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10. Pointed out by R. B. Leighton (personal communication).
11. T. A. Maxwell, E. P. Otto, M. D. Picard, and R. C. Wilson [*Geology* **1**, 9 (1973)] have suggested that meteorite impacts have melted ground ice. It is very difficult, however, to accept their identification of the smooth-rimmed circular areas of chaotic terrain as impact craters coeval with the channels. I suggest instead that they result from the collapse of thick sedimentary fill overlying craters formed on a lower surface at an earlier time. The example they chose as an illustration is much less convincing as an impact crater drained southeastward when viewed as part of a wider scene (Fig. 1, arrow) instead of in the narrow-angle view they use.
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15. I am attempting to develop models for the distribution of condensates in pore space in the martian subsurface, but the problem appears too complex for satisfactory solution even with an arbitary choice of physical conditions. A feature common to many models, however, is that during a warming or a cooling cycle the solid phase with the highest melting temperature, carbon dioxide hydrate, forms at the expense of the other phases. The problem may best be attacked through a study of analogous occurrences on Earth. Clathrate hydrates of N_2 and O_2 form only at considerably higher pressures than that of carbon dioxide, so air hydrate is unimportant [S. L. Miller, *Science* **165**, 489 (1969)]. Gases of the paraffin series, however, form hydrates at conditions much like those for carbon dioxide, and large fields of solid natural gas hydrate have recently been discovered in the permafrost zone of Siberia [Yu. F. Makogon, F. A. Trebin, A. A. Trofimuk, V. P. Tsarev, N. V. Cherskiy, *Dokl. Akad. Nauk SSSR* **196**, 203 (1971)]. The process by which these form from dispersed gas-water systems has been considered by A. A. Trofimuk, N. V. Cherskiy, Yu. F. Makagon, and V. P. Tsarev [*Akad. Nauk SSR Sib. Otd. Geol. Geofiz. No. 8* (1972), p. 3] and G. D. Ginsburg [*Sb. Statei Gidrogeol. Geoterm. Vyp.* **1**, 109 (1969)]. It is even possible that hydrocarbon clathrates occur in martian permafrost if it originated by cold-trapping juvenile volatiles.
16. A less likely possibility, but one worth exploring, is that at least some flooding resulted from abnormal local internal heat flux under atmospheric conditions similar to that of the present, with water persisting at the surface because of kinetic effects or only local equilibrium. Models have been advanced in which water could persist long enough to form rivers under the far more rigorous conditions of the lunar surface [R. E. Lingenfelter, S. J. Peale, G. Schubert, *Science* **161**, 266 (1968); G. Schubert, R. E. Lingenfelter, S. J. Peale, *Rev. Geophys. Space Phys.* **8**, 199 (1970)] which, although not convincing, are not clearly impossible. With favorable underground temperatures, water production by the dissociation of carbon dioxide hydrate would proceed at a rate at which the nonequilibrium persistence of water is plausible.
17. Work done as part of the Mariner Mars 1971 Project, Jet Propulsion Laboratory, California Institute of Technology, under contract WO-8122.

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Cholera Toxin: Interaction of Subunits with Ganglioside G_{M1}

Abstract. Vibrio cholerae *exotoxin is an aggregate of two different noninterconvertible subunits (molecular weights about 15,000 and about 25,000). Only the smaller subunit reacts with ganglioside G_{M1}, a possible biological receptor. The larger subunit, found only in active toxin molecules, can be eluted with 8 molar urea from insoluble complexes of toxin, ganglioside, and cerebroside.*

The exotoxin of *Vibrio cholerae* has been purified and shown to be a protein of molecular weight about 84,000 (*1*). It has choleragenic and skin reactivity, and both of these activities are prevented if the protein is allowed to react with the monosialosyl ganglioside G_{M1} (a possible natural receptor for the toxin which is found in most tissues) at a molar ratio of ganglioside to protein of about 3.5 to 1 (*2*). Experiments suggesting that the ganglioside reacts with only one of two different types of subunit in the toxin molecule are now reported.

Electrophoresis of the native toxin in polyacrylamide gels in 6.25*M* urea at *p*H 3.2 (*3*) showed two bands (Fig. 1a): a fast band, fragment B; and a more weakly stained slower band, fragment A. If either of the two bands was eluted from the gels and subjected again to electrophoresis in the same system, the band ran true; there was no interconversion or breakdown.

