIAID. This material was prepared by lyophilization of culture filtrate of *V. cholerae* strain 569B, grown in Richard-

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son's (Richardson and Noftle, 1970) TRY medium; 100 g of this material represented the lyophilized filtrate of about 8.45 l. of crude culture medium. Cholera toxin (lot 1071), purified by the method of Finkelstein and LoSpalluto (1970), was obtained from Dr. R. S. Northrup, SEATO Cholera Research Program; it was prepared under contract for the National Institute of Allergy and Infectious Diseases by Dr. R. A. Finkelstein, The University of Texas Southwestern Medical School, Dallas, Texas. Choleragenoid was a gift from Dr. Finkelstein. Bovine brain gangliosides (grades II and III) were purchased from Sigma. Procine thyroglobulin was obtained from Miles, fetuin from Calbiochem, bovine albumin (grade A) from Pentex, guanidine·HCl (Ultra Pure) and urea from Schwarz-Mann, Sepharose 4B from Pharmacia, dicyclohexylcarbodiimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide from Pierce, and *N*-hydroxysuccinimide from Aldrich. The multichain copolymer, poly(L-lysyl-DL-alanine), which consists of a polylysyl backbone and has an alanine to lysine ratio of 15 to 1, was purchased from Miles; the molecular weight of this compound was about 37,500. Poly(L-lysine)·HCl (mol wt 160,000) was obtained from Schwarz-Mann.

Procedures. [^{125}I]Cholera toxin (5–20 μCi/μg) was prepared from toxin samples chromatographed on Sephadex G-75 by procedures described elsewhere (Cuatrecasas, 1973a). About 80% of the radioactive material prepared in this way bound specifically to liver membranes. The molecular weight of cholera toxin was assumed to be 84,000 and the $A^{1\%}_{1\,cm}$ (280 nm) 11.41 (LoSpalluto and Finkelstein, 1972). Isolated fat cells were prepared from male Sprague-Dawley rats (90–120 g) by the method of Rodbell (1966). Liver membranes were prepared by homogenization in 0.25 M sucrose followed by differential centrifugation (Cuatrecasas, 1972b). Protein content was determined by the method of Lowry *et al.* (1951) using bovine albumin as the standard.

The specific binding of [^{125}I]cholera toxin to liver membranes was performed as described previously (Cuatrecasas, 1973a,b). Liver membranes (20–100 μg of protein) were incubated for 20 min at 24° in Krebs–Ringer–bicarbonate buffer (pH 7.4), containing 0.1% albumin and the iodinated toxin (5–10 $\times$ 10^4 cpm); binding was determined by filtration on cellulose acetate (EGWP, Millipore Corp.) filters. For every determination nonspecific binding was determined by including control samples in which native toxin (5 μg/ml) was added to the membranes before adding [^{125}I]toxin. The presence of free gangliosides in column effluents was determined by measuring the ability of these samples to block the binding of [^{125}I]cholera toxin to liver membranes (Cuatrecasas, 1973a,b). The iodoprotein was incubated with the sample for 50 min at 24° in Krebs–Ringer–bicarbonate buffer (pH 7.4), containing 0.1% albumin before determining specific binding. By these methods it is possible to detect less than 50 ng/ml of crude bovine brain gangliosides (type III, Sigma).

Lipolysis by fat cells was studied by determining the concentration of glycerol in the medium by the method of Ryley (1955). The bioassay of cholera toxin was based on the potent lipolytic action of the toxin on fat cells. Fat cells (2–8 $\times$ 10^5 cells/ml) were incubated at 37° in Krebs–Ringer–bicarbonate buffer containing 3% albumin; samples (0.1 ml) were analyzed for glycerol at various time periods between 90 and 160 min (Cuatrecasas, 1973c). Since the absolute lipolytic responses varied between experiments, the activity of unknown toxin samples were expressed on the basis of comparisons with standard curves obtained with native cholera toxin.

Protein was analyzed by electrophoresis in 7.5% polyacrylamide disc gels (7.5 $\times$ 0.5 cm) at pH 7.0 in 0.1 M sodium phosphate buffer, both in the presence and absence of sodium dodecyl sulfate (Weber and Osborn, 1969). Protein was detected by staining 1–2 hr with Coomassie Brilliant Blue (0.25% in methanol–water–acetic acid (5:5:1, v/v)), and gels were destained overnight in water–acetic acid–methanol (35:3:2, v/v). Molecular weights were estimated from electrophoretic mobilities of standard proteins (cytochrome *c*, ovalbumin, serum albumin) in gels containing 0.1% sodium dodecyl sulfate.

Preparation of Ganglioside–Agarose Derivatives. Poly(L-lysine) and the branched, multichain copolymer of L-lysine ("backbone") and DL-alanine ("side arms") were coupled to cyanogen bromide activated agarose by slight modifications (Sica *et al.*, 1973a,b) of previously described methods (Cuatrecasas, 1970). These polymers were used to increase the number of potentially modifiable functional groups (α-amino groups in the copolymer, ϵ-amino groups on the homopolymer) on the agarose, to place these groups at a considerable distance from the agarose backbone, and to enhance the likelihood of multipoint attachment of the soluble polymer on the agarose, which would increase the stability of linkage of subsequently substituted ligands. The derivatives used contained about 1.2 mg of copolymer/ml of agarose and about 1.4 mg of poly(L-lysine)/ml of agarose. Albumin was also used as a macromolecular spacer for the same reasons described above. Albumin was coupled to cyanogen bromide activated agarose in the absence (native) or presence (denatured) of 10 M urea, as described recently (Sica *et al.*, 1973a,b); these derivatives contain 2–3 mg of albumin/ml of gel. 3,3′-Diaminodipropylamine, fetuin, and thyroglobulin were coupled to agarose with cyanogen bromide (Cuatrecasas, 1970); these agarose derivatives contained about 10 μmol, 6 mg, and 8 mg, respectively, of the ligand and proteins per ml of packed gel.

Gangliosides were coupled through carboxyl groups of the terminal sialic acid residues to amino groups of the derivatized agaroses by utilizing a water-soluble carbodiimide reagent or dicyclohexylcarbodiimide, by preparing an active *N*-hydroxysuccinimide ester of the ganglioside, or by preparing an activated, mixed anhydride of the ganglioside.

Coupling with Carbodiimides. The derivatized agarose (containing amino groups) (25 ml) was washed and suspended in 50 ml of 50% (v/v) aqueous dioxane. Brain gangliosides (type III, Sigma) (50 mg) were added and the suspension was gently shaken at 24° for 15 min. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (100 mg) was added and the suspension was shaken for 6 hr at 24°, and another 100-mg portion of the carbodiimide was added. After shaking for another 12 hr, the gel was washed with 500 ml of water, 500 ml of 75% (v/v) aqueous methanol, 250 ml of 6 M guanidine·HCl, and 500 ml of water. The content of ganglioside, as judged by the recovery of unreacted ganglioside, was about 0.5 mg/ml of gel. Coupling was also performed in an organic solvent by reacting 20 mg of ganglioside and 5 mg of dicyclohexylcarbodiimide in 10 ml of dioxane for 30 min at 15°. This was added to 20 ml of albumin–agarose suspended in dioxane in a total volume of 40 ml. After reacting for 15 hr at 24°, the gel was washed with 500 ml of dioxane, 500 ml of 90% (v/v) methanol, and 250 ml of 6 M guanidine·HCl.

N-Hydroxysuccinimide Ester. Ganglioside (20 mg) was reacted with 2.5 mg of *N*-hydroxysuccinimide and 2.5 mg of dicyclohexylcarbodiimide for 30 min at 15° in 10 ml of dioxane. The solution was then added to 20 ml of albumin–agarose suspended in dioxane (total volume, 40 ml). After

shaking for 15 hr at 24°, the gel was washed as above described (water-soluble carbodiimide reaction).

Mixed Anhydride. A 100-μl portion of 0.1 M *N*-methylmorpholine in tetrahydrofuran was added to a solution of anhydrous tetrahydrofuran containing 20 mg of ganglioside. After stirring the solution for 10 min at 0°, 100 μl of 0.1 M isobutyl chloroformate (Vaughan and Osato, 1952) in tetrahydrofuran was added and the reaction was allowed to continue for 20 min at 0°. The reaction mixture was added to 20 ml of the amino agarose derivative suspended in dioxane (total volume, 40 ml). After reacting for 15 hr at 24° the gel was washed as above described.

Although all of the methods above described resulted in effective adsorbents, the best results were consistently obtained with derivatives prepared with the water-soluble carbodiimide and with the mixed anhydride.

