Proc. Natl. Acad. Sci. USA
Vol. 73, No. 8, pp. 2654–2658, August 1976
Biochemistry

Small fragments from the A subunit of cholera toxin capable of activating adenylate cyclase

(active peptide fragments/protease)

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Communicated by Lewis Thomas, May 21, 1976

ABSTRACT **Exposure of cholera toxin to membrane particles prepared from sarcoma 180 cells gives rise to a variety of fragments which are capable of activating adenylate cyclase [ATP:pyrophosphate-lyase (cyclizing), EC 4.6.1.1]. A major component of these fragments has an apparent molecular weight in the 8,000–10,000 range. The smallest stimulatory fragment has a molecular weight of approximately 1400. The small size of the fragments is confirmed by Sephadex gel filtration, in the presence of either sodium dodecyl sulfate or formic acid. These fragments are produced from holotoxin or its A subunit by protease(s) found in sarcoma membrane particles. Production of fragments appears optimal in 40–60 min at 30° and pH 7, and is prevented by protease inhibitors. The ability of the small fragments to activate adenylate cyclase is reversed by anti-holotoxin, but not anticholeragenoid, antibodies. These fragments require NAD for the activation of adenylate cyclase and are fully active after heating at 90° for 5 min (pH 7).**

Field suggested that the biological functions of cholera toxin might be mediated by adenylate cyclase [ATP:pyrophosphate-lyase (cyclizing), EC 4.6.1.1] activation (1). Subsequently, Gill found that NAD is necessary for this activation by the holotoxin (2). A number of studies have suggested that the holotoxin is composed of four to six identical B subunits with molecular weights of 9,500–14,000 and an A subunit with a molecular weight of about 28,000 (3, 4). The A subunit can be further dissociated by sulfhydryl reagents into A1 (20,000–24,000 daltons) and A2 (4000–7500 daltons) subunits (5).

There is agreement that the activation of adenylate cyclase by cholera toxin is exclusively a function of the A subunit (6). Concentration of the toxin at the cell surface is accomplished by the B subunits, which are known to bind to a specific monosialoganglioside, GM1 (7–9).

Recently we have been studying the interaction of cholera toxin and adenylate cyclase in membrane particles from sarcoma 180 cells. In these cells, as has been found with the avian erythrocyte, NAD is indispensable for the activation of adenylate cyclase by cholera toxin (10, 11). We have also found that in sarcoma 180 cells the catecholamine receptor can be entirely desensitized by prior exposure to isoproterenol or inactivated by propranolol without diminishing the response to holotoxin (11).

Here we report that incubation of holotoxin with membrane particles prepared from sarcoma 180 cells gives rise to a variety of fragments, which are capable of activating adenylate cyclase. The smallest of these has an approximate molecular weight of 1400. These fragments originate in cholera toxin, since they can be inactivated by antibody directed against the holotoxin and also they can be prepared from purified A subunit in the presence of sarcoma membrane particles. These fragments require NAD for the activation of adenylate cyclase and are heat stable, characteristics also found in the A1 subunit of cholera toxin. These findings compel a reevaluation of the designation of the active moiety of cholera toxin and pose intriguing questions concerning the postulated enzymatic nature of the cyclase activation step.

MATERIALS AND METHODS

Cholera toxin was purchased from Schwarz/Mann. [2,8-^{3}H] ATP (26 Ci/mmol) was purchased from New England Nuclear Corp. Horse anticholera was a gift from Dr. Nobuya Ohtomo

