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# Activity of Covalently Cross-Linked Cholera Toxin with the Adenylate Cyclase of Intact and Lysed Pigeon Erythrocytes

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Reaction of cholera toxin with *NN'*-bis(carboximidomethyl)tartaramide dimethyl ester produced several cross-linked species that had subunit B (which binds to the cell surface) and peptides A1 (which activates adenylate cyclase) and A2 all covalently joined together. This cross-linked material had activity with pigeon erythrocytes that was comparable in all respects with that of native toxin. It activated the adenylate cyclase of whole cells, showing a characteristic lag phase, and this activation was increased if the cells had been preincubated with ganglioside GM1, but abolished if the protein had been preincubated with the ganglioside. It activated the enzyme in lysed cells more strongly and without the lag phase. These results show that the toxin is active even when peptide A1 cannot be released from the rest of the molecule.

Cholera toxin activates adenylate cyclase (EC 4.6.1.1) on the innner plasma membrane of many different types of eukaryotic cell. The first action of the toxin is to bind to the cell rapidly and irreversibly, and then the adenylate cyclase activity inside begins to rise after a lag phase of about 1 h. The receptor on the cell surface is almost certainly ganglioside GM1* (van Heyningen *et al.*, 1971; Fishman & Brady, 1976).

The toxin is made up of five B subunits (mol.wt. 11600) and one A subunit (itself two polypeptide chains joined by a disulphide bond: mol.wts., A1 about 22000 and A2 about 5000) (van Heyningen, 1974, 1976*a*; Finkelstein *et al.*, 1974; Lönnroth & Holmgren, 1975; Sattler *et al.*, 1975). Subunit B binds to the ganglioside on the cell surface, but has no other activity. Subunit A has the direct effect on adenylate cyclase (van Heyningen & King, 1975; Gill & King, 1975; Bitensky *et al.*, 1975; Sahyoun & Cuatrecasas, 1975). Both subunit A and peptide A1 alone are active by themselves in whole cells, although rather weakly, and show the characteristic lag phase. But they are much more active in cells that have been lysed, or with detergent-solubilized cyclase (van Heyningen, 1976*b*), and then there is no lag phase. This result has led to the idea that the lag phase is due to the time taken for peptide A1 to cross the membrane and arrive at receptor inside the cell. It is now widely believed (Sahyoun & Cuatrecasas, 1975; Gill, 1976*a*; van Heyningen, 1976*a*,*b*; Fishman & Brady, 1976) that the function of subunit B is to deliver subunit A to its site of action and that peptide A1 becomes released from the rest of the molecule and is then inserted into the cytoplasm, where it is free perhaps to react with a receptor that may be the adenylate cyclase itself, or perhaps to catalyse some reaction leading to activation of the cyclase. Several co-factors are required for the reaction in solution or in lysed cells, for example $NAD^+$, ATP and an unidentified cytoplasmic factor (Gill, 1975). A thiol compound is also necessary, and this is thought to be so that the disulphide bond between peptides A1 and A2 can be broken.

The present paper describes experiments on the activity of derivatives of cholera toxin in which the subunits have been cross-linked covalently so that peptide A1 is no longer able to dissociate from the rest of the molecule. There have been two reports of cross-linking of the toxin before: by van Heyningen (1976*a*), who used artificial disulphide bonds formed with methyl 4-mercaptobutyrimidate, and by Gill (1976*a*), who used dimethyl suberimidate. Both of these studies were for structural analysis and no activity measurements were reported.

In this work, the cross-linking reagent was *NN'*-bis(carboximidomethyl)tartaramide dimethyl ester dihydrochloride [$CH_3$—O—C(=$NH_2^+Cl^-$)—$CH_2$—NH—CO—CH(OH)—CH(OH)—CO—NH—$CH_2$—C(=$NH_2^+Cl^-$)—O—$CH_3$], a reagent introduced by Coggins *et al.* (1976) that reacts with lysine residues in the protein. The advantage of this reagent is that the cross-link is stable, but, because it has a vicinal diol in the middle, it can easily be cleaved with periodate. This makes it possible to determine the composition of cross-linked species, and also to do controls for possible loss of activity during the cross-linking reaction itself.