Preparation of Water-Soluble Polymers Containing Gangliosides. Gangliosides were coupled to the branched-chain copolymer of lysine and alanine with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; 20 mg of poly(L-lysyl-DL-alanine) was dissolved in 7 ml of water and 5 ml of methanol and 40 mg of ganglioside (dissolved in 10 ml of 50% aqueous methanol) were added. The mixture was stirred at 24° for 15 min and two 40-mg portions of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide were added at 6-hr intervals. After stirring for an additional 12 hr, the reaction mixture was dialyzed against 4 l. of H_2O for 15 hr and lyophilized. The sample was then chromatographed on a column (2.4 × 70 cm) of Sephadex G-75 equilibrated with 25% aqueous methanol containing 0.005 M HCl; the flow rate was 15 ml/hr, 4-ml fractions were collected and the elution was monitored by continuously recording the absorbance at 256 nm. The peak in the void volume was collected, lyophilized, and rechromatographed on a column (1.6 × 26 cm) of Sephadex G-100 equilibrated with 6 M guanidine·HCl (12 ml/hr, 4 ml/fraction). The material present in the first peak was dialyzed against four changes of H_2O (4 l.) for 24 hr and lyophilized. The yield was 22 mg.

Results

General Properties of Affinity Adsorbents. Whereas [^{125}I]-cholera toxin does not bind to columns containing unsubstituted agarose, a substantial portion of the radioactivity does adsorb to columns containing fetuin–agarose or A-DADA-gang[1] (Figure 1). About 80% of the radioactivity in the iodinated toxin preparation can bind specifically to liver membranes before chromatography. About 30% of the radioactive material applied to a fetuin–agarose column is not adsorbed, and about 20% of this material can still bind selectively to liver membranes (Figure 1B). The ganglioside–agarose adsorbent appears to be more effective than that which contains fetuin since 15–20% of the radioactive material which is applied to the column appears in the breakthrough of the column, and virtually none of this material can bind selectively to liver membranes (Figure 1C). It appears that about 15–20% of the total radioactive content of [^{125}I]cholera toxin represents radioactivity on denatured or contaminating protein. The specificity of the adsorptive process is further illustrated by demonstrating that incubation of the [^{125}I]-cholera toxin with gangliosides before chromatography effectively prevents the subsequent adsorption of radioactivity to the column (Figure 1D).

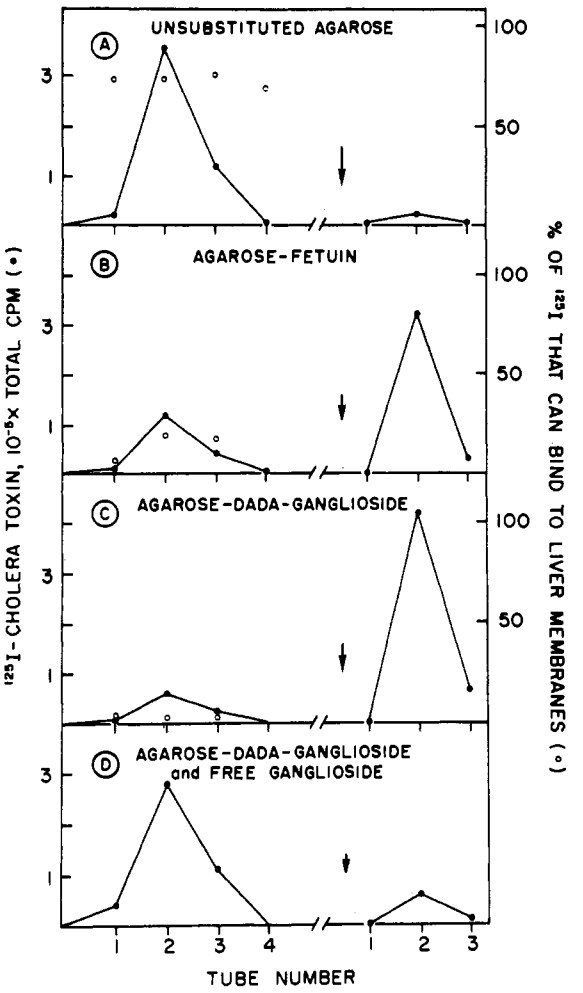

FIGURE 1: Affinity chromatography of ^{125}I-labeled toxin from *V. cholerae*. Pasteur pipets containing 0.5 ml of the indicated gel were washed for 4 hr at 24° with 50% (v/v) methanol followed by 40 ml of 7 M guanidine·HCl and 40 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% albumin. The samples (0.5 ml) applied on the columns contained 5.1 × 10^5 cpm of ^{125}I-labeled cholera toxin (6.7 Ci/g)) in Krebs–Ringer–bicarbonate buffer, containing 0.1% albumin. The columns were washed at 24° with 20 ml of the same buffer (note break in axis) before eluting with 7 M guanidine·HCl (arrows). The total radioactivity in each fraction (0.5–1 ml) was determined. Column D was exactly as that described in C except that the sample was incubated with 100 μg/ml of gangliosides for 40 min at 24° before application to the column. After elution the gel remaining in the column contained less than 5% of the radioactivity applied on the column. The proportion of radioactivity capable of binding to liver membranes was determined by incubating at 24° for 20 min with membrane samples (200 μg of protein/ml) in Krebs–Ringer bicarbonate buffer, containing 0.1% albumin, and determining binding by membrane filtration as described in the text. Binding was not determined in eluted samples because of the presence of guanidine (see text), or in expt D because of the presence of free gangliosides. The derivative described in C and D was that prepared with a water-soluble carbodiimide.

In the experiments described in Figure 1, elution of the adsorbed [^{125}I]toxin was achieved with 7 M guanidine·HCl. Experiments were performed to determine whether milder conditions could be utilized to elute the toxin from such columns (Table I). The strength with which the toxin is adsorbed is evident from the inability to achieve elution with 0.1 M acetic acid, 2 N NaCl, and 3 M guanidine·HCl containing

[1] Abbreviations used are: A-PLL-gang, ganglioside–poly(L-lysine)–agarose; A-PLL-Ala-gang, ganglioside–poly(L-lysyl-DL-alanine)–agarose; A-DADA-gang, ganglioside–diaminodipropylamine–agarose; A-NatAlb-gang, ganglioside–native albumin–agarose; A-DenatAlb-gang, ganglioside–denatured albumin–agarose.

TABLE I: Conditions for Eluting ^{125}I-Labeled Cholera Toxin from an Affinity Column.[a]

Elution Condition	% of ^{125}I-Labeled Toxin Eluted from the Column
7 M Guanidine·HCl	100
5 M Guanidine	86
4 M Guanidine·HCl	38
3 M Guanidine·HCl	11
2 M Guanidine·HCl	3
3 M Guanidine·HCl + 1 M NaCl	13
3 M Guanidine·HCl + 40% (w/v) ethylene glycol	16
0.1 M Acetic acid	4
0.1 N HCl	87

[a] Columns containing 1 ml of A-Alb-gang diluted 1:10 with unsubstituted Sepharose 4B were used as described in Figure 1 for chromatography of samples (0.5 ml) containing 3.2×10^5 cpm of ^{125}I-labeled cholera toxin. Elution was attempted with Krebs–Ringer-bicarbonate buffer (pH 7.4), containing 0.1% albumin and the indicated compounds. The samples containing acetic acid and HCl did not contain a buffer but did contain 0.1% albumin.

1 N NaCl. Even 4 M guanidine·HCl results in the elution of only about one-third of the bound toxin. Nearly quantitative elution, however, can be obtained with higher concentrations (5 M) of guanidine·HCl or with 0.1 N HCl.

Samples which had been chromatographed on A-DADA-gang columns such as that described in Figure 1 contained detectable amounts of free gangliosides. Because the presence of this compound in the samples can interfere with assays of the breakthrough material, and it can also potentially interfere with adsorption of the toxin to the column, experiments were performed to determine if other derivatives were less susceptible to this "leakage" phenomenon (Table II). Since the presence of free gangliosides in the column breakthrough samples is meaningful only when considered in relation to the concentration and effectiveness of the selective adsorbent, the various derivatives were diluted serially with unsubstituted agarose and their ability to extract [^{125}I]cholera toxin was compared. As predicted from the considerations described earlier, it is clear that the leakage of free gangliosides from the adsorbents which contain macromolecular spacers is much less marked than that which occurs with A-DADA-gang. Of equal importance, however, is the fact that the adsorbents containing the polymeric spacers are inherently much more effective in extracting the toxin. It is notable that these derivatives are quite effective even when diluted 50-fold with unsubstituted agarose. The best derivative appears to be A-NatAlb-gang; with this adsorbent some adsorption is detectable even after a 600-fold dilution. Furthermore, leakage is not a problem in this case since no significant free ganglioside is detectable in effluents of columns containing a 10-fold diluted adsorbent. Experiments of this type, which are quite useful in comparing the relative effectiveness of a variety of adsorbents, indicate that fetuin–agarose is quite inferior to any of the ganglioside–agarose derivatives since virtually no adsorption occurs to adsorbents diluted 1:10 with unsubstituted agarose (Table II).