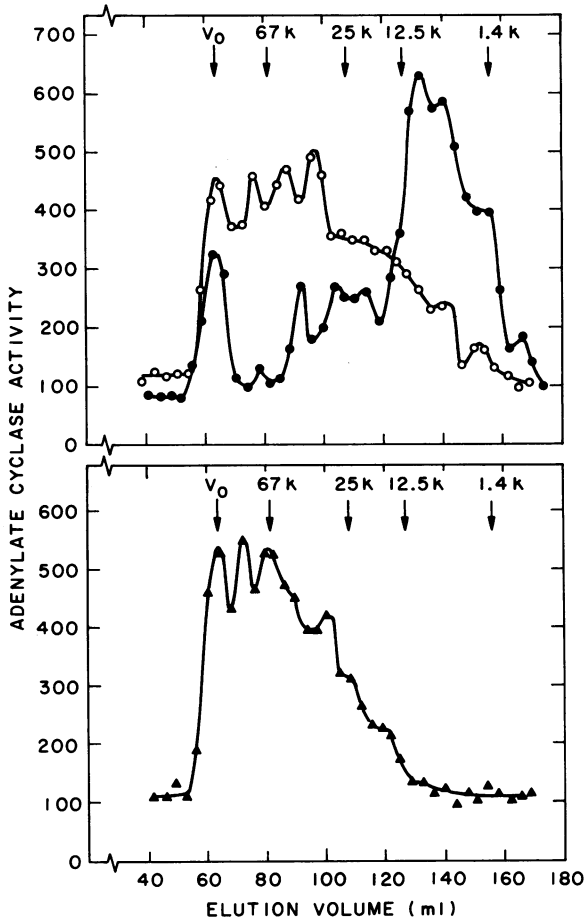

FIG. 1. Production of small fragments by sarcoma membrane particles; dependence on protease activity. Holotoxin (500 μg) in 1.6 ml of buffer A was incubated for 60 min at 30° in the presence (●) or absence (○) of membrane particles (0.64 g) or protease inhibitors (▲). Supernatant (0.9 ml) was analyzed by Sephadex G-100 gel filtration in the presence of 0.2 M formic acid–NH_4OH (pH 7.5). Aliquots (10 μl) of the designated fractions were examined for adenylate-cyclase-activating ability. V_0 is void volume.

Abbreviations: cAMP, cyclic adenosine 3′:5′-monophosphate; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate.

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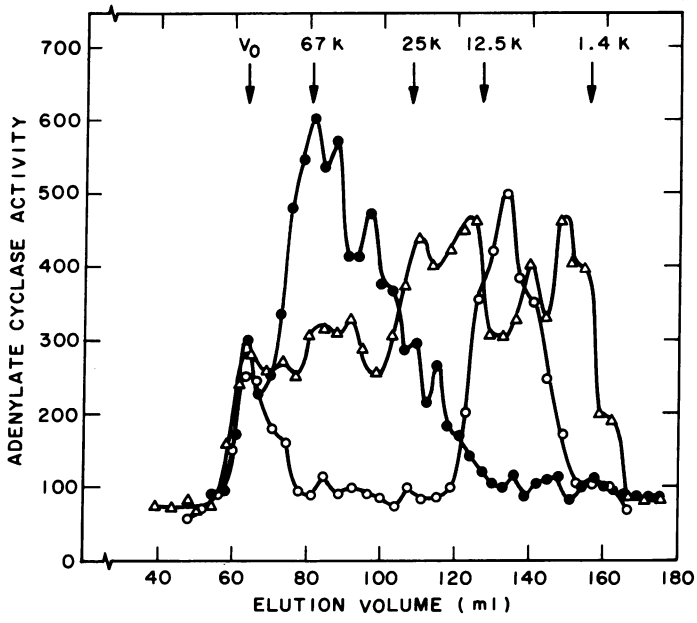

FIG. 2. Time dependence of the production of small fragments. Conditions and reactants are the same as for Fig. 1 except that the times of incubation were 3 min (●), 20 min (△), and 40 min (○).

(Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan). Horse anticholeragenoid (anti-B) was a gift from the USA cholera panel, National Institutes of Health, Bethesda, Md. (12). Bovine pancreatic trypsin (10,000 units/mg) was purchased from Sigma Chemical Co. Sarcoma 180 cells were a gift from Dr. Alan Sartorelli. The cells were maintained in the ascites form in Yale CD-1 mice and transplanted as described previously (13). Sarcoma 180 cells were harvested and homogenates were prepared with a motor-driven Teflon on glass homogenizer (0°) in a volume of 20 mM glycylglycine buffer (pH 7.4) containing 5 mM $MgSO_4$ (buffer A) that was equal to the volume of the packed cells. Homogenized cells were washed by dilution with 10 volumes of buffer A and collected by centrifugation (7710 × *g*, 80 sec). Resulting membrane particles were suspended in a volume of buffer A equal to ⅓ the original packed cell volume (0.8 g of protein per ml).