* Abbreviations: ganglioside GM1, galactosyl-*N*-acetylgalactosaminylceramide (galactosylsialosylceramide); SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

## Methods

### *Cross-linking*

*NN'*-Bis(carboximidomethyl)tartaramide dimethyl ester dihydrochloride and the corresponding ethyl compound were prepared as described by Coggins *et al.* (1976) by using *NN'*-bis(cyanomethyl)tartaramide and *NN'*-bis(2-cyanoethyl)tartaramide (kindly given by Dr. John Coggins, University of Glasgow). Cholera toxin [prepared by the method of Finkelstein & LoSpalluto (1970) and kindly given by the National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, U.S.A., from whom it was prepared under contract] was exhaustively dialysed against 0.1M-triethanolamine/HCl buffer, pH8.4, and a 1mg/ml solution of toxin was incubated with about 50mM-reagent as described by Coggins *et al.* (1976) for various times at room temperature (20°C). The reaction was stopped by freezing the mixtures and then samples were applied directly for gel electrophoresis.

### *Gel electrophoresis*

Samples of cross-linked toxin were made 1% (w/v) in SDS, about 0.1mg/ml in phenylmethanesulphonyl fluoride (a proteinase inhibitor), and in some cases 1% (v/v) in 2-mercaptoethanol, and incubated for 2h at 37°C. (The electrophoretic pattern was the same if the samples were boiled in SDS for 3min, but this was not generally done when material was to be eluted and tested for activity.) Samples (containing up to 100$\mu$g per tube) were then applied to 10% polyacrylamide gels (2.7% cross-linked) in 0.1M-sodium phosphate buffer (pH7.2)/0.1% (w/v) SDS (Weber *et al.*, 1972) with Bromophenol Blue as a marker, and electrophoresed for about 4h at 8mA per tube. Gels were removed, and either stained with 2.5g of Coomassie Brilliant Blue R/litre in 7% (v/v) acetic acid/5% (v/v) methanol for 2h and destained electrophoretically in the same solution of acetic acid and methanol, or immediately cut into slices about 2mm thick. The slices were immersed in 100$\mu$l of water (two lots) and shaken at 20°C for a total of 24h. The combined eluate solutions were freeze-dried and the product was dissolved in water for assay, sometimes after pooling adjacent slices from the gel. Protein concentrations in these pools were estimated from the $A_{280}$. Accurate absorption coefficients of isolated subunits and of cross-linked toxin are not known; the $A^{1\%}_{1cm}$ at 280nm of whole toxin is 11.41 (LoSpalluto & Finkelstein, 1972).

Mobilities on the gel were measured relative to Bromophenol Blue and assumed to be the same on stained and unstained gels run at the same time. Molecular weights were calculated from the mobilities by using marker proteins: cytochrome *c* (mol.wt. 11700), myoglobin (17200), $\gamma$-globulin (23500 and 50000), aldolase (40000), serum albumin (67000) and phosphorylase (100000).

### *Adenylate cyclase and toxin assay*

Erythrocytes from fresh heparinized pigeon blood were centrifuged (5min, 1200*g*) to remove plasma, and buffy coat, suspended in a buffer containing 130mM-NaCl, 5mM-KCl, 2mM-$MgCl_2$ and 20mM-Hepes, adjusted to pH7.3 with NaOH. The suspension was passed through cottonwool, centrifuged again (5min, 1200*g*), and resuspended in the same buffer containing 10mM-glucose. Samples (40$\mu$l) were then put into small plastic tubes, and the subsequent treatment was different for assay with whole and lysed cells.

*Whole cells.* In some cases the cells were now incubated for 1h at 37°C with 0.1mg of ganglioside GM1/ml and then washed again with buffer. In all cases the packed cells were incubated with 10$\mu$l of toxin solution (or cross-linked toxin etc.) for various times at 37°C with shaking, then washed with ice-cold buffer, centrifuged (7min, 1200*g*), frozen in a −60°C bath, and allowed to thaw.