TABLE II: Chromatography of ^{125}I-Labeled Cholera Toxin on Various Affinity Adsorbents.[a]

Agarose Derivative	% of ^{125}I-Labeled Toxin		Ganglioside in Elution; % Inhibn
	In Breakthrough	In Elution	
Unsubstituted agarose	93	3	
Diaminodipropylamino-agarose	91	7	
A-DADA-gang			
Undiluted	21[b]	74	48
1:10	27[b]	70	20
1:50	48	41	0
A-PLL-gang			
Undiluted	18[b]	76	10
1:10	21[b]	75	5
1:50	40	46	0
1:200	68	25	0
A-PLL-Ala-gang			
Undiluted	17[b]	80	5
1:10	19[b]	78	0
1:50	20	76	0
1:200	52	42	0
A-NatAlb-gang			
Undiluted	18[b]	78	10
1:10	20[b]	81	0
1:50	21[b]	75	0
1:200	38	52	0
1:600	68	30	0
A-DenatAlb-gang			
Undiluted	24[b]	72	21
1:10	29[b]	69	0
1:50	48	47	0

[a] Columns (Pasteur pipets) containing 1 ml of the specified gel were washed with 50% (w/v) methanol, 7 M guanidine·HCl, and Krebs–Ringer–bicarbonate buffer as described in Figure 1. ^{125}I-Labeled cholera toxin (2.1×10^5 cpm), in 0.5 ml of Krebs–Ringer–bicarbonate buffer, containing 0.1% albumin, was applied to each column. The columns were washed, eluted with 5 M guanidine·HCl in 50 mM Tris·HCl (pH 7.4), containing 0.1% albumin, and all the fractions were counted as described in Figure 1. In some cases the adsorbents were diluted serially with unsubstituted agarose before use. The eluted samples were examined for the presence of free gangliosides by incubating (24°, 40 min) aliquots (20 μl) with 0.1 ml of Krebs–Ringer–bicarbonate buffer, containing 0.1% albumin and 5×10^5 cpm of fresh ^{125}I-labeled cholera toxin; the binding of this iodoprotein to liver membranes was then examined as described in the text. The presence of ganglioside is expressed by the per cent inhibition of binding. None of the breakthrough samples contained free gangliosides. Columns containing fetuin–agarose diluted 1:10 with unsubstituted agarose (not shown in table) did not adsorb significant quantities of [^{125}I]toxin, confirming the impression (Figure 1) that such adsorbents are inferior to those containing gangliosides. [b] Less than 5% of the radioactivity in these samples can bind to liver membranes when examined by the binding procedures described in the text. In contrast, approximately 80% of the total radioactivity present in the samples which were applied to the column can bind to liver membranes.

TABLE III: Affinity Chromatography of Purified Cholera Toxin on Ganglioside-Agarose.[a]

	Total Protein (mg)	Cholera Toxin		Specific Activity	
		Radioactivity (cpm)	Act.[b] (Units)	cpm/mg of Protein	Units/mg of Protein
Applied on column	2.40 (100%)	8.8×10^5 (100%)	2.4 (100%)	3.7×10^5	1.0
Breakthrough	0.76 (32%)	1.2×10^5 (14%)	0.1 (4%)	1.6×10^5	0.1
Elution	1.19 (50%)	6.0×10^5 (68%)	2.1 (88%)	5.0×10^5 (1.4)[c]	1.8 (1.8)[c]

[a] The data summarize the experiment described in Figure 2 (bottom). The purification is more marked when expressed according to protein content than when expressed by radioactivity. The apparent discrepancy is explained by the fact that the ^{125}I-labeled cholera toxin was prepared from partially purified (by gel filtration) toxin. About 85% of the iodoprotein which was added to the sample as a tracer adsorbs to affinity columns in the absence of unlabeled toxin (Figure 1 and Table II), and the breakthrough radioactivity does not bind to cell membranes. [b] Lipolytic activity is described in arbitrary units which are defined on the basis of comparison with the starting cholera toxin. [c] Purification achieved by these procedures.

The chromatographic behavior of a sample of purified cholera toxin containing a tracer quantity of [^{125}I]toxin on a column containing A-Alb-gang is presented in Figure 2 and Table III. Adsorption is prevented by incubating the toxin with gangliosides before chromatography. In the absence of gangliosides the column extracts 70% of the protein and more than 95% of the lipolytic activity. The protein which does not adsorb to the column is virtually without lipolytic activity and the radioactivity in this peak does not bind to liver membranes. Nearly 90% of the lipolytic activity applied to the column is recovered upon elution with 7 M guanidine·HCl. These experiments demonstrate that the behavior of ^{125}I-labeled and native cholera toxin on such affinity columns is very similar.

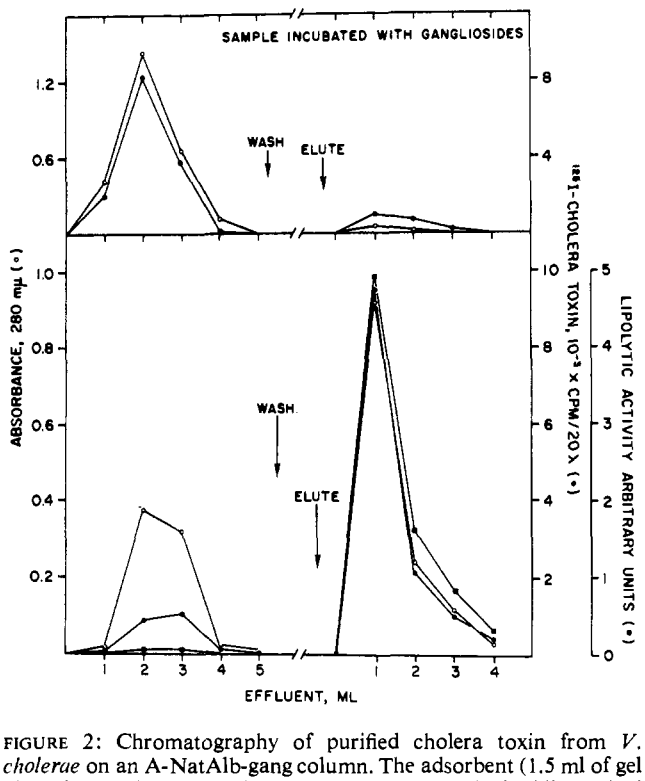

FIGURE 2: Chromatography of purified cholera toxin from *V. cholerae* on an A-NatAlb-gang column. The adsorbent (1.5 ml of gel diluted 1:5 with unsubstituted agarose) was washed while packed in a 5-ml disposable pipet with 50% (v/v) methanol for 2 days at 24°. The column was washed with 7 M guanidine·HCl for 3 hr and equilibrated with Krebs–Ringer–bicarbonate buffer. A tracer quantity of high-specific activity ^{125}I-labeled cholera toxin (14 Ci/g) was added to the sample (2.4 mg of protein in 1.4 ml of the same buffer). Chromatography was performed at 24° with (upper) or without (lower) prior incubation of the sample with 100 μg/ml of brain gangliosides. The flow rate was 3 ml/hr. After chromatography the column was washed (arrows) with 15 ml of the above buffer and eluted with 50 mM Tris·HCl (pH 7.4), containing 5 M guanidine·HCl (arrows). The eluted samples were diluted 3-fold with 50 mM Tris·HCl buffer (pH 7.4), dialyzed for 16 hr at 4°, and tested for lipolytic activity by comparison to standard curves performed with the standard, starting sample of cholera toxin.

