THE ISOLATION AND CHARACTERIZATION OF BHK AND PyBHK CELLS HIGHLY RESISTANT TO DIPHTHERIA TOXIN: EF-2 MUTANTS

by

Thomas A. Hager

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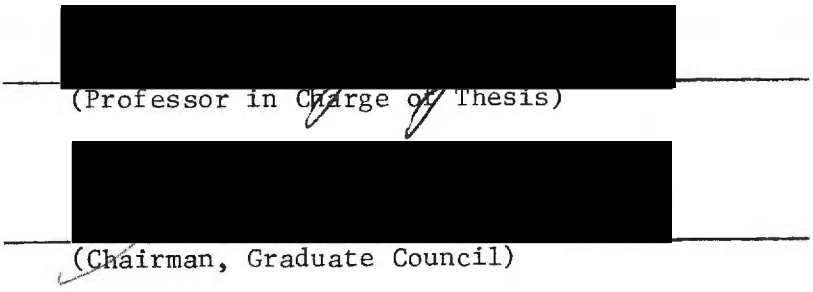

(Professor in Charge of Thesis)

(Chairman, Graduate Council)

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STATEMENT OF THE PROBLEM

Diphtheria toxin is one of the most extensively studied of the bacterial exotoxins, and one whose mode of action is best understood. A number of questions still remain to be answered, however, notably concerning the interaction of the toxin with the cell surface, the nature of postulated toxin-specific receptors, and the method by which the enzymatically active portion of the toxin molecule is transported across the plasma membrane. To investigate these questions it would be of interest to be able to compare two cell lines which differed only in their sensitivity to diphtheria toxin. Towards this end attempts have been made by at least two groups to select for mutant progeny of toxin sensitive parental cells which display a resistance to the toxin (46-51, 73).

This thesis describes the isolation in our laboratory of two such diphtheria toxin resistant cell lines from sensitive parental cells, the characterization of these new cell lines, and an investigation into the precise nature of the means by which this resistance is achieved.

INTRODUCTION

Diphtheria Toxin: In 1884, Loeffler, upon the observation that a localized infection of Corynebacterium diphtheriae produced widespread tissue damage, first made the observation that the clinical symptoms of diphtheria might be caused by a substance released from the bacteria (41). Four years later Roux and Yersin confirmed this by showing that culture filtrates of the disease producing bacillus could cause pathological changes and death in experimental animals with a pattern similar to that in a natural infection (63).

It was not until 1936 that this toxic product was purified and determined to be a protein (23). In 1951 it was discovered that the toxin was produced only by strains of C. diphtheriae lysogenized by certain temperate bacteriophages, such as β^+, which carry the tox^+ gene (25). The subsequent discovery of mutant β^+-phage which code for altered proteins antigenically similar to diphtheria toxin (CRMs) made it clear that the phage tox^+ gene actually codes for the production of toxin itself, rather than somehow turning on a host structural gene (72). Diphtheria toxin (D.T.) then is actually a phage protein. The bacterial host, however, does exert considerable control over the expression of the tox^+ gene. Different strains of C. diphtheriae lysogenized by the same tox^+ β- phage can vary greatly in the optimal yields of toxin produced (56). Additionally, bacterial iron levels have been shown to be important in toxin production; in most strains the highest toxin yields are reached only in the terminal stages of log growth when iron becomes limiting, and the levels of iron in the bacterium begin to decline (60). The mechanism by which this occurs has been investigated by Murphy et al. (55) whose results indicated there might be a repressor protein which

inhibits toxin production only in the presence of iron, presumably by acting on the operator portion of the tox^+ gene.

Diphtheria toxin is released by the bacterium as a single peptide chain of 62,000 daltons, and contains two disulfide bonds. One of these disulfides subtends an arginine rich loop of fourteen amino acids which is extremely sensitive to proteolysis. A break at this point in the molecule produces "nicked" toxin, consisting of two fragments joined by a single disulfide bridge. Exposure of nicked toxin to reducing agents breaks the disulfide bond, and the molecule separates into two large fragments, termed A and B.

Purified whole diphtheria toxin is extremely toxic to most eucaryotes, with a lethal dose consisting of only 50-100 ng/kg body weight in sensitive animals, including unimmunized humans (31). Some species however, notably rats and mice, require doses of toxin up to five orders of magnitude higher to achieve the same effect (39). Diphtheria toxin has no similar effect on procaryotes. Cell lines in culture from different species have been found to respond to toxin in a manner mirroring that found in the whole animal, i.e. mouse cell lines require much higher doses of toxin than human or hamster cells (45,14).

An early clue as to what the mechanism of action of the toxin might be was found by Strauss and Hendee in 1959 (68). They reported that protein synthesis in HeLa cell cultures was halted within 1.5 hours after exposure to the cells to lethal levels of D.T. Subsequent studies on the effect of the toxin in cell-free systems revealed that a low molecular weight dialyzable component of cell extracts was required for protein inhibition to take place; this component was determined to be NAD^+ (17). In 1967 Collier found that elongation factor 2 (EF-2) was

totally inactivated by reaction with toxin and NAD^+ (13). The inactivation of EF-2 has since been shown to be the cause of D.T. modulated inhibition of protein synthesis, and the major biochemical lesion of the toxin (14).

A theory of the means by which toxin inactivates EF-2, the action of the toxin at the molecular level, was first postulated by Honjo et al. in 1968 (32). They added NAD^+ radiolabeled in various parts of the molecule to cell extracts, and found that in the presence of toxin all portions of the NAD^+ molecule, with the exception of the nicotinamide moiety, were stably attached to EF-2. Their conclusion was that the adenosine diphosphate ribose (ADPR) portion of NAD^+ was covalently attached to EF-2 in the presence of toxin according to the formula:

$$NAD^+ + \text{EF-2} \rightleftharpoons \text{ADPREF-2} + \text{Nicotinamide} + H^+$$

This has since been confirmed in a number of laboratories.

The complete toxin molecule, although toxic for animals and in whole cell studies, is not itself enzymatically active (27,33). It was found by Collier and Cole in 1969 that enzymatic activity is dependent upon the separation of Fragments A and B (15). Early cell-free studies showing ADP-ribosylating abilities of whole toxin were probably due to varying amounts of nicked toxin present in all toxin preparations, coupled with reduction by thiols which are routinely added to such systems.

Fragment A (M.W. 24,000), although less than 0.01% as toxic as intact toxin in whole cell studies (50), is the enzymatically active portion of D.T. This is the NH_2 terminal fragment of the whole toxin molecule, and it is characterized by its hydrophilic, polar nature, and its stability. Fragment A retains its ADP-ribosylating ability even after exposure to temperatures of 100C or to pH values of two or twelve for several minutes (21). It is also quite resistant to proteolytic breakdown, expecially in the presence of NAD^+ (38). The complete amino acid sequence of Fragment A has been determined, there are no unusual amino acids (20). The exact nature of the active site in Fragment A is unknown; however, it has been reported that modification of a single tryptophan or tyrosine residue results in a loss of enzymatic activity (6,43).

The COOH terminal portion of whole toxin, Fragment B (M.W.38,000), is by contrast extremely unstable. The tendency of this fragment to aggregate, its insolubility in most buffers at neutral pH, and its sensitivity to proteolytic agents have hampered its study (56).

Since whole toxin is enzymatically inactive, intoxication of whole cells must involve the splitting of toxin into its A and B moieties, and the transport of Fragment A into the cell cytoplasm. The evidence at present indicates that this occurs via the following sequence of events:

(1) The whole toxin molecule reversibly binds, through its B portion, to specific sites on the cell surface. The evidence for this comes from several sources. First, a mutant form of D.T., CRM 197, containing a functional Fragment B but an inactive Fragment A, competitively inhibits the action of normal toxin on whole cells while having no

effect in cell-free assays (36). Second, cell lines derived from mice are up to 5,000 times more resistant to toxin in whole cell studies than are human cells, yet in cell-free studies both cell types are equally susceptible to Fragment A induced ADP-ribosylation (45). The simplest explanation of these two observations is that there are a limited number of specific receptors on the surface of toxin sensitive cells which bind Fragment B: these receptors are competed for by CRM 197 and are far less numerous or nonexistent on the toxin resistant mouse cell surface. A recent study measuring specific and nonspecific uptake of radiolabeled toxin has estimated that there are 4,000 such receptors on D.T. sensitive HeLa cells, and none on mouse L-cells (9). Although the evidence at present favors the existence of toxin-specific receptors in sensitive cells, the receptors themselves have never been isolated.

(2) Once the toxin is bound to the cell membrane, Fragment A must be released and transported across the lipid bilayer. The means by which the peptide and disulfide bonds linking fragments A and B are broken are not completely clear. It is believed that whole toxin may be nicked by cell-associated proteases involved in protein turnover (13). It appears that the disulfide bond is then cleaved at the inner surface of the plasma membrane by an as yet undetermined reducing agent (9). Fragment A is then released into the cell cytoplasm, while all or most of Fragment B remains associated with the membrane (9). The means by which Fragment A crosses the membrane is still under lively discussion, however it appears that there are two independent mechanisms. The first involves the toxin receptors, is highly specific, is unaffected by all inhibitors of energy metabolism and uncoupling agents

(with the exception of NaF (22,51)) and may involve the aid of a hydrophobic region of Fragment B (18,64). The second means is by classical endocytosis, this is nonspecific and occurs in both sensitive and resistant cells (8,9,64). Endocytotically internalized D.T. is generally degraded, and is believed to contribute to cellular intoxication only at very high toxin concentrations (9,64).

(3) Having reached the cytosol, Fragment A inactivates the cellular EF-2 and halts protein synthesis. The ADP-ribosylation of EF-2 begins within minutes of the entry of Fragment A (29), although there is generally at least a one hour lag period before a noticeable decrease in protein synthesis can be detected. This lag period is thought to be due both to the time required for transport across the membrane, and to the fact that EF-2 is in excess in the cell and must be cycled off of the ribosomes to become available for ADP-ribosylation (26,28). It has been estimated by two methods that as little as one molecule of internalized Fragment A is sufficient to kill a cell (22, 37,71).

The enzymatic properties of purified Fragment A have been studied extensively. It is highly specific in terms of substrate. NAD^+ is the only naturally occurring molecule capable of donating ADPR in the Fragment A catalyzed reaction (33,38). The specificity for EF-2 is also marked. EF-2 from all eucaryotes serves as the ADPR acceptor, while its procaryotic counterpart, EF-G, does not. Only at extremely high concentrations of Fragment A does even limited ADP-ribosylation of any other proteins take place. Under physiological conditions the ADP-ribosylation of EF-2 is virtually irreversible, with a K value of 10^4. In vitro, however, the removal of NAD^+ and the addition of nico-

tinamide can inhibit the reaction by acting as a competitor for the NAD^+ binding site, as can adenine. This indicates that both these moieties of NAD^+ are involved in binding to Fragment A (56). Other inhibitors of the reaction include ribosomes, which bind EF-2 thereby rendering it immune to ADP-ribosylation (28), and high salt levels (16). Fragment A does not require free sulfhydryl groups for activity (22), however, the EF-2 molecule does in order to bind to Fragment A effectively, therefore thiols are usually required for ADP-ribosylation to take place (62). Fragment A binds NAD^+ alone, but does not bind EF-2 effectively in the absence of NAD^+. There is now strong evidence that a ternary EF-2-Fragment A-NAD^+ complex is formed sequentially, with Fragment A first binding NAD^+, and then EF-2 (11). The NAD^+ is then split, ADPR is attached to EF-2, and the substrates are released, freeing Fragment A for another round (14,56).

Fragment A can also catalyze the following reaction:

$$NAD^+ + HOH \rightleftharpoons ADPR + Nicotinamide + H^+$$

This reaction, termed NAD^+ glycohydrolysis, has been observed in the laboratory, but due to the much higher affinity of EF-2 for the HOH binding site on Fragment A, is considered to be of little importance in normal intoxication (38).