Adenylate cyclase activity was assayed at 30° for 15 min in the presence of NAD (2.5 mM) and dithiothreitol (DTT, 3 mM), and expressed as pmol of cAMP formed per min/mg of protein. [^{3}H]cAMP was isolated by polyethyleneimine cellulose descending thin-layer chromatography (14). Basal adenylate cyclase activity is in the range of 50–100 pmol/min per mg of protein. Protease activity was evaluated with denatured hemoglobin as substrate (15). Polyacrylamide (10%) gel electrophoresis was done in 1% sodium dodecyl sulfate ($NaDodSO_4$) (16). Following exposure of various holotoxin or subunit preparations to membrane particles the particles were routinely removed by centrifugation (100,000 × *g*, 30 min, 4°) prior to subsequent analytical procedures. Density gradient centrifugation was done on a 3.5 ml continuous sucrose gradient (5–20%, wt/vol, in 10 mM Tris·HCl, (±0.1% $NaDodSO_4$, pH 7.4) at 50,000 rpm in a Beckman SW-56 rotor for 16 hr (17). Upward flow Sephadex G-100 gel filtration (column, 1.5 × 90.3 cm) was done at 25° in 0.2 M formic acid–NH_4OH (pH 7.5) or 10 mM Tris·HCl buffer (pH 7.5) containing 0.5% $NaDodSO_4$ and 1 mM DTT. Upward flow Sephadex G-75 gel filtration (column, 1.5 × 91.5 cm) was done at 25° in 5% (vol/vol) formic acid. Molecular weight markers were blue dextran 200 (200,000) for the void volume, bovine serum albumin (67,000), hemoglobin (64,000), chymotrypsinogen A (25,000), cytochrome *c* (12,500), cholera toxin B subunit (9500) (4), glucagon (3460), and bacitracin (1400). In the figures molecular weights of all markers are given in thousands, k. A mixture of 25 mM EDTA, 10 mM *N*-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM *p*-aminobenzamidine (final concentrations given) was used to inhibit protease activity. Protein was determined by the method of Lowry *et al.* (18).

RESULTS

Preparation of small fragments from holotoxin; dependence on protease activity

The holotoxin was exposed to sarcoma membrane particles, the particles were removed by centrifugation, and the supernatant was applied to a Sephadex G-100 column. Resulting fractions were analyzed for their ability to activate adenylate cyclase in sarcoma membrane particles (Fig. 1). We emphasize that: (*i*) The commercial preparation of holotoxin contains small amounts of stimulatory fragments having molecular weights less than the A subunit. (*ii*) Exposure of the toxin to sarcoma membrane particles greatly enhances the yield of these small fragments. (*iii*) Furthermore, the introduction of a mixture of reagents that collectively inhibit protease activity markedly diminishes the elaboration of these fragments.

We then looked for the optimal conditions necessary for elaboration of these fragments by studying the effect of various pH values and membrane preparation in the presence and absence of NAD and DTT. In addition, we examined the yield of small fragments as a function of time (Fig. 2). Optimal yields of small fragments were obtained by incubating the holotoxin with membrane particles for 40–60 min at pH 7.4 and 30°. The production of small fragments was negligible at 0°. The yield of small fragments was diminished by greater than 80% when holotoxin was incubated with homogenate. The addition of DTT or NAD did not enhance the yield of small fragments. Membrane particles (80 mg of protein) had protease activity comparable to 400 units of trypsin. The activity was reduced to negligible levels in the presence of the protease inhibitors.