Chromatography of Crude Vibrio cholerae Filtrates on Affinity

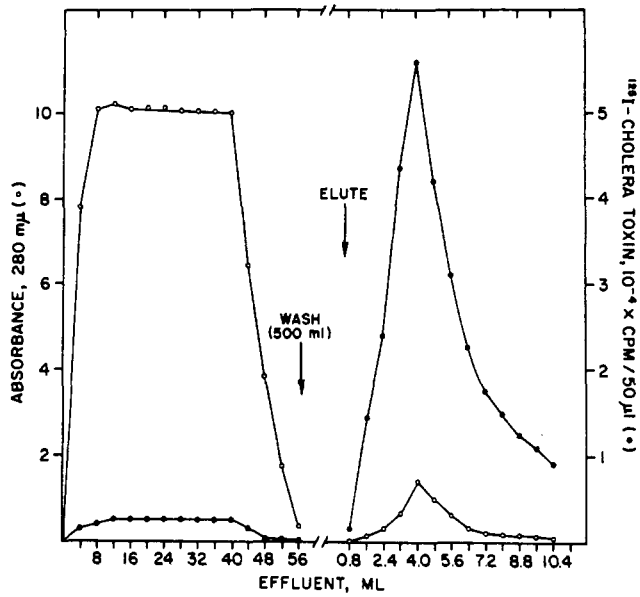

FIGURE 3: Chromatography of crude filtrate from *V. cholerae* on a column containing the adsorbent, A-NatAlb-gang. The column (5 ml of gel in a 10-ml disposable pipette) was washed at 24° for 2 days with 50% (v/v) methanol followed by 6 M guanidine·HCl (200 ml, 6 hr) and Krebs–Ringer–bicarbonate buffer (pH 7.4) (100 ml, 3 hr). Forty grams of lyophilized crude filtrate (derived from 3.4 l. of crude filtrate) was dissolved in 150 ml of distilled water and dialyzed against distilled water for 1 hr at 24° (4 l.) and for 18 hr at 4° (4 l.). The material was dialyzed for another 7 hr at 4° against 4 l. of Krebs–Ringer–bicarbonate buffer (pH 7.4). After removing precipitated material by centrifugation at 25,000*g* for 20 min, a 50-ml sample containing 4.2 mg of protein/ml was adjusted with NaCl to achieve a molarity of 0.2 M and with a tracer quantity of ^{125}I-labeled cholera toxin (11 Ci/g) to achieve 2.2×10^4 cpm/0.1 ml. The sample was applied at 4° with a flow rate of 5 ml/hr. After chromatography of the sample, the column was washed at 4° with 500 ml of Krebs–Ringer–bicarbonate buffer containing 0.8 M NaCl (arrow). Elution was achieved with 50 mM Tris·HCl (pH 7.4), containing 5 M guanidine·HCl (arrow). More than 98% of the initial biological (lipolytic) activity of the sample disappeared in the early as well as the late breakthrough effluent fractions. The various fractions of this experiment were pooled and analyzed quantitatively (Table IV).

TABLE IV: Summary of Affinity Chromatography of Crude Filtrate Cholera Toxin from *V. cholerae* on a Column of A-Nat-Alb-Gang.[a]

	Total Vol (ml)	Protein[b] mg/ml	Protein[b] Total	Cholera Toxin: From Tracer [^{125}I]Toxin cpm/mg of Protein	Cholera Toxin: From Tracer [^{125}I]Toxin Total cpm	Cholera Toxin: From Biol Act.[c] mg/ml	Cholera Toxin: From Biol Act.[c] Total cpm	Yield (%): From [^{125}I]-Toxin	Yield (%): From Act.	Purifcn[d]
Applied on column	50	4.2	210	5.2×10^4	11×10^6	0.10	5.0[g]	100	100	
Breakthrough	60	3.1	186	1.2×10^4	2.3×10^6 [e]	0.1	0.006	21[e]	0.8	
Washes	500	0.1			0.4×10^6	0	0	4	0	
Elution	9.6	0.14	1.3	4.7×10^6	6.1×10^6	0	0	55[f]	0	90

[a] The data are obtained from the experiment described in Figure 3. The eluted samples were pooled, examined for protein and total radioactivity, and then dialyzed at 24° against distilled water for 30 min. A substantial portion (9×10^5 cpm) of the radioactivity was lost by this short dialysis procedure; from other experiments it is known that dialysis of [^{125}I]toxin in guanidine·HCl, for 2 hr (24°) against distilled water results in the loss of nearly 60% of the radioactivity. The dialyzed sample was lyophilized, redissolved in 0.8 ml of water, and chromatographed (to remove the guanidine) on a Bio-Gel P2 column (1.7×45 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.4). The radioactive material, present exclusively in the void volume, was pooled, lyophilized, and redissolved in 0.4 ml for studies of biological activity and for disc gel electrophoretic experiments. [b] Determined by the method of Lowry *et al.* (1951). [c] Determined by lipolysis, comparing with standard curves performed with native cholera toxin. [d] Based on the specific activity of the ^{125}I-labeled toxin (cpm per mg of protein), without correcting for the proportion of the original radioactivity which does not correspond to active toxin (see *e*). [e] It is known that 20–25% of the ^{125}I-labeled protein in the original preparation was initially denatured or represents contaminating protein since it does not adsorb to any affinity column (Figure 1, Table III) and it does not bind to cell membranes. Therefore there is virtually quantitative adsorption of active toxin to the column in this experiment. [f] This represents at least 70% of the active toxin which adsorbed to the column. [g] On the basis of comparisons with native toxin; since the native toxin only contains (by absorbancy) about 68% of active toxin (see Table III), the estimated content should be closer to 3.5 mg.

Columns. The total material obtained from 3.4 l. of crude culture medium of *V. cholerae* was chromatographed on a 5-ml column of A-NatAlb-gang (Figure 3 and Table IV). More than 99% of the lipolytic activity and 80% (or virtually all of the active form) of the tracer ^{125}I-labeled toxin present in this material disappeared after passage through this column, and no free gangliosides could be detected in the effluent samples. After very thorough and prolonged washing, elution with 5 M guanidine·HCl resulted in the recovery of about 1 mg of protein and at least 70% of the ^{125}I-labeled toxin which had adsorbed to the column. On the basis of radioactivity, the toxin was purified more than 90-fold by this procedure.

Unfortunately, the material eluted from this column was virtually devoid of biological activity. The lack of activity in this material is not explained by the presence of gangliosides or of residual guanidine (which inhibits lipolysis) since it did not alter the lipolytic response of native cholera toxin when these were incubated together before assay. The lack of activity is similarly not explained by the presence of biologically inactive choleragenoid, which can block the binding and activity of cholera toxin (Cuatrecasas, 1973d), since preincubation of cells with the eluted material did not block the binding of ^{125}I-labeled toxin or the lipolytic response to native toxin. Data to be presented in a subsequent section suggests that the loss of activity may have resulted from dissociation of the toxin into subunits; this process is essentially irreversible when the concentration of cholera toxin is very low. In some experiments it has been possible to elute about 10–20% of the lipolytic activity adsorbed, although the reasons for such recovery in certain experiments is not known.

In the experiment described in Figure 3 it was estimated on the basis of lipolytic activity that the entire material applied to the column contained about 3.5 mg of cholera toxin (Table IV, footnote *f*). Since all of the lipolytic activity and all of the active radioactivity were removed by the column, and since the recovery of adsorbed radioactivity upon elution was about 70%, it was anticipated that elution should have yielded about 2.4 mg of protein had the purification been complete. However, only 1.3 mg of protein was present in the eluted sample. The reason for the slight but significant disparity between the quantity of protein actually eluted and that anticipated is not apparent at present. It is possible that alterations of the native toxin, not reflected in the ^{125}I-labeled material, occur during the step of concentration of the crude toxin since this exposes the protein to high ionic strength.

Since in the experiment depicted in Figure 3 all of the cholera toxin applied on the column was extracted from the sample, it was of interest to examine the binding capacity of such columns. When the quantity of sample applied was increased by 2.5 times and the adsorbent was diluted 5-fold with unsubstituted agarose, only one-third of the cholera toxin applied was adsorbed to the column (Figure 4). The toxin was effectively extracted from the first effluent fractions while virtually no extraction occurred in the last fractions. As in previous experiments, only a very small proportion of the total protein was adsorbed to the column, and elution of the radioactively labeled toxin was satisfactory. There was excellent correspondence between the appearance of radioactivity and lipolytic activity in the breakthrough fractions, pointing again to the similarity in the behavior of the labeled and native toxins. The use of ^{125}I-labeled tracers in these experiments greatly facilitates monitoring and quantitation of the chromatographic experiments.