Elongation Factor 2: Schweet and Arlinghaus were the first, in 1964, to isolate two soluble factors required for peptide chain elongation in rabbit reticulocytes (2). They originally termed these factors aminoacyl transferase 1 and 2; the nomenclature has since been changed to elongation factor 1 and 2 (EF-1 and EF-2). These two

eucaryotic enzymes were found to correlate functionally with the procaryotic factors EF-T and EF-G (24). EF-1 is required for the binding of aminoacyl-tRNA to the ribosomal A site, while EF-2 catalyzes the transfer of peptidyl-tRNA and mRNA from the A site to the P site with concurrent release of deacylated tRNA from the P site, and the hydrolysis of GTP to GDP (74). This process is termed translocation.

Since its discovery, EF-2 has been purified from a number of sources, including rabbit reticulocytes (1,42,44), rat liver (18,58), and calf brain (12). The availability of purified EF-2 allowed detailed investigation of its physical, chemical, and enzymatic properties. EF-2 preparations from various eucaryotic sources have been found to be very similar thus far. The enzyme is a single polypeptide chain with a molecular weight of 100,000; a smaller value of 70,000 has been reported for yeast EF-2 (59,70). Highly purified rat liver EF-2 contains 18 sulfhydryls and 2 disulfides per mole (62), and requires free thiols for enzymatic activity (65). The primary amino acid sequence of EF-2 has not yet been determined, however the overall mole percent amino acid composition has been reported (42). The molecule is quite acidic, with aspartic acid and glutamic acid comprising nearly 20% of the total amino acid makeup.

EF-2 has been found to comprise approximately 1% of the total soluble protein in rat liver (58). In various cells the amount of EF-2 appears to vary in direct proportion to the number of ribosomes. An early investigation indicated there were up to four molecules of EF-2 per ribosome (28), however more sensitive methods have shown the ratio of EF-2 to ribosomes to be 1.2 to 1 in a number of cell types (26). EF-2 binds to ribosomes during the process of translocation,

and in cell lysates the ribosomally bound factor has been reported to comprise up to 90% of the total cellular EF-2 with 10% free in the cytoplasm (28). Smulson and Rideau found that only 13% of the EF-2 was ribosomally bound in HeLa cells, this increased to 29% if the cells were starved (67). The discrepancy in these two studies may be due to the fact that higher concentrations of salt were used in the lysing buffer of Smulson's group; increasing salt releases ribosomally bound EF-2 (74). EDTA and guanine nucleotides, either GTP or GDP, also free EF-2 from ribosomes (28).

The details of the interaction of EF-2 with ribosomes and GTP at the molecular level and the means by which it catalyzes translocation are presently under study. It has been known for some time that EF-2 could bind to ribosomes in vitro in the presence of GTP (66). Mixing GTP or GDP with EF-2 and ribosomes results in a ternary EF-2-ribosome-GDP complex; the fact that GDP can substitute for GTP in this case indicates that GTP hydrolysis is not necessary to bind EF-2 to the ribosome (5). EF-2 also binds GTP alone, and with a lesser affinity, GDP (30). The ribosomal EF-2 binding site has been found to lie on the 60S subunit (69). Free sulfhydryl groups must be present on both EF-2 and ribosomes for this binding to take place; EF-2 does not require free sulfhydryls to bind GTP or GDP (74). The hydrolysis of GTP or GDP is necessary for translocation to take place, however the actual mechanism of translocation is unknown (3).

EF-2 is generally assayed for by three methods. The first measures EF-2 stimulation of poly(U) directed polyphenylalanine synthesis in an *in vitro* protein synthesizing system complete except for EF-2 (19). The second assay measures ribosome dependent GTP hydrolysis (24),

this is used less often due to the discovery of a GTPase activity separate from EF-2 which comigrates with it through most purification steps (30). The simplest and most quantitative method of assaying for EF-2 was developed by Gill and Dinius (26). This system utilizes the transfer of radiolabeled ADPR from NAD^+ to EF-2 in the presence of diphtheria toxin Fragment A, and is described in detail in the Materials and Methods section of this thesis.

Adenosine diphosphate ribosylated EF-2 (ADPREF-2) is formed when diphtheria toxin, Fragment A, NAD^+, and EF-2 interact; this is discussed in the diphtheria toxin section of this introduction. Some of the properties of ADPREF-2 have been reported by several groups. The ADPR molecule is covalently attached to EF-2 through its nicotinamide mononucleotide ribose moiety; ribose 5'-monophosphate remains attached to EF-2 after adenosine 5'monophosphate is released by treatment with snake venom phosphodiesterase (31,32). The free energy of hydrolysis of the link between ADPR and EF-2 has been estimated to be in the area of 4 kcal/mole at physiological pH (33). Only one ADPR is attached to each EF-2 (58,62). A fifteen amino acid tryptic peptide containing the ADPR attachment site was isolated by Robinson et al. in 1974 (61). Its sequence is Phe-Asp-Val-His-Asp-Val-Thr-Leu-His-Ala-Asp-Ala-Ile-X-Arg. ADPR is linked to the undefined, weakly basic amino acid X. There has been one case reported in which a lower molecular weight degradation product of EF-2 could be ADP-ribosylated; the entire molecule is not, therefore, required for interaction with Fragment A (18).

ADPREF-2 once formed is incapable of catalyzing GTP hydrolysis and translocation (74). Why this occurs is not understood. An early paper

by Montanaro et al. reported that ADPREF-2 could bind GTP almost as effectively as unmodified EF-2, but was not able to bind to ribosomes (54). Bermek (4) and Chuang et al. (12) have since found that ADPREF-2 does bind to ribosomes in the presence of GTP, and that native EF-2 competes with ADPREF-2 for a common site on the 60S subunit. Montanaro confirmed this in a study utilizing ricin, a toxic plant lectin which specifically inactivates the EF-2 binding site on the ribosome. Ricin inactivation of ribosomes was found to inhibit the binding of EF-2 and ADPREF-2 equally (53). The conclusion from these studies is that ADPREF-2 and EF-2 have the same or overlapping ribosomal binding sites.

Cellular Resistance to Diphtheria Intoxication: As has been mentioned, certain species and the cell lines derived from them are hundreds to thousands of times more resistant to diphtheria intoxication than D.T. sensitive lines when assayed in whole-cell or whole animal studies. Cell extracts of such naturally occurring resistant cells are found to be just as sensitive to ADP-ribosylation of EF-2 (45,46). It appears that there is a barrier of some sort preventing the uptake of Fragment A in the resistant cells; Moehring and Moehring have suggested that this may be due to a lack of toxin-specific receptors (46). These naturally occurring resistant cells are killed by high levels of toxin uptake (64). This has been supported by data obtained from studies following the fate of ^{125}I labeled toxin added to cells (7).

Toxin resistant cell lines have also been developed *in vitro* from toxin sensitive parental lines. Venter and Kaplan (73) exposed HeLa cells a single time to various levels of toxin for 96 hours, and found that cells surviving the procedure exhibited a range of increased

resistance to intoxication, with the most resistant cells requiring 10 times more toxin to inhibit protein synthesis than the parental cells. Moehring and Moehring have extensively studied diphtheria toxin resistant KB cells (from human epidermoid carcinoma) which they isolated from toxin sensitive parental cells by a sequence of exposures to increasing toxin concentrations (47). They observed a range of resistance in different clonal isolates which led them to suggest that the development of resistance may be a multistep process. The most resistant mutant KB cells required 10^5 - 10^6 times as much toxin as the parental lines to inhibit protein synthesis to an equal extent. Interestingly, these toxin resistant mutants also displayed an increased resistance to certain viruses (48,49). All of these toxin resistant mutants resemble naturally occurring resistant cells in that high concentrations of toxin are able to inhibit protein synthesis, and extracts from the resistent cells react to toxin exactly as do the sensitive parental extracts. The resistance therefore appears to be mediated at the cell membrane; these cells have been termed permeability variants (47,57).

Moehring and Moehring also isolated various clones of Chinese Hamster Ovary (CHO) cells surviving a single large dose of toxin (51). Some of these clones were determined to be permeability variants with a range of increased resistance to toxin, the maximum being about 10^5 greater than the parental cells. However, clones of CHO cells were also recovered which were unaffected in both whole cell and cell-free assays by high concentrations of toxin. It was found that the EF-2 of these cells could not be ADP-ribosylated, and the evidence indicated that there was a mutation at the level of EF-2 which conferred the resistance of toxin. These cells were termed "presumptive translational mutants."

MATERIALS AND METHODS

1) Cell and Culture Conditions:

Stocks of baby hamster kidney (BHK) and polyoma virus transformed baby hamster kidney (PyBHK) cells were provided by Jules Hallum. All cells were maintained in Eagle's Minimum Essential Medium (MEM) with Hank's Salts, supplemented with 10% fetal calf serum, and 50 ug/ml gentamicin. Cells were incubated at 37 C in a 5% CO_2 atmosphere.

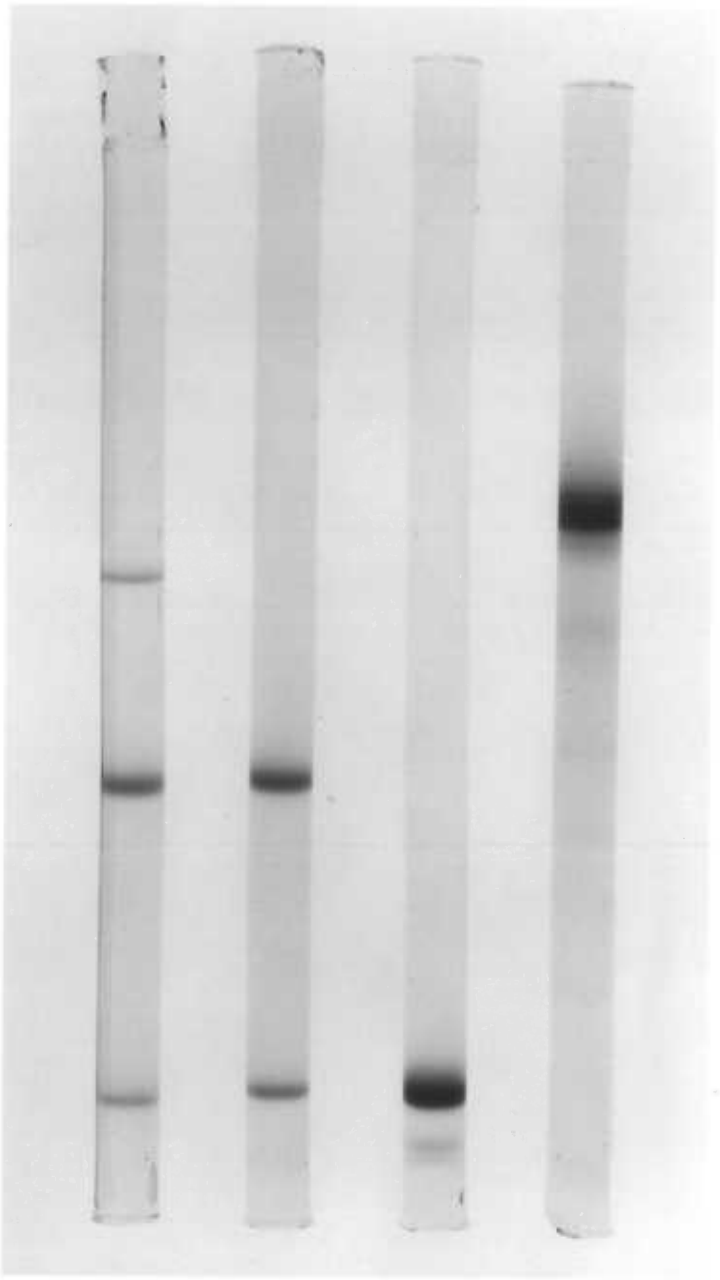
Cells were lifted by removing the growth medium and washing the cells twice with 0.25% trypsin in versene (0.5 mM EDTA, 140 mM NACl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1 mM glucose, 1.5 mM KH_2HPO_4), the washed cells were then incubated at 37 C until all cells were lifted. Cells were then suspended in growth medium and counted on a hemocytometer to determine cell numbers for assays or plating.

