MICROBIOLOGICAL REVIEWS, Sept. 1984, p. 199–221
0146-0749/84/030199-23$02.00/0
Copyright © 1984, American Society for Microbiology

Vol. 48, No. 3

Bacterial Toxins: Cellular Mechanisms of Action

JOHN L. MIDDLEBROOK* AND REBECCA B. DORLAND

Department of Basic Toxin Research, Pathology Division, U.S. Army Medical Research Institute of Infectious Diseases, Ft. Detrick, Frederick, Maryland 21701

INTRODUCTION 199
DIPHTHERIA TOXIN 201
PSEUDOMONAS EXOTOXIN A 207
PSEUDOMONAS EXOENZYME S 209
SHIGELLA TOXIN 209
CHOLERA TOXIN 210
E. COLI ENTEROTOXINS 213
PERTUSSIS TOXIN 213
ANTHRAX TOXIN 213
TETANUS TOXIN 214
BOTULINUM TOXIN 215
CONCLUDING REMARKS 215
ACKNOWLEDGMENTS 215
LITERATURE CITED 215

INTRODUCTION

The elucidation of the cellular mechanisms of action of the bacterial protein toxins remains a complex problem, as evidenced by the wealth of current and often contradictory literature. Some general patterns have emerged, however, indicating that these toxins may be conveniently classified as either membrane damaging (phospholipases, hemolysins, lysins, etc.) or intracellular acting. The latter are the subject of this review and seem to share a common mechanism of action involving (i) binding to specific receptors on the plasma membranes of sensitive cells, (ii) internalization or translocation across the membrane barrier, and (iii) interaction with an intracellular target. Although these three steps are closely interrelated, it is the purpose of this review to consider the internalization and intracellular activity stages of toxin action. The reader is referred to a related review which emphasizes toxin-receptor interactions (40).

The process of toxin binding and internalization (or entry) appears similar in many respects to that by which protein hormones and other biologically efficacious macromolecules interact with cells. Since it seems unlikely that mammalian cells possess "suicide receptors" for bacterial toxins, a reasonable possibility is that these toxins parasitize pathways for molecules normally taken up by cells. Some support for this hypothesis derives from the striking structural similarities between bacterial exotoxins and glycoprotein hormones. Both types of molecules display a dichain or two-component construction: the A or alpha chains possess the biological activity, whereas the B or beta chains mediate receptor binding. Researchers have shown that several bacterial toxins, including diphtheria toxin (25), *Pseudomonas* exotoxin A (24, 108, 220), tetanus toxin (73, 120), cholera toxin (47), and *Shigella* cytotoxin (165), are synthesized in inactive proenzyme form and must undergo proteolytic cleavage and reduction to release active A fragment. The structural characteristics and activation mechanisms of individual toxins will be discussed in greater detail below. More specifically, amino acid sequence homologies have been demonstrated among the A and B chains of cholera toxin, thyrotropin, luteinizing hormone, human choriogonadotropin, follicle-stimulating hormone, and, more recently, interferon (105, 157). Such structural similarities suggest similar uptake and processing mechanisms.

Although the necessity for internalization of certain hormones and growth factors is still in doubt, many of the bacterial toxins, either as intact molecules or as biologically active fragments, undoubtedly must enter the cell to reach specified targets. Obvious examples include diphtheria and *Pseudomonas* A toxins, which block protein synthesis by inactivating a cytoplasmic protein (25, 79); cholera toxin, which stimulates adenylate cyclase (21, 52); and *Shigella* toxin, which acts upon the 60S ribosomal subunit (179). The protein toxins are, however, relatively large molecules (diphtheria and *Pseudomonas* A toxins, for example, have molecular weights [MW] of 60,000 and 66,000, respectively, whereas intact cholera toxin weighs 82,000 daltons) and few precedents exist for the entry of such large molecules into cells. Among these is the recently described process of adsorptive or concentrative endocytosis, the means by which a large number of macromolecules, including low-density lipoprotein (LDL) (4, 18), epidermal growth factor (EGF) (20, 60, 193), transcobalamin II (231), insulin (7, 123, 193), alpha-2 macroglobulin (224), triiodothyronine (23), and Semliki Forest virus (71, 115), are internalized by cells (reviews of adsorptive endocytosis have been published [54, 174]). The process is receptor mediated and thus is also known as receptor-mediated endocytosis (RME). It proceeds via specialized "endocytic organelles" known as coated pits. Originally described by Roth and Porter (182) in mosquito oocytes, these structures now have been identified in nearly all eucaryotic cell types or lines examined. Coated pits constitute approximately 2% of the normal fibroblast plasma membrane. The pits are short, usually indented, membrane segments characterized by an electron-dense fuzzy cytoplasmic coat, composed primarily of the protein clathrin (176).

Early, and now classic, studies of endocytosis delineated the uptake pathway of serum LDL (reviewed in reference

* Corresponding author.

56). The LDL complex, a primary source of plasma membrane cholesterol, has an MW of 2×10^6 to 3.5×10^6 and is made up of an outer layer of protein subunits (apoprotein B) and an inner core of neutral lipid, largely cholesteryl esters. The molecule binds through the apoprotein B moiety to specific cell surface receptors, over 70% of which are localized in coated pits. After ligand binding, the pits invaginate to form intracellular coated vesicles. The vesicles migrate into the cytoplasm where they rapidly lose their coats and fuse with primary lysosomes. Within the lysosome, the apoprotein B component is hydrolyzed, and free cholesterol is released from its esterified form by acid lipases. The cholesterol migrates freely across the lysosomal membrane and is eventually found either in association with the plasma membrane or in a re-esterified storage form in vesicles. The entire process is very rapid, reaching completion in 10 to 15 min as determined by serial electron micrographs.

It now appears that many proteins undergo internalization in a closely related manner. The receptors for most of these molecules, however, are not prelocalized in membrane-coated regions, as in the case of LDL, but are randomly distributed (225). Studies using fluorescence microscopy indicate that, subsequent to binding, the ligand-receptor complexes cluster into coated pits (124). Concomitant pharmacological experiments suggested that the clustering step is mediated by transglutaminase, an enzyme that catalyzes protein cross-linking by the generation of ϵ-(gamma-glutamyl) lysine bonds (28, 124). Amines such as ammonium chloride, methylamine, and dansylcadaverine, which inhibit purified transglutaminase in vitro, block the clustering and internalization of these ligands. After clustering into coated regions, the membrane invaginates to form short-lived cytoplasmic coated vesicles. These rapidly lose their coat and, in uncoated form, have been designated "receptosomes" (224). The receptosomes move through the cytoplasm by saltatory motion, reaching the GERL (Golgi-endoplasmic reticulum-lysosome system) in 15 to 20 min and finally, after 30 to 60 min, fusing with primary lysosomes.

Singer et al. (202) described a mechanism for the internalization of lectins or antibodies against specific cell surface components. This process, though not mediated by coated pits and vesicles, does involve an essential clustering step. Fluorescence studies indicated that the binding of a multivalent ligand results in the cross-linking of receptor molecules into small clusters and induces the interaction of the cluster with submembranous arrays of actin- and myosin-containing stress fibers. The interaction presumably takes place through some integral membrane protein (designated X) and results in cell surface patching and capping; invagination and vesiculation follow.

In contrast to receptor-mediated forms of endocytosis, cells may also internalize exogenous molecules non-specifically, via fluid-phase pinocytosis (these processes are comparatively reviewed in reference 199). This process, by definition nonsaturable and low affinity, is inefficient in molecular transport, though L-929 cells have been shown to interiorize the equivalent of their entire cell surface area every 125 min and cultured macrophages every 33 min (204). Willingham and Pastan (225) describe two types of fluid-phase uptake, designated macro- and micropinocytosis. The first, macropinocytosis, involves the folding over of membrane ruffles or blebs onto the adjacent cell surface, such as to entrap large vesicles of extracellular fluid. Intracellularly, the macropinosome undergoes repeated attack by lysosomes ("piranhalysis") until it is completely consumed. The more familiar micropinocytosis involves the pinching off of small membrane invaginations (caveolae) to form intracellular micropinosomes, which fuse directly with lysosomes. Neither macro- nor micropinosomes appear to localize in the GERL.

An alternative to an endocytic molecular transport mechanism is a direct traversal of the plasma membrane, either by some receptor-mediated process or by nonspecific hydrophobic diffusion. The latter, in view of the substantial size of most bacterial toxins and protein hormones, seems unlikely on the grounds of energetic feasibility. There are precedents, however, for the direct transfer of proteins across membranes. A large number of secretory proteins, including immunoglobulin light chains (10), the G protein of vesicular stomatitis virus (82), albumin (208), parathyroid hormone (64), prolactin (110), and growth hormone (110), are synthesized with short N-terminal or internal hydrophobic "signal sequences." These allow the protein to penetrate the membrane of the endoplasmic reticulum at specific recognition sites and direct transfer into the cisternae. After completion of transfer, these sequences are cleaved by specific intracisternal signal peptidases. Equivalent "signals" are hypothesized to function in the transfer of cytoplasmically synthesized proteins into the mitochondria or chloroplasts.

Among the bacterial protein toxins, evidence exists for a number of internalization mechanisms. In some cases, results strongly suggest a receptor-mediated endocytic pathway. In others, internalization appears to proceed by direct membrane transfer. Here, however, the concept of "productive" as opposed to "nonproductive" uptake becomes important. Productive uptake of toxin (or hormone) molecules is that which leads to expression of biological activity. In many instances, vast discrepancies are found between the number of available cell surface binding sites for toxin and the number of toxin molecules required to elicit biological effect, suggesting that many of these binding sites are nonproductive. As few as 4 to 10 molecules of intracellular cholera A fragment, for example, can effect maximal stimulation of adenylate cyclase (48), whereas in the case of diphtheria toxin, a single cytoplasmic A molecule is sufficient to cause cell death (228). Under such circumstances, it is conceivable that the productive internalization pathway is a minor undetectable subsidiary of a bulk surface clearance and disposal mechanism. On the other hand, since the receptors recognized by toxins are probably reserved for some other ligand or function, perhaps it is no surprise that many more molecules are delivered intracellularly than are required to attain a maximal biological response. A possible means of addressing this problem involves correlation of observed morphological and biochemical processes to the biological effects, under the assumption that, if perturbation of one leads to perturbation of the other, the observed process is biologically relevant. Specific instances will be discussed in detail below.

The intracellular activities of many bacterial toxins are enzymatic, and, in those instances where the biochemical events have been defined, the similarities among them are striking. Clearly, the key term is ADP-ribosylation, that is, toxin-catalyzed cleavage of endogenous NAD and covalent attachment of the adenosine diphosphoribose moiety to a cellular substrate. In some instances (diphtheria and *Pseudomonas* A toxins, for example), ADP-ribosylation inactivates a critical component of the cellular biosynthetic machinery. In others (cholera, *Escherichia coli*, or pertussis toxins), ADP-ribosylation leads to a marked increase in a cellular metabolic activity. Although the increase is not lethal to

individual cells, it has disastrous consequences in animals. It is evident, however, that it is the expression of enzymatic activity by the toxins which results in the primary biochemical lesions.

DIPHTHERIA TOXIN

Diphtheria toxin, the exotoxin of beta-lysogenized strains of *Corynebacterium diphtheriae*, is lethal for many animal species and cytotoxic to a wide range of cultured cell lines (extensively reviewed in references 25 and 169). Like many of the bacterial protein toxins, plant lectins, and protein hormones, diphtheria toxin is a bipartite molecule, composed of a B subunit (MW 39,000) which mediates binding to a specific cell surface receptor and an A subunit (MW 21,150) which possesses enzymatic activity. For full expression of enzymatic activity, fragment A must be separated from B by a process of proteolytic "nicking" and disulfide bond reduction. The toxin, though synthesized by the bacterium as an intact single polypeptide chain, is nicked by proteases in the bacterial culture supernatant or, in cell culture experiments, by enzymes present in the medium serum supplement.

The biological activity of diphtheria toxin is initiated by a receptor-binding event: Boquet and Pappenheimer (14) identified 4,000 binding sites per cell in HeLa cultures, whereas Middlebrook et al. (132) found 100,000 to 200,000 binding sites per cell in the highly diphtheria toxin-sensitive Vero cells. The toxin-receptor interaction was specific, saturable, and of high affinity ($K_d = 10^{-9}$ in Vero cells), and pharmacological experiments indicated that the observed binding was biologically relevant. Isolated fragment B, CRM 197 (a mutant toxin molecule containing a functional B but inactive B component), or nucleotide triphosphate compounds blocked both toxin-receptor binding and toxin-induced cytotoxicity. Furthermore, in a number of tested cell lines, the amount of observed toxin-receptor binding was shown to correlate to toxin sensitivity. Recent work suggests that the receptor itself is a high-molecular-weight glycoprotein. (For details, see the related review [40].)

Since the cellular target for diphtheria toxin is known to be cytoplasmic elongation factor 2 (EF-2), it is evident that, subsequent to receptor binding, either the intact toxin or a biologically active fragment must enter the cell. Currently, two major mechanisms have been proposed for this translocation to the cytoplasm: direct plasma membrane traversal and RME. The first, originally proposed by Pappenheimer and co-workers (15, 170, 171), generally involves (i) a rapid, reversible binding to specific cell surface receptor mediated by the positively charged C-terminal tail of fragment B; (ii) a slow and irreversible conformational change, which results in penetration of the phospholipid bilayer by a hydrophobic segment of fragment B; (iii) the formation of a channel through which fragment A, in unfolded form, is inserted into the cytoplasm; and (iv) specific proteolytic cleavage and reduction to release fragment A from the membrane-anchored B chain.

Studies of fragment B structure have had a fundamental bearing on the direct membrane traversal model of toxin internalization. Boquet et al. (16) initially showed that native diphtheria toxin, fragment B, and CRM 45 (a 45,000-MW premature chain termination mutant of diphtheria toxin lacking the B fragment carboxyl-terminal tail sequence) all bound substantial quantities of Triton X-100 after denaturation in 0.1% sodium dodecyl sulfate. This indicated the presence of sizable hydrophobic regions in all of these molecules. Fragment A, with or without sodium dodecyl sulfate treatment, did not bind Triton X-100, nor did undenatured native toxin, indicating that the presumptive hydrophobic region is masked in the intact molecule. Furthermore, undenatured CRM 45 and the CRM 45 B fragment inserted readily into Triton X-100 micelles. More direct evidence for the hydrophobicity of fragment B recently was obtained by Lambotte et al. (103), using amino acid sequence analysis. These studies identified two types of lipid-associating domains. The first, located in the N-terminal region of the fragment, is apolipoprotein-like in structure, with a large polar face, presumably capable of interacting with hydrophilic headgroups of membrane phospholipids, and a narrow apolar face capable of stabilizing hydrophobic interactions with the membrane lipid core. The second domain, found in the B fragment midregion, is structurally related to the integral membrane proteins, possessing a large polar face suited to interact with the phospholipid hydrocarbon core. This segment was calculated to be 3.5 nm in length, the approximate thickness of the membrane phospholipid bilayer, and thus could function as a transverse domain in a membrane penetration mechanism. The 8,000-MW C terminus of the B fragment appears to have no lipid-associating properties and is thought to mediate the initial toxin-receptor binding event by means of electrostatic interactions.

It has also been shown, using model lipid bilayer systems, that the insertion of toxin causes channel formation. CRM 45-induced channels have been demonstrated in both artificial lipid bilayers and unilammelar liposomes (192), whereas Donovan et al. (32) have shown that diphtheria toxin is capable of forming ion-conducting transmembrane channels in model membranes. However, the low conductance of the channels formed suggested that channel diameter was only about 0.5 nm, too small for the passage of fragment A, even in extended, denatured form. Thus, the observed channels may not be directly involved in the intoxication pathway, but may be merely an offshoot of toxin insertion into the membrane.

Some further suggestive evidence for a direct membrane traversal internalization mechanism derives from cell line sensitivity studies. Pappenheimer and Moynihan (173) compared the sensitivities of cultured cells from a variety of species and tissues to whole diphtheria toxin, CRMs 45 and 197, and isolated fragment A. The cells showed a wide range of diphtheria toxin sensitivities, with over a 10^6-fold difference between the most and the least sensitive types. Sensitivities to the mutant CRM 45, which, as mentioned above, lacks a portion of the fragment B sequence and thus cannot bind to the receptor, were low and relatively uniform. The exception was rat Schwann cells, which were about 10-fold more sensitive to CRM 45 than any other cell type. A possible interpretation is that the high lipid content of the myelinated Schwann cell membrane facilitates the process of insertion and direct transfer.

Though the direct membrane penetration hypothesis can by no means be ruled out, many results from recent diphtheria toxin studies are suggestive of internalization by RME. The RME route for diphtheria toxin, paralleling those processes described above for hormones and certain viruses, would involve (i) binding of the toxin to specific cell surface receptors, (ii) internalization of the toxin (or, more likely, toxin-receptor complexes) into intracellular endocytic vesicles, (iii) migration of toxin-containing vesicles into the cytoplasm to fuse with primary lysosomes or other acidic vesicles, and (iv) release of fragment A or proteolytic degradation of toxin or toxin-receptor complexes or both.

Investigations into the binding and internalization of diphtheria toxin were greatly facilitated after the observation of Middlebrook et al. (132) that Vero cells, a cultured fibroblastic line derived from African green monkey kidney, possess large numbers of specific, high-affinity toxin receptors. These studies were performed with biologically active ^{125}I-labeled diphtheria toxin of high specific activity. At 4°C, the ^{125}I-labeled toxin-cell association increased with time to a steady state, whereas at 37°C the association was biphasic, increasing to a peak at 1 to 2 h and decreasing thereafter. Subsequent work indicated that this biphasic pattern reflected a progressive process of cell surface binding, internalization, degradation, and excretion of degradative products into the extracellular medium (35), with a concomitant cessation of protein synthesis and inhibition of further toxin uptake (131). More precise biochemical studies delineated the kinetics of toxin internalization (35). In these experiments, cells were prebound with ^{125}I-labeled diphtheria toxin at 4°C and then rapidly warmed to 37°C. At various times after the temperature transfer, cells samples were treated with a combination of pronase and inositol hexaphosphate which removed any toxin still exposed at the cell surface, and the amounts of releasable (surface bound) and resistant (internalized) toxin were determined. Analogous methods have been used to study internalization rates of other bioactive macromolecules, including LDL (55), alpha-2 macroglobulin (30), and EGF (2, 20). In the case of diphtheria toxin, the half-time of internalization was approximately 25 min. Under similar incubation conditions, LDL (55), EGF (20), and alpha-2 macroglobulin (30) are much more rapidly cleared, disappearing from the cell surface in <10 min. Platelet-derived growth factor, on the other hand, is internalized even more slowly, with a half-time of 1 h (70). As internalization progressed, the intracellular diphtheria toxin was degraded, as measured by trichloroacetic acid-soluble radioactivity in the culture supernatant. The amount of trichloroacetic acid-soluble radioactivity in the medium increased with time of incubation at 37°C to a plateau level around 3 to 4 h, at which point 60 to 70% of the original cell-associated radioactivity had been excreted in degraded form, primarily ^{125}I-labeled monoiodotyrosine.

A number of earlier diphtheria toxin uptake studies have shown that both sensitive and resistant cells appear to adsorb and degrade substantial amounts of toxin by an apparently nonspecific pinocytic mechanism. Boquet and Pappenheimer (14) found that HeLa and L-929 cells, which differ in diphtheria toxin sensitivity by nearly four orders of magnitude, took up ^{125}I-labeled diphtheria toxin at the same rate and to the same extent. However, after a 5-h incubation at 37°C, a 10^{-5} M concentration of diphtheria toxin was required to reduce protein synthesis in L-929 cells by 50%, or 2×10^6 internalized toxin molecules per cell. Under the same conditions, only a 10^{-9} M concentration of toxin was sufficient to reduce HeLa cell synthesis by 50%, an estimated 200 molecules per cell. Since cytoplasmic extracts of these cell lines have identical sensitivities to the enzymatically active fragment A (as determined by in vitro ADP-ribosylation assays), it seems unlikely that the observed pinocytic uptake is biologically relevant.