Reversible Denaturation of Cholera Toxin. The binding of cholera toxin to the affinity columns is so strong that to achieve elution it is necessary to use buffers which are likely to unfold, and possibly denature, the protein (Table I). Because of this, and because the protein eluted from columns

TABLE V: Effect of Various Conditions on the Reversible Denaturation of Cholera Toxin.[a]

Preincubn Condn	Concn of Denaturant: Preincubn	Concn of Denaturant: Incubn	Sp Binding of Toxin (cpm)
Krebs–Ringer bicarbonate			17,600
Acetic acid	0.1 M	1 mM	2,800
HCl	20 mM	0.2 mM	1,600
NaOH	20 mM	0.2 mM	3,500
	10 mM	0.1 mM	9,800
Guanidine·HCl[b]	5 M	50 mM	2,200
	3 M	30 mM	2,400
	2 M	20 mM	17,500
Urea[b]	6 M	60 mM	9,300
	5 M	50 mM	14,100
	4 M	40 mM	16,000

[a] [^{125}I]Cholera toxin (0.25 μg/ml, 14 Ci/g) was preincubated in plastic tubes for 30 min at 24° under various conditions designed to obtain protein denaturation. The solutions were then diluted 10-fold with Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin. Samples (50 μl) of these were then incubated for 30 min at 24° in 0.45 ml of Krebs–Ringer–bicarbonate containing 0.1% (w/v) albumin and 50 μg of liver membrane protein. Specific binding of the toxin to membranes was determined by filtration as described in the text. [b] Addition of 0.7 M guanidine·HCl or 1 M urea to the membrane incubation mixture does not decrease the binding of ^{125}I-labeled toxin.

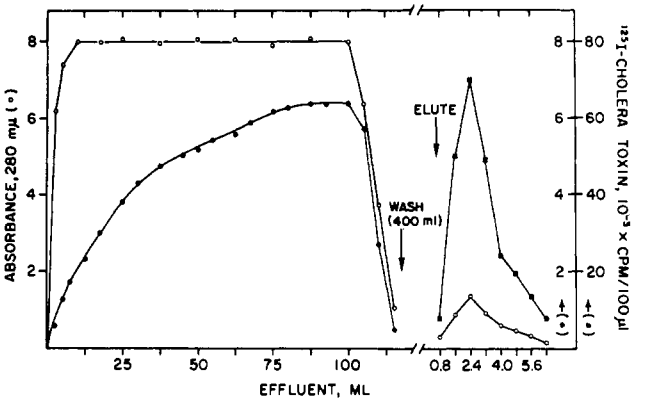

FIGURE 4: Chromatography of crude culture filtrate cholera toxin from *V. cholerae* on a column (5 ml) containing A-NatAlb-gang diluted fivefold with unsubstituted agarose. The column was washed and chromatographed as described in Figure 3. The sample was 120 ml of a culture filtrate prepared as described in Figure 3 except that the content of tracer ^{125}I-labeled cholera toxin was 7.2×10^3 cpm/100 μl. The flow rate was 10 ml/hr. All other conditions were as described in Figure 3. The loss of biological (lipolytic) activity in the chromatographed breakthrough effluent paralleled precisely the loss of radioactivity (●) in the same samples; more than 90% of the biological activity disappeared in the first 15 ml while there was virtually no loss of activity in the last 30 ml.

on which crude samples were chromatographed (Figures 3 and 4, Table IV) yielded essentially inactive toxin preparations, the ability of cholera toxin to renature after removal of denaturants was examined (Table V). Brief exposure of [^{125}I]-cholera toxin to acidic and basic conditions, and to relatively low concentrations of urea and guanidine·HCl, diminishes profoundly the ability of the iodoprotein to bind to liver membranes upon dilution or neutralization of the denaturant; similar effects are observed if urea and guanidine·HCl are removed by dialysis. The ^{125}I-labeled toxin which is eluted from affinity columns such as those depicted in Figure 1 and Tables I and II does not bind at all to liver membranes if tested after removal of guanidine·HCl. These results suggest that the ^{125}I-labeled toxin is undergoing an irreversible unfolding or denaturation. The conditions which cause this irreversible effect occur with concentrations of urea and guanidine·HCl which are lower than those (Table I) which are required to elute the toxin which is adsorbed to a ganglioside–agarose column. This suggests that ganglioside binding greatly stabilizes the tertiary or quaternary structure of the protein.

The effects described above seemed somewhat contradictory to the results of the experiments described in Figure 2, where guanidine·HCl elution of a chromatographed sample of purified toxin yielded active toxin. The possibility was examined that the irreversible denaturation described above is dependent on the concentration of toxin used in such experiments. Samples of native cholera toxin (0.1–0.5 mg/ml) containing a tracer of ^{125}I-labeled toxin were exposed for 25 min at 24° to (a) 0.1 M phosphate buffer (pH 7.4), (b) distilled water, (c) 0.1 N HCl, (d) 7 M guanidine·HCl, and (e) 7 M urea under conditions similar to those described in Table V. The samples were then diluted fivefold and dialyzed overnight against large volumes of Krebs–Ringer–bicarbonate buffer. Virtually no radioactivity was lost during the period of dialysis, and the lipolytic activity of all the samples was equal to that of the sample exposed only to phosphate buffer. It is clear that at these concentrations of cholera toxin, which are about 10,000 times higher than those described in Table V, denaturation of the toxin by exposure to these solvents is readily reversible.

There is some evidence that the denaturation described above involves a process of dissociation of cholera toxin into subunits. As described above, exposure of high concentrations (0.5 mg/ml) of toxin to 7 M guanidine·HCl followed by dialysis does not result in the loss of the ^{125}I-labeled toxin which is added as a tracer. In contrast, exposure of tracer quantities (10 ng/ml) of ^{125}I-labeled toxin to 7 M guanidine·HCl results in the rapid loss of radioactivity upon dialysis, even when 0.1% albumin is added to the sample to prevent adsorption to the dialysis membrane. Under these conditions 60% of the radioactivity is lost after dialysis for 2 hr at 24°, and about 85% is lost after dialysis for 24 hr at 4°. Further evidence for the dissociation into subunits comes from disc gel electrophoretic experiments in 0.5% sodium dodecyl sulfate, which are presented in a later section. These results are consistent with findings of LoSpalluto and Finkelstein (1972), who described reversible dissociation of cholera toxin into subunits of about 15,000 molecular weight upon exposure to 6 M urea or to a pH 3.6; these experiments were performed at concentrations of toxin varying from 2.5 to 4 mg per ml.

Ganglioside–Agarose and the Lipolytic Response to Cholera Toxin. The ganglioside–agarose derivatives described in this report are quite effective in removing ^{125}I-labeled toxin from buffer solutions when the derivatized beads are added and incubated in suspension. The derivatives can be diluted with unsubstituted agarose, and adsorption is generally complete after incubating for 15 min at 24°. The derivatives are also quite potent in protecting fat cells against the metabolic effects of the toxin provided that the beads are added to the

TABLE VI: Effect of Ganglioside-Agarose Derivatives on the Lipolytic Activity of Cholera Toxin.[a]

Ganglioside Derivative[b]	Concn[c] (μl)	Modification of the Lipolytic Response: Added before Toxin	Modification of the Lipolytic Response: Added after Toxin
		% of Cholera Toxin Response	
None		100	100
A-PLL-gang	20	4	103
	100	7	90
A-PLL-Ala-gang	20	2	107
	100	5	101
A-NatAlb-gang	20	12	104
	100	3	96

[a] Isolated fat cells were incubated at 37° for 160 min in Krebs–Ringer–bicarbonate buffer (pH 7.4), containing 3% albumin, the indicated agarose derivative, and cholera toxin (50 ng/ml). In some cases the insoluble ganglioside derivative was added to the cells 10 min after addition of the toxin. Lipolysis was measured by the release of glycerol into the medium as described in the text. [b] In the absence of cholera toxin the agarose derivatives did not alter lipolysis. [c] Microliters of packed agarose beads per milliliter of incubation medium.

cells before the toxin (Table VI). Addition of the adsorbent 10 min after addition of the toxin has no effect on the lipolytic response to the toxin. These results are consistent with the nearly irreversible nature of the binding of cholera toxin to cell membranes (Cuatrecasas, 1973a,b).

Effect of Water-Soluble Polymers Containing Gangliosides. The water-soluble copolymer of poly(L-lysine) (backbone) and -(DL-alanine) (side branches) which contains covalently linked gangliosides effectively inhibits the binding of ^{125}I-labeled cholera toxin to liver membranes (Table VII). This ganglioside polymer is effective in concentrations which in the final incubation medium are as low as 0.1 μg/ml.