2) Toxins:

Diphtheria Toxin:

Diphtheria toxin used in the initial isolation of resistant cell lines was obtained from Connaught Laboratories (Lot D-290, 3 mg/ml). This preparation was subsequently found to contain a contaminant which inhibited protein synthesis in toxin resistant cells when applied at high concentration in our inhibition of protein synthesis assay. therefore this toxin was replaced by highly purified diphtheria toxin (13 mg/ml) obtained from Barbara H. Iglewski. This purified toxin migrated on SDS polyacrylamide gels in the presence of reducing agents as three bands correlating with whole toxin and fragments A and B. (Fig. 1), On a mg protein basis the Connaught toxin and the purified toxin were found to have approximately equal toxicity for sensitive cells; and the purified

Fig. 1: SDS-Polyacrilamide Gel Electrophoresis of Toxins. Gels were run as described in Materials and Methods. 50 ug total protein was loaded on each gel. Left to Right: Purified diphtheria toxin; nicked and reduced purified D.T.; D.T. Fragment A; Pseudomonas aeruginosa Exotoxin A.



toxin did not inhibit protein synthesis at high concentrations in diphtheria toxin resistant cells. Purified toxin was used, therefore in all inhibition of protein synthesis assays.

Diphtheria Toxin Fragment A:

Fragment A for cell-free ADP-ribosylation experiments was prepared from 10 ml Connaught diphtheria toxin (Lot D-290, 3 mg/ml) by adding 10 ug/ml trypsin, and dithiothreitol (DTT) to a final concentration of 0.05 M. This solution was vortexed and incubated at 37 C for ten minutes. 30 ug/ml soybean trypsin inhibitor was then added, dissolved by vortexing, and incubated an additional ten minutes at 37 C. This was then cooled to room temperature, and precipitating Fragment B removed by centrifugation at 5,000 x g for ten minutes in a clinical centrifuge.

2.0 mls of the supernatant from the above procedure was loaded onto a 2.5 x 30 cm Sephadex G-100 column and eluted with buffer (50 mM Tris-HCl, pH 8.0; 1 mM EDTA, 1 mM DTT). 1 ml fractions were collected. Fractions containing ADP-ribosylating activity were pooled and dialyzed against 2 L distilled H_2O overnight, lyopholized until dry, and resuspended in distilled H_2O to a concentration of 2.0 mg/ml. Electrophoresis of this preparation on 10% SDS polyacrylamide gels showed a heavy band comigrating with Fragment A, and several faint bands (Fig. 1).

Pseudomonas aeruginosa exotoxin A:

Pseudomonas aeruginosa exotoxin A (PAT) was provided by Barbara H. Iglewski. This purified toxin produced a single major band

upon polyacrilamide gel electrophoresis (Fig. 1); its concentration was determined to be 2 mg protein per ml (in 10 mM Tris, pH 8.0) and had a mouse median lethal dose of 0.2 ug/20 g mouse when injected intraperitoneally.

Activated PAT for use in cell-free ADP-ribosylation experiments was prepared by mixing equal volumes of PAT and a solution of 0.1 g DTT in 0.5 ml 8 M urea. This was incubated at 24 C for 15 minutes, and used immediately.

3) Radioactive Reagents:

^{14}C-NAD labeled in the adenine moiety (25 uCi/ml, spec. act. 302 mCi/mmole); ^{3}H-Leucine (1.0 mCi/ml, spec. act. 105 Ci/mmole); and ^{3}H mixed amino acids (1.0 mCi/ml) were obtained from the Amersham Corporation.

4) Isolation of Diphtheria Toxin Resistant Cell Lines:

Cells were seeded in 25 cm^2 tissue culture flasks at a concentration of 1 X 10^6 cells per flask, and allowed to adhere overnight. The medium was then replaced with 9.6 ml fresh medium, with 0.4 ml various toxin dilutions as indicated in the legends to figures. The toxin was removed after various time periods (generally four hours unless otherwise indicated), the cells washed twice with medium, fresh medium added, and the cells incubated. Dead cells were washed off every other day, and the medium replaced. After 5-7 days growth, flasks containing 1-20 surviving colonies of cells were kept, the others discarded. These surviving cells were grown, stocks frozen at -70 C in growth medium containing 20% fetal calf serum and 10% glycerol at a concentration of 10^7 cells/ml; these cells could then be re-exposed

to higher concentrations of toxin. The final passaged cells, BHKR and PyBHKR, were grown from single cells by cloning in 96-well plates. This involved diluting lifted cells in growth medium to a concentration of 10 cells/ml, and adding 0.2 ml of this suspension to each well of a 96-well plate. After five hours incubation to allow settling, a microscopic inspection of each well was made, and wells containing a single cell were marked. Incubation continued for five days with medium changes every two days, at which time the cloned colonies were lifted and transferred to 25 cm^2 flasks for further growth.

5) Cloning in Soft Agar:

7.0 ml of 0.5% agar in growth medium containing 2% fetal calf serum was placed in each of twelve 60 X 15 mm tissue culture plates, and allowed to harden. This was overlaid with 1.5 ml of 0.3% agar in growth medium containing 2% fetal calf serum and 6.6 X 10^3 freshly lifted cells per ml, three plates of each of the cell types tested. The top agar layer was allowed to harden at room temperature for 15 minutes, the plates were then placed in a 37 C incubator with a 5% CO_2 atmosphere. After six days the plates were examined microscopically for cells able to clone in agar.

6) Protein Synthesis Inhibition Assay:

Cells were seeded in Linbro 6-well plates (30 mm^2 each) at a concentration of $5x10^5$ cells/plate, and allowed to attach overnight. The medium was then removed and the cells washed with medium containing 1/10 the normal concentration of amino acids. This was followed by incubation at 37 C, 5% CO_2, for three hours in a mixture of 0.8 ml of the 1/10 amino acid medium and 0.1 ml of toxin diluted in Tris-Glu buffer (25 mM Tris-HCl pH 7.2, 140 mM NACl, 5mM KCl, 0.7 mM Na_2HPO_4, 6 mM

glucose, pH adjusted to 7.4 with 1 N HCl). Control plates received no toxin. At the end of this time period 0.1 ml ^{3}H amino acids diluted to 4 uCi/ml in 1/10 amino acid medium was added to each plate, and incubation containued for two hours. The medium was then decanted and 1.0 ml 0.25% trypsin in H_2O was added per plate. After lifting, the cells were lysed by freezing at -20 C overnight, and thawing the next day. Proteins in the lysate were precipitated by the addition of trichloroacetic acid to a final concentration of 10%, heating at 90 C for 15 minutes, and cooling in an ice bath. The precipitated proteins were collected on 0.45 u Millipore filters and the incorporated radioactivity counted in a Beckman LS-200B scintillation counter. The triplicate samples were averaged and expressed in graphs as a percent of control protein synthesis.

7) Kinetics of ^{3}H Amino Acid Incorporation into Proteins:

The procedure here is basically that used in the protein synthesis inhibition assay described above; 0.9 ml 1/10 amino acid medium was added per plate, no toxin was added, and the ^{3}H amino acids were added at the beginning of the assay instead of at hour three. The uptake of label was stopped after various time periods by removing the media and lifting the cells, and the radioactivity incorporated into protein counted as outlined above.

8) Karyotypic Analysis:

Subconfluent monolayers of cells were exposed to 1 ug/ml colcemid in growth medium for four hours. Colcemid arrests the cell cycle in metaphase, and cells thus treated have a tendency to lift off of the flask. To recover these cells the medium was decanted and the cells free in the decanted medium were collected by centrifugation at

5,000 x g in a clinical centrifuge. The remaining cells were lifted as usual, and all cells were pooled in a hypotonic solution of one part growth medium and four parts distilled water for one hour. The cells were then spun down and resuspended in a fixative of one part methyl alcohol and three parts glacial acetic acid overnight at -20 C. The fixed cells were stained with Giemsa, and metaphases viewed under oil immersion.

9) Tumor Induction and Recovery of Cells:

Five week old male Golden Hamsters were inoculated subcutaneously and intrascapularly, two each with a suspension of 10^6 freshly lifted PyBHK cells in 0.5 ml growth medium, and two with an equal number of PyBHKR cells in the same manner. The hamsters carrying the PyBHK cells were caged separately from the PyBHKR inoculated animals. Visual examinations for tumor growth were made three times per week. Eight weeks post-inoculation large, palpable tumors were found on the backs of all four animals.

The hamsters were killed by breaking their necks by hand, and the tumors removed by dissection under a sterile hood. Care was taken to remove only tissue that was within the tumor mass. The tumors from each animal were handled separately. The majority of the removed tissue was immediately frozen at -70 C. The remainder was adapted to cell culture by sterilly mincing the tissue with a spatula in ten ml warm 0.25% trypsin in Tris-Glu buffer. The minced cells were then stirred for three minutes, large cell aggregates were allowed to settle out for five minutes, and the supernatant containing free cells was collected with a pasteur pipette, placed in a 50 ml centrifuge tube, and kept on

ice. This trypsinization process was repeated three times, after which the remaining large cell aggregates were discarded. The collected free cells were spun out of suspension, the Tris-Glu decanted, the cells washed in an equal volume of growth medium, centrifuged, and resuspended in growth medium containing 20% fetal calf serum. The cells were then ready to be seeded onto 25 cm^2 tissue culture flasks, $2x10^6$ cells per flask, and were thereafter grown as usual.

10) EF-2 Assay (Cell-Free ADP-Ribosylation Assay):

The method is basically that of Gill and Dinius (34), who have found that this method results in a quantitative assay of total cellular EF-2.

Washed, packed cells or frozen tumor tissue were weighed, and 0.5 g lysed in 2 ml 0.25 M sucrose either by sonication in a Biosonik IV sonicator in the case of free cells, or by twelve strokes with a hand homogenizer (Thomas) in the case of frozen tissue. Sonication was found to repture the nuclear membrane of cells leading to an increase in undesirable poly-ADP-ribosylating enzymes. Sonication was necessary, however, because it was found free cells could not be effectively lysed by the hand homogenizer. 1.5 ml of the resulting lysate was removed to a small tube to which 0.25 ml 4 M NaCl with 20 mM DTT was added, along with 0.4 g washed, activated charcoal. The mixture was shaken at 4 C for 15 minutes, then centrifuged at 27,000 rpm in a type 30 rotor in the ultracentrifuge for 65 minutes. This removed all cell debris and charcoal, with the EF-2 remaining in the supernatant. This supernatant was collected and stored at -70 C.

To assay the EF-2, 50 ul of the supernatant was added to

300 ul histamine buffer (0.11 M histamine; 90 mM Tris-HCl, pH 8.0; 70 mM DTT; 0.017 mM EDTA; pH adjusted to 8.0 by the addition of 1 N HCl) with or without the addition of Fragment A (7.5 ug/ml final concentration in the reaction mix). This was equilibrated at 37 C by placing in a waterbath for a few seconds, after which 0.125 uCi ^{14}C-NAD (4.14×10^{-4} umoles) in a volume of 5 ul was added to each tube. This was vortexed and incubated 10 minutes at 37 C. The reaction was halted by the addition of several volumes of 5% trichloroacetic acid, the proteins collected by filtration in 0.45 u Millipore filters, and the protein-associated radioactivity counted on a low background gas flow counter, Nuclear Chicago.

11) Assay for Cell-Free Protein Synthesis:

Preparation of Rabbit Reticulocyte Cell Lysate:

The procedure was basically that of Pelham and Jackson (71). One large male white rabbit was made anemic by injections of 1.2% N-acetylphenyl-hydrazine subcutaneously according to the following schedule:

Day 1: 2.0 ml

Day 2: 2.0 ml

Day 3: 1.8 ml

Day 4: 1.6 ml

Day 5: 2.0 ml

Day 6: 2.0 ml

Day 7: rest

On day 8 the rabbit was bled dry via cardiac puncture with a heparinized syringe. Care was taken to bleed the animal as sterilly as possible to reduce the chance of contaminating the reticulocytes with

RNAses. The following procedure was done in the cold:

Reticulocytes were packed by spinning at 5,000 x g in a clinical centrifuge; the serum was discarded and the cells washed three times in an equal volume of ice-cold saline solution (130 mM NaCl; 5 mM KCl; 7.5 mM $MgCl_2$). The packed cells from the final wash were then lysed in an equal volume of cold sterile distilled H_2O. The lysate was then spun at 4,000 rpm in a low speed centrifuge for 15 minutes to remove cell membranes. The supernatant from this step was aliquotted into 2 ml freezing vials, 1.8 ml/vial, and immediately frozen at -70 C.