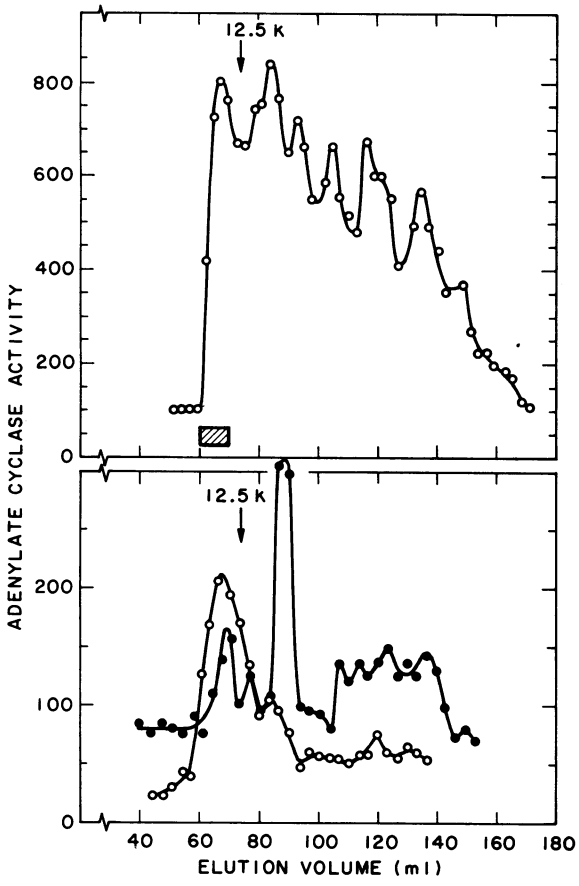

FIG. 3. Preparation of A1 subunit and gel filtration behavior of small fragments in the presence of $NaDodSO_4$. Holotoxin (2 mg) was dissolved in 1 ml of 1% $NaDodSO_4$ containing 0.4 mM EDTA (pH 7.5) and 16 mM DTT. The mixture was incubated at 40° for 30 min and applied to a Sephadex G-100 column equilibrated with 10 mM Tris·HCl buffer (pH 7.5) containing 0.5% $NaDodSO_4$ and 1 mM DTT (upper panel, O). The hatched bar indicates those pooled fractions which were rechromatographed and repooled to prepare the A1 subunit. Following removal of $NaDodSO_4$ by cooling, the A1 subunit (48 μg) was incubated at 30° for 60 min (lower panel) in the presence (●) or absence (O) of membrane particles. Particles were sedimented, and the supernatants were incubated in 1% $NaDodSO_4$ (containing 0.4 mM EDTA, pH 7.5, and 16 mM DTT) and rechromatographed on Sephadex G-100 as above. Each fraction was diluted with an equal volume of water, chilled at 0° for 3 hr, and centrifuged to remove precipitated $NaDodSO_4$, and the supernatants (10 μl aliquots) were examined for adenylate-cyclase-activating ability.

Preparation of small fragments from A or A1 subunit and their behavior in gel filtration in the presence of $NaDodSO_4$ or formic acid

In order to clarify whether these fragments were significantly smaller than A1 or were really larger fragments whose emergence from the column had been delayed by nonspecific interactions with Sephadex, we examined other types of separation conditions. The A1 subunit was prepared by incubation of holotoxin with $NaDodSO_4$ and DTT followed by Sephadex gel filtration (Fig. 3). (Although the A1 subunit was contaminated with B subunit, it migrated more rapidly than the A subunit in $NaDodSO_4$ polyacrylamide gel electrophoresis.) Most of the $NaDodSO_4$ was then precipitated by cooling at 0° overnight. After the A1 subunit was incubated with sarcoma membrane particles, the particles were removed by centrifugation. The resultant supernatant was incubated with DTT and $NaDodSO_4$ and analyzed on Sephadex G-100 in the continued presence of DTT and $NaDodSO_4$. There was a marked increase in the yield of low-molecular-weight fragments which activate adenylate cyclase only when A1 was incubated with sarcoma membrane particles (Fig. 3). In contrast, the apparent molecular weight of A1 subunit prepared by Sephadex G-100 gel filtration was not altered by repeat gel filtration in the presence of $NaDodSO_4$.