*Lysed cells.* The packed cells were suspended in an equal volume of buffer, frozen in the −60°C bath, allowed to thaw, incubated with toxin as above, and then washed in buffer.

In both cases, the adenylate cyclase activity of the packed 'ghosts' was then assayed by incubating 25$\mu$l for 30min at 37°C while shaking with 75$\mu$l of assay medium [40mM-NaCl, 3mM-EDTA, 10mM-$MgCl_2$, 0.2mM-dithiothreitol, 2mM-ATP, 0.5mM-papaverine, 1mg of serum albumin/ml, 12mM-phosphoenolpyruvate, 50$\mu$g of pyruvate kinase (EC 2.7.1.40)/ml and 50$\mu$g of adenylate kinase (EC 2.7.4.3)/ml, all adjusted to pH8 with NaOH with Phenol Red as an internal indicator]. The reaction was stopped by boiling the solution for 2min. The tubes were cooled rapidly in ice, centrifuged (3000*g*), and triplicate 20$\mu$l samples removed for assay of cyclic AMP. All assays (except when every slice from a gel was assayed on the same day) were done at least in duplicate. Production of cyclic AMP was linear with time up to 1h.

Cyclic AMP was assayed essentially by the competitive protein-binding method of Brown *et al.* (1971) in 0.05M-Tris / HCl / 8mM-theophylline / 6mM-2-mercaptoethanol, pH7.4, by using a cyclic AMP-binding protein from bovine adrenals. The assay was calibrated with standard cyclic AMP and was linear in the range 0–10pmol; no determinations were made outside this range.

## Results

### *Cross-linking of the toxin*

Both the dimethyl ester of *NN'*-bis(carboximidomethyl)tartaramide and the corresponding ethyl