TABLE VII: Inhibition of Binding of ^{125}I-Labeled Cholera Toxin to Liver Membranes by Water-Soluble Poly(L-lysyl-DL-alanine) Copolymer Containing Gangliosides.[a]

Concn of Copolymer[b] (μg/ml)	Sp Binding of ^{125}I-Labeled Cholera Toxin (cpm)
None	34,130
100	2,160
10	1,730
1	8,000
0.1	31,900

[a] ^{125}I-Labeled cholera toxin (3.6×10^6 cpm/ml, 8 Ci/g) was incubated for 20 min at 24° in Krebs–Ringer–bicarbonate buffer, containing 0.1% albumin, with varying concentrations of the branched copolymer of L-lysine and DL-alanine which contained covalently attached gangliosides. Samples (50 μl) of these were then added to 0.2 ml of the same buffer containing liver membranes (0.3 mg of protein/ml). Specific binding was determined after incubating for 15 min at 24°. [b] The concentration given is that of the preincubation mixture; the concentrations in the binding studies were 10-times lower.

TABLE VIII: Effect of Soluble Poly(L-lysyl-DL-alanine) Copolymer Containing Covalently Attached Ganglioside on the Lipolytic Activity of Cholera Toxin.[a]

Order of Addn of Toxin and Polymer[b]	Polymer Concn (μg/ml)	Lipolysis[c]
No additions		12
Cholera toxin		46
Polymer added before toxin	100	13
	10	11
	2	12
	0.5	14
	0.1	30
Polymer added 5 min after toxin	100	30
	10	38
Polymer added 15 min after toxin	100	43
	10	44

[a] The experiment was performed essentially as described in Table VI. [b] In the absence of cholera toxin the polymer did not alter lipolysis. [c] Micromoles of glycerol released per millimole of cell triglyceride.

The ganglioside-containing polymer is also quite effective in blocking the lipolytic effect of cholera toxin on fat cells (Table VIII). However, as with the insoluble ganglioside derivatives, marked effects are observed only if the polymer is added to the cells before the addition of cholera toxin. These soluble derivatives appear to be more potent than the comparable insoluble agarose derivatives. Nearly complete inhibition of activity is achieved with concentrations of the polymer as low as 0.5 μg/ml.

Disc Gel Electrophoretic Characterization of Cholera Toxin and Choleragenoid. In the course of these studies the disc gel electrophoretic behavior of cholera toxin and of choleragenoid, an inactive, immunologically identical toxin derivative which competitively blocks the activity of cholera toxin (Finkelstein and LoSpalluto, 1969, 1970; Cautrecasas, 1973d), were investigated. The purified cholera toxin, as obtained from the SEATO Cholera Research Program (see Experimental Section), consists of three bands on sodium dodecyl sulfate disc gel electrophoresis (Figure 5, left). The molecular weights of these components, as judged by comparisons with standard proteins of known molecular weight, are about 66,000 (band A), 36,000 (band B), and 8000 (band C). Choleragenoid consists of two bands which correspond nearly identically with bands A and C of the toxin (Figure 5, left); no band corresponding to band B can be detected in choleragenoid.

Chromatography of cholera toxin and choleragenoid on Sephadex G-75 (0.1 M sodium phosphate buffer, pH 7.4) immediately before electrophoresis results in the loss of band C in both cases. This gel filtration procedure leads to an increase in the specific biological activity of the toxin. Furthermore, as described earlier (Cuatrecasas, 1973a), iodination of cholera toxin is greatly improved if the iodination is performed after Sephadex G-75 chromatography. These results indicate that in the purified samples the low molecular weight

component (band C) is probably not associated with the protein of bands A and B, and that it is not biologically active. The Sephadex G-75 purified proteins demonstrate single bands on disc gel electrophoresis in the absence of sodium dodecyl sulfate (Figure 5, right). The protein in this cholera toxin band can be eluted from the disc gel with 0.1 M sodium phosphate buffer (pH 7.4) with excellent recovery of biological activity. In agreement with the findings of LoSpalluto and Finkelstein (1972), choleragenoid appears to be less anionic than cholera toxin. The above studies suggest that active, native cholera toxin is composed of bands A and B while native choleragenoid is composed only of band A.

Reduction and carboxymethylation of cholera toxin results in the loss of bands A and B on sodium dodecyl sulfate disc gel electrophoresis (Figure 5, left). Band C becomes relatively more intense, and a new band appears which has a mobility intermediate with that of bands B and C and has a molecular weight of about 27,000. Reduction and carboxymethylation of choleragenoid results in the loss of band A; only band C is detected in such preparations. These results, together with those to be described shortly, suggest that reduction and carboxylation results in the conversion of band A (in both toxin and choleragenoid) to components having a molecular weight of about 8000 while band B is converted to components having molecular weights of about 27,000 and 8000.

More than 90% of the radioactivity of ^{125}I-labeled preparations of cholera toxin purified by Sephadex G-75 (Cuatrecasas, 1973a) is recovered in band B on sodium dodecyl sulfate disc gel electrophoresis (Figure 6, I). Since the protein iodinated in these preparations consists almost exclusively of components corresponding to bands A and B, it is notable that the radioactivity is so selectively confined to band B. Reduction and carboxymethylation of the protein which is eluted from band B results in a shift of 48% of the radioactivity from band B to a position corresponding to a molecular weight of about 27,000 (Figure 6, IV); 37% of the radioactivity, however, is recovered in the position of band C, and 15% is apparently not chemically altered since it remains in the position of band B. Reduction and carboxymethylation of the protein present in band A, which is purified by elution from the disc gels, results in a complete conversion of radioactivity (and protein) from band A to band C (Figure 6, III). It appears clear that the protein of band A can be dissociated to subunits of very low molecular weight, and that band B can change from a component of molecular weight about 36,000 to two components having molecular weights of about 27,000 and 8000. Since the latter conversion is accompanied by the appearance of two new protein-staining bands, it is reasonable to suggest that band B (mol wt 36,000) is composed of one subunit of mol wt 27,000 and one of about 8000. Because of the nearly equal distribution of radioactivity between the two new bands, it is apparent that the iodine content of band B is localized relatively preferentially to the portion of the molecule which is represented by the low molecular weight component.

Exposure of ^{125}I-labeled cholera toxin to 6 M guanidine·HCl, followed by removal of the denaturant by chromatography on Bio-Gel P2, does not change the pattern on subsequent disc gel electrophoresis from that shown in Figure 6 (I). The radioactivity appears almost exclusively in band B whether or not the electrophoresis is performed in the presence or absence of carrier native cholera toxin. The proteins of bands A and B maintain relatively high molecular weights in the presence of sodium dodecyl sulfate, suggesting that the conversion of these proteins to low molecular weight components

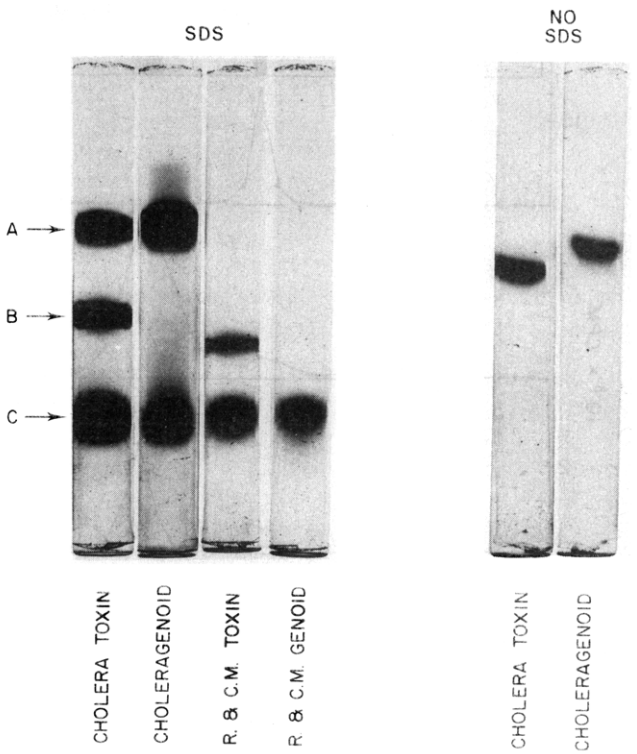

FIGURE 5: Disc gel electrophoretic patterns of native and reduced and carboxymethylated (RCM) cholera toxin and choleragenoid (genoid) in the presence (left) and absence of 0.1% sodium dodecyl sulfate. Samples of cholera toxin (60 μg) and of choleragenoid (20 μg) were incubated at 37° for 30 min in the presence of 20 mM sodium phosphate buffer, 0.5% sodium dodecyl sulfate, and 2% sucrose; electrophoresis was performed at 24° for 3.5 hr at 5 mA/tube. The patterns were not significantly altered by increasing the concentration of detergent in the sample to 2%. Electrophoresis in the absence of sodium dodecyl sulfate (right) was performed at 4° for 6 hr (6 mA/tube) with 6 μg of cholera toxin and of choleragenoid; both had been previously chromatographed on a column of Sephadex G-75 as described in the text. Reduction was performed by incubating samples (0.2 mg) in 0.1 ml of 0.1 M sodium phosphate buffer (pH 7.4), containing 6 M guanidine·HCl and 30 mM dithiothreitol for 2 hr at 24°; carboxymethylation was accomplished by the addition of 0.13 M iodoacetamide and incubating for 30 min at 24°. The samples were chromatographed on a column (10 ml) of Bio-Gel P2 equilibrated with 10 mM sodium phosphate buffer (pH 7.0). The protein-containing material in the void volume was pooled, lyophilized, and dissolved in 0.1 ml of the sodium dodecyl sulfate containing buffer described above; 20-μl samples were applied on the disc gels. By comparisons with gels containing standard proteins the molecular weight of bands A, B, C, and the new band in the reduced and carboxymethylated toxin gel were about 66,000, 36,000, about 8000, and 27,000, respectively. Band C is virtually absent in sodium dodecyl sulfate disc gels of toxin and choleragenoid samples chromatographed on Sephadex G-75.