Similar results were obtained by Bonventre et al. (12) with HEp-2 and L-929 cells and by Saelinger et al. (183, 184) with HEp-2 cells and actively phagocytic mouse or guinea pig macrophages. Both studies supported the view that pinocytic uptake of toxin does not necessarily lead to cytotoxicity. In the latter study, it was proposed that sensitive cells possess two distinct and independent mechanisms of diphtheria toxin entry: a nonproductive pathway in which the majority of toxin molecules are taken up by nonspecific pinocytosis and degraded (though a few may occasionally escape proteolytic destruction); and a productive pathway, in which a minor fraction of toxin molecules are taken up specifically, possibly through fragment B–receptor-mediated direct traversal of the plasma membrane.

Data from other laboratories have further bearing upon the questioned biological relevance of pinocytic toxin uptake. Whereas EF-2 extracts from mouse L-929 cells are sensitive to diphtheria toxin, the intact cells are almost completely toxin resistant. However, treatment of these cells with poly-L-ornithine, a compound known to stimulate cellular protein uptake, markedly increased their sensitivity to toxin (137). Somewhat similar experiments by Wolstenholme et al. (227) investigated the effects of hypertonic salt solutions on the interaction of diphtheria toxin fragment A with HeLa cells. Though intact cells are ordinarily resistant to isolated A fragment, in the presence of 0.85 M $MgSO_4$, an inducer of intracytoplasmic uptake and vacuolation, cytotoxicity was achieved. Studies with toxin-resistant mutant cell lines also demonstrate intoxication via pinocytosis. Although the majority of experimentally generated diphtheria toxin-resistant cells are EF-2 mutants, resistant by virtue of an altered nonribosylatable form of EF-2 (37, 62, 145) (discussed in detail below), some "permeability" mutants have been identified (37, 138, 141, 143, 145). Comparisons of the effects of whole diphtheria toxin and purified A fragment on sensitive KB cells and permeability-type resistant KB mutants showed that, whereas the mutants were at least 100,000-fold resistant to whole toxin, both cell types were equally sensitive to fragment A (143). Intoxication under these conditions most likely results from the rare escape of an active toxin molecule or fragment from some compartment of the pinocytic pathway. Such escapes may occur at best at a rate of 1 in 2,000 pinocytosed molecules (14), though other estimates are considerably less optimistic. Bonventre et al. (12) calculated that <1 in 10^6 toxin molecules escape the macrophage phagosome. Nonetheless, the results indicate that pinocytosis may be a viable, though highly inefficient, mechanism for the productive internalization of diphtheria toxin.

In further studies on this subject, Morris and Saelinger (147) found that most diphtheria toxin molecules entered toxin-resistant LM cells through non-clathrin-coated regions of the plasma membrane and rapidly moved to the lysosome. This pathway was unlike the entry of *Pseudomonas* exotoxin A in the same cell line (LM cells are sensitive to exotoxin A) (146) or diphtheria toxin in Vero cells. In both of those cases, toxin was seen sequentially in coated pits, coated vesicles, the Golgi region of the cell, and finally in lysosomes. Morris and Saelinger reached the conclusion that diphtheria toxin-resistant cells have a defect in the entry process at some step between toxin binding and delivery of fragment A to the cytoplasm. Essentially this same hypothesis was proposed earlier by Didsbury et al. (31) based on biochemical studies of labeled diphtheria toxin binding to LM cells.

A primary advantage in the use of the Vero cell system is the ability to study a receptor-mediated entry process. The internalization and degradation described, therefore, do not result from nonspecific pinocytosis, but are direct corollaries of toxin-receptor binding. Certain drugs or chemicals which block either the internalization or the degradation step of the uptake process concomitantly block cytotoxicity, strongly implying that the observed pathway is biologically relevant. The plant lectin concanavalin A, for example, was shown to prevent the internalization of diphtheria toxin without inhib-

iting toxin-receptor binding (133); simultaneously, concanavalin A blocked toxicity (38, 133). These results have been confirmed electron microscopically (134). Early autoradiographic studies (172) were unable to demonstrate the migration of surface-bound diphtheria toxin into the cell interior. However, these studies used the toxin-resistant L-929 cell line and the relatively toxin-insensitive HeLa line, neither of which is optimal for uptake studies. In subsequent autoradiographic studies with Vero cells, intracellular toxin was evident (36). After a 2-h incubation at 37°C, >90% of the silver grains in control cells were associated with the cytoplasm, as opposed to 39% in the concanavalin A-treated sample, indicating a lectin-mediated inhibition of internalization. Furthermore, at neither 37 nor 4°C was there autoradiographic evidence for the preferential localization of diphtheria toxin over coated membrane regions. In both cases, membrane-associated silver grains showed a random distribution over the cell surface. It is possible that this may explain the relatively slow internalization or diphtheria toxin (half-time, 25 min) in comparison to LDL, EGF, and alpha-2 macroglobulin, molecules that are known to internalize through coated pits.

The toxin degradation process observed in Vero cells is blocked by the lysosomotropic agent chloroquine (107a). Chloroquine simultaneously protected cells from the activity of diphtheria toxin in cytotoxicity and protein synthesis assays. The ability of chloroquine to inhibit degradation of exogenous proteins has been widely exploited. It has been shown to prevent the degradation of a large number of macromolecules internalized by RME mechanisms (reviewed in reference 54), among them LDL (57), EGF (20, 99), human choriogonadotropin (5), transcobalamin II (231), growth hormone (75), thrombin (191), insulin (116), and platelet-derived growth factor (70). Not only chloroquine, but a wide range of alkylamines, protect cells from diphtheria toxin (34). Ammonium chloride has been found by several laboratories to be a potent blocker of toxin activity (36, 83, 98, 130). Mekada et al. (126) showed that diphtheria toxin-induced inhibition of protein synthesis in FL cells was prevented by methylamine, ethylamine, *n*-propylamine, isopropylamine, chloroquine, or ammonium chloride; similar results were obtained by Dorland (34) in Vero cells, using a wider range of alkylamines and substituted ethylenediamine compounds. The mechanism by which these compounds exert their toxin-protective effects remains unclear, though a number of possibilities will be discussed below.

The lysosomotropic effects of chloroquine and the related alkylamines are believed to result from the weakly basic character of these compounds (29). Within the environment of acidic vesicles, these agents become protonated. The vesicular membrane is impermeable to protonated forms of the amines, which thus accumulate to high concentrations within the organelle. A number of physiological activities have been attributed to the lysosomotropic amines. Chloroquine, for example, is known to increase lysosomal pH (29, 162), induce cytoplasmic vacuolation (163), inhibit the activity of lysosomal enzymes (notably cathepsin B) (223), enhance the fusion of lysosomes with other cellular organelles (61), and block the degradation of both endogenous (223) and exogenous (54) proteins. Recent studies also have demonstrated an effect of chloroquine on the Golgi apparatus (177): chloroquine treatment prevented the transfer of endocytosed insulin to the lysosomes and thus enhanced protein accumulation in the Golgi vesicles.

Chloroquine, ammonium chloride, and various amine compounds have been shown, in addition to their intracellular effects, to prevent the internalization of certain surface-bound macromolecules. Among these are alpha-2 macroglobulin and EGF (124), lysosomal enzymes (185), and the toxic lectin modeccin (190). There is strong evidence that these blocks of internalization result from an inhibition of membrane-associated transglutaminase, and hence, as discussed above, a block of ligand-receptor clustering before endocytosis. Such a mechanism does not appear to obtain, however, in the case of diphtheria toxin. Neither dansylcadaverine nor bacitracin, both effective transglutaminase and internalization inhibitors in other systems (28, 122), had a measurable effect on diphtheria toxin uptake (36). Furthermore, none of the toxin-protective lysosomotropic amines studied affected the internalization of radiolabeled toxin, though all, with the exception of ammonium chloride, blocked toxin degradation (34). Furthermore, in this series of experiments, ethylenediamine had no effect on either toxin degradation or biological activity, in contrast to the ethylenediamine derivatives, which blocked both. A suggested explanation is that a degree of lipophilicity is required for amine potency, implying an intracellular site of action.

Whereas internalization and degradation of ^{125}I-labeled diphtheria toxin appeared to proceed normally in the presence of ammonium chloride, a fraction of potentially active toxin molecules was maintained in a position accessible to neutralizing antitoxin for prolonged periods at 37°C (36, 184). The initial interpretation of such data was that ammonium chloride protects cells by preventing the internalization of biologically relevant toxin molecules or that only a small fraction of toxin-specific receptors are productive receptors. Experiments by Draper and Simon (39), however, cast doubt on these explanations. Their work, using V79 cells, showed that whereas ammonium chloride did maintain diphtheria toxin in an antibody-accessible position at 37°C, this effect was abolished at 4°C. These results were confirmed subsequently in Vero cells, using both ammonium chloride and a wide range of alkylamines (34). Based on these additional data, a likely interpretation is that, in the presence of ammonium chloride, toxin becomes sequestered in intracellular vesicles. Thus, at 37°C, either antitoxin is similarly endocytosed and neutralizes the toxin molecules intravesicularly or the toxin-containing vesicles are recycled to the membrane surface, where the antitoxin-toxin interaction occurs. Precedents for the recycling of intracellular vesicles to the plasma membrane exist. Schneider et al. (194) have shown that surface-bound antiplasma membrane immunoglobulins are returned to the cell surface after internalization into endocytic vesicles. Furthermore, in recent studies where inositol hexaphosphate (a compound capable of detaching diphtheria toxin from its receptor [35]) was substituted for neutralizing antibody in the same experimental design, protection was observed at 37°C, but not at 4°C. Since inositol hexaphosphate is highly charged and presumably does not readily enter the cell, these results further support the idea that toxin contained in intracellular vesicles is recycled to the cell surface for neutralization. Although results obtained by Tietze et al. (214) in another ligand-receptor system indicated that both chloroquine and ammonium chloride block receptor recycling, neither drug affected the recycling of diphtheria toxin receptors (J. L. Middlebrook, unpublished data). In any case, at 4°C, presumably neither endocytosis nor exocytosis takes place, such that either antitoxin cannot enter the cell or the toxin-containing intracellular vesicles cannot return to the cell surface. None of the available data, however, rule out the possibility that biologically relevant toxin remains on the cell surface in the

presence of ammonium chloride but, at 4°C, is either masked or conformationally altered such as to prevent recognition by antibody.

It is well known that the inactive native form of diphtheria toxin must undergo proteolytic cleavage and reduction before the release of active fragment (see above). In vitro trypsinization is commonly used to activate intact toxin preparations. It is possible that, in vivo, lysosomal enzyme-catalyzed cleavage is necessary for the release of fragment A or perhaps larger active fragments capable of both ADP-ribosylating activity and lysosomal membrane penetration. An example of such a fragment might be a CRM 45-like molecule, which possesses both a complete fragment A and a truncated B segment able to embed in detergent micelles (16). Such a mechanism may be analogous to that of certain viral uptake systems. Early reports indicated that the uncoating of enveloped viruses occurs in the lysosomes of infected cells (198). A recent study by Miller and Lenard (136) demonstrated that the infectivity of vesicular stomatitis, Semliki Forest, Sendai, and influenza viruses is blocked by lysosomotropic amines in a manner that does not interfere with their endocytic internalization. The conclusion was that these viruses were uncoated ("activated") in lysosomes before cytoplasmic replication. Of interest in this context is the discovery by Moehring and Moehring (142) that a number of diphtheria toxin-resistant KB cell sublines were concurrently resistant to RNA viruses, such as poliovirus, Mengo virus, Newcastle disease virus, and vesicular stomatitis virus.

Some support for a lysosomal activating mechanism for diphtheria toxin recently was generated by the work of Middlebrook and Leatherman (135) with rabbit reticulocytes. These cells, which lack defined lysosomes, were insensitive to intact diphtheria toxin, implying either an absence of the required "nicking enzyme" or an improper packaging of this enzyme such that it can no longer interact effectively with toxin. Protein synthesis in the reticulocytes was inhibited by nicked diphtheria toxin, and this inhibition was prevented by ammonium chloride, chloroquine, or methylamine, though less effectively than in other cell lines. Whereas the reticulocytes did appear to have some lysosomal enzyme activity, both electron microscopic examinations and sucrose density gradient analyses suggested that these enzymes were not localized in defined organelles. These studies thus indicated that lysosomes or lysosome-like organelles may be necessary for effective activation of endocytosed diphtheria toxin and further demonstrated that the level of action of the alkylamines is not, or is not solely, at the nicking or activation step.

Although it is difficult to demonstrate differential sensitivity to nicked and intact toxins in most cell lines, due to the nicking activity of proteases in the medium serum supplement, Sandvig and Olsnes (188) recently showed a twofold-greater activity or nicked toxin in Vero cells in inhibition of protein synthesis studies. The effect was enhanced when nicked or intact toxin molecules were introduced directly across the plasma membrane into the cytoplasm by a technique to be discussed below. Under these conditions, the nicked toxin was at least 30-fold more active than the intact form. Further experiments seemed to rule out nicking at the cell surface as a means of toxin activation, although no evidence was obtained for an efficient intracellular nicking process.

The effects of nicked and intact toxin preparations also have been compared in cultured cells derived from patients with I-cell disease, a lysosomal anomaly resulting from a deficiency of lysosomal enzymes. The speculation was that these cells might be toxin resistant by virtue of a missing nicking enzyme. These cells were, however, equally sensitive to both nicked and intact toxin preparations (R. B. Dorland, unpublished data).

A spectrofluorometric technique devised by Okhuma and Poole (162) has proven valuable in studying the mechanism of action of lysosomotropic agents. In these experiments, cells were allowed to internalize fluorescein isothiocyanate-conjugated dextran and the relative fluorescence intensity of the sample was measured before and after addition of the experimental lysosomotropic agent. Under these conditions, an increase in fluorescence intensity was taken to reflect an increase in intralysosomal pH. Since lysosomal proteases have pH optima in the range of 4.5 to 4.8, enzyme inhibition at increased pH may be a primary mechanism of action of the lysosomotropic amines. Recent experiments using this technique demonstrated that ammonium chloride and the toxin-protective alkylamines increased the pH of Vero cell lysosomes by approximately two pH units, whereas related nonprotective compounds had no pH effect (34).

In the diphtheria toxin system, however, putative lysosomal enzyme inhibition at increased pH may be less significant than the effect of pH on the interaction of diphtheria toxin with the lysosomal or endosomal membrane. A persistent problem of the RME model of toxin internalization is the question of how fragment A (MW 21,150) translocates to the cytoplasm. Endocytosis indisputably still leaves the internalized toxin inside a membrane-limited vesicle, a lipid bilayer away from its cytoplasmic target. A possible way around this difficulty was suggested by the work of Helenius et al. (71) on the infection of BHK-21 cells by Semliki Forest virus. Semliki Forest virus has been shown to enter sensitive cells by a receptor-mediated endocytic mechanism. Furthermore, virus infection is blocked by addition of ammonium chloride or lysosomotropic amines. This lysosomotropic amine-mediated protection was bypassed, however, if viral infection occurred at an acidic extracellular pH. Similarly, in a model membrane system, Semliki Forest virus at a pH of 6 or less was found to fuse with target liposomes and to transfer viral nucleocapsid into the liposome interior (222). These results were interpreted to indicate viral penetration to the host cytoplasm through the lysosomal membrane.

Equivalent results were obtained, initially by Draper and Simon (39) and confirmed by Sandvig and Olsnes (187) and Dorland (34), in studies of diphtheria toxin entry into sensitive cells. Similarly, King and Cuatrecasas (99) found that low pH reversed the methylamine-induced block of the biological activity of EGF. In the diphtheria studies, at an extracellular pH below 4.7, amine-mediated protection was abrogated. Analogous results have been obtained with CRM 45 (192). The work of Donovan et al. (32) in a model lipid membrane system showed that both diphtheria toxin-induced membrane channel formation and intrabilayer insertion were greatest at low pH. These studies in toto imply that, at low pH, diphtheria toxin (or active fragment) is able to penetrate the plasma membrane directly, and whereas the protective amines block some step of the normal endocytic pathway, they do not affect the low-pH bypass route. Under normal conditions, internalized toxin contacts a low-pH environment in the lysosome or some equivalent acidified vesicle. Penetration thus occurs across the vesicular, rather than the plasma, membrane.

The hypothesis that diphtheria toxin enters the cytoplasm from a lysosome or other acidified vesicle is further supported by recent studies with carboxylic ionophores (160, 216).

The compound monensin has been shown to dissipate intracellular proton gradients by the transmembrane exchange of a proton for a monovalent cation (usually K^+) and thus could readily produce an increase in lysosomal pH (162). The experiments of Marnell et al. (114) showed that Vero cells were protected from the action of diphtheria toxin by both monensin and a related ionophore, nigericin. The protection, like that induced by ammonium chloride and the lysosomotropic amines, was bypassed by low extracellular pH, and combination studies implied that monensin and ammonium chloride block the same step of the toxin uptake process. Similar results were obtained by Sandvig and Olsnes (189).

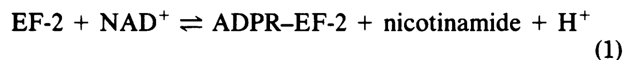
Later work more strongly suggests that toxin enters the cytoplasm from an acidic endocytic vesicle rather than from a lysosome. Early electron microscopic analyses indicated that the toxic lectin ricin entered the cytoplasm directly from the endocytic vesicle after receptor-mediated internalization (160). It was recently shown that such prelysosomal endocytic vesicles (endosomes), as well as lysosomes, can be rapidly acidified intracytoplasmically by a process that requires both ATP and magnesium (127). Further investigations compared endosomes and lysosomes isolated from a class of mutant CHO-K1 cells simultaneously resistant to diphtheria toxin and *Pseudomonas* exotoxin A and to infection by Sindbis, Semliki Forest, and vesicular stomatitis viruses (127). In these "cross-resistant" cells, ATP-dependent acidification of endosomes was defective, whereas ATP-dependent acidification of lysosomes proceeded normally. In non-cross-resistant mutants, insensitive to diphtheria toxin but sensitive to virus infection, both endosomes and lysosomes acidified normally. These results support the conclusion that both active toxin and viral nucleocapsid enter the cytoplasm from an acidic endocytic vesicle.

In view of the fact that as little as one molecule of fragment A in the cytoplasm is sufficient to kill a cell (228), it is conceivable that studies of the uptake of the relatively high concentrations of ^{125}I-labeled diphtheria toxin are delineating a bulk toxin disposal pathway, whereas the minor productive uptake pathway proceeds undetected. On the other hand, most recent examinations of the diphtheria toxin internalization process have attempted to correlate biochemical uptake with biological activity. With such correlations and a wide range of morphological, biochemical, and pharmacological techniques, the bulk of the evidence now indicates that diphtheria toxin productively enters cells by RME. One notable exception was a study by Keen et al. with epifluorescence video intensification microscopy (93). In those experiments, rhodamine-labeled diphtheria toxin and fluorescein-labeled alpha-2 macroglobulin were found in the same clusters, suggesting coated-pit-mediated internalization. Identical results were obtained with toxin-resistant mouse 3T3 cells, leading to the suggestion that sensitive and resistant cells bind and internalize diphtheria toxin by similar mechanisms. This interpretation was based on the assumption that the fluorescent clusters represented coated pits and vesicles. Based on the more recent work of Morris and Saelinger at the ultrastructural level (147) (discussed above), this assumption was probably not valid.