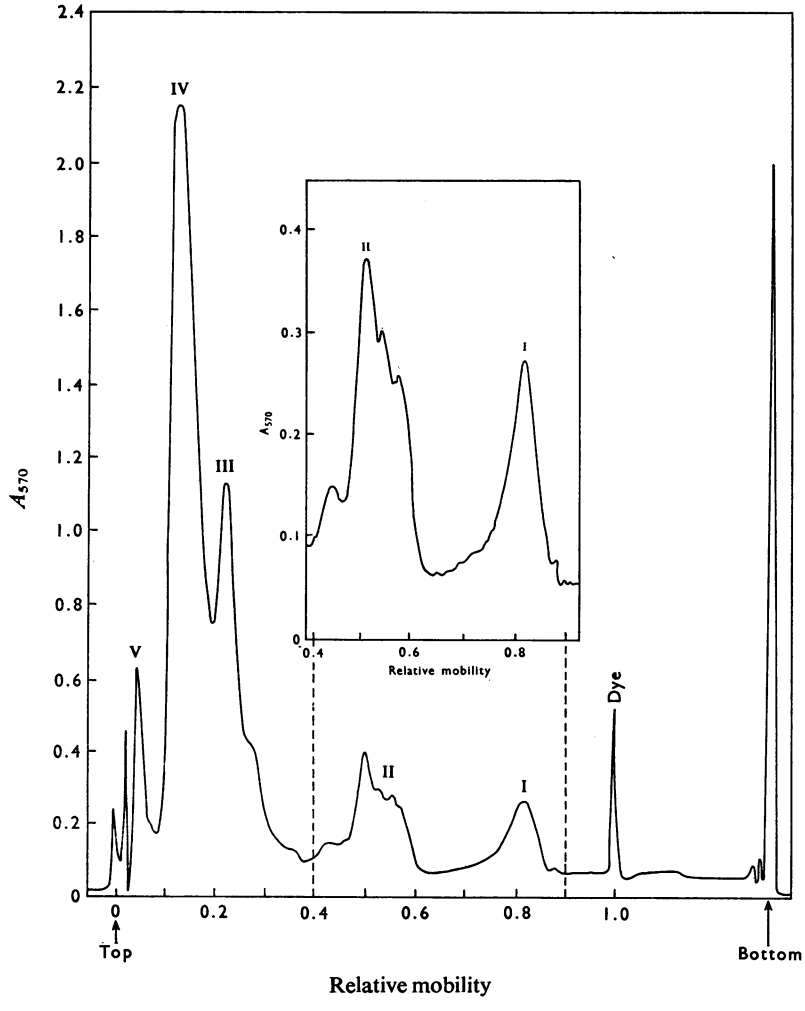


Fig. 1. *Densitometric trace of a polyacrylamide gel of cross-linked toxin*
The protein was cross-linked for 1.5h at 37°C with *NN'*-bis(carboximidomethyl)tartaramide dimethyl ester. About 100$\mu$g was applied to the gel, which was run in SDS as described by Weber *et al.* (1972), stained in Coomassie Brilliant Blue, destained electrophoretically and scanned in a Gilford model 2520 scanner with 0.1mm slit. The mobilities are relative to Bromophenol Blue, whose position on the gel (5.2cm from the top) was marked before staining. The insert shows a portion of the same scan at higher sensitivity.

compound (Coggins *et al.*, 1976) produced good yields of cross-linked toxin, but the products formed were complex and not always easy to identify. Fig. 1 shows a tracing taken from a densitometric scan of polyacrylamide-gel electrophoresis in SDS of cross-linked material formed when toxin was allowed to react with the methyl compound for 1.5h at 37°C. The result was similar with the ethyl compound, but the yield of cross-linked material was smaller. The reaction was probably complete within about 30min at 37°C; residual amounts of free subunits B and A did not disappear even if it was allowed to continue to react overnight. The gel shown was run in the absence of thiol reducing agent. When the cross-linked sample was treated with 2-mercaptoethanol before electrophoresis, it made no difference to the bands formed, except sometimes for small changes in the relative intensities.

Samples of cross-linked material eluted from the gels and run again under the same conditions had the same mobility as before, so the aggregation was not reversible. Treatment of the eluted cross-linked material with 10mM-$NaIO_4$ for 1h at room temperature followed by 20mM-Tris/HCl, pH7 (to react with

excess of $NaIO_4$), liberated free subunits A and B, as shown by electrophoresis.

Electrophoresis of similar samples after reduction with 2-mercaptoethanol in 12.5% polyacrylamide gels containing SDS and 8M-urea (a system particularly suitable for peptides of molecular weight less than about 10000; Swank & Munkres, 1971) showed that there was no sign of any free peptide A2 after 10min reaction and that there was no noticeable further reaction in the next 2h.

It is not easy to say definitely what the structure of each band of cross-linked toxin might be, especially as a cross-linked protein cannot be fully unfolded in SDS, so that its mobility may not be accurately related to molecular weight. The fastest visible band (mobility about 0.85, labelled I in Fig. 1) can only be free subunit B, since there is nothing smaller (except peptide A2, which does not show up well in this system). Running slightly slower than subunit B is a set of three bands (labelled II in Fig. 1, and shown in the enlarged part of the scan): presumably complexes $B_2$, A and $B_3$. There are some faint intermediate bands, perhaps complexes AB, $B_4$ and $B_5$. Towards the top of the gel there is a strong band (III) that might be complex $AB_4$, (mobility about 0.5) with a shoulder, perhaps complex $AB_3$. Strongest of all is a band (IV) that runs with a mobility corresponding to a molecular weight of about 85000 and so is probably complex $AB_5$ (in other words whole toxin). The mobility of these slower bands suggests that they must be larger than complex $B_5$ and so must contain some subunit A, and, furthermore, they produced both subunits A and B after treatment with $NaIO_4$. There is also some material (V) whose mobility corresponds to a greater molecular weight than that of whole toxin and so that must be intermolecular aggregates of several toxin molecules. Such intermolecular bonding was also shown by treating RNA polymerase with this reagent (Coggins *et al.*, 1977).