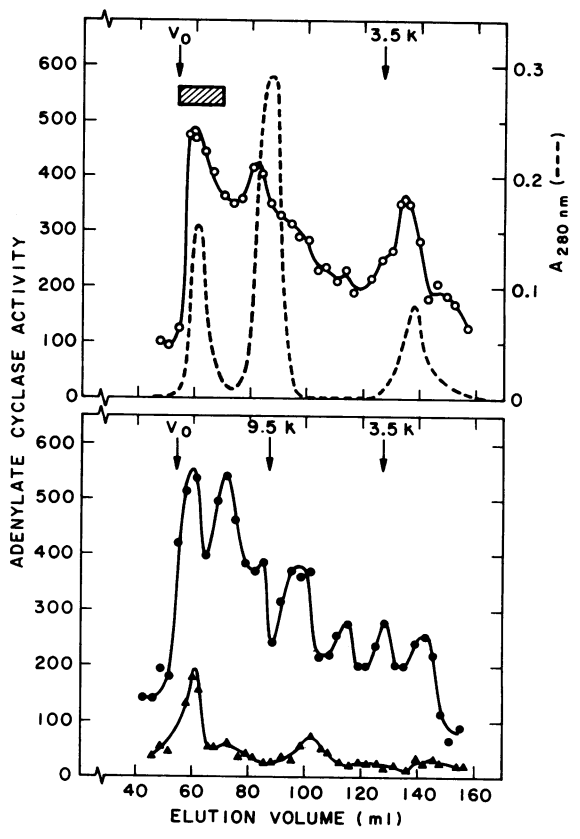

FIG. 4. Preparation of A subunit and gel filtration behavior of the small fragments in the presence of formic acid. (Upper panel) Holotoxin (5 mg) in 1 ml of 5% formic acid was applied to a Sephadex G-75 column equilibrated with 5% formic acid (O). The hatched bar (A subunit) indicates the fractions which were pooled, lyophilized, redissolved in 5% formic acid, and neutralized with NaOH. (Lower panel) This A subunit (250 μg) was then incubated with membrane particles in the presence (▲) or absence (●) of protease inhibitors. The resultant mixtures were adjusted to pH 2.1 with formic acid, the particles were sedimented, and the supernatants were applied to a Sephadex G-75 column equilibrated with 5% formic acid. The fractions were brought to pH 7.5 by dilution with 10 volumes of 0.2 M Tris–0.2 M NaOH (pH 12.0) and examined (10 μl aliquots) for their adenylate-cyclase-activating ability.

We then prepared A subunit using gel filtration in the presence of formic acid (Fig. 4). The A subunit purified in this manner gave a single band on polyacrylamide gel electrophoresis (4). The A subunit was neutralized and incubated with sarcoma membrane particles. The pH was then adjusted to 2.1 with formic acid, the membrane particles were sedimented, and the supernatant was analyzed on Sephadex G-75. Sarcoma membrane particles greatly increased the yield of small, adenylate-cyclase-activating fragments from this A subunit (Fig. 4). This increase was prevented by the mixture of protease inhibitors.

We emphasize that the small fragments of A subunit that can activate adenylate cyclase appear heterogeneous in size; that the bulk of the fragments produced by the sarcoma membrane particles have an apparent molecular weight in the 8,000–10,000