Historically, diphtheria toxin was the first bacterial toxin identified as an ADP-ribosyl (ADPR) transferase, and in many ways it has served as a prototype for later studies on other toxins. In addition, recent studies have brought to light many details on diphtheria toxin-catalyzed ADP-ribosylation, making it the best characterized of all bacterial toxin ADPR transferases. Since there are excellent reviews (25, 169) discussing the basics of diphtheria toxin enzymology, we will briefly discuss those points and cover at length the more recent work.

As mentioned before, diphtheria toxin is a proenzyme; to express enzymatic activity, the A and B fragments must separate, a process somehow transpiring during internalization by target cells. Alternatively, fragment separation can be effected in vitro by treatment with a reductant and a denaturant. Although some earlier studies discussed enzymatic activity of the whole toxin, it later became evident that contaminating fragment A was probably responsible for the results. In any event, once on its own, fragment A acquires characteristics ideally suited for its nefarious ways. For example, isolated fragment A is extremely resistant to thermal denaturation, its enzymatic activity essentially unaffected by boiling for several minutes. Second, something about the structure or conformation of fragment A renders it resistant to or unrecognized by those cellular degradative mechanisms normally used to catabolize exogenous proteins. This observation was made in an interesting study by Yamaizumi et al. (229), in which they inserted whole diphtheria toxin, diphtheria toxin fragment A, CRM 197 fragment A, or diphtheria toxin fragment A plus anti-fragment A immunoglobulin G into the cytoplasm of mammalian cells. Whether delivered as part of the intact toxin or as the purified chain, diphtheria toxin fragment A was very long-lived, 80% surviving 24 h after introduction. In contrast, diphtheria toxin fragment B, CRM 197 fragment A, or diphtheria toxin fragment A plus anti-A immunoglobulin G had cytoplasmic half-lives of about 3, 3, and 5 h, respectively. Other in vitro experiments suggested that the binding of NAD to diphtheria toxin fragment A conferred considerable protection from proteolysis, and this in part may explain the above results.

Once free in the cytoplasm, diphtheria toxin fragment A is ready for business. Simply written, the reaction catalyzed by diphtheria toxin fragment A is as follows:



$$\text{EF-2} + \text{NAD}^+ \rightleftharpoons \text{ADPR–EF-2} + \text{nicotinamide} + \text{H}^+ \quad (1)$$

Since the reaction results in formation of a covalent bond, the equilibrium lies far to the right. Nevertheless, the reaction is reversible, so that lowering the pH and adding excess nicotinamide in the presence of fragment A will remove ADPR and regenerate active EF-2 (25). Although earlier studies provided evidence that diphtheria toxin fragment A works catalytically rather than stoichiometrically, this point was elegantly reinforced in a recent study by Yamaizumi et al. (228). Erythrocyte ghosts were loaded with known amounts of diphtheria toxin and fluorescein-conjugated bovine serum albumin and fused with toxin-resistant L-929 cells. By means of a fluorescence-activated cell sorter, L cells which fused with only one reticulocyte were isolated and their cloning efficiency was determined. The results indicated that a single molecule of fragment A was sufficient to kill an L cell. Clearly, fragment A must act catalytically for one molecule to bring about cell death.

The nucleotide specificity for ADP-ribosylation is quite stringent. NAD is the only naturally occurring nucleotide tested which gave any believable biological effect. The synthetic derivatives 3-thiocarboxamide pyridine-AD and 3-acetyl-AD did serve as substrates in the inactivation of protein synthesis, but analogs such as NADH, NADP, NADPH, and NMN were inactive (59).

Although several mechanistic possibilities exist, the scheme best supported by kinetic studies is the formation of

a ternary intermediate of fragment A, NAD, and EF-2. Experiments designed to detect a covalent ADPR-fragment A intermediate produced negative results (43). NAD does bind tightly (K_d = 8 μM), but reversibly, to fragment A. Under the conditions tested, EF-2 did not bind significantly to fragment A. Presumably, EF-2 binds to a site adjacent to the NAD on fragment A and ADP-ribosylation proceeds by nucleophilic attack at the acceptor site.

Since diphtheria toxin fragment A inactivates EF-2 from all eucaryotic sources tested (25), it was of considerable interest to identify the site of ADPR covalent attachment. Using [^{14}C]NAD, Maxwell and co-workers (180) were able to isolate a short, radiolabeled peptide from ADP-ribosylated rat liver EF-2. Upon sequencing the peptide, they found that the ADPR was attached to an unusual residue termed "amino acid X." Subsequently, Van Ness et al. (218) isolated a sufficient amount of ADP-ribosylated amino acid X to permit a structural determination by nuclear magnetic resonance analysis. The proposed structure was 2-(3- carboxyamido-3(trimethylammonio)propyl)histidine, given the trivial name diphthamide. Clearly, such a residue must have undergone multistep post-translational modification. As suggested by Van Ness et al. (218), the messenger-coded diphthamide precursor is almost certainly histidine. In all probability, at least three subsequent enzymatic reactions would be necessary to generate diphthamide.

Diphtheria toxin fragment A-catalyzed ADP-ribosylation is highly specific. Although there is a low level of self-ADP-ribosylation by the toxin, virtually the only cellular constituent labeled by fragment A and radioactive NAD is EF-2. Interestingly, fragment A does not label the "G" protein ADP-ribosylated by cholera toxin. Although it was reasonable to suspect that the specificity of the reaction could be defined solely by the unusual structure of diphthamide, such is apparently not the case. Van Ness et al. (217) found that diphtheria toxin did not ADP-ribosylate the diphthamide-containing tryptic fragment of EF-2, suggesting that there are other features of EF-2 structure required for specific recognition by diphtheria toxin.

Using nuclear Overhauser enhancement nuclear magnetic resonance, Oppenheimer and Bodley sought to further define the nature of the ADP-ribose–diphthamide bond (167). Their results suggested that the ADP-ribose is attached to the N-1 of diphthamide via an a glycoside linkage. This configuration would require fragment A-catalyzed ADP-ribosylation to proceed via inversion about the site of bond formation. Such a conclusion is intriguing in light of similar studies with cholera (166) and *E. coli* (149) enterotoxins, both of which also ADP-ribosylate with inversion.

One interesting spinoff of work with diphtheria toxin has been the insights gained into diphthamide biosynthesis. This information was acquired largely during studies with diphtheria toxin-resistant cells. Working independently, three laboratories developed toxin-resistant sublines from diphtheria toxin-sensitive parent cells (37, 62, 63, 139, 140, 144, 145). Two general classes of resistant cells were obtained (144). Class I, permeability mutants, were only rarely isolated. In these cells, apparently one or more elements in the binding and internalization pathway were altered such that diphtheria toxin was delivered much less efficiently into the cytoplasm. Class II mutants were isolated far more frequently. Phenotypically speaking, class II cells were composed of two distinct subclasses (140, 145). Cells in the first subclass (type IIa) were not inhibited in their growth rate even by very high concentrations of diphtheria toxin. Similarly, protein synthesis in type IIa cell lines was unaffected by concentrations of diphtheria toxin several orders of magnitude above the maximally effective doses for wild-type cells. Finally, cell extracts containing EF-2 prepared from class IIa cells were not susceptible to diphtheria toxin-catalyzed ADP-ribosylation. In contrast, growth of cells in the second subclass (type IIb) was inhibited by diphtheria toxin, although not completely as in the parent lines. Similarly, protein synthesis in class IIb cells was inhibited by diphtheria toxin, although never more than 50%, regardless of toxin concentration. As one might have expected, toxin-catalyzed ADP-ribosylation was observed when extracts from class IIb cells were used. However, when normalized on the basis of total cellular protein, the incorporation of radiolabeled ADPR did not exceed 50% of the level attained with parent cells.

Cell hybridization experiments with the resistant sublines produced interesting, but sometimes confusing, results. Fusion of CHO-derived class IIa cells with CHO parents produced hybrids with the characteristics of class IIb mutants; i.e., at saturating concentrations of diphtheria toxin protein synthesis was 50% inhibited and EF-2 was ADP-ribosylated to 50% of the level in wild-type cells (140). Similar fusion experiments with class IIb cells generated hybrids in which diphtheria toxin inhibited protein synthesis as maximum of 75% and catalyzed a like level of ADP-ribosylation (140). The resistance marker, therefore, behaves in a codominant fashion. To explain these results, it was suggested that CHO cells have two functional copies of the structural gene for EF-2. The class IIa and IIb cells thus represent the homozygous (R/R) and heterozygous (R/S) states of resistance, respectively (140).

These observations and interpretations were brought into question by data from other laboratories. Draper et al. (37) obtained diphtheria toxin-resistant CHO cells which were not susceptible to either toxin-induced protein synthesis inhibition or ADP-ribosylation. When fused with parent cells, the hybrids were fully sensitive to diphtheria toxin, indicating that the resistance was recessive. This group also isolated cell lines which behaved phenotypically as class IIb cells. However, in hybridization experiments, resistance in these lines was codominant, in agreement with the work cited above (140). Gupta and Siminovitch (62, 63) also obtained diphtheria toxin-resistant cell lines which acted phenotypically as class IIa cells, yet the resistance was recessive in hybridization experiments. No class IIb cells were isolated in these experiments.

These apparent contradictions were resolved by some elegant work from the Moehrings laboratory (139). As increasing numbers of diphtheria toxin-resistant lines were studied, it became clear that two entirely different mutations could result in the phenotypic behavior classified as type IIa. One, the originally described EF-2 structural gene mutant, produced cells in which the resistance factor behaved codominantly in hybridization experiments. The other was a mutation in the enzyme system responsible for the post-translational synthesis of diphthamide. Cell lines bearing this mutation were thus termed MOD mutants. This latter resistance factor acted recessively during hybridization experiments. It seems reasonable to suspect that the IIa-type cells isolated by Draper et al. (37) and Gupta and Simonovitch (62, 63) were actually MOD mutants. This suspicion could be easily confirmed by using the reconstitution system recently developed by Moehring et al. (139). This involves mixing cytoplasmic extracts of wild-type and mutant cells in the presence of an energy production source. In this system, "cryptic" EF-2 in extracts from MOD mutants can be post-

translationally converted to a fully active state by the wild-type modifying enzymes and thus can be ADP-ribosylated by diphtheria toxin. In contrast, extracts from true structural gene mutants cannot be converted to a substrate for ADP-ribosylation.

PSEUDOMONAS EXOTOXIN A

Pseudomonas exotoxin A, a 66,000-MW protein product of toxicogenic strains of *Pseudomonas aeruginosa*, has been implicated as a lethal factor in burn victim bacteremias and in infections of other immunologically compromised patients (178, 212). The toxin has been shown by Iglewski and Kabat (79) to possess an enzymatic activity apparently identical to that of diphtheria toxin; i.e., both molecules catalyze the NAD-dependent ADP-ribosylation of EF-2. Furthermore, both molecules are originally secreted in a proenzyme form. It was previously stated that intact diphtheria toxin must undergo proteolytic cleavage and reduction to attain full activity. Analogously, intact *Pseudomonas* toxin must be activated, either by limited proteolysis to release a 26,000-MW active fragment (24) or by reduction in the presence of urea or other chaotropic agents (108, 219). The latter treatment enhances ADP-ribosylating activity about 30-fold and is believed to expose the buried fragment A active site. Despite their enzymological relationship, *Pseudomonas* exotoxin and diphtheria toxin have no discernible immunological cross-activity and display very different cell line specificities (129) and markedly different patterns of inhibition by various drugs and chemicals (130). Direct competition studies have demonstrated that the two toxins bind to different cell surface receptors, and further evidence suggests that their internalization pathways are dissimilar.

A number of difficulties have arisen in the study of *Pseudomonas* exotoxin interactions with cultured cells, most prominently the lack of a sensitive cell line equivalent to the Vero line used in much of the diphtheria toxin work. The most *Pseudomonas* exotoxin-sensitive cell lines identified to data are those of mouse or rat cell origin (129): L-929 and 3T3 cells, for example, have tissue culture 50% lethal dose values of 0.10 and 0.15 ng/ml, respectively. In contrast, these lines are almost totally resistant to diphtheria toxin. However, their *Pseudomonas* exotoxin sensitivity is about 10-fold less than that of Vero cells to diphtheria toxin or on the same order as that of BHK-21 cells (diphtheria toxin tissue culture 50% lethal dose, 0.15 ng/ml). In BHK-21 cells, specific diphtheria toxin-cell association at 4°C could not be demonstrated, presumably due to a paucity of cell surface receptors (132). Similar results were obtained by Middlebrook et al. (134), using ^{125}I-labeled toxin and monolayer L-929 cells: specific binding was not statistically demonstrable at 4°C, though substantial specific uptake was shown at 37°C. At this temperature, toxin uptake increased rapidly for about 3 h and then remained at a plateau for at least 8 h, in marked contrast to the biphasic uptake pattern of diphtheria toxin in Vero cells. The plateau was shown to represent continued active uptake of extracellular *Pseudomonas* exotoxin even in the absence of cellular synthesis, which is shut down by toxin after approximately 60 min. Again, this is in marked contrast to the effects of diphtheria toxin in Vero cells, which cease active toxin uptake concomitantly with the toxin-induced cessation of protein synthesis. One possible explanation of these results may be that the maintenance of *Pseudomonas* exotoxin "receptors" on the membrane surface does not require protein synthesis, perhaps due to efficient receptor recycling, whereas continuous protein synthesis is essential for maintenance of diphtheria toxin receptor levels (131). Kinetic studies (128) indicated that the initial internalization of *Pseudomonas* exotoxin is very rapid. In LM cell fibroblasts, prebound toxin was rendered inaccessible to antitoxin neutralization within 2.5 min of transfer to 37°C. In comparable electron microscopic studies, Fitzgerald et al. (45) showed that <20% of the original cell-associated toxin remained at the cell surface after 10 min. The 60-min lag period that then occurs before the onset of protein synthesis inhibition presumably represents the translocation of toxin or active fragment to the cytoplasm, either by a process of conformational change and direct membrane penetration or by a receptor-mediated endocytic mechanism.

Whereas the biological relevance of an observed uptake process is always in doubt in systems where only one or a few molecules are needed to elicit toxicity, considerable electron microscopic and pharmacological evidence supports an endocytic internalization mechanism for *Pseudomonas* exotoxin A. Using the immunocytochemical technique of Sternberger (205), Leppla and Dorland (107) found that at 4°C in L-929 cells receptor-bound *Pseudomonas* exotoxin was localized over indented regions of the plasma membrane. Similar autoradiographic experiments with ^{125}I-labeled *Pseudomonas* exotoxin showed the silver grains localized over identifiable coated pits. The electron microscopic studies of Fitzgerald et al. (45), using a ferritin-conjugated antibody sandwich technique, demonstrated, in contrast, that at 4°C cell-associated *Pseudomonas* exotoxin was randomly distributed over 35 to 50% of the plasma membrane surface. At 37°C, the surface-bound toxin clustered into coated regions of the membrane and was rapidly internalized. In these studies, clustering was inhibited in cells treated with methylamine, ammonium chloride, or chloroquine, all of which concomitantly protected cells from toxin-induced inhibition of protein synthesis. Such results imply that, like alpha-2 macroglobulin and EGF, *Pseudomonas* exotoxin binds to randomly distributed surface receptors which are subsequently clustered into coated pits by a transglutaminase-mediated cross-linking reaction. The lysosomotropic amines, shown by Maxfield et al. (124) to inhibit both the clustering of receptor-bound alpha-2 macroglobulin and EGF and the activity of solubilized transglutaminase, may protect cells from *Pseudomonas* exotoxin by preventing its migration into coated pits and subsequent entry into the productive uptake pathway.

Several researchers have used a pharmacological approach in attempts to establish the biological relevance of the observed endocytic pathway. In early experiments, Middlebrook and Dorland (130) compared the effects of a large number of chemicals and drugs on *Pseudomonas* exotoxin- and diphtheria toxin-induced cytotoxicity. Results showed that none of the compounds examined protected HeLa or HEp-2 cells from *Pseudomonas* exotoxin, but in several cases cytotoxicity was potentiated by drug treatment. Conversely, many of the compounds protected cells from diphtheria toxin; no incidences of potentiation were observed. The metabolic inhibitors sodium fluoride and sodium arsenite both markedly potentiated *Pseudomonas* exotoxin-induced cytotoxicity, with maximum effect occurring at concentrations of 3 to 6 mM and 3×10^{-5} to 6×10^{-5} M, respectively. Similarly, the local anesthetics lidocaine and chlorpromazine potentiated cytotoxicity, though the related procaine butacaine and nonanol had no effect. Michael and Saelinger (128) similarly observed a sodium fluoride-induced potentiation of *Pseudomonas* exotoxin-mediated cytotoxicity in LM cells, but, under the conditions of

their assay, sodium arsenite was *Pseudomonas* exotoxin protective. The differential responses of *Pseudomonas* exotoxin- or diphtheria toxin-treated cells to drugs strongly suggests differential mechanisms of cellular intoxication. Since the majority of the tested drugs had no or little effect on the enzymatic activities of the toxins as determined by in vitro ADP-ribosylation assays, indications are that these differences lie at the binding or internalization step of the intoxication process or both.

In the Middlebrook and Dorland studies (130), ammonium chloride was not found to affect *Pseudomonas* exotoxin-induced cytotoxicity. However, later work by Michael and Saelinger (128) with LM mouse fibroblasts demonstrated that ammonium chloride does have a *Pseudomonas* exotoxin-protective effect. For expression of protective activity, however, the ammonium chloride treatment must be followed by addition of specific antitoxin. The requirement for serial ammonium chloride-antitoxin treatment implies that ammonium chloride blocks the internalization of toxin and is suggestive evidence for a transglutaminase-mediated process. A number of lysosomotropic amines have been shown to protect cells from both *Pseudomonas* and diphtheria toxins (34, 107, 146). Generally, higher concentrations of amines were required to protect cells from *Pseudomonas* exotoxin than from diphtheria toxin, again implying dissimilar entry or activation mechanisms. Whereas it may be that the *Pseudomonas* exotoxin-protective mechanism of these agents involves a block of internalization via transglutaminase inhibition, other possibilities arise. Primary alkylamines, for example, do not block ligand-receptor complex internalization in the EGF system (100) and have no measurable effects on the internalization of diphtheria toxin in Vero cells (34). This suggests that the protective effects of alkylamines derive from their action on lysosomes or endosomes.

Classically, RME involves a migration of pinched-off endocytic vesicles to the perinuclear region of the cytoplasm, where they fuse with lysosomes. This fusion process is relatively slow, usually occurring 15 to 30 min after vesicular internalization (54). Some experimental evidence suggests that *Pseudomonas* exotoxin, like diphtheria toxin, must undergo some lysosomal processing step to express biological activity. Like diphtheria toxin, cell-associated ^{125}I-labeled *Pseudomonas* exotoxin is rapidly degraded at 37°C as evidenced by the appearance of trichloroacetic acid-soluble fragments in the culture medium (Leppla et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, B10, p. 17). This implies that a substantial fraction of the internalized toxin does reach the lysosomes. The biological relevance of this process is uncertain. However, the pharmacological studies of Fitzgerald et al. (45) suggest that the productive uptake pathway may include a lysosomal step. In these experiments, researchers found that methlyamine and chloroquine were fully protective if added up to 10 min after toxin treatment. Partial protection was observed if the drugs were added at 30 min or later, with degree of protection decreasing as time elapsed after toxin addition. This was interpreted to indicate a dual role for the lysosomotropic agents: an early block of internalization through prevention of ligand-receptor complex clustering and a late block of toxin processing at the lysosomal level. Further support for a dual role of amines was obtained in electron microscopic studies by Morris et al. (146). Clustering of *Pseudomonas* exotoxin was observed within 30 s after warming toxin-prebound cells to 37°C. Primary amines such as ammonium chloride or methylamine blocked the clustering, whereas the tertiary amine chloroquine did not.