*Activity*

Figs. 2(*a*) and 2(*b*) show the activity of material eluted from slices of a gel run at the same time as the one in Fig. 1 and measured with whole and lysed cells respectively. There is unlikely to be exact correspondence between the slices from the one unstained gel and the bands on a different stained gel, so the mobilities in Figs. 2(*a*) and 2(*b*) may not agree exactly with those in Fig. 1. Nevertheless, it is clear that material running in the positions of peptides $AB_5$, $AB_4$, and probably free subunit A is active in both systems, There is no sign of any activity that can be put down to peptides $AB_2$ or AB, but these bands were very faint on the gel, so there probably was not enough material eluted. The relative activities of a given fraction measured with whole cells and with lysed cells are about the same as usually seen with

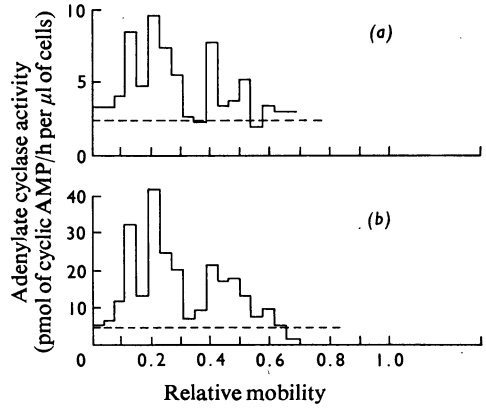


Fig. 2. *Activity of material eluted from gels measured with whole and lysed pigeon erythrocytes*

Toxin (100μg) was applied to a gel run at the same time as and under conditions identical with the one shown in Fig. 1. The relevant part of the gel was then sliced into 18 slices each about 2mm thick. The slices were immersed in two lots of 100μl of water and shaken at 20°C for a total of 24h. The combined eluate solutions were freeze-dried and the product was dissolved in 50μl of water. Samples (10μl) were preincubated at 37°C for 90min with whole cells and for 30min with lysed cells, and the adenylate cyclase activity of the cells then assayed as described in the Methods section. ----, Adenylate cyclase activity of cells preincubated in buffer alone without toxin.

ordinary native toxin (e.g. van Heyningen & King, 1975; Gill & King, 1975). Toxin samples eluted from these gels must have been significantly contaminated with SDS eluted from the gels at the same time. However, SDS is known not to inactivate toxin (indeed, rather to activate it; van Heyningen, 1976*a*; Gill, 1976*b*) and the concentration of SDS in each fraction must have been roughly the same. The activity of toxin eluted from gels has been measured before (e.g. Gill & King, 1975).

Fig. 3 shows a dose–response curve for cross-linked material corresponding to complex $AB_4$, whose protein concentration had been measured from the $A_{280}$; it shows a sharp change in activity with concentration at an $A_{280}$ of around 0.001.

The behaviour of cross-linked toxin towards whole and lysed cells is similar to that of native toxin. For example, Fig. 4 shows a time course for preincubation of whole and lysed cells with cross-linked toxin for various times before assay of the adenylate cyclase. There is a lag phase with whole cells, such as is observed with almost all systems and cholera toxin, but this phase is absent with lysed cells. Fig. 4 also shows the effect of cross-linked toxin on

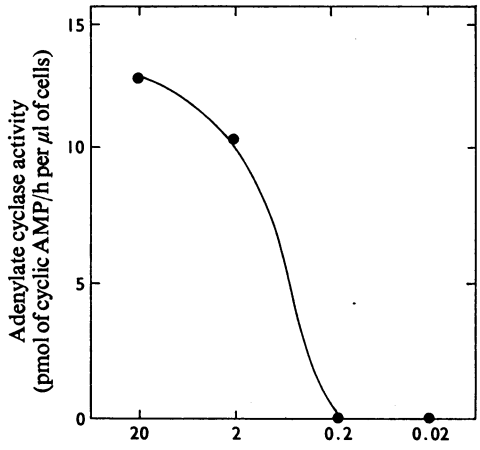


Fig. 3. *Variation of adenylate cyclase activity in whole pigeon erythrocytes after preincubation with different concentrations of cross-linked toxin*

A sample of cross-linked toxin corresponding to peak III in Fig. 1 was eluted from slices of gels run as in Fig. 2. The eluates from three adjacent slices corresponding to the whole peak were pooled and the protein concentration was estimated from the $A_{280}$. A portion (10$\mu$l) of the pool (one-twentieth of the total) and suitable dilutions of this were then incubated for 60min at 37°C with whole pigeon erythrocytes (40$\mu$l) that had previously been treated with ganglioside and washed. The adenylate cyclase activity of the cells was then assayed as described in the Methods section.