The ganglioside that inactivates cholera toxin is soluble in water but can be made insoluble by forming a 1 : 9 (by weight) complex with cerebroside (*4*), and this product still reacts with the toxin. Brain ganglioside G_{M1} (1 mg) and brain cerebroside (9 mg) (supplied by N. Gascoyne) were dissolved in 2 ml of a mixture of chloroform and methanol (1 : 1, by volume) and dried in a vacuum. The residue was suspended in 1 ml of 0.1*M* tris-HCl buffer, *p*H 7.0, containing 1 mg of cholera toxin (from Dr. R. A. Finkelstein, lot No. 12.9.72), and incubated at 37°C for 45 minutes. More than 90 percent of the toxin was adsorbed to the ganglioside-cerebroside. The complex was removed by centrifugation, washed twice with buffer, suspended in 0.4 ml 8*M* urea, 0.25*M* tris-HCl, *p*H 7.0, and incubated at 37°C for 3 hours. Electrophoresis of the supernatant after this incubation showed that essentially only fragment A had been eluted (Fig. 1b). (There was a slight trace of fragment B, but the amount was not increased even after incubation for a total of 5 hours, although the elution of fragment A was complete after 3 hours.) The rest of the toxin molecule (entirely fragment B) was eluted after prolonged incubation at 37°C in 6*M* guanidine hydrochloride, *p*H 7.0, an observation compatible with a recent report that whole toxin could be eluted with "guanidine" from ganglioside insolubilized by covalent bonding to agarose gels (*5*).

If fragment A was dialyzed against 0.1*M* tris-HCl, *p*H 7.0, and treated again with ganglioside-cerebroside, it was not readsorbed. However, fragment B could be readsorbed after dialysis. These results suggest that binding of the whole toxin molecule to the ganglioside is through fragment B. Electrophoresis in the urea gels of choleragenoid (a "natural" cholera toxoid, also a gift from Dr. Finkelstein) showed that it contained material running with fragment B only (Fig. 1c). Fragment A was absent. Thus it seems that it is the protein of choleragenoid that reacts with ganglioside, an observation which could explain the observed blocking of cholera toxin by choleragenoid.

Fragment A (isolated after elution from ganglioside-cerebroside or by eluting directly from the polyacrylamide gels) has some skin reactivity (*6*), about 1 percent of that of native toxin. It is not possible to know what the reactivity would have been had it not been necessary to incubate the material first in 8*M* urea, under which conditions most proteins are denatured. Obviously it would also be desirable to test the activity of fragment A in the ligated intestinal loop, but it has not yet been possible to accumulate enough biologically active material (after treatment with 8*M* urea) to carry out such a test. What distinguishes the activity of fragment A in the skin from that of the native toxin is that it is not decreased at all by prior incubation with ganglioside G_{M1} even at a molar ratio of ganglioside to total protein of about 10,000 to 1.

Although the low *p*H gels in urea distinguish between the two fragments, they give no information about their size or nature. Thin-layer gel chromatography on Sephadex G-200 in 6*M* guanidine hydrochloride (*7*) can give such information. Native toxin was dissociated into two proteins visible after

staining, one of molecular weight about 22,000 and one about 15,000 (measured after reduction and carboxymethylation in guanidine). Choleragenoid gave only the 15,000 molecular weight species, and material eluted with urea from the ganglioside-cerebroside complex (fragment A) showed only the 22,000 molecular weight material. This result confirms the proposition that intact toxin is made up of the subunits (fragment B) of choleragenoid plus fragment A.

Gel electrophoresis in sodium dodecyl sulfate (SDS) (*8*) also gives information about the fragments although molecular weights can only be calculated from mobilities in this system under conditions where the disulfide bonds in the proteins are completely reduced (*9*). When cholera toxin (donated by Dr. Finkelstein; or by Wellcome Laboratories, lot No. VT2096D) was separated in 10 percent gels in the presence of 2-mercaptoethanol (*10*), it gave three bands: one of molecular weight about 68,000 (present only when exposure to SDS had been very brief), one about 21,000, and one whose molecular weight was too low to measure accurately in this system (*8*), that is, less than about 15,000. If the 68,000 molecular weight band was eluted and again subjected to gel electrophoresis, it was degraded into the lowest molecular weight material, but the 21,000 molecular weight band remained as an entity and did not interconvert or break down.