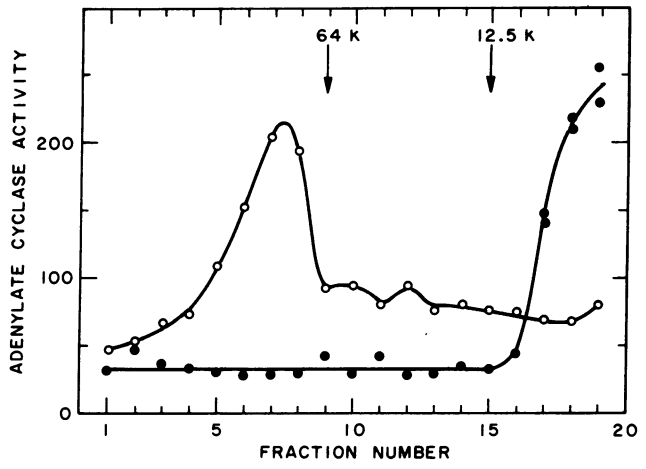

FIG. 5. Sedimentation behavior of small fragment(s) on a continuous sucrose density gradient; reversal of aggregation by $NaDodSO_4$. Small fragment fraction (300 μl) (see Fig. 2 △, elution volume, 148 ml) was incubated at 40° for 30 min in the presence (●) or absence (○) of 1% $NaDodSO_4$ (containing 0.4 mM EDTA, pH 7.5, and 16 mM DTT), chilled at 0° overnight, and sedimented to remove precipitated $NaDodSO_4$. Resulting supernatants were mixed with hemoglobin and cytochrome *c* (− $NaDodSO_4$) or cytochrome *c* alone (+ 0.1% $NaDodSO_4$) as standards, and layered on a 3.5 ml continuous sucrose density gradient. Following centrifugation, the contents were collected from the bottom in 19 equal fractions which were chilled at 0° for 3 hr, centrifuged to remove precipitated $NaDodSO_4$, and examined (10 μl aliquots) for adenylate-cyclase-activating ability.

range (as judged by elution behavior from Sephadex); and that the smallest fragment that displays adenylate-cyclase-activating ability appears smaller then glucagon (3460 daltons) and has roughly the same size as bacitracin (1400 daltons) (Figs. 1 and 2).

Behavior of small fragments in sucrose density gradients; aggregation reversed by $NaDodSO_4$

The smallest toxin fragments which retained the ability to activate adenylate cyclase were eluted from Sephadex G-100 and sedimented on a continuous sucrose density gradient (5–20%). Under these conditions the small fragments formed aggregates that sedimented more rapidly than hemoglobin. When this procedure was repeated in the presence of $NaDodSO_4$, however, the fragments did not aggregate but sedimented less rapidly than cytochrome *c* (12,500 daltons) (Fig. 5).

In addition to the small fragments there is also evidence that the A subunit of the cholera toxin tends to aggregate and loose activity when stored in neutral aqueous solutions at 5°. Activity can be fully restored by exposure to 1% $NaDodSO_4$ (25°, 1 hr) and subsequent removal of detergent by cooling at 0° for 3 hr followed by centrifugation.

Antigenic and stability properties of small fragments

When small fragments were combined with antiholotoxin antibody prior to admixture with membrane particles for assay of adenylate cyclase, they were rendered completely inactive. Antibody directed against the B subunits did not interfere with the activity of small fragments (Table 1). Small fragments were found to be fully active after heating at pH 7 and 90° for 5 min.

Table 1. Antigenic behavior of small fragments

Column fractions*	Adenylate cyclase activity (pmol of cAMP per min/mg of protein) after treatment with antibody preparation: None	Anti-holotoxin	Anti-cholera-genoid
Control	33	32	32
Control + 0.1 mM *l*-epinephrine	83	83	90
Holotoxin	134	28	129
A1	308	30	400
Small fragments			
~12,500	287	34	358
~3,000	292	28	362

* Fraction of Sephadex G-100 gel filtration (see Fig. 2, △, 20 min) were used as follows: control, elution volume (V_e) = 39 ml; holotoxin, V_e = 73 ml; A1, V_e = 110 ml; ~12,500, V_e = 125 ml; ~3,000, V_e = 148 ml. The concentration of antiholotoxin used reversed adenylate cyclase activation by 10 μg/ml of holotoxin. The concentration of anticholeragenoid was enough to neutralize from 2 to 5 μg/ml of purified choleragen as assayed by rabbit skin permeability. In each case the antibody was added to the toxin or toxin fragment prior to evaluation of adenylate-cyclase-activating ability.

DISCUSSION

Previous work (10) indicated that membranes from sarcoma 180 cell could elaborate macromolecular adenylate cyclase activating factor (MCAF) having an apparent molecular weight of 26,000. Further work indicates that these membranes can produce fragments of cholera toxin with molecular weights as low as 1400 which can activate adenylate cyclase. We suggest that the small fragments originate from the A or A1 subunits of cholera toxin and not the sarcoma cells because they are inactivated by antibody to holotoxin, need NAD for activation of adenylate cyclase (as do the holotoxin and the A subunit), and are heat stable (as is the A subunit). The commercial holotoxin preparations are contaminated by small amounts of adenylate-cyclase-activating fragments whose quantity is greatly increased by exposure to sarcoma membrane particles. The effect of sarcoma membrane particles is virtually prevented by protease inhibitors. Since the yield of small fragments is greatly increased by membrane particles, and indeed diminished by long incubation (60 min) in the presence of cytoplasm, we suggest that the enzymes responsible for small fragment production are associated with a particulate fraction and that fragment damaging activities are found in the cytoplasm.