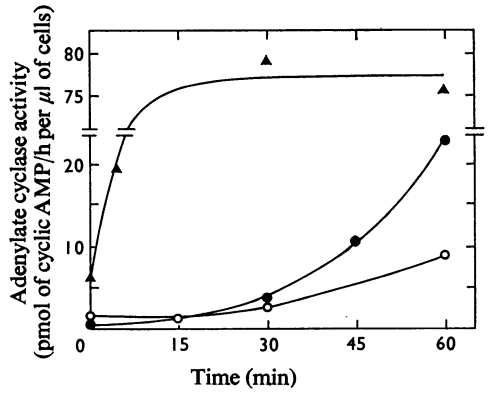


Fig. 4. *Time course showing effect of preincubation of whole and lysed pigeon erythrocytes with cross-linked toxin on their adenylate cyclase activity*

Cross-linked toxin eluted from gels as described in Fig. 3 was incubated with lysed cells (▲) and with whole cells preincubated with ganglioside (●) and without ganglioside (○) for various times at 37°C. The cross-linked toxin concentration in the incubation corresponded to a final $A_{280}$ of about 0.02. The adenylate cyclase activities of the whole and lysed cells after incubation were then measured as described in the Methods section.

cells that had been preincubated with ganglioside GM1 and then washed before exposure to toxin, so increasing the effective number of available binding sites for subunit B (Gill & King, 1975). The lag phase is still present, but the activity of the toxin is enhanced 3-fold. There was no comparable effect with subunit A itself eluted from the gels (although there is a lag phase in this system). Cross-linked toxin that had been preincubated with excess ganglioside before exposure to cells was inactive. Native and cross-linked toxin were both inactive after incubation with periodate.

*Cell lysis*

The preparation of the erythrocytes for assay inevitably leads to a little lysis. Roughly what proportion of cells had lysed could be judged from the haemoglobin concentration in the supernatant (measured from the $A_{430}$) when the cells were washed in buffer compared with the concentration when the same volume of cells was frozen and thawed (for lysis) before washing in the same volume of buffer. Such an experiment showed a ratio ($A_{430}$ of nonlysed supernatant/$A_{430}$ of lysed supernatant) of 8% in the very worst samples; most were considerably better (about 3%). There was no apparent correlation between adenylate cyclase activity and extent of lysis.

## Discussion

*NN'*-Bis(carboximidomethyl)tartaramide dimethyl ester is an efficient cross-linking reagent for cholera toxin. It produces covalently aggregated subunits that do not dissociate in SDS, but that can be made to dissociate back into the component subunits A and B by breaking the covalent link with $IO_4^-$. It joins peptides A1 and A2 of subunit A as well as joining subunit A to subunit B and joining between subunits B. These results are similar to the earlier cross-linking experiments of van Heyningen (1976*a*) and of Gill (1976*a*), except that Gill (1976*a*) found less cross-linking between the rest of the molecule and peptide A1. Thus in his system, treatment of the cross-linked material with 2-mercaptoethanol made a marked change in the pattern of bands seen and produced some aggregates containing subunit B bound to peptide A2 only. These and other experiments suggest that in the native molecule it is peptide A2 that is closest to the B subunits and that binds to it non-covalently, and the experiments described in

the present paper are compatible with this result in that time courses after reduction never showed any sign of free peptide even after a short incubation time. However, since in general the pattern of cross-linked material was not altered by reduction, the cross-linking reagents must have linked the two peptides. It is not surprising that *NN'*-bis(carboximidomethyl)-tartaramide dimethyl ester and dimethyl suberimidate behave differently, since the tartaric acid derivative is longer than dimethyl suberimidate, although it contains the same groups that react with the protein, and also it has two amide bonds likely to more rigid than the flexible hydrocarbon chain of dimethyl suberimidate. Comparison with the cross-linking experiments using methyl 4-mercaptobutyrimidate is not possible, since that reagent introduces disulphide bonds as cross-links, so that its effect is reversed by reduction.