Activation of the Cell Lysate:

To 750 ul of the cell lysate was added the following:

50 ul 10 mg/ml creatine phosphokinase

45 ul 0.3 M creatine phosphate in 10 mM Tris ph 7.5

50 ul 2 M KCl

100 ul 20 minus 1 amino acid stock (0.5 mM)

20 ul 1 M Tris-HCl, pH 7.2

5 ul 0.2 M DTT

5 ul 50 mM GTP

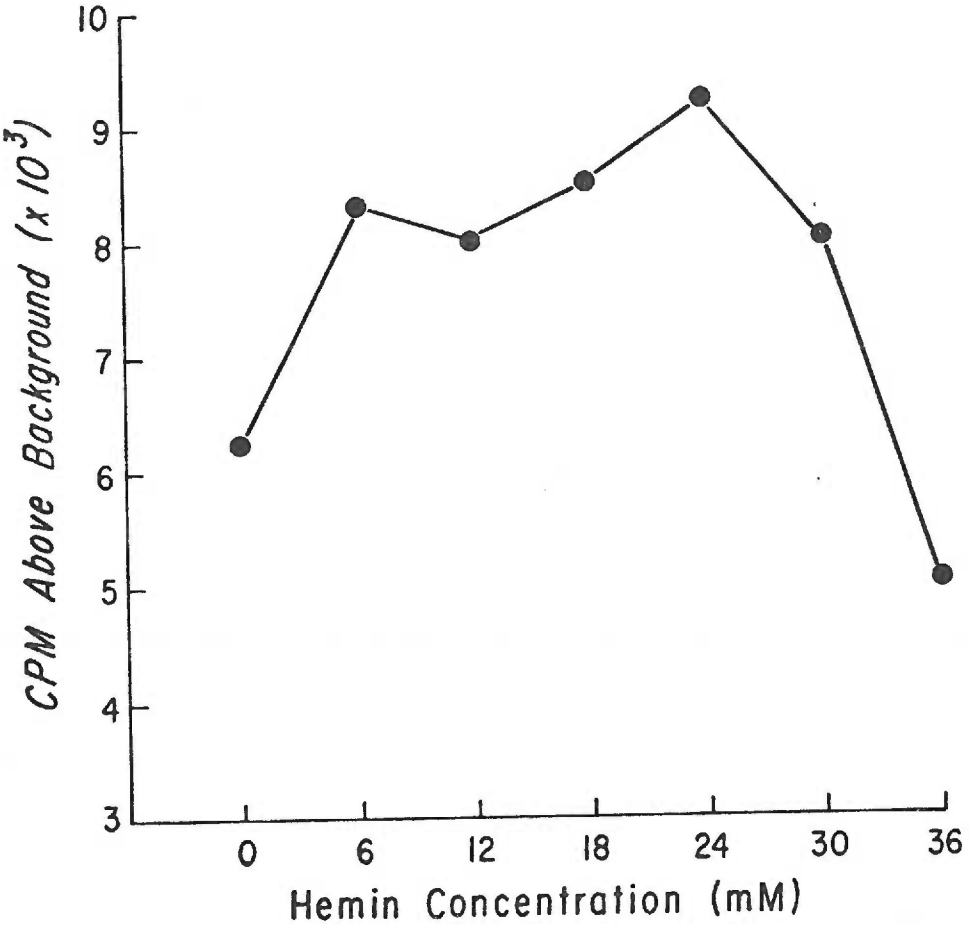
24 ul 1 mM hemin*

20 ul 50 mM $MgCl_2$*

*Optimal hemin and $MgCl_2$ concentrations must be determined for each batch of lysate; the values reported here were optimal for my system (Figs. 2,3).

The lysate is "activated" by the addition of the above components, it contains everything it needs to synthesize protein <u>in vitro</u>. This activation may be done prior to the actual assay and the

Fig. 2: Effect of Hemin Concentration on Cell-Free Protein Synthesis. The assay procedure is as described in Materials and Methods, incubation was for 1.5 hours. Mg^{++} concentration was 1 mM. Background was determined by assaying the reaction mixture at zero incubation time.


CPM Above Background (x 10³)
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7
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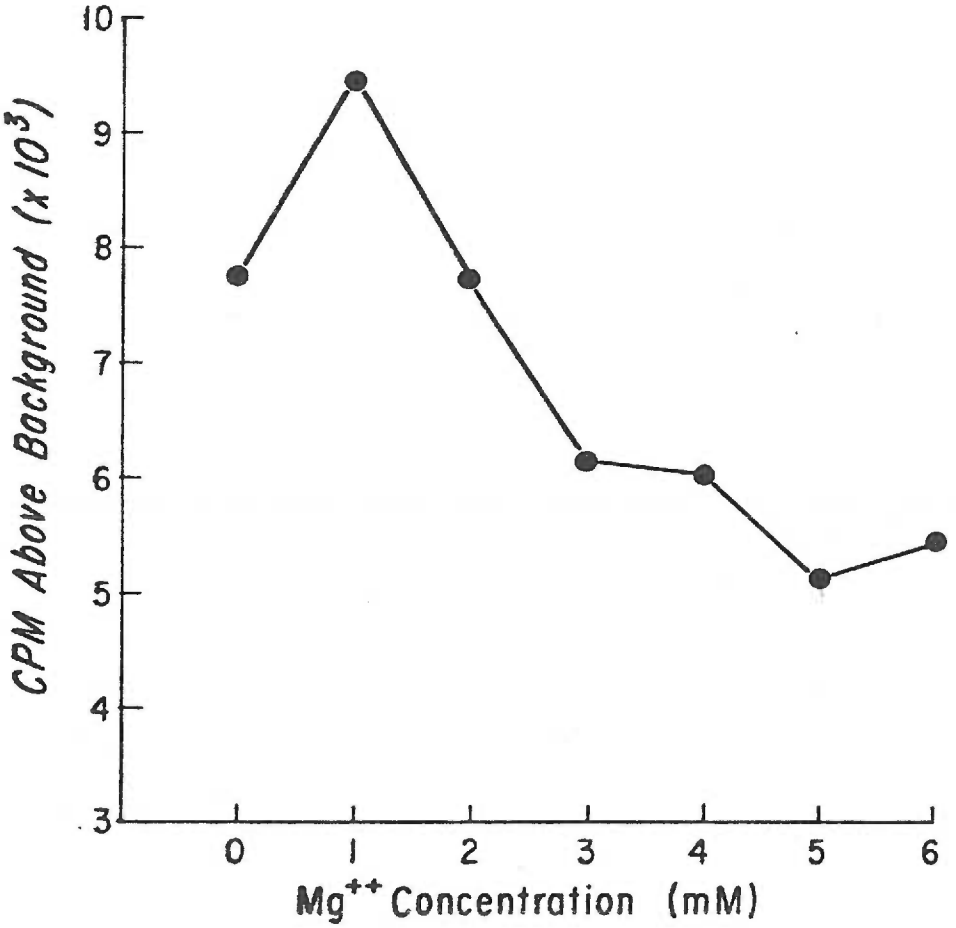
5
4
3
0
6
12
18
24
30
36
Hemin Concentration (mM)

Fig. 3: Effect of Mg^{++} Concentration on Cell-Free Protein Synthesis. The assay procedure is as described in Materials and Methods, incubation was for 1.5 hours. Hemin concentration was 24 mM. Background was determined by assaying the reaction mixture at zero incubation time.


CPM Above Background (x 10³)
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7
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5
4
3
0
1
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4
5
6
Mg++ Concentration (mM)

activated lysate stored at -70 C.

Assay Procedure:

The cell lysate should be thawed slowly and transferred to ice while there is still an ice chunk in the vial; this has been found to reduce background counts.

To 40 ul of the activated lysate was added 5 uCi ^{3}H-Leucine (4.76×10^{-4} u moles) in a volume of 5 ul; any additional components added to the system such as Fragment A were in a volume to give a final 50 ul reaction mix. All components were added with sterile tips to avoid RNAse contamination. The reaction mix was vortexed and incubated for up to three hours at 30 C.

The incorporation of ^{3}H-Leucine into protein was measured by removing 5 ul of the reaction mix at various times, and adding it to 1 ml cold H_2O. The protein was then precipitated by the addition of 1 ml 60% trichloroacetic acid, vortexing, and cooling on ice for ten minutes. This was then filtered on 0.45 u Millipore filters, dried, and counted in a Beckman LS-200B scintillation counter.

12) Determination of Protein Concentrations:

Protein concentrations were determined by the dye-binding technique of Bradford (10).

The protein reagent was prepared by dissolving 100 mg Coomasie Brilliant Blue G-250 in 50 ml 95% EtOH, adding to this 100 ml 85% H_3PO_4, and diluting to one liter with distilled H_2O. This was stored at room temperature. The reagent must be made fresh each month.

Protein concentrations were assayed by preparing samples of the unknown which contained 1 to 10 ug protein in a volume of 0.1 ml; this usually required several dilutions to be tested. At the same time

bovine serum albumin was prepared in concentrations of 2.5, 5.0, 7.5, and 10.0 ug in 0.1 ml for the standard curve. All samples were assayed in duplicate.

To each of the above samples were added 1.0 ml of the protein reagent with immediate mixing. After five minutes to allow the dye-binding reaction to go to completion, and before one hour, the optical absorbance of the samples was read at 595 nm in 1.0 ml cuvettes in a standard spectrophotometer.

Protein concentrations of the unknows were determined from the BSA standard curve.

13) SDS-Polyacrylamide Gel Electrophoresis:

The procedure is based on the discontinuous system of Laemmli (40).

Stock solutions:

A: 30 g acrylamide, 0.8 g N'-N'-bis-methylene-acrylamide made up to 100 ml with H_2O; stored dark at 2 C.

B: 18.15 g Tris Base +50 ml H_2O; adjusted to pH 8.8 with 1N HCl; made up to 100 ml with H_2O.

C: 10% SDS in H_2O.

D: 3 g Tris Base + 20 ml H_2O; adjusted to pH 6.8 with 1 N HCl; made up to 50 ml with H_2O.

Protein samples were diluted 1:1 into the following solution: 2.5 ml solution D, 4 ml solution C, 1 ml B-mercaptoethanol, 2 ml glycerol, 0.1 ml 0.1% bromphenol blue in H_2O, 0.4 ml H_2O, and heated to 100 C for two minutes in a boiling water bath. Samples generally contained 100 ug protein in a total volume of 200 ul.

7.5% separating gels were prepared by mixing 12.5 ml A, 6.25 ml B, 0.5 ml C, 12.5 ul N, N, N', N'-Tetramethylethylenediamine (TEMED), bringing the volume to 49.5 ml with H_2O, adding 0.5 ml 10% ammonium persulphate, pouring the gels, and overlaying with butanol. After polymerizing for not less than 3 hours, 3% stacking gels were prepared by mixing 1 ml A, 1.25 ml D, 0.1 ml C, 5 ul TEMED, bringing the volume to 9.9 ml with H_2O, then adding 0.1 ml 10% ammonium persulphate. 0.3 ml of this solution was poured on top of the separating gel (washed free of butanol) and overlaid with butanol. This was allowed to polymerize for not less than 30 minutes, and gels were run no later than one hour after polymerization.

After carefully washing all butanol off of the stacking gel, 100 ul of prepared protein sample was loaded. Electrophoresis was carried out at 2.5 ma per gel until the marker dye reached the bottom of the gel (approximately 3 hrs.). The electrophoresis apparatus was kept cool by water jacketing with cold tap water.