In the case of *Pseudomonas* exotoxin, a reduction of extracellular pH does not effect a bypass of the amine-mediated protection (R. K. Draper, unpublished data), suggesting that a pH-mediated conformational change does not direct the transmembrane passage of *Pseudomonas* exotoxin. However, the virus and diphtheria toxin-cross-resistant CHO-K1 mutants discussed by Merion et al. (127) (discussed above) were simultaneously resistant to *Pseudomonas* exotoxin A. Since these cells were shown to possess a defective endosomal acidification system, it is a possibility that *Pseudomonas* toxin also enters the cytoplasm from an acidified endosome.

The *Pseudomonas* exotoxin results, in general, strongly suggest a receptor-mediated endocytic uptake mechanism which, unlike that of diphtheria toxin, proceeds through the medium of coated pits and vesicles. Once in the endocytic vesicle, however, the events that occur during the 60-min lag period preceding inhibition of protein synthesis are vague. Some pharmacological studies imply a requirement for lysosomal exotoxin interaction, though it is equally possible that active *Pseudomonas* exotoxin escapes from the endocytic vesicle itself or is released during the vesicle-lysosome fusion process. On the other hand, ultrastructural evidence implicates the Golgi region as possibly playing a role in the release process (146). Again, the data do not rule out the possibility of a minor undetected productive pathway involving, for example, a small number of toxin molecules directly penetrating the plasma membrane.

Iglewski and Kabat (79) originally reported that *Pseudomonas* exotoxin inhibited in vitro protein synthesis, that this inhibition required the presence of NAD, and that radiolabeled adenine from NAD was covalently attached to a protein having the same molecular weight as EF-2. This and other evidence led Iglewski and Kabat to suggest that *Pseudomonas* exotoxin acts intracellularly in the same manner as diphtheria toxin fragment A. Their proposal was strongly supported by subsequent experiments. Most convincing was a simple, but compelling, experiment in which the ADP-ribosylation catalyzed by either diphtheria or *Pseudomonas* toxin was reversed by the other toxin (80). It had previously been shown that the ADP-ribosylation catalyzed by diphtheria toxin was reversed by removal of NAD, followed by addition of nicotinamide and toxin and acidification (see reaction 1). Taking advantage of this information, Iglewski et al. (80) not only showed that *Pseudomonas* exotoxin could reverse the ADP-ribosylation catalyzed by diphtheria toxin (and vice versa), but also that the main small-molecular-weight product formed was NAD. These ingenious experiments provide extremely strong evidence that *Pseudomonas* and diphtheria toxins ADP-ribosylate the diphthamide residue in EF-2 in a stereochemically identical fashion.

The structure-activity relationships of *Pseudomonas* exotoxin are less well defined than those of diphtheria toxin. Evidence for an enzymatically active (ADP-ribosylating) fragment of *Pseudomonas* exotoxin has been obtained. This fragment was found in the later stages of *P. aeruginosa* cultures (24) and upon purification was found to have a 26,000 MW. This fragment had several properties in common with diphtheria toxin fragment A, including pH optimum, ionic strength dependence, kinetic constants, and nucleotide specificities. Clearly, however, the fragments were not identical; antibody to diphtheria toxin fragment A did not neutralize the *Pseudomonas* endotoxin enzymatic fragment, thermal stabilities were different, and amino acid compositions were dissimilar.

An alternative pathway to "activation" of the latent enzymatic activity of *Pseudomonas* exotoxin has been identified. When the toxin was treated in vitro with a combination of a denaturant and a reductant, the enzymatic activity increased 50- to 100-fold (108, 220). Treatment of the toxin with either denaturant or reductant alone produced only a slight increase in enzymatic activity. Examination of denaturant/reductant-treated toxin by sodium dodecyl sulfate-polyacrylamide gel electrophoresis did not reveal the generation of any new fragments. Furthermore, all ADP-ribosylating activity migrated with an apparent molecular weight corresponding to intact toxin. Since neither activated intact *Pseudomonas* exotoxin nor the 26,000-MW fragment have been isolated from intoxicated cells or tissues, it remains unclear which of the two is the active species in vivo.

PSEUDOMONAS EXOENZYME S

Some strains of *P. aeruginosa* produce another enzyme which appears to ADP-ribosylate eucaryotic proteins (81). This enzyme, termed exoenzyme S, is distinct from *Pseudomonas* exotoxin as evidenced by a lack of neutralization using *Pseudomonas* exotoxin antisera, by differential thermal stabilities, by inactivation upon urea/reductant treatment, and by markedly different sodium dodecyl sulfate gel patterns of *Pseudomonas* exotoxin- versus exoenzyme S-produced ADP-ribosylated cellular products (81). Unfortunately, little more is known about the enzymology of exoenzyme S. Although there was an initial hint that one target cellular protein was EF-1 (81), this possibility has not been confirmed with a purified preparation. The relationship of the enzyme's activity to pathogenesis is unclear, since there have been no published reports that exoenzyme S is toxic to either cells or animals. The enzyme was apparently produced by certain strains of *P. aeruginosa* in an animal model of infection (9). In another study, it was found that approximately 10% of clinical isolates produced exoenzyme S-like activity and an additional 30% produced both exoenzyme S and *Pseudomonas* exotoxin A activities (203). However, since this is not an overwhelming correlation between morbidity and exoenzyme S production, further work is required before we know whether this enzyme is an important pathogenic element or merely an interesting protein.

SHIGELLA TOXIN

The *Shigella* organisms induce a severe diarrheic and dysenteric syndrome (shigellosis) which is mediated by a protein exotoxin (95). Cytotoxic, neurotoxic, and enterotoxic activities have all been demonstrated in *Shigella* culture media or bacterial lysates, and all of these activities are apparently due to the same protein toxin (41). A number of workers have studied the effects of partially purified Shiga toxin in cultured cell systems and have shown that in some cases it is highly cytotoxic, most notably to HeLa cells (67, 97). The primary biological activity appears to be inhibition of protein synthesis (17, 213), brought about by inactivation of the 60S subunit of the eucaryotic ribosome (179). Using HeLa cultures, Keusch et al. (97) found that an overnight incubation with as little as 0.5 ng/ml resulted in the killing of 50% of the cell population, whereas Olsnes and Eiklid (164), using a HeLa S_3 line, found 0.1 ng/ml was required to inhibit protein synthesis to a similar degree. In contrast, a number of cell lines are highly resistant to Shiga toxin. Hale and Formal (67) demonstrated that Henle 407 cells, a line derived from human embryonic intestine, are insensitive to Shiga toxin; Olsnes and Eiklid (164) showed that CHO, L, BHK, and human melanoma cells were unresponsive to toxin-induced inhibition of protein synthesis. One possible mechanism for such resistance is a lack of cell surface receptors for the toxin. Henle 407 cells, though resistant to exogenous cytotoxin, are sensitive to intracellular toxin secreted in situ by invasive strains of *Shigella dysenteriae* 1. In vivo, microcolony growth and localized toxin production within invaded cells of the colonic epithelium may lead to extensive cellular injury, expressed clinically as colitis and dysentery. Shiga toxin-specific receptors have been demonstrated directly on HeLa and rat liver cell membranes and are thought to be glycoproteins, with a short-chain oligosaccharide moiety instrumental in binding specificity (96). Olsnes et al. (165) suggest that the high potency of Shiga toxin in HeLa S_3 cells is a function of the large number of cell surface receptors (about 10^6 per cell) and the high toxin-receptor association constant (10^{10} M). In general, with cell lines sensitive to exogenous cytotoxin, it appears that the biological activity is preceded by a surface-receptor binding step.

The structure of the native cytotoxin has only recently been elucidated. In 1975, McIver et al. (125) isolated a purely cytotoxic activity from bacterial lysates, using isoelectric focusing techniques. Further purification of the *Shigella* cytotoxin has been performed by Olsnes and Eiklid (164) in conjunction with biochemical characterization of the native molecule. Their results indicate that the cytotoxin has an MW of 65,000 and contains an enzymatically active heavy chain or A subunit (MW 30,800) and a B oligomer consisting of six to seven B chains (MW 5,000) (165). The A subunit contains a trypsin-sensitive region which, when cleaved, yields two fragments, A_1 and A_2, attached by a single disulfide bond. It appears that the Shiga cytotoxin, like diphtheria toxin and *Pseudomonas* exotoxin, is synthesized in an inactive proenzyme form and requires either proteolytic cleavage and reduction or denaturation to express full biological activity. The biological activity, inhibition of protein synthesis, is attributed to the A_1 fragment which, though active in cell-free protein synthesis systems, is not toxic to whole cells. One possibility, by analogy to diphtheria toxin, is that toxin-receptor binding is mediated by the B portion of the molecule, without which only minimal (fluid pinocytic) uptake occurs. Fragment B-receptor binding, however, has not as yet been demonstrated. Olsnes et al. (164) suggest that the failure of such experiments may be due to conformational changes in solution. Keusch (94) proposes a functionally tripartite cytotoxin molecule, containing an enzymatically active fragment (A), a sequence directing transmembrane passage (E), and a receptor-binding component (B). It is possible that the A_2 fragment, like the A_2 moiety of cholera toxin (see below), plays a role in binding or internalization, but as yet the relevant studies have not been performed.

A number of lines of evidence suggest that Shiga toxin enters sensitive cells by a process of RME. It has been shown that a receptor-binding step is a prerequisite for the expression of biological activity, and the extreme toxicity of Shiga toxin (50% lethal dose, 50 to 100 molecules per cell) (165) has been attributed to the concentrative effect of the receptor interaction. After the addition of Shiga toxin to a cell population, there is a lag period of up to 3 h preceding the shutdown of protein synthesis (67). A lag is also seen when sensitive cells are treated with diphtheria or *Pseudomonas* toxins or with the toxic lectin abrin or ricin. It presumably represents the time required for binding and subsequent translocation of toxin of its cytoplasmic site of action.

A number of pharmacological studies carried out in the laboratory of Keusch (94) indicate that translocation proceeds by RME and further suggest an involvement of lysosomes or endosomes. Initially, these studies demonstrated that a variety of functionally unrelated metabolic inhibitors, including NaF, iodoacetate, KCN, 2,4-dinitrophenol, antimycin A, oligomycin, puromycin, cycloheximide, and actinomycin D, all blocked the effects of Shiga toxin to some extent in HeLa cell toxicity assays. The results were interpreted to suggest a common effect on some active cellular process requiring both a metabolic energy source and continuing protein synthesis. A likely candidate was endocytosis. Since endocytosis involves the invagination of membrane segments to form vesicles and the subsequent movement of vesicles into the cytoplasm, various elements of the cytoskeleton are essential components of this uptake process. Keusch's experiments showed that both colchicine, a microtubule inhibitor, and cytochalasin B, which interferes with microfilament function, partially protected cells from the action of Shiga toxin. Although this is certainly a possibility, the data do not correlate with those obtained in other endocytic systems. The uptake of Semliki Forest virus, for example, shown to occur by absorptive endocytis in coated pits, is insensitive to colcemid, a microtubule inhibitor, and only slightly sensitive to cytochalasin B (115). Neither colchicine nor cytochalasin B had a detectable effect on the internalization of abrin or ricin by HeLa cells (186) or on the cytotoxicity of *Pseudomonas* exotoxin A (130). Similarly, colchicine had no effect on the cytotoxic activity of diphtheria toxin, but in the same system cytochalasin B showed a partial degree of protection (130). The endocytosis of LDL by cultured human fibroblasts was shown to be partially inhibited by colchicine (168), as was the degradation of LDL in lysosomes. Cytochalasin D, but not B, exerted a similar degradative block in the LDL system. Sandvig and Olsnes (186) showed that the degradation of ricin in HeLa cells was inhibited by colchicine. One possible explanation for these results is that disruption of cytoskeletal elements prevents the fusion of ligand-containing endocytic vesicles with lysosomes, thus precluding normal processing and degradation. A similar situation may obtain with Shiga toxin.

The suggestion that the lysosomes play a role in intoxication by *Shigella* toxin is reinforced by experiments with chloroquine, which is somewhat protective in cytotoxicity assays (94). As discussed above, chloroquine-induced inhibition appears to correlate to a receptor-mediated endocytic mode of uptake (54). Steroids, which exert, among other activities, a lysosomal membrane-stabilizing effect (29), also partially protect cells from the action of Shiga toxin. In both cases, however, the tested drugs have a number of different effects on cell function and no definitive conclusion can be drawn.

Results obtained with amines and other transglutaminase-inhibitory compounds were similarly suggestive. In these experiments, cells were exposed to Shiga toxin for 30 min in the presence of amines (ammonium chloride, methylamine, ethylamine, propylamine, or butylamine), dansylcadaverine, bacitracin, or putrescine. When specific antibody was added after the washout of toxin and experimental chemical, partial protection was observed, suggesting that the chemical compounds maintained toxin in a position accessible to exogenous antibody. In the absence of amines, antibody rescue did not occur if antibody was added as late as 10 min after toxin treatment. However, as discussed above with diphtheria toxin, these experiments do not differentiate between toxin held at the cell surface and that trapped in intracellular vesicles.

Whereas the data are in some cases indicative of a rapid receptor-mediated endocytic internalization of Shiga toxin, results are by no means conclusive. Furthermore, as in the toxin systems discussed above, we are left with the problem of how the molecule enters the cytoplasm from a membrane-limited endocytic vesicle. Keusch (94) proposed a signal sequence function for the toxin "E" moiety, analogous to the internal signal sequence described by Lingappa et al. for chicken albumin (111). Olsnes et al. (165) have demonstrated that the toxin is synthesized by the bacterium in an inactive zymogen form which requires proteolytic cleavage or denaturation to express enzymatic activity. The protective effects of chloroquine and the lysosome-stabilizing steroids may indicate that such an activation routinely takes place in the lysosomes. It is also conceivable that a lysosomal protease may expose or activate a putative E sequence.

Regarding the enzymatic activity of Shiga toxin, little can be said. That so few molecules are required to kill a target cell and the structure-activity (i.e., dichain) features of the toxin both support the hypothesis that Shiga toxin acts enzymatically. However, until demonstrated directly, the whole concept is obviously speculative.

CHOLERA TOXIN

A number of review articles over the past 10 years have dealt in depth with the mechanism of action of cholera toxin, from the classical work by Finkelstein (44) to more recent synopses by Gill (48), Moss and Vaughan (156), and Holmgren (76). The toxin is the causative agent of clinical cholera, the disease brought on after colonization of intestinal cells with toxin-secreting *Vibrio cholerae* bacteria. The end result of the infection is a massive diarrhea and dehydration, due to elevation of cellular cyclic AMP (cAMP) and disruption of normal ion fluxes in the intestinal epithelium.

The toxin itself is a large protein molecule, now known to be composed of three subunits: A_1 (MW 29,000), A_2 (MW 5500), and B (MW 11,500). Subunit A_1 has been shown to possess enzymatic activity and is well known to effect the toxin-specific biological response by ADP-ribosylating a component of the adenylate cyclase system, the GTP-regulatory protein (21, 52). Subunit A_2 is thought to function in the toxin internalization process (51), whereas subunit B binds specifically to a cholera toxin-specific cell surface receptor, in part consisting of ganglioside G_{M1} (148). Biochemical cross-linking (47) and electron microscopic studies have shown that there are five B subunits per toxin molecule, arranged in a ring around a central core which contains the enzyme A_1. A_2 connects A_1 to the B ring through a single disulfide bond, which must be severed for expression of maximal enzymatic activity. Almost all cultured cell lines are sensitive to cholera toxin and display a wide range of responses, mostly attributable to increases in intracellular cAMP levels. These include alterations in morphology and surface adherence characteristics, shifts in membrane protein profiles, phosphorylation of specific surface proteins, stimulation of steroidogenesis and certain hormone-mediated responses, and inhibition of mitogenesis. As in the cases of the toxins discussed above, the cholera toxin target is indisputably intracellular: adenylate cyclase, a multisubunit enzyme, is localized on the cytoplasmic surface of the plasma membrane. Thus, for the A_1 enzyme to interact with its substrate molecule, it must pass a membrane barrier. Indications are that membrane passage may take 15 to 60 min in intact cells, this being the length of the lag period

between toxin addition and intracellular adenylate cyclase stimulation. This lag period is absent in experiments with cell lysates and either "nicked" or "unnicked" toxin preparations, strongly suggesting that the activation or release or both of A_1 from holotoxin and its enzymatic reaction are virtually instantaneous. The limiting factor, therefore, must be cross-membrane transfer.

Subunit A_1, on its own, has little or no detectable effect on intact cells. There appears at this time little doubt that the effects of cholera toxin are mediated via cell surface receptors, with the B subunit responsible for the receptor binding. Initially a single B moiety binds to a single G_{M1} molecule. Than lateral diffusion in the plane of the membrane occurs until all five B subunits are associated with five corresponding G_{M1} gangliosides. Craig and Cuatrecases (27) have shown, using fluorescein-labeled toxin conjugates, that the cholera toxin-receptor complex is mobile in the plane of the lymphocyte membrane, redistributing in a temperature-dependent manner to form patches and caps. Patching and capping were blocked by specific antitoxin, which concomitantly blocked the toxin-induced stimulation of cyclase, suggesting that the observed redistribution is a necessary step in the intoxication process. Antitoxin added after patching and capping had taken place was unable to prevent the biological effect of cholera toxin.

The proposed biological significance of the toxin-receptor complex redistribution step may relate to the mobile receptor theory of hormone-adenylate cyclase interaction (6). One aspect of the theory, reviewed by Rodbell (181), argues that the binding of hormone to receptor forms an activated complex, which then, by means of lateral diffusion, collides with and acts upon the cyclase. An analogous situation may obtain with cholera toxin. The lag between toxin binding and cyclase activation is dependent upon both temperature and cell membrane viscosity and is extended in cells with reduced levels of G_{M1} gangliosides, presumably due to proloned periods of lateral diffusion (49). The prolonged lag in G_{M1}-poor cells is reduced by addition of exogenous ganglioside.

Craig and Cuatrecasas (27) argue that, since the hydrophobic tail of the G_{M1} ganglioside is too short (only 3 nm) to extend across the plasma membrane and interact with the submembrane cytoskeleton, capping may proceed through the association of receptor with an integral membrane protein. The possible importance of a membrane protein component was further supported by the work of Hagmann and Fishman (66), who demonstrated that the pretreatment of toxin-sensitive cells with inhibitors of protein synthesis blocked both the appearance of subunit A_1 in the cytoplasm and toxin degradation. The effect could not be attributed to either a reduction in toxin-receptor binding or a depletion of intracellular adenylate cyclase. The results thus imply that some membrane protein is involved in the translocation of receptor-bound toxin.

Antibody neutralization studies (49) show that the toxin very rapidly undergoes "eclipse"; that is, within 10 to 60 s of surface binding, the toxin is no longer susceptible to antibody neutralization, though electron microscopic studies show that substantial amounts of toxin remain exposed at the plasma membrane for up to 24 h. It appears at this stage that either all or part of the receptor-bound toxin becomes embedded in the membrane such that it is no longer available for antibody binding or undergoes some conformational change such that it loses its antigenic recognizability or both.

Considerable experimental evidence exists for an alteration in the conformation of cholera toxin subsequent to surface receptor binding. Early work by Gill (47) suggested that the five-point binding of toxin to gangliosides holds the toxin flat against the membrane surface in such a manner that the internal hydrophobic associations of the B subunits are replaced by interactions with integral membrane components. The B ring thus unfolds and becomes embedded in the membrane, forming a channel through which the hydrophilic A subunit can enter the cytoplasm. This hypothesis, somewhat similar to the direct membrane traversal mechanism proposed for diphtheria toxin A fragment, finds some support in the direct demonstration that cholera toxin indeed forms ion-conducting channels in G_{M1}-containing artificial lipid membranes (152, 215). Though no estimations were made of the channel size, presumably the A_1 subunit would best traverse a channel in elongated, unfolded form. Again, like diphtheria fragment A, cholera A_1 has been shown to readily renature to full biological activity after boiling in sodium dodecyl sulfate (47).