Since the aggregated species does not liberate peptide A1 on incubation with SDS and 2-mercaptoethanol for 2h at 37°C it seems very unlikely that the peptide A1 could be liberated at the surface of the cell.

The observed pattern of bands is compatible with the proposed $AB_5$ structure for native toxin. There has been some disagreement about the number of B subunits, but perhaps this is no longer a matter for speculation now that the molecular weight of subunit B is very accurately known from published amino acid sequences (Lai *et al.*, 1976; Nakashima *et al.*, 1976; Kurosky *et al.*, 1977).

The cross-linked toxin, from which peptide A1 cannot escape, has all the activity normally associated with intact native toxin. It shows the same time course as native toxin with whole and lysed cells, and a similar dose–response curve. It presumably interacts with the membrane gangliosides in the same way, since its activity is enhanced by increasing the number of binding sites after preincubation of the cells with ganglioside. This experiment also shows that the activity was not due to any lysed cells that might be present in the mixture (in any case unlikely, since only a small proportion were lysed), since lysed cells would not show a lag phase nor any stimulation by preincubation with ganglioside. Similarly the cross-linked material was inactivated when preincubated with ganglioside, presumably because, as with native toxin, all the binding sites on subunit B were occupied with ganglioside, leaving nothing free to react with more ganglioside in the cell membrane.

It would have been interesting (and was the original reason for the choice of this particular cross-linking reagent) to have determined the activity of material after breaking the cross-links with $IO_4^-$, so allowing the subunits to separate as in native toxin. However, unfortunately neither toxin nor cross-linked material was active after incubation with $IO_4^-$.

These results are difficult to reconcile with theories of cholera-toxin action that propose that peptide A1 is released from the complex and inserted into the cytoplasm, where it can then interact with some receptor on the inner plasma membrane. It is not surprising that the cross-linked material (like free peptide A1) was active with lysed cells, where transport through the cell membrane is not necessary, but the activity with whole cells is surprising.

The inescapable conclusion is that the peptide A1, which, as it is active by itself, must have the ability to interact with cyclase or some system linked to the cyclase, can also interact when held on the outside of the cell. Since the evidence is very strong that it cannot work by acting directly on some receptor on the outside of the cell, a possibility is that it can protrude through the membrane to the inside. Once even part of it is through, it can perhaps catalyse some reaction involving some component free in the cytoplasm. There is good evidence that the toxin works enzymically in some way (Gill, 1976*b*). It is known to catalyse the breakdown of $NAD^+$ into nicotinamide and ADP-ribose (Moss *et al.*, 1976, 1977) and presumably such catalysis would not necessarily involve the whole of subunit A becoming free in solution.

Another possibility is suggested by the interesting results of Matuo *et al.* (1976), who have evidence that all the activity of subunit A can be exerted by one small peptide (perhaps as little as ten residues long) that can be cleaved from the whole subunit by proteolytic attack. Possibly part of the A1 chain protrudes into the cytoplasm, where it is attacked by intracellular proteinases which cleave off an active fragment. The active fragment could then diffuse freely in the cytoplasm in the same sort of way as has been suggested for the whole peptide A1.

Both of these theories are in agreement with earlier ideas (Gill, 1976*a*; van Heyningen, 1976*a*) in that they involve conformational change in the whole molecule leading to penetration of the membrane by at least part of peptide A1. This conformational change and/or the penetration of the membrane is thought to be the reason for characteristic lag phase observed only with whole cells. So it is not surprising that such a lag phase is observed with cross-linked toxin as well as with native toxin or with subunit A. The lag phase may also be due to lateral movement of the toxin–ganglioside complex in the membrane (Sahyoun & Cuatrecasas, 1975).

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