may be dependent on or facilitated by reductive cleavage of disulfide bonds. It is very possible that unusually strong noncovalent forces resistant to disruption by the detergent are involved in these subunit interactions, and that reduction and alkylation of intrachain disulfide bonds may interfere critically with the noncovalent subunit interactions.

The distribution of radioactivity in samples purified by affinity chromatography from crude culture media of *V. cholerae* indicates that most of the radioactivity is present in a band corresponding to the electrophoretic mobility of band C (Figure 6, II). The manner by which this conversion occurs during the process of purification is not apparent from the present studies, although the possibilities of proteolytic cleavage or of subunit disaggregation with the loss of the

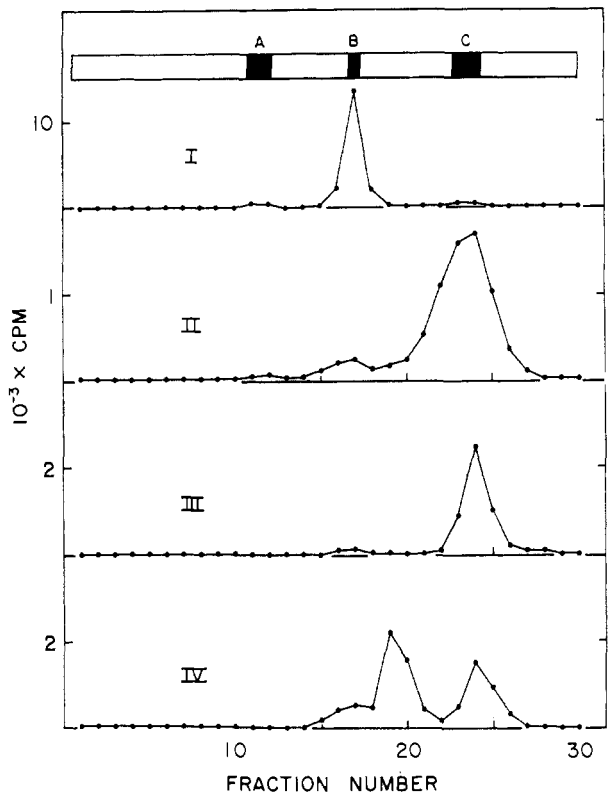

FIGURE 6: Distribution of radioactivity on sodium dodecyl sulfate disc gel electrophoresis of native (I), affinity column purified (II), and reduced and carboxymethylated (III and IV) samples of ^{125}I-labeled cholera toxin. In disc gel I, 25 μg of cholera toxin containing 2.3×10^4 cpm of ^{125}I-labeled toxin, prepared from a toxin sample which had been chromatographed on Sephadex G-75 (see text), was electrophoresed as described in Figure 5. Disc gel II is from a sample of crude *V. colerae* culture medium toxin purified by affinity chromatography on a A-NatAlb-gang column (Figure 3 and Table IV). The gels were sliced into sections (2–3 mm) and counted in a gamma counter. The samples in III and IV were obtained from samples eluted from disc gels similar to that described in I. Five gels, each containing 100 μg of toxin and about 1.8×10^5 cpm of ^{125}I-labeled toxin, were sliced (3 mm) and counted. The radioactive samples corresponding to bands A and B were pooled separately and eluted by adding 2 ml of 0.1 M sodium phosphate buffer containing 7 M guanidine·HCl and shaking at 37° for 1 hr. The supernatant obtained by centrifugation was adjusted with dithiothreitol to 40 mM and incubated at 24° for 80 min. Iodoacetamide (0.15 M) was added and the incubation was continued for 30 min at 24°. Four milliliters of distilled water was added and the samples were dialyzed against distilled water for 3 hr at 24°. The material was lyophilized and dissolved in 0.1 ml of the 0.5% sodium dodecyl sulfate buffer (Figure 5); 3.0×10^4 cpm was recovered from band A, and 1.6×10^5 cpm from band B. Samples of the reduced and carboxymethylated elutions A (gel III) and B (gel IV) were electrophoresed as described above. The gels were stained for protein, sliced, and counted; protein bands similar to those seen in Figure 5 were present at the positions of all the major radioactive peaks.

component of mol wt 27,000 must be considered. The possibility that the latter peptide was degraded or that it remained adsorbed to the column cannot be excluded.

Discussion

The present studies demonstrate that columns containing ganglioside–agarose derivatives can selectively extract cholera toxin even when the toxin is present in concentrations as low as 10^{-11} M. The most effective adsorbents are those which contain macromolecular spacers (*i.e.*, poly(amino acid) polymers, albumin) interposed between the agarose backbone and the covalently attached ganglioside. Some of these derivatives are still effective after 600-fold dilution with unsubstituted agarose. Such macromolecular adsorbents have recently proved most useful in the purification of estrogen receptors from the uterus (Sica *et al.*, 1973a,b). It has been possible to purify estrogen receptors about 100,000-fold in a single step by using 20-fold diluted poly(L-lysyl-DL-alanine)–agarose derivatives containing covalently attached estradiol.

Among the specific advantages of the derivatives having macromolecular spacers is a high degree of ligand substitution which permits the use of the adsorbent in diluted form, and this results in a decrease in the nonspecific protein adsorptive properties of the gel. Furthermore, the ligand is separated from the agarose backbone by large distances, which in the case of the branched amino acid copolymer may be as great as 150 Å. The high probability that the interposing macromolecule is anchored to the insoluble polymer by multiple points greatly increases the overall chemical stability of the attached ligand by stabilizing the basic cyanogen bromide attachment of the unit to the agarose. This enhanced stability may be quite important in very high-affinity systems such as the present one in which leakage of even small quantities of the ligand may seriously interfere with the specific adsorptive behavior of the proteins. These derivatives, particularly those prepared with albumin, may in addition present a more favorable microenvironment for the specific ligand–protein interaction. This appears to be the case with the estradiol–agarose derivatives (Sica *et al.*, 1973b).

The ability of gangliosides to bind extremely tightly to cholera toxin, and thus to prevent the binding and the biological effects of the toxin in various tissues (Cuatrecasas, 1973a,b), suggested that gangliosides may be useful therapeutic agents for clinical cholera. However, the demonstration that free gangliosides may be incorporated spontaneously into cell membranes, and that this can ultimately result in increased binding of the toxin and in enhanced biological effects (Cuatrecasas, 1973b), suggests that the *in vivo* use of such agents may be dangerous if not ineffective. For this reason the insoluble and soluble polymeric ganglioside derivatives described in the present report may be more rational agents with which to explore the possible therapeutic value of gangliosides in clinical cholera.

The present studies suggest that native cholera toxin is composed of two major protein components having molecular weights of 66,000 and 36,000. If the active, native molecule is composed of one of each of these, its molecular weight would be 102,000. LoSpalluto and Finkelstein (1972) have estimated the molecular weight of this protein to be about 84,000, as determined by sedimentation velocity and equilibrium measurements. The latter authors estimated the molecular weight of choleragenoid to be about 58,000, which is relatively close to the value (66,000) calculated in the present studies. The 66,000 molecular weight component of cholera toxin, as well as that of choleragenoid, can be completely dissociated by reduction and alkylation into components having a molecular weight under 10,000. The cholera toxin component of 36,000 molecular weight appears to dissociate, upon reduction and alkylation, into a component of mol wt 27,000 and one of molecular weight about 8000. Although LoSpalluto and Finkelstein (1972) have estimated that cholera toxin has five to six and choleragenoid four to five disulfide bonds, it is not entirely clear in the present studies whether the subunit dissociation observed upon reduction and alkylation represents dissociation of disulfide-linked subunits or disruption of non-

covalent interactions which are unusually resistant to the action of sodium dodecyl sulfate. The fact that ^{125}I-labeled cholera toxin exposed to high concentrations of guanidine·HCl is lost extremely rapidly through dialysis membranes suggests strongly that this subunit structure is not basically maintained through disulfide bond linkage. Furthermore, LoSpalluto and Finkelstein (1972) presented evidence that urea and acid dissociate cholera toxin to subunits having a molecular weight of about 15,000.