Gels were fixed overnight in destain solution (10% HAc, 25% MeOH in H_2O), stained 8 hours in "heavy stain" (25% MeOH, 10% HAc, 0.05% Coomasie Brilliant Blue R), followed by 24 hours in "light stain" (10% MeOH, 10% HAc, 0.005% Coomasie Brilliant Blue R), and destained in destain solution.

RESULTS

1) Selection of Diphtheria Toxin Resistant Cells:

BHK and PyBHK cells are very sensitive to diphtheria toxin as measured in our whole cell protein synthesis inhibition assay. A toxin concentration of less than $3x10^{-3}$ ug/ml is sufficient to cause a 50% or greater decrease in protein synthesis in our five hour assay system (Figs. 4 & 5). These parent lines were exposed to media containing diphtheria toxin in varying concentrations for four hours. The surviving cells were grown in toxin-free media and then re-exposed to greater concentrations of toxin to test for subsequent resistance of survivors to intoxication.

With BHK cells very little increase in toxin resistance was seen with cells surviving exposure to $1.2x10^{-2}$ ug/ml and $1.2x10^{-1}$ ug/ml toxin (Fig. 4). At this point increasing the amount of toxin to which the cells were exposed resulted invariably in 100% cell death. It therefore became necessary to re-expose the cells to $1.2x10^{-2}$ ug/ml toxin. A single colony of cells survived this procedure. These cells were found to have a greatly increased resistance to toxin. Single cell clones from this colony were subsequently obtained as described in Materials and Methods. and all clones tested were found to be totally resistant to intoxication in our five-hour protein inhibition assay. One clone was selected for further experimentation and termed BHKR (Fig. 4). Further exposure of BHKR cells to as much as 750 ug/ml toxin for up to 24 hours had no discernable lethal effect.

PyBHK cells reacted differently in their shift towards toxin resistance upon exposure to diphtheria toxin. Cells surviving prior exposure to $1.2x10^{-1}$ ug/ml toxin showed little increase in their resistance

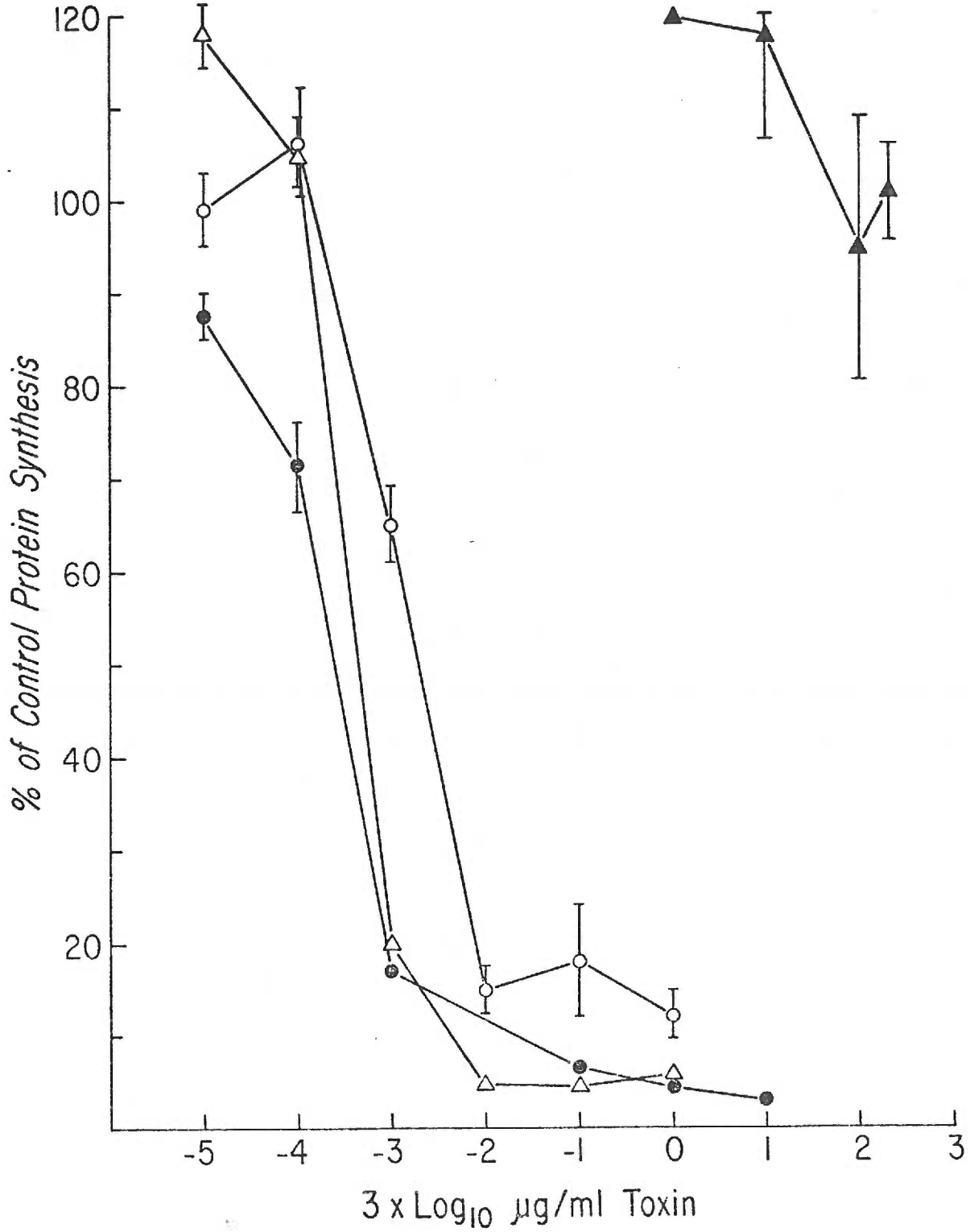
Fig. 4: Increasing resistance of BHK cells to diphtheria toxin. The assay procedure is described in Materials and Methods. Inhibition of protein synthesis by diphtheria toxin in:


, BHK parent cells;


, First passage cells surviving prior exposure to 1.2×10^{-2} ug/ml toxin;


, Second passage cells surviving prior exposure to 1.2×10^{-1} ug/ml toxin; and


, Single cell clone of third passage cells surviving prior exposure to 1.2×10^{-2} ug/ml toxin (BHKR cells).


120
100
80
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40
20
% of Control Protein Synthesis
-5
-4
-3
-2
-1
0
1
2
3
3 x Log10 µg/ml Toxin

Fig 5: Increasing resistance of PyBHK cells to diphtheria toxin. The assay procedure is as described in Materials and Methods. Inhibition of protein synthesis by diphtheria toxin in:

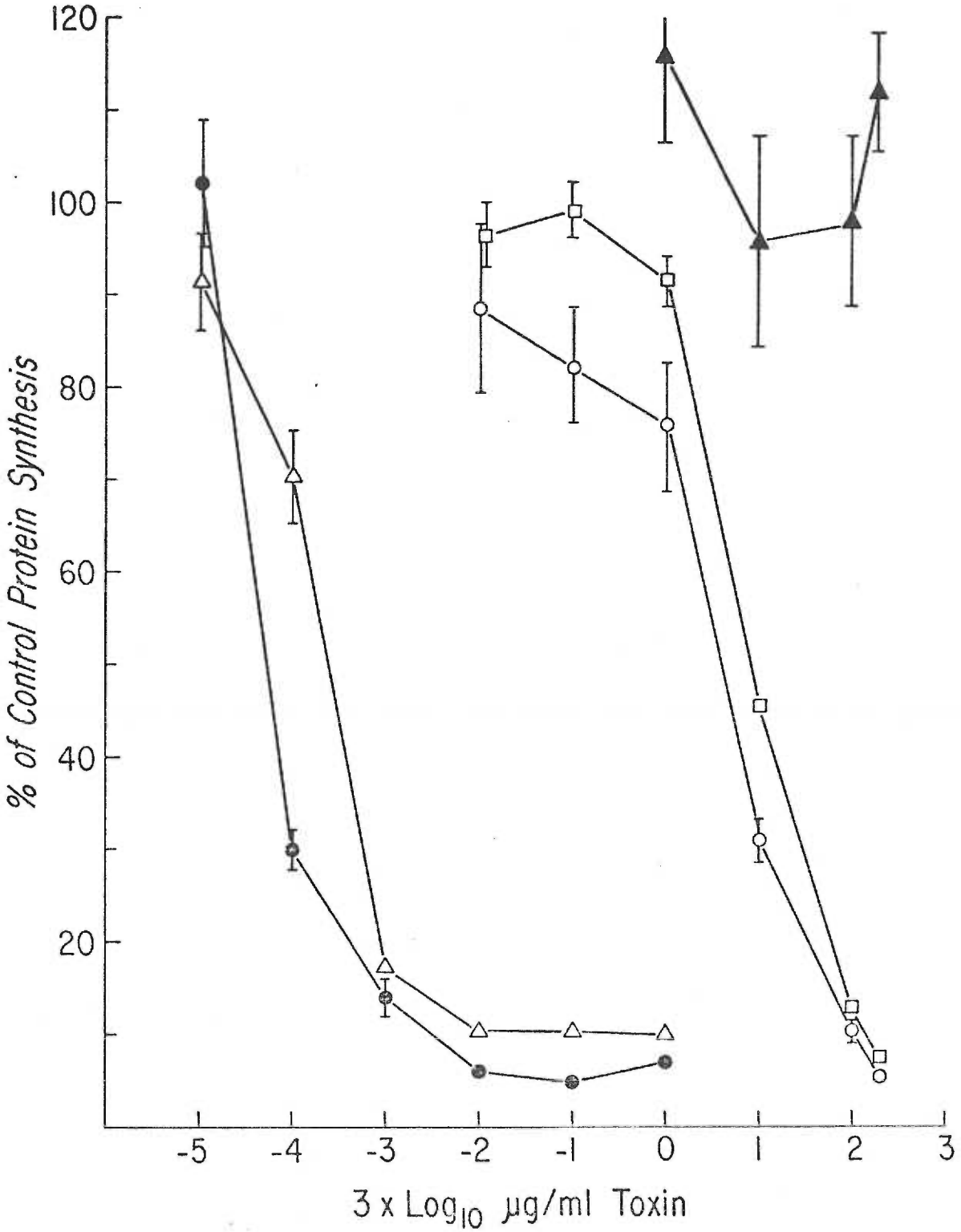
●—● , PyBHK parent cells;

△—△ , First passage cells surviving prior exposure to 1.2×10^{-1} ug/ml toxin;

○—○ , Second passage cells surviving prior exposure to 1.2 ug/ml toxin;

□—□ , Third passage cells surviving prior exposure to 12 ug/ml toxin; and

▲—▲ , A single clone from fourth passage cells surviving prior exposure to 120 ug/ml toxin (PyBHKR cells).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% of Control Protein Synthesis
-5
-4
-3
-2
-1
0
1
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3
3 x Log10 µg/ml Toxin

to inhibition of protein synthesis by diphtheria toxin (Fig. 5). However, exposure of these cells to 1.2 ug/ml yielded survivors with a greatly increased resistance to inhibition of protein synthesis. There was, therefore, a more rapid shift from toxin sensitivity to toxin resistance in PyBHK as compared to BHK cells. Subsequent exposure to these toxin resistant cells to 12 and 120 ug/ml toxin further increased this resistance. Many survivors resulted from the final toxin exposure of the PyBHK resistant cells. The cells were therefore plated for isolation of single cell clones in 96-well tissue culturing trays. The resulting toxin resistant clones varied in their resistance to intoxication by diphtheria toxin (Fig. 6). The clone with the highest resistance to toxin was termed PyBHKR and used in further experiments (Fig. 5). Further exposure of these PyBHKR cells to toxin concentrations of up to 750 ug/ml for up to 24 hours had no discernable lethal effect on the cells.

BHKR and PyBHKR cells react similarly in our inhibition of protein synthesis assay (Figs. 4 & 5). Toxin concentrations of up to 600 ug/ml have no effect on the abilities of these lines to synthesize protein over a five-hour period; this is 10^5 times as much toxin as is necessary to inhibit protein synthesis by more than 50% in the toxin sensitive BHK and PyBHK parental lines.