We emphasize that the apparent smallness of these fragments is not an artefact of elution behavior because: (*i*) their elution behavior in Sephadex gel filtration is not altered by the presence of formic acid or $NaDodSO_4$; (*ii*) the yield of these fragments is greatly increased by sarcoma membrane particles; (*iii*) this increase in yield is prevented by protease inhibitors. The larger aggregates which can be formed spontaneously in aqueous solutions of fragments obtained from the A1 subunit are very different from A1, since the former are dissociated by $NaDodSO_4$ and the latter is not.

Aggregation behavior is shown by the A subunit as well as the small fragments. This can be accompanied by relative loss of adenylate-cyclase-activating ability which is recovered following exposure of the aggregates to $NaDodSO_4$. It is intriguing that the smallest fragment which retains adenylate-cyclase-activating ability also retains antigenic determinants found in the holotoxin in a peptide which may be no larger than 12 amino acid residues.

There are many critical questions which remain unanswered. Does the activation of adenylate cyclase require the smaller fragments or can it also be accomplished by A1 subunit unaltered by proteolysis? How general is the phenomenon of small fragment production: can it be found in a variety of normal as well as cancer cell membrane particles? Are the known subunits of holotoxin (e.g., A1) repeating polymers of the smallest active fragment? Is the activation of adenylate cyclase by toxin or its fragments an enzymatic reaction? Although the smallest fragment size argues *against* the possibility of enzymatic activity it does not exclude it. Further studies will be needed for the purification, amino acid composition and sequence, and specific activity and the stoichiometric ratios between A1 and the smallest fragments. These data should further enhance understanding of the mechanism of action of cholera toxin and adenylate cyclase regulation.

This work was supported by U.S. Public Health Service Grants CA 13444 and GM 12463 and by American Cancer Society Grant BC 106.

1. Field, M., Plotkin, G. R. & Silen, W. (1968) *Nature* **217,** 469–471.
2. Gill, D. M. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 2064–2068.
3. Van Heyningen, S. (1976) *J. Infect. Dis.* **133,** S5–S13.
4. Lai, C. Y., Mendez, E. & Chang, D. (1976) *J. Infect. Dis.* **133,** S23–S30.
5. Finkelstein, R. A., Boesman, M., Neoh, S. H., LaRue, M. K. & Delaney, R. (1974) *J. Immunol.* **113,** 145–150.
6. Van Heyningen, S. & King, S. A. (1975) *Biochem. J.* **146,** 269–271.
7. Cuatrecasas, P. (1973) *Biochemistry* **12,** 3547–3558.
8. Cuatrecasas, P. (1973) *Biochemistry* **12,** 3558–3566.
9. Cuatrecasas, P. (1973) *Biochemistry* **12,** 3577–3581.
10. Bitensky, M. W., Wheeler, M. A., Mehta, H. & Miki, N. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 2572–2576.
11. Wheeler, M. A., Solomon, R. A., Cooper, C., Hertzberg, L., Mehta, H., Miki, N. & Bitensky, M. W. (1975) *J. Infect. Dis.* **133,** S89–S96.
12. Finkelstein, R. A. (1970) *Infect. Immun.* **2,** 691–697.
13. Agrawal, K. C., Booth, B. A. & Sartorelli, A. C. (1968) *J. Med. Chem.* **11,** 700–703.
14. Keirns, J. J., Wheeler, M. A. & Bitensky, M. W. (1974) *Anal. Biochem.* **61,** 336–348.
15. Davis, N. C. & Smith, E. L. (1955) in *Methods of Biochemical Analysis,* ed. Glick, D. (Interscience Publishers, Inc., New York), Vol. II, pp. 215–258.
16. Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10,** 2602–2617.
17. Martin, B. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236,** 1372–1379.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.