Mullin et al. (157) argue that the cholera toxin-G_{M1} interaction is in many ways equivalent to that of thyrotropin with its receptor. Thyrotropin, which possesses sequence homologies to cholera toxin in both the alpha and beta subunits, similarly binds by virtue of its beta moiety to a cell surface ganglioside. Binding elicits a conformational change in thyrotropin which, after the extrusion of water molecules, results in close contact of the hormone with the membrane lipid bilayer. This in turn perturbs the plasma membrane, altering both the surface exposure of various membrane components and the character of the transmembrane ion fluxes. These changes allow the active alpha subunit of the hormone to penetrate the membrane and interact with its cytoplasmic target, adenylate cyclase. Fluorescence studies of the thyrotropin or cholera toxin interaction with gangliosides indicate that the initial binding is inhibited by salts, implying a preliminary electrostatic event. The formed ligand-receptor complex, however, is impervious to salt, suggesting a subsequent hydrophobic event. Mullin et al. (157) proposed that the ganglioside may act as an "emulsifying agent," overcoming the considerable energy barrier presented to the entry of a large polar ligand into the polar lipid bilayer.

As mentioned above, subunit A_1 alone has little effect on intact cells, and reconstitution experiments have demonstrated that both the ganglioside binding B subunits and fragment A_2 are required for expression of biological activity. The role of A_2 in the toxin uptake process is unknown. It was originally believed that A_2 functioned simply to connect A_1 to the multiunit B ring; however, more recent evidence suggests an A_2 requirement for translocation. Gill et al. (51) proposed that A_2 may act like a secretory protein signal sequence in directing the transmembrane passage of A_1. The case for a receptor-mediated direct membrane translocation process as the biologically relevant uptake mechanism for cholera toxin seems fairly strong. A number of studies (22, 58, 85, 86) have shown that cholera toxin or toxin-receptor complex undergoes adsorptive endocytosis into the GERL of neuronal or neuroblastoma cells. Toxin taken up in this manner does not elicit a biological effect (22), suggesting that the observed endocytosis is not the biologically significant uptake pathway. One possibility is that this process reflects the recycling of plasma membrane components. Gonatas et al. (58), in experiments with cholera toxin-horseradish peroxidase conjugates, found that the conjugates were detectable on the neuroblastoma cell surface as long as 24 h after addition, implying either a very slow rate of surface clearance or an efficient and continuous recycling process.

Pharmacological studies further suggest that the endocytic process described above is not biologically productive. Though electron micrographs demonstrated that the endocytosis of cholera toxin did not appear to involve coated pits, Gill et al. (51) tested the effects of dansylcadaverine on the uptake of cholera toxin by CHO cells. Pretreatment with dansylcadaverine appeared to partially reduce the morphological response (elongation) of the cells to toxin. However, the cellular response to dibutyryl cAMP was similarly reduced by dansylcadaverine, suggesting that the observed effect was at the level of intracellular cAMP rather than toxin entry. On the other hand, experiments by Hagmann and Fishmann (66) showed that dansylcadaverine blocked both the biological activity and the degradation of cholera toxin, implying a legitimate role of transglutaminase in toxin uptake. In view of the previous discussion, it seems possible that dansylcadaverine has differential effects on the two pathways of cholera toxin intake: a block of biological activity at the cAMP level in the productive pathway and a block of degradation in the nonproductive (endocytic?) pathway.

Additional evidence that the biologically relevant uptake of cholera toxin does not proceed by adsorptive endocytosis into lysosomes derives from experiments with ammonium chloride and chloroquine (51). Both compounds (see above) have been shown to affect the lysosomes, and their concomitant abilities to protect cells is taken to indicate a lysosomal role in toxin activation or transmembrane passage. Neither compound inhibited the activity of cholera toxin in CHO cells.

In general, intact cell studies indicate that a substantial fraction of surface-bound cholera toxin at saturation is nonproductive. Relatively few molecules need interact with target cells to elicit maximal biological effect. Donta (33) estimated that 50 molecules of toxin per cell were sufficient to induce morphological and steroidogenic changes in Y-1 adrenal cells. Holmgren et al. (77) found maximal inhibition of mitogenesis in thymocytes at 10 molecules per cell. In broken-cell preparations, the cyclase is activated by A_1 concentrations as low as 10^{-11} M, though intact cells may possess vast numbers of surface receptor sites. Fibroblasts, for example, have 5×10^5 to 1×10^6 receptors per cell for cholera toxin. The discrepancy between cell surface binding and concentration required to elicit biological effect may reflect inefficiency of uptake; that is, if productive uptake is a very rare event, extensive surface receptor binding may be a statistical necessity. Alternatively, there may be functional and nonfunctional forms of toxin receptor, the former of which alone is able to effect the translocation and cytoplasmic release of A_1.

Regarding the enzymatic activity of cholera toxin, once again the story is ADP-ribosylation. One of the first clues was provided in the report by Gill that NAD is a necessary cofactor for cholera toxin-catalyzed adenylate cyclase activation (46). This observation was followed by work showing tht cholera toxin has an NAD glycohydrolase activity (150, 151, 155). The reaction occurred in water but was promoted by guanidine, arginine, or, even more efficiently, arginine methyl ester. The NADase activity was dependent on the type of buffer used and was markedly enhanced in dithiothreitol. One curious feature of the reaction was the inhibitory effect of gangliosides (151). G_{M1}, G_{M2}, G_{M3}, and G_{D1a} all inhibited the NAD hydrolysis reaction catalyzed by either intact cholera toxin or the A promoter. Fluorescence studies indicated that only G_{M1} bound to isolated B promoters, whereas all four gangliosides bound to promoter A. It seems probable, therefore, that a ganglioside binding site exists on the A promoter and its occupancy results in an inhibition of glycohydrolase activity. Whether the inhibition is steric or allosteric is not clear. Also uncertain is the biological significance of ganglioside association with A promoter.

The demonstration that cholera toxin ADP-ribosylated a component of the adenylate cyclase system came simultaneously from two laboratories (21, 52). Using radiolabeled NAD as a probe, both groups observed toxin-catalyzed incorporation of radioactivity into a 42,000-MW membrane component. This component was identified as the GTP-binding regulatory subunit of the adenylate cyclase system. Subsequent studies have confirmed and expanded upon these original reports to yield the following model. The catalytic component of adenylate cyclase is membrane bound and located on the cytoplasmic side. The enzyme can exist in two forms, active and inactive. There is a regulatory component (referred to variously as the G protein, the G/F protein, or the N_s protein) of the cyclase which has a binding site for GTP. The G-GTP complex interacts with the catalytic component to form a ternary system active in the synthesis of cAMP from ATP. Hydrolysis of GTP to GDP converts the G protein into a form which no longer activates the catalytic unit.) To reactivate the system enzymatically, G-GDP disassociation occurs followed by rebinding of GTP to the original site.

Since it is the hydrolysis of GTP which initiates the process of cyclase deactivation, it follows that inhibition of GTPase activity would lead to a persistent activation of the cyclase catalytic component. One predictable means of effecting persistent activation would be the substitution of a hydrolysis-resistant analog such as the beta-gamma-linked imide derivative [Gpp(NH)p] for GTP. Indeed, when Gpp(NH)p was added, the activation of cyclase was prompt and essentially irreversible. Functionally, ADP-ribosylation by cholera toxin seems to convert the regulatory G protein to a state incapable of GTP hydrolysis. For example, the ability of GTP to activate adenylate cyclase is markedly enhanced by pretreatment with cholera toxin. In contrast, cholera toxin pretreatment has very little effect on the activation stimulated by Gpp(NH)p (112).

Several lines of evidence indicate that the substrate for cholera toxin-catalyzed ADP-ribosylation is the G protein. Incubation of membranes from a variety of sources with cholera toxin and [^{32}P]NAD results in labeling of a 42,000-dalton protein; with some cells a second protein (52,000 daltons) is also labeled. These labeled proteins copurify with G-reconstituting activity by several molecular sizing techniques (88, 161). Particularly compelling was the observation that membranes from wild-type S-49 cells, which have G-protein activity, were labeled by toxin and [^{32}P]NAD, whereas membranes from subline S-49 lacking G-protein activity were not (84).

The ADP-ribosylation reaction depends upon the presence of several components. Cholera toxin, NAD, and a membrane source of G protein are required, as well as GTP or a GTP analog and magnesium. In addition, a macromolecule in the cytosol markedly enhances ADP-ribosylation. The exact identity of this component is not clear, but it behaves like a 20,000-dalton protein. Enomoto and Gill (42) and Malbon and Gill (113) tested several proteins (including calmodulin) for their ability to substitute in the reaction with negative results.

The acceptor specificity for cholera toxin-catalyzed ADP-ribosylation is not as stringent as it is with diphtheria toxin. Many guanine nucleotide-binding regulatory proteins are

recognized and ADP-ribosylated by cholera toxin (26). For example, transducin, the signal-carrying regulatory protein in retinal outer rod segments, was ADP-ribosylated (1). The reaction was enhanced by Gpp(NH)p and inhibited by GTP or GDP. There was a good correlation between the level of labeling and the loss of transducin GTPase activity.

Cytoskeletal proteins also appear to be targets for ADP-ribosylation by cholera toxin. Both tubulin and intermediate filament proteins were radiolabeled by cholera toxin and [^{32}P]NAD (68, 87). It appeared that labeling required a GTP cofactor. However, labeling only occurred in lysed cell preparations. No evidence was obtained for toxin-catalyzed ADP-ribosylation of cytoskeletal proteins in intact cells.

E. COLI ENTEROTOXINS

There is now much evidence that most cases of so-called traveler's diarrhea are caused by infection with certain toxigenic strains of *E. coli*. From a pathogenic standpoint, the symptoms appear to be caused by one or the other of two distinct enterotoxin types. The first, referred to as the heat-labile enterotoxin (LT), is very similar to cholera toxin in both its structure and mode of action. As discussed in the related review (40), LT apparently binds to the same G_{M1}-containing receptor that is recognized by cholera toxin. Although little direct information is available on the internalization of LT by target cells, presumably the mechanism is essentially the same as that of cholera toxin. Much more reliably, we know that the enzymatic activity of LT is remarkably like that of cholera toxin. When added to cell membranes, LT activated the adenylate cyclase in a time- and concentration-dependent manner (50). Activation required NAD, ATP, and a soluble component from the cells. The reaction was inhibited by antibody against cholera toxin. Further studies demonstrated that LT catalyzes the hydrolysis of NAD to ADP-ribose and nicotinamide and that hydrolysis is stimulated by denaturant treatment of the toxin or addition or arginine methyl ester, arginine, or guanidine (153). Finally, Gill and Richardson showed that LT ADP-ribosylates the 42,000-dalton, GTP-binding regulatory protein that is ADP-ribosylated by cholera toxin (53).

The second enterotoxin produced by *E. coli* is referred to as the heat-stable toxin. This toxin is quite small (approximately 2,000 MW) (3) and apparently elicits its enterotoxic response by stimulating guanylate cyclase. Whether or not heat-stable toxin enters cells to carry out its action is not clear. Furthermore, no enzymatic activity has been reported for heat-stable toxin. Considering the toxin's size, it is much more likely to act stoichiometrically than catalytically, but this remains to be determined.

PERTUSSIS TOXIN

Pertussis toxin is isolated and purified from the culture medium of *Bordetella pertussis*. The toxin elicits a number of responses in a variety of cell types (69, 89, 90, 158). These responses apparently stem from toxin-induced effects on the adenylate cyclase system which ultimately lead to increased concentrations of cAMP. The pentapeptide structure of pertussis toxin (discussed in detail in the related review [40]) is complex, but functionally conforms to the A-B, enzymatic binding subunit model (210). The 28,000-MW S promoter (generically, the A portion) is active in ADP-ribosylation. In broken-cell preparations, it is as biochemically effective as intact toxin (210). The B oligomer (four peptides) blocks the biological action of the whole toxin (211), apparently by competing for receptor occupancy. Though lacking intrinsic enzymatic activity, the B oligomer alone will elicit insulin-like or mitogenic responses from cells. These responses presumably result from B-oligomer-induced cross-linking or aggregation of cell surface (receptor?) proteins. As such, they seem analogous to cellular responses to concanavalin A or antireceptor antibodies (211).

Essentially nothing is known about the nature of the receptor for pertussis toxin. Little more is known about the mechanism by which pertussis toxin enters target cells. Tamura et al. (211) found that the A promoter added alone to cells did not evoke a response. However, if cells were pretreated with the B oligomer, washed, and then exposed to the A promoter, a toxin-like response was observed. Apparently, after binding to the cells, the B oligomer remains on the surface in a state recognized by the A promoter. This experiment demonstrates the important role of the oligomer in delivering the enzymatically active (see below) A promoter to its supposed intracellular site of action. In comparison to many other bacterial toxins, however, these results are unusual. In most cases, it is not possible to successively add the binding and enzymatic components to cells and obtain the toxic response.

Although pertussis toxin affects the adenylate cyclase system, several studies have shown that its mode of action is different from that of cholera toxin. Incubation of C6 glioma cell membranes with pertussis toxin, NAD, and ATP converts the adenylate cyclase to a state in which the activating effects of beta-adrenergic agonists or GTP or both were markedly enhanced. The degree of enhancement correlated with the level and rate of toxin-catalyzed ADP-ribosylation of a 41,000-MW membrane protein (91, 92). This protein was clearly distinct from the 45,000-MW G protein ADP-ribosylated by cholera toxin, as shown by direct comparative experiments (11, 91). Equilibrium dialysis and photoaffinity labeling experiments demonstrated that this 41,000-MW protein has a specific binding site for guanine nucleotides (11). Thus, there appear to be two regulatory GTP binding proteins associated with the adenylate cyclase enzyme system. One, the G protein, is susceptible to ADP-ribosylation by cholera or *E. coli* enterotoxin. The other, termed the G_i or N_i protein, can be ADP-ribosylated by pertussis toxin.

Similar studies were performed in other cell lines such as 3T3 fibroblasts (158), adipocytes (159), or the neuroblastoma × glioma hybrid cell line NG108-15 (19, 101). The use of the NG108-15 line was of particular interest, since these cells have alpha-adrenergic, cholinergic, muscarinic, and opiate receptors, all of which inhibit adenylate cyclase by a GTP-dependent mechanism. Treatment of these cells by pertussis toxin led to an uncoupling of the cyclase-inhibitory response in all four receptor systems (19, 101).

Pertussis toxin catalyzes the hydrolysis of NAD to ADP-ribose and nicotinamide (154). Expression of this activity requires treatment with a thiol reagent. The K_m for the reaction was approximately 25 μM as compared to 4 to 8 mM for cholera or *E. coli* enterotoxin. However, pertussis toxin did not transfer ADP-ribose to small-molecular-weight guanidine compounds such as arginine.

ANTHRAX TOXIN

There is good evidence for the involvement of a toxin in the pathogenesis of anthrax infections (109). Toxic activities can be demonstrated in the broth of *Bacillus anthracis* cultures. Anthrax toxin can be resolved into three components termed protective antigen, edema factor, and lethal factor (109). Each of the three is a protein, with an MW of

approximately 80,000 (105). Alone, none of the components elicits observed biological responses. However, when combined with protective antigen and injected intradermally, edema factor produces edema. Likewise, when combined with protective antigen, lethal factor is lethal to animals; rats are the most sensitive. These and other experiments suggested that anthrax toxin has the now familiar A-B, enzymatic-binding structure, with protective antigen acting as the B fragment and either lethal factor or edema factor acting as the active A fragment. This speculation was partially supported in recent work by Leppla (105). In that study, it was shown that the combination of edema factor and protective antigen elevated cellular cAMP to extraordinarily high levels. Neither component alone brought about this response, and addition of excess lethal factor blocked the cAMP increase, presumably by competing with edema factor for interaction with protective antigen. Interestingly, rather than stimulating the target cell adenylate cyclase, it appeared that edema factor itself was forming the cAMP; i.e., edema factor is an adenylate cyclase. The adenylate cyclase activity of edema factor was stringently dependent on a soluble, heat-stable component of the cells. The cellular cofactor is probably calmodulin (106), as was shown for an adenylate cyclase from *Bordetella pertussis* (74, 226). Thus far, the (enzymatic?) activity of lethal factor leading to death is unknown.

TETANUS TOXIN

Tetanus and botulinum toxins are among the most toxic substances known to humans. Their extremely potent toxicities strongly suggest a very specific and highly efficient mode of action. Unfortunately, at present, very little is known about the cellular or molecular events leading to toxicity. Clinically, the time course and symptoms of poisoning by tetanus and botulinum toxins are quite different. However, there are several structural aspects of the toxins which, bolstered by some recent toxicity data, indicate more similarities than differences.

As discussed in the related review (40), tetanus toxin is a dichain protein composed of an approximately 100,000-MW heavy chain and an approximately 50,000-MW light chain. Like diphtheria toxin, tetanus toxin is synthesized by the bacterium as a single chain. However, due to the action of bacterial proteases, the toxin is routinely recovered from culture supernatants in a nicked form. The two resulting chains are held together by a disulfide bond and noncovalent interactions. Separated, the heavy and light chains are nontoxic (118, 119, 121), thus following the usual structure-activity pattern of A-B dichain toxins. There is a second protease-sensitive site in the heavy chain which, when cleaved, splits the chain approximately in half. In the absence of reducing agents, two new chains can be isolated. One, consisting of the entire light chain linked to a 50,000-dalton amino-terminal polypeptide of the heavy chain, has been (unfortunately) termed fragment B. The remaining 50,000-MW portion of the heavy chain was called fragment C. Unlike the separated heavy and light chains of tetanus, isolated fragment B was toxic to animals. However, the pattern of death due to fragment B was particularly interesting. Upon intramuscular injection, the animals developed a flaccid paralysis 48 to 72 h after challenge, much like that seen in botulinum toxin poisoning (72). In contrast, the flaccid paralysis induced by botulinum toxin can occur as early as 4 h after toxin administration. More recently, it was shown that intravenous injection of intact tetanus toxin produced a very rapid (30-min) flaccid paralysis indistinguishable from the symptoms of botulinum toxin (117). Of special note was the observation that theophylline delayed the time to death due to intravenously injected tetanus toxin. Theophylline was earlier reported to partially protect mice from botulinum toxin-induced death (78). Together, these observations suggest that tetanus and botulinum toxins might have similar (enzymatic?) mechanisms of action when delivered to (or into) the same neuronal cells.

Tetanus toxin causes spastic paralysis by blocking the release of neurotransmitters from inhibitory synapses in spinal cord motoneurons. Despite the structural analogy of tetanus toxin to other A-B dichain bacterial toxins, there is no evidence that either chain is an enzyme. Neither is there direct evidence that tetanus toxin must enter the presynaptic neuron to express biological activity. However, since several indirect experiments have shown that tetanus toxin conforms to the basic A-B model, we presently believe that this is the best working hypothesis. Certainly, it is clear that part of tetanus toxin's mechanism of action involves binding to peripheral nerve terminals followed by internalization and retrograde axonal transport (195, 207). Next, tetanus toxin is released from the postsynaptic dendrites and taken up by the presynaptic nerve terminals (196, 197). Thus, the process by which tetanus toxin reaches its ultimate site of action involves binding, internalization, and transport within a cell. This transport seems to occur within smooth vesicles, cisternae, and tubules. Upon reaching the cell body the toxin appears to localize within lysosomes. This sequence of events was visualized in electron microscopic studies carried out by Schwab, Thoenen and co-workers (195–197, 206, 207).