BHKR and PyBHKR cells kept in continuous culture for up to three months in the absence of toxin have shown no detectable decrease in toxin resistance as measured by our inhibition of protein synthesis assay (Fig. 7). This indicated that, <u>in vitro</u> at least, toxin resistance was a stable, heritable quality of the resistant cell lines. We therefore refer to the change resulting in toxin resistance as a mutation.

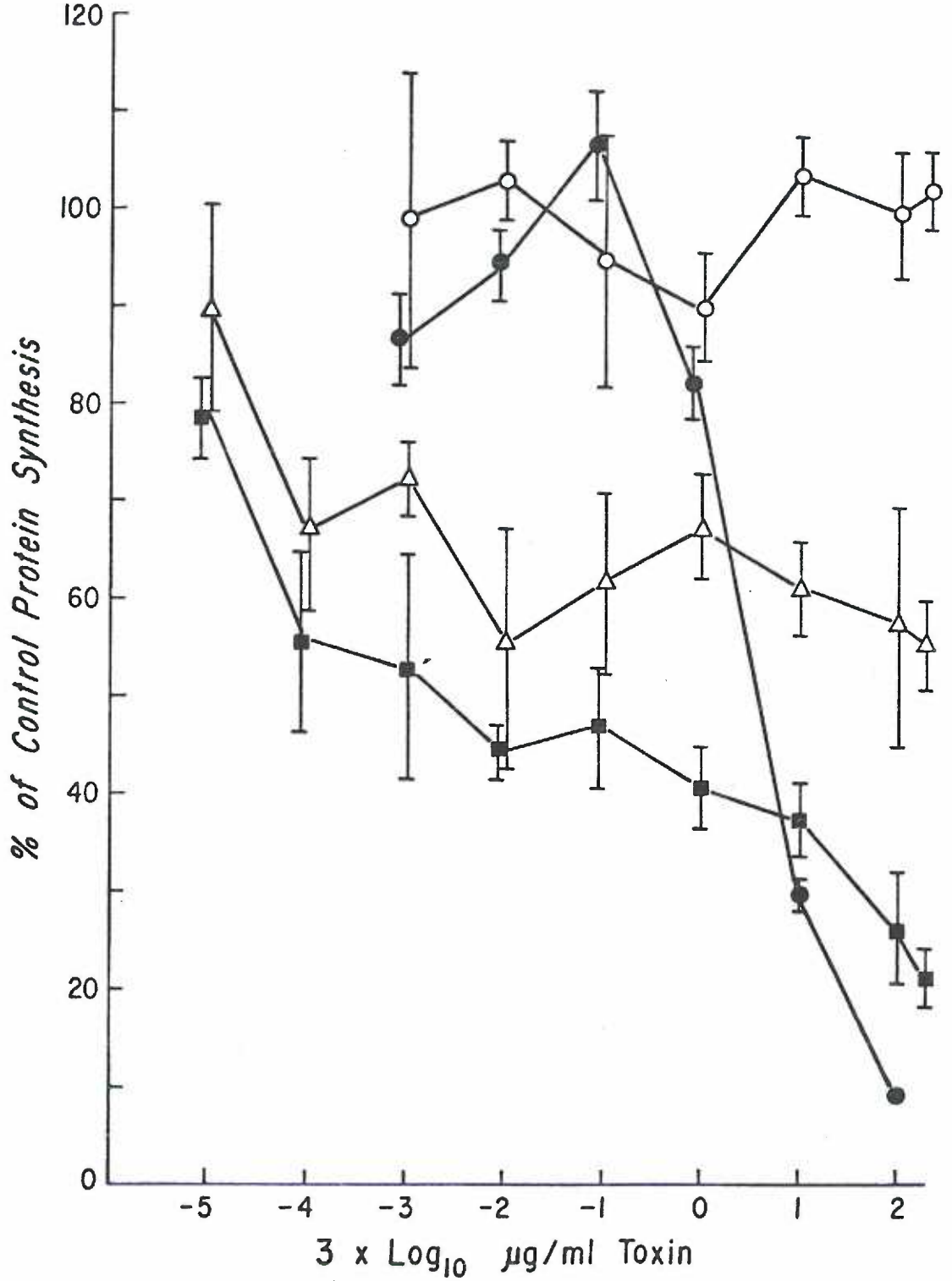
Fig. 6: Varying Resistance of PyBHK Single Cell Clones to Diphtheria Toxin. The isolation and assay procedures are described in Materials and Methods. All clones were from PyBHK cells surviving exposure to 120 ug/ml toxin.

 = Clone C3

 = Clone F2

 = Clone G6

 = Clone E11 (PyBHKR cells)


120
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0
% of Control Protein Synthesis
-5
-4
-3
-2
-1
0
1
2
3 x Log10 µg/ml Toxin

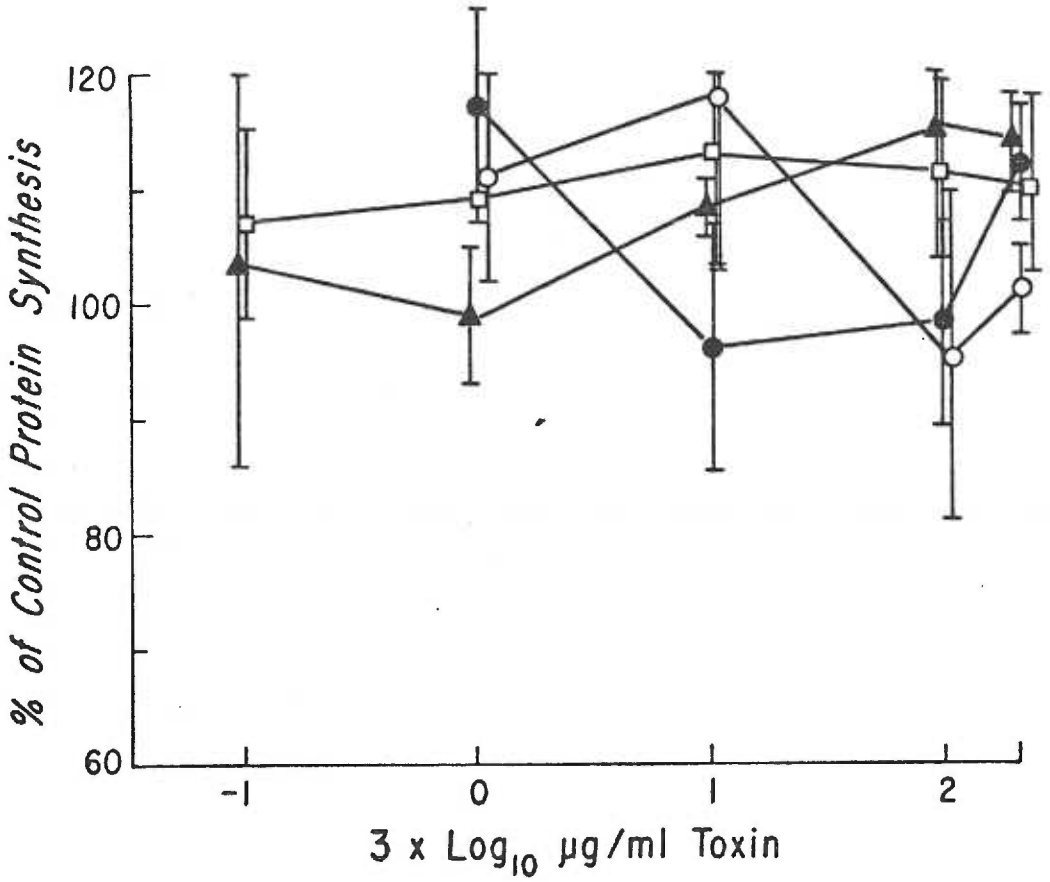
Fig. 7: Stability of Toxin Resistance Over Time. Toxin resistant cells were assayed as described in Materials and Methods. Cells were kept in continuous culture in the absence of toxin between the time periods indicated.

○—○ = BHKR, assayed 2/13/78

□—□ = BHKR, assayed 5/26/78

●—● = PyBHKR, assayed 2/13/78

▲—▲ = PyBHKR, assayed 5/26/78


120
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% of Control Protein Synthesis
-1
0
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3 x Log10 µg/ml Toxin

2) Characterization of BHKR and PyBHKR Cell Lines:

An immediate question was whether the BHKR and PyBHKR cells were actually descendants of the starting BHK and PyBHK cell lines, or whether we had selected for a toxin resistant contaminating cell line. A comparison of the toxin resistant to sensitive cells by several methods was therefore undertaken to answer this question. Additionally this comparison would to some extent characterize the biological properties of the new BHKR and PyBHKR cell lines.

Cell Morphology:

Upon microscopic examination, PyBHK cells display a morphology which is similar to that of other virally transformed cells. They appear as squamous or somewhat rounded short fibroblasts, with a tendency to overlap and form piles. Cell monolayers are unordered, with no tendency of the cells to align themselves with each other. The cell cytoplasm appears granular. PyBHKR cells are at this level indistinguishable from PyBHK cells morphologically.

BHK cells are more fibroblastic in appearance, single cells are elongated and tend to orient themselves in parallel to one another. Confluent monolayers of BHK cells present a typical whorled pattern of tightly aligned cells, with little overlap or piling occurring. BHKR cells are, once again, indistinguishable from BHK cells, when compared at this level.

Cloning in Soft Agar:

One of the classical means of differentiating between transformed and untransformed cells is to compare their abilities to divide and form clonal colonies in medium containing agar. The relative abilities of BHK, PyBHK, BHKR and PyBHKR cells to form such clones was

investigated as a further means of characterizing the toxin resistant cell lines and defining their relationship to the parental lines.

Cells were suspended in soft agar as described in Materials and Methods. A concentration of 2% fetal calf serum in the soft agar was determined by experimentation to give the best differential effect between BHK and PyBHK cells in terms of their cloning abilities (Table 1). BHK cells, as reported, clone efficiently at higher serum levels (52). It can be seen from the results presented in Table 1 that the toxin resistant progeny cell lines each maintained an ability to clone in soft agar similar to that of the parental line from which they were derived.

Rates of Protein Synthesis and Cell Division:

The two parental and two diphtheria toxin resistant progeny cell lines were further compared by measuring the rate at which each cell type incorporated labeled amino acids into an acid-precipitable product as described in Materials and Methods. The results in Figure 8 show that the progeny cell lines synthesize protein under these conditions at a rate comparable to the parental strains. This finding, coupled with the finding that all four cell lines grow and divide in culture at rates which do not differ appreciably (Table 2), indicates that the mutation conferring toxin resistance did not alter the protein synthesis machinery of the resistant cell lines in a way which greatly affected the rate of protein synthesis, or the ability of the cells to thrive in vitro.

Karyotypic Analysis:

Karyotypic analysis was performed on both sensitive and resistant cell lines to eliminate the possibility that toxin resistant mouse cells, also used in this laboratory, had contaminated the sensitive

Table 1

Cloning Abilities of Parental and Mutant Cells in Soft Agar[1]

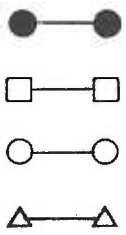
Cell Type	Serum Level	% of Cells Forming Clones[2]	Average Number of Cells/Clone[3]
BHK	10%	90	>20
	5%	75	20
	2%	40	12
BHKR	10%	50	15
	5%	30	15
	2%	20	10
PyBHK	10%	99	>>20
	5%	98	>>20
	2%	99	>20
PyBHKR	10%	99	>>20
	5%	99	>20
	2%	99	>20

[1]Assay conditions were as described in Materials and Methods.

[2]Plates were examined microscopically and 100 total single cells and/or clones counted per plate.

[3]Clones with more than twenty cells could not be assayed efficiently for cell number

Fig. 8: Rates of Protein Synthesis. The assay procedure is described in Materials and Methods.