It is notable that one of the two protein components of cholera toxin appears to be identical in molecular weight (66,000) with the single protein species of choleragenoid. Furthermore, both appear to dissociate completely to components with about the same molecular weight (8000) upon reduction and alkylation. It has been proposed (Finkelstein and LoSpalluto, 1969; Finkelstein, 1970) that cholera toxin and choleragenoid are antigenically identical. Although choleragenoid is devoid of biological activity, it binds to the same cell membrane sites (gangliosides) as cholera toxin and the affinity of binding of these two derivatives is indistinguishable (Cuatrecasas, 1973d). Choleragenoid is therefore a potent competitive antagonist of cholera toxin binding and activity (Cuatrecasas, 1973d). These observations together suggest that the 66,000 molecular weight component of cholera toxin is very similar to if not identical with the choleragenoid protein, and that this protein species is alone responsible for the specificity and affinity of binding of cholera toxin (or of choleragenoid) to cell membranes and to gangliosides. The smaller (mol wt 36,000) subunit of cholera toxin, which alone does not appear to bind to cell membranes, probably does not modify the nature of the initial binding interaction of the protomer with cells. This subunit, however, probably endows the toxin molecule with properties which, once bound, induce profound biological effects on cells. Principally because of the existence of a prolonged lag phase before the onset of the biological effects of cholera toxin, it has been postulated (Cuatrecasas, 1973c) that the cholera toxin- and choleragenoid–receptor complexes as initially formed are both very similar and biologically inactive, and that the toxin- but not the choleragenoid–receptor complex is capable of undergoing a subsequent rearrangement within the membrane which converts it into a biologically active form. The present studies suggest that the larger toxin subunit may in effect act to deliver to specific regions of the membrane the smaller subunit which is somehow responsible for the conversion of an initially inactive toxin–cell complex into an active form.

The results of the present studies suggest that cholera toxin can be readily dissociated, probably first into the two major subunits (A and B), by acid, urea, or guanidine·HCl. This dissociation appears to be readily reversible, with reestablishment of biological activity, provided that the concentration of the components is sufficiently high. At very low concentrations (less than 0.1 μg/ml) reassociation is negligible, as witnessed by the inability of the [^{125}I]toxin to bind to membranes after exposure to these denaturants. Since after the exposure of low concentrations of ^{125}I-labeled toxin to these denaturants the disc gel electrophoretic pattern (in sodium dodecyl sulfate) is unchanged, it is very unlikely that the denaturation represents irreversible dissociation of the 66,000 and 36,000 molecular weight components. These results are consistent with the view that the 36,000 molecular weight component is itself incapable of binding and thus eliciting biological effects on cells. It is unlikely that the relatively irreversible nature of the denaturation observed at low concentrations of cholera toxin is based on the inability of the 66,000 and 36,000 molecular weight components to reassociate rather than on difficulties related to the reacquisition of tertiary structure by the individual components. The recent demonstration by LoSpalluto and Finkelstein (1972) that cholera toxin and choleragenoid are reversibly dissociated by guanidine·HCl at protein concentrations varying from 2.5 to 4 mg per ml supports these suggestions.

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Addendum

Following submission of this manuscript, we noticed a recent pertinent report on the electrophoretic behavior and structure of cholera toxin which is in close agreement with many of the findings and conclusions described independently in the present studies (Finkelstein *et al.*, 1972). These authors describe the resistance of the toxin and of choleragenoid to dissociation by sodium dodecyl sulfate in the absence of reducing agents. The toxin molecule is described as consisting of a 56,000 molecular weight protein, which is probably identical with choleragenoid, and a 28,000 molecular weight fraction which appears to be unique to the toxin. Under certain conditions the native molecules can be dissociated into subunits having a molecular weight of about 14,000.

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Biosynthesis of Slaframine, (1*S*,6*S*,8a*S*)-1-Acetoxy-6-aminooctahydroindolizine, a Parasympathomimetic Alkaloid of Fungal Origin. I. Pipecolic Acid and Slaframine Biogenesis†

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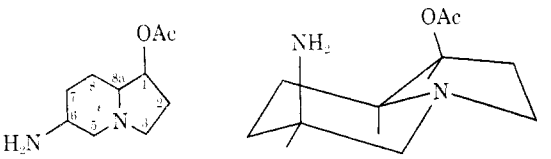
ABSTRACT: *Rhizoctonia leguminicola*, a fungus that causes black spot disease of red clover, produces an alkaloid that induces excessive salivation in animals. This alkaloid, termed slaframine, has been isolated and characterized as (1*S*,6*S*,-8a*S*)-1-acetoxy-6-aminooctahydroindolizine. Using techniques of radioisotope incorporation and dilution, the biosynthesis of this piperidine alkaloid was studied in growing or resting cultures of *R. leguminicola*. Appropriate radioactive test substances were added to such cultures and, following incubation, slaframine or related compounds were subsequently isolated and examined for radioactivity. DL-[1-^{14}C]Lysine and DL-[6-^{14}C]lysine were equally incorporated into slaframine; such incorporation was effectively blocked by pipecolic acid. Both ring-labeled and carboxyl-labeled pipecolic acid were equally incorporated into slaframine and were approximately 1000 times more effective than lysine as precursors of slaframine. Tritiated 1-ketooctahydroindoindolizine, 1-hydroxyoctahydroindolizine, and 1,6-dihydroxyoctahydroindolizine were readily utilized in slaframine formation, and an NADPH dependent reduction of 1-ketooctahydroindoindolizine to 1-hydroxyoctahydroindolizine was demonstrated in cell-free extracts of *R. leguminicola* cells. Such extracts also catalyzed the acetyl-CoA dependent formation of slaframine from 6-amino-1-hydroxyoctahydroindolizine. A tentative scheme of slaframine biosynthesis stemming from the metabolism of lysine and pipecolic acid is presented.

This laboratory became interested some years ago in the isolation and identification of a mycotoxin elaborated by the fungus, *Rhizoctonia leguminicola*. Consumption by ruminants of legumes such as red clover infested by this fungus results in excessive salivation and can pose problems of some consequence in animal husbandry. General accounts dealing with such aspects together with research on the isolation, chemistry, and physiology of this salivation factor have appeared (Broquist and Snyder, 1971; Aust *et al.*, 1968; Spike and Aust, 1971) and document early work in this field from this and other laboratories.

Chemical studies have now established that this salivation factor, termed slaframine, is (1*S*,6*S*,8a*S*)-1-acetoxy-6-aminooctahydroindolizine (Aust *et al.*, 1966; Gardiner *et al.*, 1968); and its total synthesis has recently been achieved (Cartwright *et al.*, 1970).

In early attempts to increase the yield of slaframine in growing cultures of *R. leguminicola*, and prior to elucidation



of structure, a systematic examination of the effect of amino acids on slaframine synthesis revealed that both DL-[6-^{14}C]-lysine and DL-[1-^{14}C]lysine were incorporated into the alkaloid (Aust, 1965). Brief accounts of progress to date have appeared (Snyder and Broquist, 1968, 1969; Guengerich, 1971) which indicate a role of pipecolic acid in slaframine biosynthesis and will be discussed more fully herein. Using techniques of radioisotope incorporation and dilution, evidence will be presented that slaframine biosynthesis deriving from the lysine metabolism of *R. leguminicola* may include the transformations postulated in Figure 1.

Materials and Methods

Radioactive Lysines. DL-[1-^{14}C]- and [6-^{14}C]lysines were purchased from New England Nuclear. DL-[4,5-^{3}H]Lysine was obtained from Volk Chemical Co.

[^{14}C]Pipecolic Acids, [4,5-^{3}H]Pipecolic Acid. Variously labeled pipecolic acids were synthesized from appropriately labeled lysine in a manner analogous to the synthesis of proline from ornithine (Hamilton, 1952). To DL-lysine·HCl (3.0 mmol) and the desired labeled lysine (*e.g.*, 3 ml of DL-[4,5-^{3}H]lysine·HCl, or 30 μl of DL-[1-^{14}C]lysine·HCl, or 30

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