As mentioned in the related review (40), there are several studies which suggest that tetanus toxin might bind to and utilize the receptor-uptake system normally used by thyroid-stimulating hormone (65, 104). Tetanus toxin binds to membranes from thyroid cells with characteristics quite similar to those for thyrotropin binding. Significantly, tetanus toxin did not bind to membranes from thyroid tumor cells deficient in thyrotropin receptors (65). Whereas these experiments are interesting, they do not link binding (and internalization?) of toxin with the biologically relevant response, i.e., inhibition of neurotransmitter release.

Attempts have been made to develop cell culture models for studying the mechanism of action of tetanus toxin. Pearce et al. found that the toxin inhibited the release of gamma-aminobutyric acid from primary cerebellar cells (175). Bigalke et al. (8) were able to demonstrate tetanus toxin-induced inhibition of acetylcholine release by primary nerve cell cultures. Using a similar cell preparation, Yavin et al. (230) studied cellular uptake of labeled toxin. As judged by competition of unlabeled toxin, nonspecific binding/uptake was high, approximately 40%. Furthermore, approximately 70% of the cell associated toxin could not be disassociated by conditions which completely reversed toxin binding to neural membranes. These results are difficult to understand, in that the toxin-cell binding was performed at 0 to 4°C, a temperature well below that normally required for the internalization of bacterial toxins.

There would be many advantages to having available an established cell line suitable for tetanus toxin mechanistic studies. Wendon and Gill (221) demonstrated that tetanus toxin inhibited the release of acetylcholine from the neuroblastoma-glioma hybrid cell line NG108-15. Attempts to relate this response with ADP-ribosylation or phosphorylation met with negative results. Nevertheless, these cells provide a promising system for biochemical studies with the toxin.

Boquet and Duflot (13) examined the ability of tetanus toxin or tetanus toxin-derived fragments to interact with asolectin vesicles and to bind Triton X-100. Tetanus toxin fragment B was the most effective component tested at forming channels in the vesicles, but only at a pH below 5.0. Intact tetanus toxin apparently did form channels, but much less effectively than fragment B. Neither light chain nor fragment C had any detectable channel-forming capacity. Boquet and Duflot also noted a very large, low pH-induced increase in detergent binding to tetanus toxin, but not to fragment C or light chain. These results are consistent with the notion that tetanus toxin requires lowered pH to insert into or cross membranes as is apparently the case with diphtheria toxin (see above).

BOTULINUM TOXIN

Botulinum neurotoxin is a large protein (150,000 MW) which acts on peripheral nerves to block the release of acetylcholine. Its basic structure resembles that of tetanus toxin (heavy chain, approximately 100,000 daltons; light chain, approximately 50,000 daltons [200, 209]. Similarly, it has a propensity to bind gangliosides. A number of studies have examined the binding stage of botulinum toxin's action (discussed in the related review [40]). However, there is no direct information on how, or indeed if, botulinum toxin enters the neuron. Quite recently, one intriguing report (201) showed that chloroquine or hydrochloroquine delayed the time of onset of botulinum toxin-induced neuromuscular blockade. These drugs had to be added before or simultaneously with the toxin to be effective. In view of the well-established effect of chloroquine on toxins which enter target cells by RME, these results suggest that botulinum toxin enters the cell to inhibit neurotransmitter release.

There is no evidence as yet that botulinum toxin has any enzymatic activity. However, since it is exceedingly potent and bears structural and kinetic analogies to other bacterial toxins, an enzymatic mechanism of action is highly probable.

CONCLUDING REMARKS

Striking similarities are exhibited by bacterial toxins in three general areas: (i) an A-B, enzymatic-bindng structure, (ii) an entry into target cells via an RME route, and (iii) an ADP-ribosylating activity at the biochemical level. Another possible similarity is a low pH-induced conformational change which exposes regions of the toxin capable of inserting into or through membranes.

One subject not covered here is the growing field of work on toxin hybrids. In this approach, the enzymatic chain of a toxin is linked to a molecule which should direct the conjugate or "hybrid" to specific cell types. Most frequently, the targeting half of a hybrid has been a polypeptide hormone, such as insulin, an antibody against a specific surface antigen on the target cell. The use of hybrids holds tremendous potential. With the advent of modern genetics, there is reason to believe that hybrids could be constructed genetically. To use the example above, the genes for insulin and a toxin enzymatic chain could be juxtapositioned on a plasmid so as to code for the entire hybrid. Furthermore, the binding fragments of toxin, to date unexplored, may be highly useful in this regard. For example, the specificity shown by toxin B chains can be used to target drugs to certain tissues. The B chains also could be used, ironically, enough, to target antitoxin agents to holotoxin-sensitive cells.

Modern toxin research has provided important information on both the pathogenesis of various infections and the nature of basic cellular or metabolic processes. Consider, for example, how much less we would know about the adenylcyclase system without cholera and pertussis toxins as probes. In this vein, we hope that information will soon be forthcoming on the molecular mechanisms of action of botulinum and tetanus toxins. It is most likely that determining how these toxins work biochemically will lead to important insights into the process of neurotransmitter release. Furthermore, with the availability of highly purified heavy chains or nontoxic CRMs, one could consider the exciting possibility of targeting therapeutic agents to the peripheral nerves or the central nervous system.

ACKNOWLEDGMENTS

We thank Dennis Leatherman, Lourdes Rivera, Lynn Trusal, and Selene Watiwat for their valuable assistance in preparing this manuscript.

LITERATURE CITED

1. **Abood, M. E., J. B. Hurley, M.-C. Pappone, H. R. Bourne, and L. Stryer.** 1982. Functional homology between signal-coupling proteins. Cholera toxin inactivates the GTPase activity of transducin. J. Biol. Chem. **257**:10540–10543.
2. **Aharanov, A., R. M. Pruss, and H. R. Herschman.** 1978. Epidermal growth factor. Relationship between receptor regulation and mitogenesis in 3T3 cells. J. Biol. Chem. **253**:3970–3977.
3. **Aimoto, S., T. Takao, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani.** 1982. Amino-acid sequence of heat-stable enterotoxin produced by human enterotoxigenic *Escherichia coli*. Eur. J. Biochem. **29**:257–263.
4. **Anderson, R. G. W., M. S. Brown, and J. L. Goldstein.** 1977. Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. Cell **10**:351–364.
5. **Ascoli, M., and D. Puett.** 1978. Inhibition of the degradation of receptor-bound human choriogonadotropin by lysosomotropic agents, protease inhibitors, and metabolic inhibitors. J. Biol. Chem. **253**:7832–7838.
6. **Bennett, V., E. O'Keefe, and P. Cuatrecasas.** 1975. Mechanism of action of cholera toxin and the mobile receptor theory of hormone receptor-adenylate cyclase interactions. Proc. Natl. Acad. Sci. U.S.A. **72**:33–37.
7. **Bergeron, J. J. M., R. Sikstrom, A. R. Hand, and B. I. Posner.** 1979. Binding and uptake of ^{125}I-insulin into rat liver hepatocytes and endothelium. An *in vivo* radioautographic study. J. Cell Biol. **80**:427–433.
8. **Bigalke, H., W. Dimpfel, and E. Habermann.** 1978. Suppression of ^{3}H-acetylcholine release from primary nerve cell cultures by tetanus and botulinum-A toxin. Naunyn-Schmiedeberg's Arch. Pharmacol. **303**:133–138.
9. **Bjorn, M. J., O. R. Pavlovskis, M. R. Thompson, and B. H. Iglewski.** 1979. Production of exoenzyme S during *Pseudomonas aeruginosa* infections of burned mice. Infect. Immun. **24**:837–842.
10. **Blobel, G., and B. Dobberstein.** 1975. Transfer of protein across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J. Cell Biol. **67**:835–851.
11. **Bokoch, G. M., T. Katada, J. K. Northup, E. L. Hewlett, and A. G. Gilman.** 1983. Identification of the predominant substrate for ADP-ribosylation by islet activating protein. J. Biol. Chem. **258**:2072–2075.
12. **Bonventre, P. F., C. B. Saelinger, B. Ivins, C. Woscinski, and M. Amorini.** 1975. Interaction of cultured mammalian cells with [^{125}I]diphtheria toxin. Infect. Immun. **11**:675–684.
13. **Boquet P., and E. Duflot.** 1982. Tetanus toxin fragment forms channels in lipid vesicles at low pH. Proc. Natl. Acad. Sci. U.S.A. **79**:7614–7618.

14. **Boquet, P., and A. M. Pappenheimer, Jr.** 1976. Interaction of diphtheria toxin with mammalian cell membranes. J. Biol. Chem. **251**:5770–5778.
15. **Boquet, P., M. S. Silverman, and A. M. Pappenheimer, Jr.** 1977. Interaction of diphtheria toxin with mammalian cell membranes, p. 501–509. *In* J. P. Revel, U. Henning, and C. F. Fox (ed.), Progress in clinical and biological research, vol. 17. Cell shape and surface architecture. Alan R. Liss, Inc., New York.
16. **Boquet, P., M. S. Silverman, A. M. Pappenheimer, Jr., and W. B. Vernon.** 1976. Binding of Triton X-100 to diphtheria toxin, crossreacting material 45, and their fragments. Proc. Natl. Acad. Sci. U.S.A. **73**:4449–4453.
17. **Brown, J. E., M. A. Ussery, S. H. Leppla, and S. W. Rothman.** 1980. Inhibition of protein synthesis by Shiga toxin. Activation of toxin and inhibition of peptide elongation. FEBS Lett. **117**:84–88.
18. **Brown, M. S., and J. L. Goldstein.** 1976. Receptor-mediated control of cholesterol metabolism. Science **191**:150–154.
19. **Burns, D. L., E. L. Hewlett, J. Moss, and M. Vaughan.** 1983. Pertussis toxin inhibits enkephalin stimulation of GTPase of NG108-15 cells. J. Biol. Chem. **258**:1435–1438.
20. **Carpenter, G., and S. Cohen.** 1976. ^{125}I-labeled human epidermal growth factor. Binding, internalization, and degradation in human fibroblasts. J. Cell Biol. **71**:159–171.
21. **Cassel, D., and T. Pfeuffer.** 1978. Mechanism of cholera toxin action: covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system. Proc. Natl. Acad. Sci. U.S.A. **75**:2669–2673.
22. **Charalampous, F. C., J. O. Gonatas, S. van Heyningen, and N. K. Gonatas.** 1982. Cholera toxin covalently linked with horseradish peroxidase does not activate the adenylate cyclase of neuroblastoma cells. Exp. Cell. Res. **137**:449–452.
23. **Cheng, S.-Y., F. R. Maxfield, J. Robbins, M. C. Willingham, and I. H. Pastan.** 1980. Receptor-mediated uptake of 3,3′,5-triiodo-L-thyronine by cultured fibroblasts. Proc. Natl. Acad. Sci. U.S.A. **77**:3425–3429.
24. **Chung, D. W., and R. J. Collier.** 1977. Enzymatically active peptide from the adenosine diphosphate-ribosylating toxin of *Pseudomonas aeruginosa*. Infect. Immun. **16**:832–841.
25. **Collier, R. J.** 1975. Diphtheria toxin: mode of action and structure. Bacteriol. Rev. **39**:54–85.
26. **Cooper, D. M. F., R. Jagus, R. L. Somers, and M. Rodbell.** 1981. Cholera toxin modifies diverse GTP-modulated regulatory proteins. Biochem. Biophys. Res. Commun. **101**:1179–1185.
27. **Craig, S. W., and P. Cuatrecasas.** 1975. Mobility of cholera toxin receptors on rat lymphocyte membranes. Proc. Natl. Acad. Sci. U.S.A. **72**:3844–3848.
28. **Davies, P. J. A., D. R. Davies, A. Levitski, F. R. Maxfield, P. Milhaud, M. C. Willingham, and I. Pastan.** 1980. Transglutaminase is essential in receptor-mediated endocytosis of alpha-2 macroglobulin and polypeptide hormones. Nature (London) **283**:162–167.
29. **De Duve, C., T. de Barsy, B. Poole, A. Trouet, P. Tulkens, and F. Van Hoof.** 1974. Lysosomotropic agents. Biochem. Pharmacol. **23**:2495–2531.
30. **Dickson, R. B., M. C. Willingham, and I. Pastan.** 1981. Binding and internalization of ^{125}I alpha-2 macroglobulin by cultured fibroblasts. J. Biol. Chem. **256**:3454–3459.
31. **Didsbury, J. R., J. M. Moehring, and T. J. Moehring.** 1983. Binding and uptake of diphtheria toxin by toxin-resistant Chinese hamster ovary and mouse cells. Mol. Cell. Biol. **3**:1283–1294.
32. **Donovan, J. J., M. I. Simon, R. K. Draper, and M. Montal.** 1981. Diphtheria toxin forms transmembrane channels in planar lipid bilayers. Proc. Natl. Acad. Sci. U.S.A. **78**:172–176.
33. **Donta, S. T.** 1974. Comparison of the effects of cholera enterotoxin and ACTH. Am. J. Physiol. **227**:109–113.
34. **Dorland, R. B.** 1982. The protection mechanism of action of amines in diphtheria toxin-treated Vero cells. Can. J. Microbiol. **28**:611–617.
35. **Dorland, R. B., J. L. Middlebrook, and S. H. Leppla.** 1979. Receptor-mediated internalization and degradation of diphtheria toxin by monkey kidney cells. J. Biol. Chem. **254**:11337–11342.
36. **Dorland, R. B., J. L. Middlebrook, and S. H. Leppla.** 1981. Effect of ammonium chloride on the receptor-mediated uptake of diphtheria toxin by Vero cells. Exp. Cell Res. **134**:319–327.
37. **Draper, R. K., D. Chin, D. Eurey-Owens, I. E. Scheffler, and M. I. Simon.** 1979. Biochemical and genetic characterization of three hamster cell mutants resistant to diphtheria toxin. J. Cell Biol. **83**:116–125.
38. **Draper, R. K., D. Chin, and M. I. Simon.** 1978. Diphtheria toxin has the properties of a lectin. Proc. Natl. Acad. Sci. U.S.A. **75**:261–265.
39. **Draper, R. K., and M. I. Simon.** 1980. The entry of diphtheria toxin into mammalian cell cytoplasm: evidence for lysosomal involvement. J. Cell Biol. **87**:849–854.
40. **Eidels, L., R. L. Proia, and D. A. Hart.** 1983. Membrane receptors for bacterial toxins. Microbiol. Rev. **47**:596–620.
41. **Eiklid, K., and S. Olsnes.** 1983. Animal toxicity of *Shigella dysenteriae* cytotoxin: evidence that the neurotoxic, enterotoxic, and cytotoxic activities are due to one toxin. J. Immunol. **130**:380–384.
42. **Enomoto, K., and D. M. Gill.** 1980. Cholera toxin activation of adenylate cyclase. Roles of nucleoside triphosphates and a macromolecular factor in the ADP-ribosylation of GTP-dependent regulatory component. J. Biol. Chem. **255**:1252–1258.
43. **Everse, J., D. A. Gardner, N. O. Kaplan, W. Galisinski, and K. Moldave.** 1970. The formation of a ternary complex between diphtheria toxin, aminoacyltransferase II, and diphosphopyridine nucleotide. J. Biol. Chem. **245**:899–901.
44. **Finkelstein, R. A.** 1973. Cholera. Crit. Rev. Microbiol. **2**:553–623.
45. **Fitzgerald, D., R. E. Morris, and C. B. Saelinger.** 1980. Receptor-mediated internalization of *Pseudomonas* toxin by mouse fibroblasts. Cell **21**:867–873.
46. **Gill, D. M.** 1975. Involvement of nicotinamide adenine dinucleotide in the action of cholera toxin *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **72**:2064–2068.
47. **Gill, D. M.** 1976. The arrangement of subunits in cholera toxin. Biochemistry **15**:1242–1248.
48. **Gill, D. M.** 1977. Mechanism of action of cholera toxin. Adv. Cyclic Nucleotide Res. **8**:85–118.
49. **Gill, D. M.** 1978. Seven toxic peptides that cross cell membranes, p. 291–332. *In* J. Jeljaszewics and T. Wadstrom (ed.), Bacterial toxins and cell membranes. Academic Press, Inc., New York.
50. **Gill, D. M., D. J. Evans, Jr., and D. G. Evans.** 1976. Mechanism of activation of adenylate cyclase *in vitro* by polymyxin-released, heat-labile enterotoxin of *Escherichia coli*. J. Infect. Dis. **133**(Suppl.):S103–S107.
51. **Gill, D. M., J. A. Hope, R. Meren, and D. S. Jacobs.** 1981. Cholera toxin's interaction with cell membranes, p. 113–122. *In* J. L. Middlebrook and L. D. Kohn (ed.), Receptor-mediated binding and internalization of toxins and hormones. Academic Press, Inc., New York.
52. **Gill, D. M., and R. Meren.** 1978. ADP-ribosylation of membrane proteins catalyzed by cholera toxin: basis of the activation of adenylate cyclase. Proc. Natl. Acad. Sci. U.S.A. **75**:3050–3054.
53. **Gill, D. M., and S. H. Richardson.** 1980. Adenosine diphosphate-ribosylation of adenylate cyclase catalyzed by heat-labile enterotoxin of *Escherichia coli*: comparison with cholera toxin. J. Infect. Dis. **141**:64–70.
54. **Goldstein, J. L., R. G. W. Anderson, and M. S. Brown.** 1979. Coated pits, coated vesicles, and receptor-mediated endocytosis. Nature (London) **279**:679–685.
55. **Goldstein, J. L., S. K. Basu, G. Y. Brunschede, and M. S. Brown.** 1976. Release of low density lipoprotein from its cell surface receptor by sulfated glycosaminoglycans. Cell **7**:85–95.
56. **Goldstein, J. L., and M. S. Brown.** 1977. The low-density lipoprotein pathway and its relation to arteriosclerosis. Annu. Rev. Biochem. **46**:897–930.
57. **Goldstein, J. L., G. Y. Brunschede, and M. S. Brown.** 1975.

Inhibition of the proteolytic degradation of low density lipoprotein in human fibroblasts by chloroquine, concanavalin A and Triton WR1339. J. Biol. Chem. **250**:7854–7862.