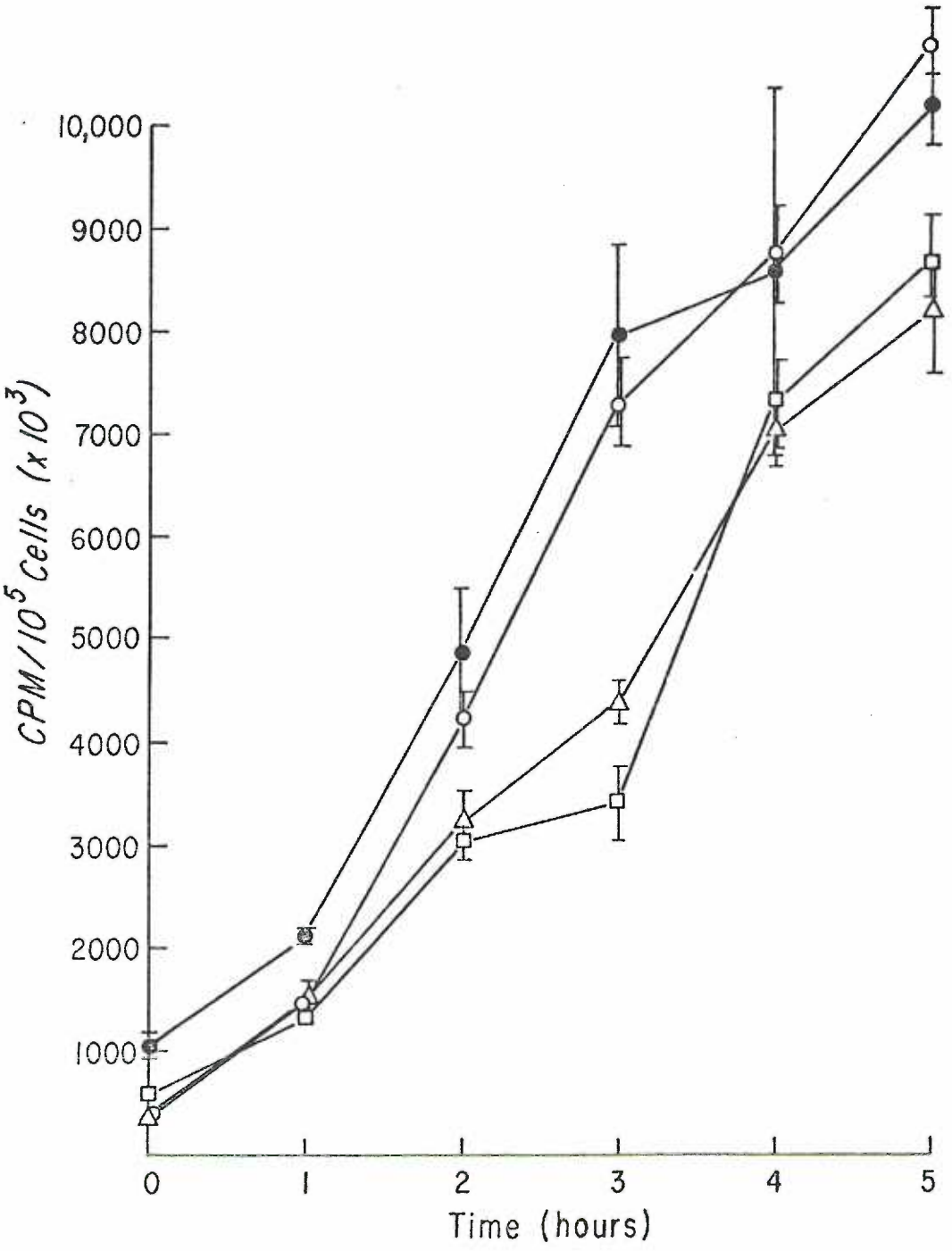

10,000
9000
8000
7000
6000
5000
4000
3000
2000
1000
CPM/10^5 Cells (x 10^3)
0
1
2
3
4
5
Time (hours)

Table 2

Rates of Cell Division of Parental and Mutant Cells[1]

Time (Hours)	Log_{10} Cell Number			
	BHK	BHKR	PyBHK	PyBHKR
0	5.83	5.66	5.55	5.40
8	5.93	5.75	5.84	5.70
16	6.13	5.95	6.00	5.92
24	6.32	6.13	6.11	5.99
32	6.51	6.27	6.25	6.23
Average Doubling Time (Hours)[2]	14.88	15.05	13.22	12.48

[1] $5x10^5$ cells of each type were seeded onto 25 cm^2 tissue culture flasks in 10 ml growth medium. Time 0 was taken as the first cell count after the cells had settled overnight. Cells were lifted and counted at the times indicated as described in Materials and Methods.

[2] Determined by averaging the increase in cell number over the first 24 hours of log growth.

hamster cell lines. The analysis showed the BHK, PyBHK, and PyBHKR cells to be non-mouse. A lack of suitable metaphases in the BHKR cells prevented karyotypic analysis of that line. The PyBHK and PyBHKR cells were further found to have 84 chromosomes each (a common tetraploid number for Golden Hamsters), in a similar distirbution. This is a strong indication that in this case the PyBHKR cells were descendants of the PyBHK cells.

Induction of Tumors by PyBHK and PyBHKR Cells:

As a further means of comparing PyBHK and PyBHKR cells, as well as obtaining large amounts of cells for further study, an attempt was made to induce tumors in hamsters by subcutaneous injection of the cells. The procedure used is described in Materials and Methods.

It was found that 10^6 cells of either the PyBHK or PyBHKR cell types could induce tumors in hamsters under the described conditions (Table 3). All tumors were found upon examination after dissection to be morphologically identical: in each animal one major, compact tumor mass was seen just below the skin over the shoulder muscle, with two or three much smaller tumors nearby. The large tumors were surrounded by a membrane. When this membrane was cut away a mass of tightly packed, dark red cells was revealed. The tumor tissue was consistent throughout and there was no sign of cell deterioration in the center. The tumor mass was suffused with blood which may have been the result of severing blood vessels during dissection.

Approximately four grams of the tumor mass, well cleaned of membrane, was recovered from each animal. All tumors were handled separately. The majority of the tissue was frozen immediately, the remainder was adapted to cell culture. All four tumors survived the transfer to cell culture easily. The cells derived from PyBHK tumors were

Table 3

Induction of Tumors by PyBHK and PyBHKR Cells[1]

Cell Line	Number of Cells Injected	Frequency[2]	Latent Period[3] (days)	Tumor Size
PyBHK	$1x10^6$	2/2	40	20x32 mm
			40	22x27 mm
PyBHKR	$1x10^6$	2/2	40	20x30 mm
			40	21x28 mm

[1]The experimental conditions are as described in Materials and Methods.

[2]Number of animals displaying tumors/number of animals injected.

[3]Time between injection of cells and appearance of visually noticeable tumors.

termed PCT1 and PCT2, those from PyBHKR tumors, PRT1 and PRT2. Subsequent comparison of these cell types in an inhibition of protein synthesis assay revealed essentially no difference in the response to diphtheria toxin between the cells recovered from tumors and the inoculated PyBHK and PyBHKR cells (Fig. 9).

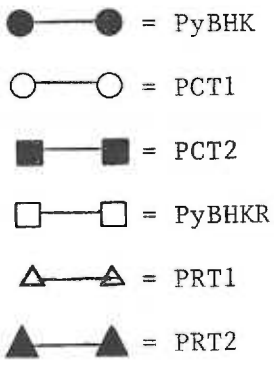
These results show that both the toxin sensitive and toxin resistant transformed cell lines can induce tumors in hamsters, once again indicating that the two are related. Further, this experiment shows that the mutation or mutations conferring toxin resistance are stable in vivo, as well as in vitro.

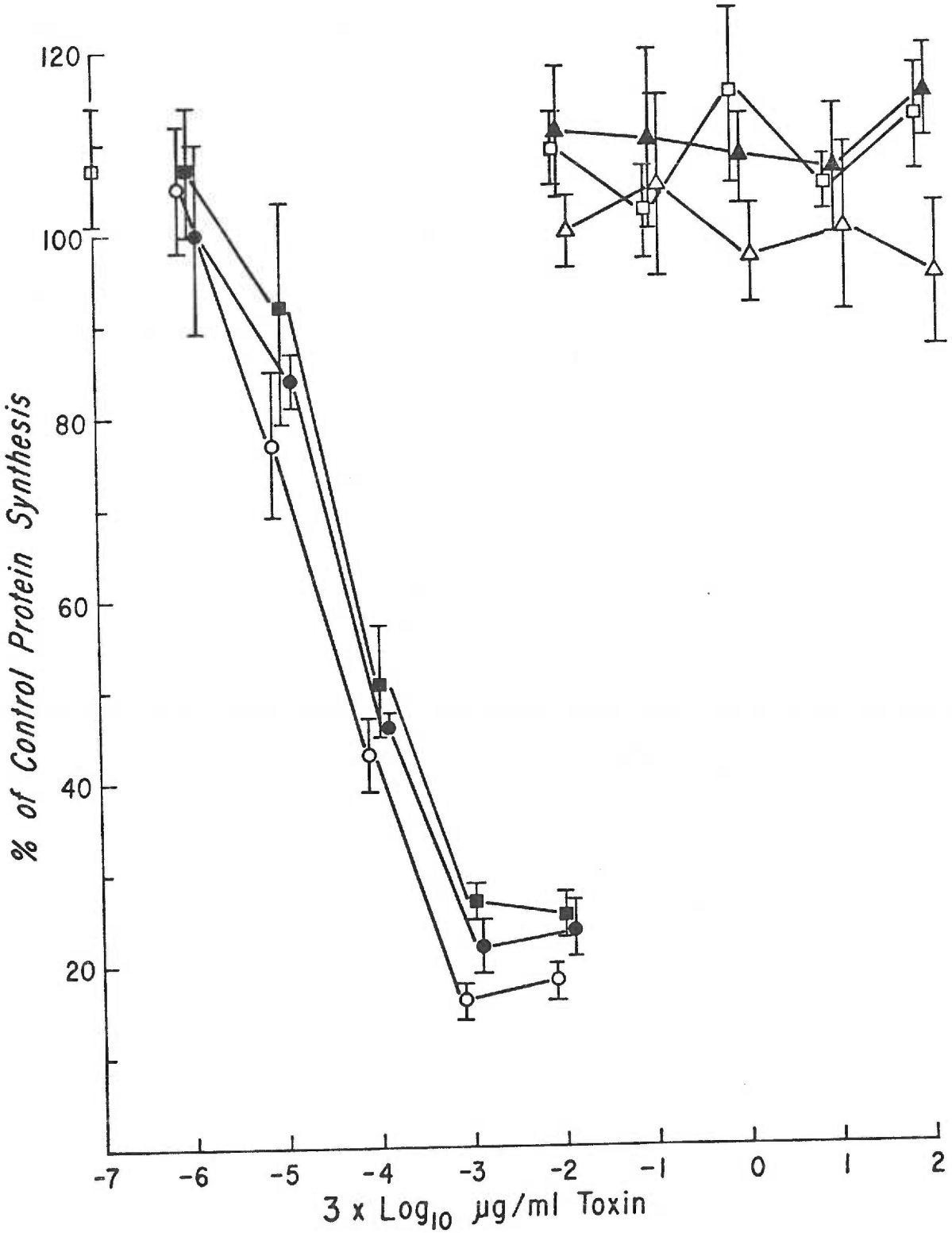
3) The Basis of Toxin Resistance:

It next became of interest to attempt to determine the level at which the BHKR and PyBHKR cell lines expressed their resistance to diphtheria intoxication.

Theoretically a resistance to the action of diphtheria toxin could arise at several points: (a) an alteration in the cell membrane could drastically decrease the binding or the uptake of toxin; (b) the ribosomal EF-2 accepting complex could be altered so that ADPREF-2 could function actively in place of EF-2; (c) a new factor in the cytoplasm could quickly neutralize internalized fragment A; (d) the EF-2 itself could be altered such that ADPREF-2 is not formed. A form of resistance involving altered NAD^+ could be theorized, but is highly unlikely due to the fact that NAD^+ is essential in a great many metabolic processes. An altered NAD^+ would be expected to be a lethal event, or at least have profound metabolic consequences which are not apparent in our mutants. In order to address these points, the following series of experiments were carried out.