Choleragenoid (or fragment B eluted from urea gels) under these conditions gave the heaviest and lightest species only, while fragment A gave the 21,000 molecular weight species plus, sometimes, a trace of material of very low molecular weight. In the absence of reducing agent, results were similar, except that fragment B formed much more of a high molecular weight species (but this could still be degraded after elution and further electrophoresis). Fragment A gave a single band which if eluted, reduced, and again subjected to electrophoresis gave the 21,000 molecular weight species and a trace of lighter material as above. Fragment A may therefore be made up of one comparatively large and one comparatively small polypeptide chain joined by disulfide bridges. These results confirm approximately the molecular weights from the thin-layer gels, but show some aggregation of fragment B in SDS to a larger species, not necessarily the same as choleragenoid.

These observations on SDS gels are similar to those of Lönnroth and Holmgren (*11*) and suggest that fragment B is their L, and fragment A is their H. They are also consistent with those of Finkelstein *et al.* (*12*), except that these workers have some evidence suggesting that a 28,000 molecular weight species was an aggregate. In my experiments, there was no evidence for any interconversion of fragments A and B under any conditions tried. LoSpalluto and Finkelstein (*13*) showed total breakdown of toxin at low *p*H to a single species of low molecular weight (14,000). Although this result cannot be explained by my observations or by those of Lönnroth and Holmgren, it may be relevant that exposure of cholera toxin to *p*H 3.6 often resulted in slow formation of a precipitate of almost all of the fragment A, leaving a supernatant which was almost pure fragment B.

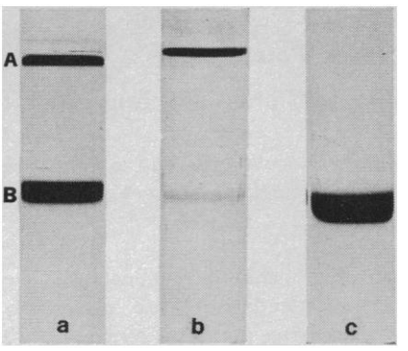

Fig. 1. Electrophoresis on polyacrylamide gels in urea (*3*). (a) Intact cholera toxin (75 μg) (lot No. 12.9.72); (b) About 20 μg of material eluted with urea after adsorption of intact toxin to ganglioside-cerebroside; (c) choleragenoid (100 μg) (lot No. 18.1.72).

In immunodiffusion experiments, choleragenoid, or fragment B eluted from SDS gels, showed reactions of identity with intact toxin against antiserum prepared against toxin in rabbits. In contrast, fragment A reacted only very weakly with the antiserum and showed nonidentity with fragment B and partial identity with intact toxin.

The conclusion is that cholera toxin seems to be composed of two types of subunit held together by weak, noncovalent forces. The intact molecule must contain several (perhaps four) subunits of fragment B plus at least one of fragment A. Since choleragenoid, which is nontoxic, is made up entirely of fragment B, fragment A must be at least partly responsible for the toxicity. There are analogies [see (*1*)] between this possible structure and that of diphtheria toxin (*14*), which has two fragments, one having the enzymic activity necessary for toxicity and the other probably being required for the toxin's entering the cell. In the case of cholera toxin, it might be fragment A that is active, and fragment B (choleragenoid) that facilitates this activity by binding to some receptor (perhaps ganglioside G_{M1} itself) in the susceptible tissue. It is known that, among other things, choleragenoid binds specifically to the membranes of the intestinal microvilli in the same way as does toxin (*15*), blocks the action of the toxin on the intact gut (*16*), blocks the stimulation of adenylate cyclase by toxin in lymphocytes (*17*), and blocks the binding of toxin by ganglioside G_{M1} (*18*). In contrast, the skin reactivity of fragment A may be irrelevant to the gut activity of cholera toxin, and fragment A might act by affecting the conformation of fragment B in such a way as to render it active in the gut.

Cholera toxin is a highly active stimulator of adenylate cyclase by a mechanism independent of the usual humoral regulators of this system (*1*). A knowledge of the structure of the toxin should make possible further mechanism studies involving modification of either the receptor subunits or the active subunit.

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