58. **Gonatas, M., A. Steiber, J. Gonatas, and T. Mommoi.** 1981. Endocytosis of cholera toxin and (^{3}H)G_{M1} ganglioside by neuroblastoma cells, p. 123–131. *In* J. L. Middlebrook and L. D. Kohn (ed.), Receptor-mediated binding and internalization of toxins and hormones. Academic Press, Inc., New York.
59. **Goor, R. S., and A. M. Pappenheimer, Jr.** 1967. Studies on the mode of action of diphtheria toxin. IV. Specificity of the cofactor (NAD) requirement for toxin in cell-free systems. J. Exp. Med. **126**:913–921.
60. **Gorden, P., J.-L. Carpentier, S. Cohen, and L. Orci.** 1978. Epidermal growth factor: morphological demonstration of binding, internalization, and lysosomal association in human fibroblasts. Proc. Natl. Acad. Sci. U.S.A. **75**:5025–5029.
61. **Gordon, A. H., P. D'Arcy Hart, and M. R. Young.** 1980. Ammonia inhibits phagosome-lysosome fusion in macrophages. Nature (London) **286**:79–80.
62. **Gupta, R., and L. Siminovitch.** 1978. Isolation and characterization of mutants of human diploid fibroblasts resistant to diptheria toxin. Proc. Natl. Acad. Sci. U.S.A. **75**:3337–3340.
63. **Gupta, R., and L. Siminovitch.** 1980. Diphtheria toxin resistance in Chinese hamster cells: genetic and biochemical characteristics of the mutants affected in protein synthesis. Somat. Cell Gen. **6**:361–379.
64. **Habener, J. F., M. Rosenblatt, B. Kemper, H. M. Kronenberg, A. Rich, and J. T. Potts, Jr.** 1978. Pre-proparathyroid hormone: amino acid sequence, chemical synthesis, and some biological studies of the precursor region. Proc. Natl. Acad. Sci. U.S.A. **75**:2616–2620.
65. **Habig, W. H., E. F. Grollman, F. D. Ledley, M. F. Meldolesi, S. M. Aloj, M. C. Hardegree, and L. D. Kohn.** 1978. Tetanus toxin interactions with the thyroid: decreased toxin binding to membranes from a thyroid tumor with a thyrotropin receptor defect and *in vivo* stimulation of thyroid function. Endocrinology **102**:844–851.
66. **Hagmann, J., and P. H. Fishman.** 1981. Inhibitors of protein synthesis block action of cholera toxin. Biochem. Biophys. Res. Commun. **98**:677–684.
67. **Hale, T. L., and S. B. Formal.** 1980. Cytotoxicity of *Shigella dysenteriae* 1 for cultured mammalian cells. Am. J. Clin. Nutr. **33**:2485–2490.
68. **Hawkins, D. J., and E. T. Browning.** 1982. Tubulin adenosine diphosphate ribosylation is catalyzed by cholera toxin. Biochemistry **21**:4474–4478.
69. **Hazeki, O., and M. Ui.** 1981. Modification by islet-activating protein of receptor-mediated regulation of cyclic AMP accumulation in isolated rat heart cells. J. Biol. Chem. **256**:2856–2862.
70. **Heldin, C.-H., A. Westeson, and B. Westermark.** Interaction of platelet-derived growth factor with its fibroblast receptor. Demonstration of ligand degradation and receptor modulation. J. Biol. Chem. **257**:4216–4221.
71. **Helenius, A., J. Kartenbeck, K. Simons, and E. Fries.** 1980. On the entry of Semliki Forest virus into BHK-21 cells. J. Cell Biol. **84**:404–420.
72. **Helting, T. B., H.-J. Ranneberger, R. Vollerthun, and V. Neubauer.** 1978. Toxicity of papain-digested tetanus toxin. Pathological effect of fragment B in the absence of spastic paralysis. J. Biol. Chem. **253**:125–129.
73. **Helting, T. B., and O. Zwisler.** 1974. Enzymatic breakdown of tetanus toxin. Biochem. Biophys. Res. Commun. **57**:1263–1270.
74. **Hewlett, E., and J. Wolff.** 1976. Soluble adenylate cyclase from the culture medium of *Bordetella pertussis*: purification and characterization. J. Bacteriol. **127**:890–896.
75. **Hizuka, N., P. Gordon, M. A. Lesniak, E. Van Obberghen, J.-L. Carpentier, and L. Orci.** 1981. Polypeptide hormone degradation and receptor regulation are coupled to ligand internalization. A direct biochemical and morphological demonstration. J. Biol. Chem. **256**:4591–4597.
76. **Holmgren, J.** 1981. Actions of cholera toxin and the prevention and treatment of cholera. Nature (London) **292**:413–417.
77. **Holmgren, J., L. Lindholm, and I. Lonnroth.** 1974. Interaction of cholera toxin and toxin derivatives with lymphocytes. I. Binding properties and interference with lectin-induced cellular stimulation. J. Exp. Med. **139**:801–819.
78. **Howard, B. D., C.-S. Wu, and C. B. Gundersen, Jr.** 1976. Antagonism of botulinum toxin by theophylline. Biochem. Biophys. Res. Commun. **71**:413–415.
79. **Iglewski, B. H., and D. Kabat.** 1975. NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. Proc. Natl. Acad. Sci. U.S.A. **72**:2284–2288.
80. **Iglewski, B. H., P. V. Liu, and D. Kabat.** 1977. Mechanism of action of *Pseudomonas aeruginosa* exotoxin A: adenosine diphosphate-ribosylation of mammalian elongation factor 2 in vitro and in vivo. Infect. Immun. **15**:138–144.
81. **Iglewski, B. H., J. Sadoff, M. J. Bjorn, and E. S. Maxwell.** 1978. *Pseudomonas aeruginosa* exoenzyme S: an adenosine diphosphate ribosyltransferase distinct from toxin A. Proc. Natl. Acad. Sci. U.S.A. **75**:3211–3215.
82. **Irving, R. A., F. Toneguzza, S. H. Rhee, T. Hofmann, and H. P. Ghosh.** 1979. Synthesis and assembly of membrane glycoproteins: presence of leader peptide in unglycosylated precursor of membrane glycoprotein of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. **76**:570–574.
83. **Ivins, B., C. B. Saelinger, P. F. Bonventre, and C. Woscinski.** 1975. Chemical modulation of diphtheria toxin action on cultured mammalian cells. Infect. Immun. **11**:665–674.
84. **Johnson, G. L., H. R. Kaslow, and H. R. Bourne.** 1978. Genetic evidence that cholera toxin substrates are regulatory components of adenylate cyclase. J. Biol. Chem. **253**:7120–7123.
85. **Joseph, K. C., S. U. Kim, A. Steiber, and N. K. Gonatas.** 1978. Endocytosis of cholera toxin into neuronal GERL. Proc. Natl. Acad. Sci. U.S.A. **75**:2815–2819.
86. **Joseph, K. C., A. Steiber, and N. K. Gonatas.** 1979. Endocytosis of cholera toxin in GERL-like structures of murine neuroblastoma cells pretreated with G_{M1} ganglioside. Cholera toxin internalization into neuroblastoma GERL. J. Cell Biol. **81**:543–554.
87. **Kaslow, H. R., V. E. Groppi, M. E. Abood, and H. R. Bourne.** 1981. Cholera toxin can catalyze ADP-ribosylation of cytoskeletal proteins. J. Cell Biol. **91**:410–413.
88. **Kaslow, H. R., G. L. Johnson, V. M. Brothers, and H. R. Bourne.** 1980. A regulatory component of adenylate cyclase from human erythrocyte membranes. J. Biol. Chem. **255**:3736–3741.
89. **Katada, T., T. Amano, and M. Ui.** 1982. Modulation by islet-activating protein of adenylate cyclase activity in C6 glioma cells. J. Biol. Chem. **257**:3739–3746.
90. **Katada, T., and M. Ui.** 1980. Slow interaction of islet-activating protein with pancreatic islets during primary culture to cause reversal of alpha-adrenergic inhibition of insulin secretion. J. Biol. Chem. **255**:9580–9588.
91. **Katada, T., and M. Ui.** 1982. ADP ribosylation of the specific membrane protein of C6 cells by islet-activating protein associated with modification of adenylate cyclase activity. J. Biol. Chem. **257**:7210–7216.
92. **Katada T., and M. Ui.** 1982. Direct modification of the membrane adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. Proc. Natl. Acad. Sci. U.S.A. **79**:3129–3133.
93. **Keen, J. H., F. R. Maxfield, M. C. Hardegree, and W. H. Habig.** 1982. Receptor-mediated endocytosis of diphtheria toxin by cells in culture. Proc. Natl. Acad. Sci. U.S.A. **79**:2912–2916.
94. **Keusch, G. T.** 1981. Receptor-mediated endocytosis of *Shigella* cytotoxin, p. 95–112. *In* J. L. Middlebrook and L. D. Kohn (ed.), Receptor-mediated binding and internalization of toxins and hormones. Academic Press, Inc., New York.
95. **Keusch, G. T., and M. Jacewicz.** 1975. The pathogenesis of *Shigella* diarrhea. V. Relationship of Shiga enterotoxin, neurotoxin, and cytotoxin. J. Infect. Dis. **131**:(Suppl.):S33–S39.
96. **Keusch, G. T., and M. Jacewicz.** 1977. Pathogenesis of *Shigella* diarrhea. VII. Evidence for a cell membrane toxin receptor

involving beta 1 4-linked N-acetyl-D-glucosamine oligomers. J. Exp. Med. **146**:535–546.

97. **Keusch, G. T., M. Jacewicz, and S. Z. Herschman.** 1972. Quantitative microassay in cell culture for enterotoxin of *Shigella dysenteriae* 1. J. Infect. Dis. **125**:539–541.
98. **Kim, K., and N. B. Groman.** 1965. In vitro inhibition of diphtheria toxin action by ammonium salts and amines. J. Bacteriol. **90**:1552–1556.
99. **King, A. C., and P. Cuatrecasas.** 1982. Exposure of cells to an acidic environment reverses the inhibition by methylamine of the mitogenic response to epidermal growth factor. Biochem. Biophys. Res. Commun. **106**:479–485.
100. **King, A. C., L. Hernaez-Davis, and P. Cuatrecasas.** 1980. Lysosomotropic amines cause intracellular accumulation of receptors for epidermal growth factor. Proc. Natl. Acad. Sci. U.S.A. **77**:3283–3287.
101. **King, A. C., L. Hernaez-Davis, and P. Cuatrecasas.** 1981. Lysosomotropic amines inhibit mitogenesis induced by growth factors. Natl. Acad. Sci. U.S.A. **78**:717–721.
102. **Kurose, H., T. Katada, T. Amano, and M. Ui.** 1983. Specific uncoupling by islet-activating protein, pertussis toxin, of negative signal transduction via alpha-adrenergic, cholinergic, and opiate receptors in neuroblastoma × glioma hybrid cells. J. Biol. Chem. **258**:4870–4875.
103. **Lambotte, P., P. Falmagne, C. Capiau, J. Zaren, J.-M. Ruysschaert, and J. Dirkx.** 1980. Primary structure of diphtheria toxin fragment B: structural similarities with lipid-binding domains. J. Cell Biol. **87**:837–840.
104. **Ledley, F. D., G. Lee, L. D. Kohn, W. H. Habig, and C. Hardegree.** 1977. Tetanus toxin interactions with thyroid plasma membranes. J. Biol. Chem. **252**:4049–4055.
105. **Ledley, F. D., B. R. Mullin, G. Lee, S. M. Aloj, P. H. Fishman, L. T. Hunt, M. O. Dayhoff, and L. D. Kohn.** 1978. Sequence similarity between cholera toxin and glycoprotein hormones: implications for structure-activity relationship and mechanism of action. Biochem. Biophys. Res. Commun. **69**:852–858.
106. **Leppla, S. H.** 1982. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations in eukaryotic cells. Proc. Natl. Acad. Sci. U.S.A. **79**:3162–3166.
107. **Leppla, S. H., and R. B. Dorland.** 1981. Uptake mechanisms for ADP-ribosylating toxins, p. 65–79. *In* J. L. Middlebrook and L. D. Kohn (ed.), Receptor-mediated binding and internalization of toxins and hormones. Academic Press, Inc., New York.

107a. **Leppla, S. H., R. B. Dorland, and J. L. Middlebrook.** 1980. Inhibition of diphtheria toxin degradation and cytotoxic action by chloroquine. J. Biol. Chem. **255**:2247–2250.

108. **Leppla, S. H., O. C. Martin, and L. A. Muehl.** 1978. The exotoxin of *Pseudomonas aeruginosa*: a proenzyme having an unusual mode of activation. Biochem. Biophys. Res. Commun. **81**:532–538.
109. **Lincoln, R. E., and D. C. Fish.** 1970. Anthrax toxin, p. 361–414. *In* T. C. Montie, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. 3. Bacterial protein toxins. Academic Press, Inc., New York.
110. **Lingapa, V. R., A. Devillers-Thierry, and G. Blobel.** 1977. Nascent prehormones are intermediates in the biosynthesis of authentic bovine pituitary growth hormone and prolactin. Proc. Natl. Acad. Sci. U.S.A. **74**:2432–2436.
111. **Lingapa, V. R., J. R. Lingappa, and G. Blobel.** 1979. Chicken ovalbumin contains an internal signal sequence. Nature (London) **281**:117–121.
112. **Londos, C., Y. Salomon, M. Lin, J. P. Harwood, M. Schram, J. Wolff, and M. Rodbell.** 1974. 5′ Guanylimidophosphate, a potent activator of adenylate cyclase systems in eukaryotic cells. Proc. Natl. Acad. Sci. U.S.A. **71**:3087–3090.
113. **Malbon, C. C., and D. M. Gill.** 1979. ADP-ribosylation of membrane proteins and activation of adenylate cyclase by cholera toxin in fat cell ghosts from euthyroid and hypothyroid rats. Biochim. Biophys. Acta **586**:518–527.
114. **Marnell, M. H., M. Stookey, and R. K. Draper.** 1982. Monensin blocks the transport of diphtheria toxin to the cell cytoplasm. J. Cell Biol. **93**:57–62.
115. **Marsh, M., and A. Helenius.** 1980. Adsorptive endocytosis of Semliki Forest virus. J. Mol. Biol. **142**:439–454.
116. **Marshall, S., and J. M. Olefsky.** 1979. Effects of lysosomotropic agents on insulin interactions with adipocytes. Evidence for a lysosomal pathway for insulin processing and degradation. J. Biol. Chem. **254**:10153–10160.
117. **Matsuda, M., M. Sugimoto, K. Ozutsumi, and T. Hirai.** 1982. Acute botulinum-like intoxication by tetanus neurotoxin in mice. Biochem. Biophys. Res. Commun. **104**:799–805.
118. **Matsuda, M., and M. Yoneda.** 1974. Two antigenically active, complementary polypeptide fragments of tetanus neurotoxin, p. 363–373. *In* A. Ohsaka, K. Hayaski, and Y. Sawai (ed.), Animal, plant and microbial toxins, vol. 1. Biochemistry. Plenum Press, New York.
119. **Matsuda, M., and M. Yoneda.** 1974. Dissociation of tetanus neurotoxin into two polypeptide fragments. Biochem. Biophys. Res. Commun. **57**:1257–1262.
120. **Matsuda, M., and M. Yoneda.** 1975. Isolation and purification of two antigenically active "complementary" polypeptide fragments of tetanus neurotoxin. Infect. Immun. **12**:1147–1152.
121. **Matsuda, M., and M. Yoneda.** 1977. Antigenic substructure of tetanus neurotoxin. Biochem. Biophys. Res. Commun. **77**:268–274.
122. **Maxfield, F. R., P. J. A. Davies, L. Klemper, M. C. Willingham, and I. Pastan.** 1979. Epidermal growth factor stimulation of DNA synthesis is promoted by compounds that inhibit its clustering in coated pits. Proc. Natl. Acad. Sci. U.S.A.**76**:5731–5735.
123. **Maxfield, F. R., J. Schlessinger, Y. Shechter, I. Pastan, and M. C. Willingham.** 1978. Collection of insulin, EGF, and alpha-2 macroglobulin in the same patches on the surface of cultured fibroblasts and common internalization. Cell **14**:805–810.
124. **Maxfield, F. R., M. C. Willingham, P. J. A. Davies, and I. Pastan.** 1979. Amines inhibit the clustering of alpha-2 macroglobulin and EGF on the fibroblast cell surface. Nature (London) **277**:661–663.
125. **McIver, J., G. F. Grady, and G. T. Keusch.** 1975. Production and characterization of exotoxin(s) of *Shigella dysenteriae* type 1. J. Infect. Dis. **131**:559–566.
126. **Mekada, E., T. Uchida, and Y. Okada.** 1981. Methylamine stimulates the action of ricin toxin but inhibits that of diphtheria toxin. J. Biol. Chem. **256**:1225–1228.
127. **Merion, M., P. Schlesinger, R. M. Brooks, J. M. Moehring, T. J. Moehring, and W. S. Sly.** 1983. Defective acidification of endosomes in chinese hamster ovary cell mutants "cross-resistant" to toxins and viruses. Proc. Natl. Acad. Sci. U.S.A. **80**:5315–5319.
128. **Michael, M., and C. B. Saelinger.** 1979. Toxicity of *Pseudomonas* toxin for mouse LM cell fibroblasts. Curr. Microbiol. **2**:103–108.
129. **Middlebrook, J. L., and R. B. Dorland.** 1976. Response of cultured mammalian cells to the exotoxins of *Pseudomonas aeruginosa* and *Cornybacterium diphtheriae*: differential cytotoxicity. Can. J. Microbiol. **23**:183–189.
130. **Middlebrook, J. L., and R. B. Dorland.** 1977. Differential chemical protection of mammalian cells from the exotoxins of *Cornybacterium diphtheria* and *Pseudomonas aeruginosa*. Infect. Immun. **16**:232–239.
131. **Middlebrook, J. L., and R. B. Dorland.** 1981. Receptor-mediated internalization of diphtheria toxin, p. 15–29. *In* J. L. Middlebrook and L. D. Kohn (ed.), Receptor-mediated binding and internalization of toxins and hormones. Academic Press, Inc., New York.
132. **Middlebrook, J. L., R. B. Dorland, and S. H. Leppla.** 1978. Association of diphtheria toxin with Vero cells: demonstration of a receptor. J. Biol. Chem. **253**:7325–7330.
133. **Middlebrook, J. L., R. B. Dorland, and S. H. Leppla.** 1979. Effects of lectins on the interaction of diphtheria toxin with mammalian cells. Exp. Cell Res. **121**:95–101.
134. **Middlebrook, J. L., R. B. Dorland, S. H. Leppla, and J. D. White.** 1980. Receptor-mediated binding and internalization of

Pseudomonas exotoxin A and diphtheria exotoxin by mammalian cells, p. 463–470. *In* D. Eaker and T. Wadstrom (ed.), Natural toxins, Pergamon Press, New York.

135. **Middlebrook, J. L., and D. L. Leatherman.** 1982. Differential sensitivity of reticulocytes to nicked and unnicked diphtheria toxin. Exp. Cell Res. **138**:175–182.
136. **Miller, D. K., and J. Lenard.** 1981. Antihistaminics, local anesthetics, and other amines as antiviral agents. Proc. Natl. Acad. Sci. U.S.A. **78**:3605–3609.
137. **Moehring, J. M., and T. J. Moehring.** 1967. The response of cultured mammalian cells to diphtheria toxin. II. The resistant cell: enhancement of toxin action by poly-L-ornithine. J. Exp. Med. **127**:541–545.
138. **Moehring, J. M., and T. J. Moehring.** 1976. Comparison of diphtheria intoxication in human and nonhuman cell lines and their resistant variants. Infect. Immun. **13**:221–228.
139. **Moehring, J. M., T. J. Moehring, and D. E. Danley.** 1980. Posttranslational modification of elongation factor 2 in diphtheria-toxin-resistant mutants of CHO-K1 cells. Proc. Natl. Acad. Sci. U.S.A. **77**:1010–1014.
140. **Moehring, T. J., D. E. Danley, and J. M. Moehring.** 1979. Codominant translational mutants of Chinese hamster ovary cells selected with diphtheria toxin. Somat. Cell Genet. **5**:469–480.
141. **Moehring, T. J., and J. M. Moehring.** 1972. Response of cultured mammalian cells to diphtheria toxin. IV. Isolation of KB cells resistant to diphtheria toxin. Infect. Immun. **6**:487–492.
142. **Moehring, T. J., and J. M. Moehring.** 1972. Response of cultured mammalian cells to diphtheria toxin. V. Concurrent resistance to ribonucleic acid viruses in diphtheria toxin-resistant KB cell strains. Infect. Immun. **6**:493–500.
143. **Moehring, T. J., and J. M. Moehring.** 1976. Interaction of diphtheria toxin and its active subunit, fragment A, with toxin-sensitive and toxin-resistant cells. Infect. Immun. **13**:1426–1432.
144. **Moerhing, T. J., and J. M. Moehring.** 1977. Selection and characterization of cells resistant to diphtheria toxin and *Pseudomonas* exotoxin A: presumptive translational mutants. Cell **11**:447–454.
145. **Moehring, T. J., and J. M. Moehring.** 1979. Characterization of the diphtheria toxin-resistance system in Chinese hamster ovary cells. Somat. Cell Genet. **5**:453–468.
146. **Morris, R. E., M. D. Manhart, and C. B. Saelinger.** 1983. Receptor-mediated entry of *Pseudomonas* toxin: methylamine blocks clustering step. Infect. Immun. **40**:806–811.
147. **Morris, R. E., and C. B. Saelinger.** 1983. Diphtheria toxin does not enter resistant cells by receptor-mediated endocytosis. Infect. Immun. **42**:812–817.
148. **Moss, J., P. H. Fishman, V. C. Manganiello, M. Vaughan, and R. O. Brady.** 1976. Functional incorporation of ganglioside into intact cells: induction of choleragen responsiveness. Proc. Natl. Acad. Sci. U.S.A. **73**:1034–1037.
149. **Moss, J., S. Garrison, N. J. Oppenheimer, and S. H. Richardson.** 1979. NAD-dependent ADP-ribosylation of arginine and proteins by *Escherichia coli* heat-labile enterotoxin. J. Biol. Chem. **254W**:6270–6272.
150. **Moss, J., V. C. Manganiello, and M. Vaughan.** 1976. Hydrolysis of nicotinamide adenine dinucleotide by choleragen and its A promoter: possible role in the activation of adenylate cyclase. Proc. Natl. Acad. Sci. U.S.A. **73**:4424–4427.
151. **Moss, J., J. C. Osborne, Jr., P. H. Fishman, H. B. Brewer, Jr., M. Vaughan, and R. O. Brady.** 1977. Effect of gangliosides and substrate analogues on the hydrolysis of nicotinamide adenine dinucleotide by choleragen. Proc. Natl. Acad. Sci. U.S.A. **74**:74–78.
152. **Moss, J., R. L. Richards, C. R. Alving, and P. H. Fishman.** 1977. Effect of the A and B protomers of choleragen on release of trapped glucose from liposomes containing or lacking ganglioside G_{M1}. J. Biol. Chem. **252**:797–798.
153. **Moss, J., and S. H. Richardson.** 1978. Activation of adenylate cyclase by heat-labile *Escherichia coli* enterotoxin. Evidence for ADP-ribosyltransferase activity similar to that of choleragen. J. Clin. Invest. **61**:281–285.
154. **Moss, J., J. Stanley, D. L. Burns, J. A. Hsia, D. A. Yost, G. A. Myers, and E. L. Hewlett.** 1983. Activation by thiol of the latent NAD glycohydrolase and ADP-ribosyltransferase activities of *Bordetella pertussis* toxin (islet activating protein). J. Biol. Chem. **258**:11879–11882.
155. **Moss, J., and M. Vaughan.** 1976. Evidence for ADP-ribosyltransferase activity with arginine as an acceptor. J. Biol. Chem. **252**:2455–2457.
156. **Moss, J., and M. Vaughan.** 1979. Activation of adenylate cyclase by choleragen. Annu. Rev. Biochem. **48**:581–600.
157. **Mullin, B. R., P. H. Fishman, G. Lee, S. M. Aloj, F. D. Ledley, R. J. Winand, L. D. Kohn, and R. O. Brady.** 1976. Thyrotropin-ganglioside interactions and their relationship to the structure and function of thyrotropin receptors. Proc. Natl. Acad. Sci. U.S.A. **73**:842–846.
158. **Murayama, T., T. Katada, and M. Ui.** 1983. Guanine nucleotide activation and inhibition of adenylate cyclase as modified by islet-activating protein, pertussis toxin, in mouse 3T3 fibroblasts. Arch. Biochem. Biophys. **221**:381–390.
159. **Murayama, T., and M. Ui.** 1983. Loss of the inhibitory function of the guanine nucleotide regulatory component of adenylate cyclase due to its ADP ribosylation by islet-activating protein, pertussis toxin, in adipocyte membranes. J. Biol. Chem. **258**:3319–3326.
160. **Nicolson, G. L.** 1974. Ultrastructural analysis of toxin binding and entry into mammalian cells. Nature (London) **251**:628–630.
161. **Northup, J. K., P. C. Sternweis, M. D. Smigel, L. S. Schleifer, E. M. Ross, and A. G. Gilman.** 1980. Purification of the regulatory component of adenylate cyclase. Proc. Natl. Acad. Sci. U.S.A. **77**:6516–6520.
162. **Okhuma, S., and B. Poole.** 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. U.S.A. **75**:3327–3331.
163. **Okhuma, S., and B. Poole.** 1981. Cytoplasmic vacuolation of mouse peritoneal macrophages and uptake into lysosomes of weakly basic substances. J. Cell Biol. **90**:656–664.
164. **Olsnes, S., and K. Eiklid.** 1980. Isolation and characterization of *Shigella shigae* cytotoxin. J. Biol. Chem. **255**:284–289.
165. **Olsnes, S., R. Reisbig, and K. Eiklid.** 1981. Subunit structure of *Shigella* cytotoxin. J. Biol. Chem. **256**:8732–8738.
166. **Oppenheimer, N. J.** 1978. Structural determination and stereospecificity of choleragen-catalyzed reaction of NAD^+ with guanidines. J. Biol. Chem. **253**:4907–4910.
167. **Oppenheimer, N. J., and J. W. Bodley.** 1981. Diphtheria toxin: site and configuration of ADP-ribosylation of diphthamide in elongation factor 2. J. Biol. Chem. **236**:8579–8581.
168. **Ostlund, R. E., Jr., B. Pfleger, and G. Schonfeld.** 1979. Role of microtubules in low density lipoprotein processing by cultured cells. J. Clin. Invest. **63**:75–84.
169. **Pappenheimer, A. M., Jr.** 1975. Diphtheria toxin. Annu. Rev. Biochem. **46**:69–94.
170. **Pappenheimer, A. M., Jr.** 1979. Transport of diphtheria toxin A fragment across the plasma membrane, p. 669–674. *In* J. P. Revel, U. Henning, and C. F. Fox (ed.), Progress in clinical and biological research, vol. 31. Transmembrane signalling. Alan R. Liss, Inc., New York.
171. **Pappenheimer, A. M., Jr.** 1979. Interaction of protein toxins with mammalian cell membranes, p. 187–192. *In* D. Schlessinger (ed.), Microbiology—1979. American Society for Microbiology, Washington, D.C.
172. **Pappenheimer, A. M., Jr., and R. Brown.** 1968. Studies on the mode of action of diphtheria toxin. VI. Site of the action of toxin in living cells. J. Exp. Med. **127**:1073–1086.
173. **Pappenheimer, A. M., Jr., and M. R. Moynihan.** 1981. Diphtheria toxin: a model for translocation of polypeptides across the plasma membrane, p. 31–51. *In* J. L. Middlebrook and L. D. Kohn (ed.), Receptor-mediated binding and internalization of toxins and hormones. Academic Press, Inc., New York.
174. **Pastan, I. H., and M. C. Willingham.** 1981. Receptor-mediated

endocytosis of hormones in cultured cells. Annu. Rev. Physiol. **43**:313–332.

175. **Pearce, B. R., A. L. Gard, and G. R. Dutton.** 1983. Tetanus toxin inhibition of K^+-stimulated (^{3}H)GABA release from developing cell cultures of rat cerebellum. J. Neurochem. **40**:887–890.
176. **Pearse, B. M. F.** 1976. Clathrin: a unique protein associated with intracellular transfer of membrane by coated vesicles. Proc. Natl. Acad. Sci. U.S.A. **73**:1255–1259.
177. **Posner, B. I., B. A. Patel, M. N. Khan, and J. J. M. Bergeron.** 1982. Effect of chloroquine on the internalization of ^{125}I-insulin into subcellular fractions of rat liver. Evidence for an effect of chloroquine on Golgi elements. J. Biol. Chem. **257**:5789–5799.
178. **Pruitt, B. A.** 1974. Infections caused by *Pseudomonas* species in patients with burns and in other surgical patients. J. Infect. Dis. **130**(Suppl.):S8–S13.
179. **Reisbig, R., S. Olsnes, and K. Eiklid.** 1981. The cytotoxic activity of *Shigella* toxin. Evidence for catalytic inactivation of the 60S ribosomal subunit. J. Biol. Chem. **256**:8739–8744.
180. **Robinson, E. A., O. Henrikson, and E. S. Maxwell.** 1974. Elongation factor 2. Amino acid sequence at the site of adenosine diphosphate ribosylation. J. Biol. Chem. **249**:5088–5093.
181. **Rodbell, M.** 1980. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. Nature (London) **284**:17–22.
182. **Roth, T. F., and K. R. Porter.** 1964. Yolk protein uptake in the oocyte of the mosquito, *Aedes aegyptii*. J. Cell Biol. **20**:313–332.
183. **Saelinger, C. B., P. F. Bonventre, and J. Imhoff.** 1975. Interaction of the toxin of *Cornybacterium diphtheria* with phagocytes from susceptible and resistant species. J. Infect. Dis. **131**:431–438.
184. **Saelinger, C. B., P. F. Bonventre, B. Ivins, and D. Strauss.** 1976. Uptake of diphtheria toxin and its fragment A moiety by mammalian cells in culture. Infect. Immun. **14**:742–751.
185. **Sando, G. N., P. Titus-Dillon, C. W. Hall, and E. F. Neufield.** 1979. Inhibition of receptor-mediated uptake of a lysosomal enzyme into fibroblasts by chloroquine, procaine and ammonia. Exp. Cell Res. **119**:359–364.
186. **Sandvig, K., and S. Olsnes.** 1979. Effect of temperature on the uptake, excretion and degradation of abrin and ricin by HeLa cells. Exp. Cell Res. **121**:15–25.
187. **Sandvig, K., and S. Olsnes.** 1980. Diphtheria toxin entry into cells is facilitated by low pH. J. Cell Biol. **87**:828–832.
188. **Sandvig, K., and S. Olsnes.** 1981. Rapid entry of nicked diphtheria toxin into cells at low pH. Characterization of the entry process and effects of low pH on the toxin molecule. J. Biol. Chem. **256**:9068–9076.
189. **Sandvig, K., and S. Olsnes.** 1982. Entry of the toxin proteins abrin, modeccin, ricin, and diphtheria toxin into cells. II. Effect of pH, metabolic inhibitors, and ionophores, and evidence for toxin penetration from endocytic vesicles. J. Biol. Chem. **257**:7504–7513.
190. **Sandvig, K., S. Olsnes, and A. Pihl.** 1979. Inhibitory effect of ammonium chloride and chloroquine on the entry of the toxic lectic modeccin into HeLa cells. Biochem. Biophys. Res. Common. **90**:648–655.
191. **Savion, N., J. D. Isaacs, D. Gospodarowicz, and M. A. Shuman.** 1981. Internalization and degradation of thrombin-binding sites in corneal endothelial cells. J. Biol. Chem. **256**:4514–4519.
192. **Schein, S. J., B. L. Kagan, and A. Finkelstein.** 1978. Colicin K acts by forming voltage-dependent channels in phospholipid bilayer membranes. Nature (London) **276**:159–163.
193. **Schlessinger, J., Y. Shechter, M. C. Willingham, and I. Pastan.** 1978. Direct visualization of the binding, aggregation, and internalization of insulin and epidermal growth factor in fibroblastic cells. Proc. Natl. Acad. Sci. U.S.A. **75**:2659–2662.
194. **Schneider, Y.-J, P. Tulkens, C. de Duve, and A. Trouet.** 1979. Fate of plasma membrane during endocytosis. I. Uptake and processing of anti-plasma membrane and control immunoglobulins by cultured fibroblasts. J. Cell Biol. **82**:449–465.
195. **Schwab, M. E., E. Suda, and H. Thoenen.** 1979. Selective retrograde transsynaptic transfer of a protein, tetanus toxin, subsequent to its retrograde axonal transport. J. Cell Biol. **82**:798–810.
196. **Schwab, M. E., and H. Thoenen.** 1976. Electron microscopic evidence for a trans-synaptic migration of tetanus toxin in spinal cord motoneurons: an autoradiographic and morphomeric study. Brain Res. **105**:213–227.
197. **Schwab, M. E., and H. Thoenen.** 1977. Selective trans-synaptic migration of tetanus toxin after retrograde axonal transport in peripheral sympathetic nerves: a comparison with nerve growth factor. Brain Res. **122**:459–474.
198. **Silverstein, S. C., and S. Dales.** 1968. The penetration of reovirus RNA and initiation of its genetic function in L-strain fibroblasts. J. Cell Biol. **36**:197–230.
199. **Silverstein, S. C., R. M. Steinman, and Z. A. Cohn.** 1977. Endocytosis. Annu. Rev. Biochem. **46**:669–722.
200. **Simpson, L. L.** 1981. The origin, structure, and pharmacological activity of botulinum toxin. Pharmacol. Rev. **33**:155–188.
201. **Simpson, L. L.** 1982. The interaction between aminoquinolines and presynaptically acting neurotoxins. J. Pharmacol. Exp. Ther. **222**:43–48.
202. **Singer, S. J., J. F. Ash, L. Y. W. Bourguignon, M. H. Haggeness, and D. Louvard.** 1978. Transmembrane interactions and the mechanism of transport of proteins across membranes. J. Supramol. Struct. **9**:373–389.
203. **Sokol, P. A., B. H. Iglewski, T. A. Hagar, J. C. Sadoff, A. Cross, A. McManus, B. F. Farber, and W. J. Iglewski.** 1981. Production of exoenzyme S by clinical isolates of *Pseudomonas aeruginosa*. Infect. Immun. **34**:147–153.
204. **Steinman, R. M., S. E. Brodie, and Z. A. Cohn.** 1976. Membrane flow during pinocytosis. A stereologic analysis. J. Cell Biol. **68**:665–687.
205. **Sternberger, L. A.** 1973. Enzyme immunocytochemistry, electron microscopy of enzymes, principles and methods, p. 150–192. D. Van Nostrand/Reinhold Co., Florence, Ky.
206. **Stockel, K., G. Guroff, M. E. Schwab, and H. Thoenen.** 1977. Role of gangliosides in the uptake and retrograde axonal transport of cholera and tetanus toxin as compared to nerve growth factor and wheat germ agglutinin. Brain Res. **132**:273–185.
207. **Stockel, K., M. E. Schwab, and H. Thoenen.** 1975. Comparison between the retrograde axonal transport of nerve growth factor and tetanus toxin in motor, sensory and adrenergic neurons. Brain Res. **99**:1–16.
208. **Strauss, A. W., C. D. Bennett, A. M. Donahue, J. A. Rodkey, and A. W. Alberts.** 1977. Rat liver pre-proalbumin: complete amino acid sequence of the pre-piece. J. Biol. Chem. **252**:6846–6855.
209. **Sugiyama, H.** 1980. *Clostridium botulinum* neurotoxin. Microbiol. Rev. **44**:419–448.
210. **Tamura, M., K. Nogimori, S. Murai, M. Yajima, K. Ito, T. Katada, M. Ui, and S. Ishii.** 1982. Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A-B model. Biochemistry **21**:5516–5522.
211. **Tamura, M., K. Nogimori, M. Yajima, K. Ase, and M. Ui.** 1983. A role of the B-oligomer moiety of islet-activating protein, pertussis toxin, in development of biological effects on intact cells. J. Biol. Chem. **258**:6756–6761.
212. **Tapper, M. L., and D. Armstrong.** 1974. Bacteremia due to *Pseudomonas aeruginosa* complicating neoplastic disease: a progress report. J. Infect. Dis. **130**:(Suppl.):S14–S23.
213. **Thompson, M. R., M. S. Steinberg, P. Gemski, S. B. Formal, and B. P. Doctor.** 1976. Inhibition of *in vitro* protein synthesis by *Shigella dysenteriae* 1 toxin. Biochem. Biophys. Res. Commun. **71**:783–788.
214. **Tietze, C., P. Schlesinger, and P. Stahl.** 1980. Chloroquine and ammonium ion inhibit receptor-mediated endocytosis of mannose-glycoconjugates by macrophages: apparent inhibition of receptor cycling. Biochem. Biophys. Res. Commun. **93**:1–8.
215. **Tosteson, M. T., and D. C. Tosteson.** 1978. Bilayers containing gangliosides develop channels when exposed to cholera toxin. Nature (London) **275**:142–144.
216. **Tycko, B., and F. R. Maxfield.** 1982. Rapid modification of

endocytic vesicles containing alpha-2 macroglobulin. Cell **28**:643–651.

217. **Van Ness, B. G., B. Barrowclougii, and J. W. Bodley.** 1980. Recognition of elongation factor 2 by diphtheria toxin is not solely defined by the presence of diphthamide. FEBS Lett. **120**:4–6.
218. **Van Ness, B. G., J. B. Howard, and J. W. Bodley.** 1980. ADP-ribosylation of elongation factor 2 by diphtheria toxin. J. Biol. Chem. **255**:10710–10716.
219. **Vasil, M. L., and B. H. Iglewski.** 1978. Comparative toxicities of diphtherial toxin and *Pseudomonas aeruginosa* exotoxin A: evidence for different cell receptors. J. Gen. Microbiol. **108**:333–337.
220. **Vasil, M. L., D. Kabat, and B. H. Iglewski.** 1977. Structure-activity relationships of an exotoxin of *Pseudomonas aeruginosa*. Infect. Immun. **16**:353–361.
221. **Wendon, L. M. B., and D. M. Gill.** 1982. Tetanus toxin action on cultured nerve cells: does it modify a neuronal protein? Brain Res. **238**:292–297.
222. **White, J., and A. Helenius.** 1980. pH-dependent fusion between the Semliki Forest virus membrane and liposomes. Proc. Natl. Acad. Sci. U.S.A. **77**:3273–3277.
223. **Wibo, M., and B. Poole.** 1974. Protein degradation in cultured cells. II. The uptake of chloroquine by rat fibroblasts and the inhibition of cellular protein degradation and cathepsin B1. J. Cell Biol. **63**:430–440.
224. **Willingham, M. C., F. R. Maxfield, and I. H. Pastan.** 1979. Alpha-2 macroglobulin binding to the plasma membrane of cultured fibroblasts. Diffuse binding followed by clustering into coated regions. J. Cell Biol. **82**:614–625.
225. **Willingham, M. C., and I. H. Pastan.** 1981. Receptor-mediated endocytosis of hormones in cultured fibroblasts, p. 135–148. *In* J. L. Middlebrook and L. D. Kohn (ed.), Receptor-mediated binding and internalization of toxins and hormones. Academic Press, Inc., New York.
226. **Wolff, J., G. H. Cook, A. B. Goldhammer, and S. A. Berkowitz.** 1980. Calmodulin activates prokaryotic adenylate cyclase. Proc. Natl. Acad. Sci. U.S.A. **77**:3841–3844.
227. **Wolstenholme, J., R. D. Burgoyne, and J. Stephen.** 1977. Studies on the $MgSO_4$-induced cytoplasmic uptake of proteins by cells in culture. Exp. Cell Res. **104**:377–388.
228. **Yamaizumi, M., E. Mekada, T. Uchida, and Y. Okada.** 1978. One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. Cell **15**:245–250.
229. **Yamaizumi, M., T. Uchida, K. Takamatsu, and Y. Okada.** 1982. Intracellular stability of diphtheria toxin fragment A in the presence and absence of anti-fragment A antibody. Proc. Natl. Acad. Sci. U.S.A. **79**:461–465.
230. **Yavin, E., Z. Yavin, W. H. Habig, M. C. Hardegree, and L. D. Kohn.** 1981. Tetanus toxin association with developing neuronal cell cultures. Kinetic parameters and evidence for ganglioside-mediated internalization. J. Biol. Chem. **256**:7014–7022.
231. **Youngdahl-Turner, P., I. S. Mellman, R. H. Allen, and L. E. Rosenberg.** 1979. Protein-mediated vitamin uptake. Adsorptive endocytosis of the transcobalamin II-cobalamin complex by cultured human fibroblasts. Exp. Cell Res. **118**:127–134.