Fig. 9: Assay of protein synthesis inhibition by diphtheria toxin: Sensitivities of cells recovered from tumors. The assay procedure is as described in Materials and Methods.

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% of Control Protein Synthesis
-7
-6
-5
-4
-3
-2
-1
0
1
2
3 x Log10 µg/ml Toxin

Sensitivity to Pseudomonas aeruginosa Exotoxin A:

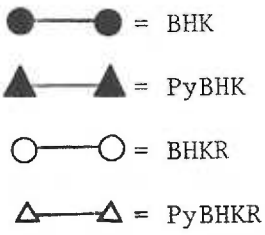
Pseudomonas aeruginosa exotoxin A (PAT) has been shown to catalyze the same intracellular ADP-ribosylation of EF-2 as diphtheria toxin (34,35). However, the cellular attachment and/or uptake of PAT appears to be different than that of diphtheria toxin, with various cell types displaying different sensitivity to the two toxins (44,45). If the BHKR and PyBHKR cells were resistant to diphtheria toxin because of a permeability barrier at the membrane level, this barrier might not exist for PAT; therefore the sensitivities of the various cell lines to PAT were compared in an inhibition of proteins synthesis assay.

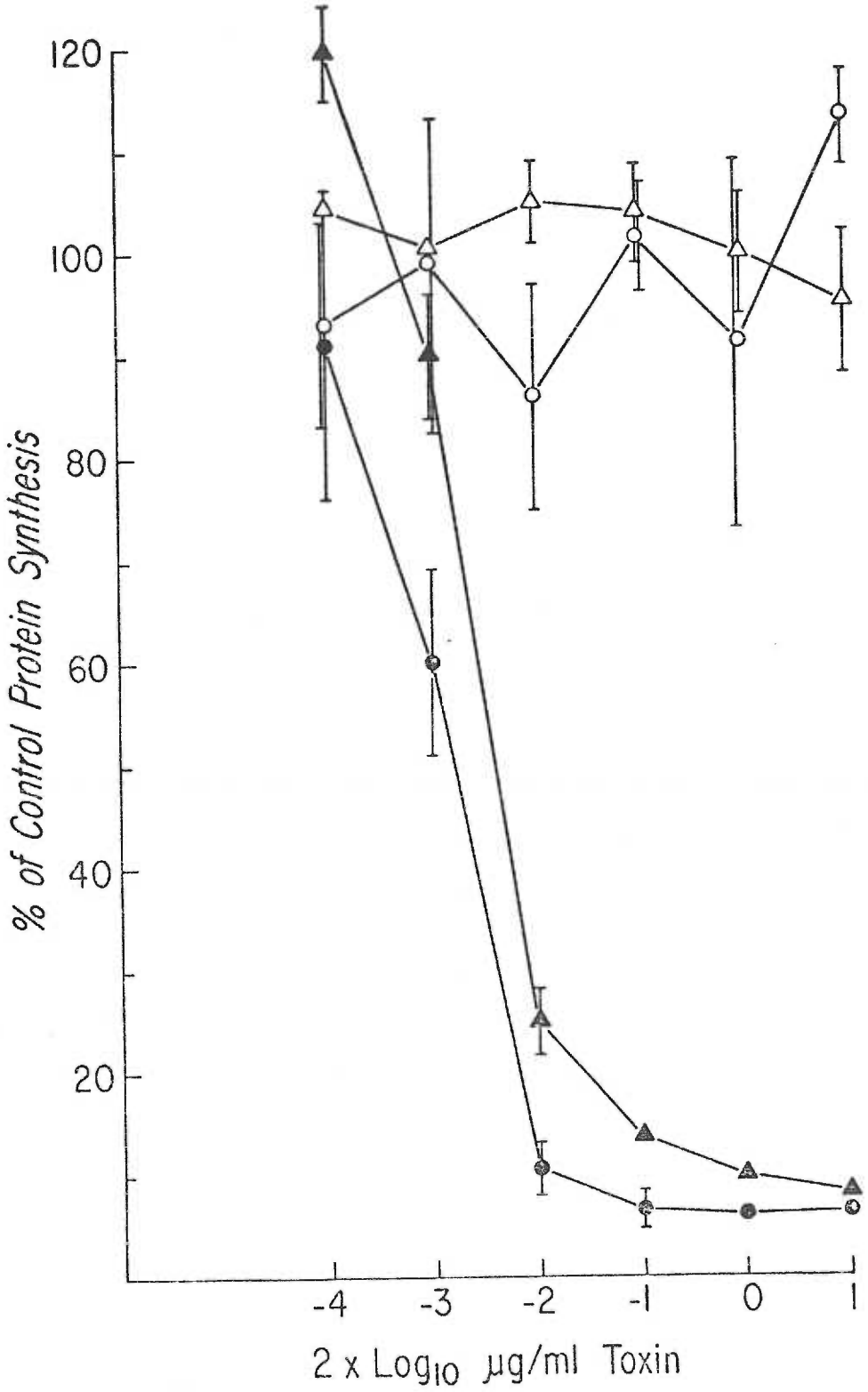
The results in Fig. 10 show that both BHK and PyBHK cells are sensitive to PAT, with concentrations of less than 2×10^{-2} ug/ml sufficient to inhibit protein synthesis by 50% under the conditions of the assay. BHKR and PyBHKR cells on the other hand were completely unaffected by up to 1000 times as much PAT. Since the modes of cellular binding and/or internalization of the two toxins differ, this result indicated that the resistance was mediated at a point common to both toxins: the ADP-ribosylation of EF-2.

Cell-Free ADP-Ribosylation:

The most direct method available for comparing the ADP-ribosylation of EF-2 in toxin sensitive vs. toxin resistant cells was one utilizing a cell-free system in which the cellular membrane barrier was removed. Gill and Dinius (34) have developed such an assay, which measures EF-2 in cell extracts by measuring the amount of labeled ADPR transferred to EF-2 when excess ^{14}C-NAD is reacted with EF-2 in the presence of excess fragment A. Under the conditions of our assay it was found that a final fragment A concentration of 6 ug/ml was in four-fold

Fig 10: Resistance of BHKR and PyBHKR cells to Pseudomonas aeruginosa exotoxin A. The assay procedure is as described in Materials and Methods.




120
100
80
60
40
20
% of Control Protein Synthesis
-4
-3
-2
-1
0
1
2 x Log10 µg/ml Toxin

excess over that necessary to completely ADP-ribosylate the EF-2 present in 1000 ug/ml BHK cell extract, 25 uCi added NAD^+ per tube was found to be in excess also (Table 4).

We used this cell-free ADP-ribosylation assay to compare the relative abilities of sensitive and resistant cells' EF-2 to be ADP-ribosylated, both in the presence of fragment A, and activated (enzymatically active) PAT. The results shown in Table 5 indicate that BHK and PyBHK EF-2 are readily ADP-ribosylated, while BHKR and PyBHKR EF-2s are not labeled by either toxin. The fewer counts in the presence of PAT may be due to a lesser amount of fully activated toxin since PAT was not shown to be in excess. These results provide strong evidence that the mutation was cytoplasmic in nature, as the major biochemical lesion caused by these two toxins, the ADP-ribosylation of EF-2, did not occur. Since ADPREF-2 was not formed in extracts from the resistant cells, the possible existence of an ADPREF-2 molecule active in mutant protein synthesis could also be rejected as a possible mode of resistance.

The failure to transfer ADPR to EF-2 in the resistant extracts could conceivably be due to a factor in the resistant cytoplasm which interferes in some way with the toxin catalyzed reaction. To test this possibility the resistant and normal cell extracts were mixed with equal amounts of either an EF-2 preparation from PyBHK cells, or bovine serum albumin (BSA). The results presented in Table 6 show that the addition of resistant cell extracts did not interfere with the transfer of labeled ADPR to the PyBHK EF-2. This indicated that there was no factor in the resistant extracts which interfered with the fragment A catalyzed reaction to an extent that would account for the complete absence of ADP-ribosylation of EF-2 in resistant cell extracts.

Table 4

Effect of Fragment A and ^{14}C-NAD Concentrations on Cell-Free ADP-Ribosylation Reaction[1]

Fragment A (µg/ml)	Acid-Precipitable Radioactivity (CPM/mg Cell Protein)
6	20,250
3	20,500
1.5	21,750
0.75	18,750
0.1	10,000
^{14}C-NAD$^+$ (µCi/tube)	
25	22,500
12.5	22,000
6.25	22,250
3.13	13,750
1.6	8,250

[1]The assay is as described in Materials and Methods, with BHK cell extract present in a final concentration of 1 mg/ml, and varying concentrations of ^{14}C-NAD$^+$ or Fragment A as indicated.

Table 5

Effect of Various Toxins on ^{14}C-ADPR Transfer to EF-2[1]

Cell Extract	TOXIN	
	Diphtheria Fragment A	Pseudomonas Exotoxin A
BHK-C	18,733	6,260
BHKR	0	0
PyBHK-C	15,714	6,205
PyBHKR	278	214

[1]Numbers are expressed in counts per minute per mg added EF-2 extract above background. Final cell extract protein concentrations in the reaction mixture: BHK, 580 ug/ml; BHKR, 440 ug/ml; PyBHK, 590 ug/ml; PyBHKR, 300 ug/ml. Both toxins were presented in final concentrations of 6 ug/ml.

Table 6

Effect of Addition of PyBHK EF-2 on Fragment A Catalyzed ^{14}C-ADPR Transfer[1]

Cell Extract	+BSA	+PyBHK EF-2
BHK	12,579	28,549
BHKR	0	15,568
PyBHK	17,619	29,883
PyBHKR	0	15,092

[1] Numbers are expressed in counts per minute per mg total protein above background. 200 ug of either BSA or PyBHK cell extract in 50 ul were added to give the following final protein concentrations in the reaction mixture: BHK, 1.2 mg/ml; BHKR, 1.12 mg/ml; PyBHK, 1.07 mg/ml; PyBHKR, 0.94 mg/ml.

Of all the possible modes of resistance to intoxication mentioned at the beginning of these section, only one appeared consistent with the data: The presence of a mutationally altered EF-2 (mEF-2) incapable of accepting ADPR in the toxin catalyzed reaction.

4) Comparison of Mutant to Normal EF-2:

Several types of mEF-2 could exist: 1) Amino acid X, the specific attachment point for ADPR in the diphtheria toxin catalyzed reaction, could be lost through deletion or substitution, 2) Amino acid X could be present, but may be buried within the mEF-2 molecule, or otherwise made unavailable for reaction with ADPR due to a configurational mutation, 3) mEF-2 could be conformationally changed so that it no longer binds to fragment A. In order to clarify which of these mechanisms might be responsible for conferring toxin resistance, a series of experiments were carried out which investigated some of the properties of mEF-2 as well as comparing mEF-2 to EF-2.

Temperature Sensitivity of mEF-2:

An interesting preliminary question was whether or not the mutation resulting in mEF-2 was temperature sensitive. To answer this, BHK, BHKR, and PyBHKR cells were grown at either 30 C or 41 C for 24 hours prior to an inhibition of protein synthesis assay. The results of that assay, shown in Fig. 11, indicate that the mutation resulting in resistance to intoxication is not temperature sensitive.

ADP-Ribosylation of mEF-2:

In order to examine the possibility that amino acid X may have been made unavailable for reaction with Fragment A through a conformational change, several experiments were performed which attempted to "open up" the mEF-2 molecule in hopes of exposing the ADPR attachment

Fig 11: Effect of Varying Growth Temperature on Diphtheria Toxin Resistance. Cells were grown at the temperature indicated for 24 hours prior to assay. The inhibition of protein synthesis assay was as described in Materials and Methods, except that it was performed at the indicated temperatures, and duplicate samples were averaged for each point. Inhibition of protein synthesis in:


, BHK, 41C

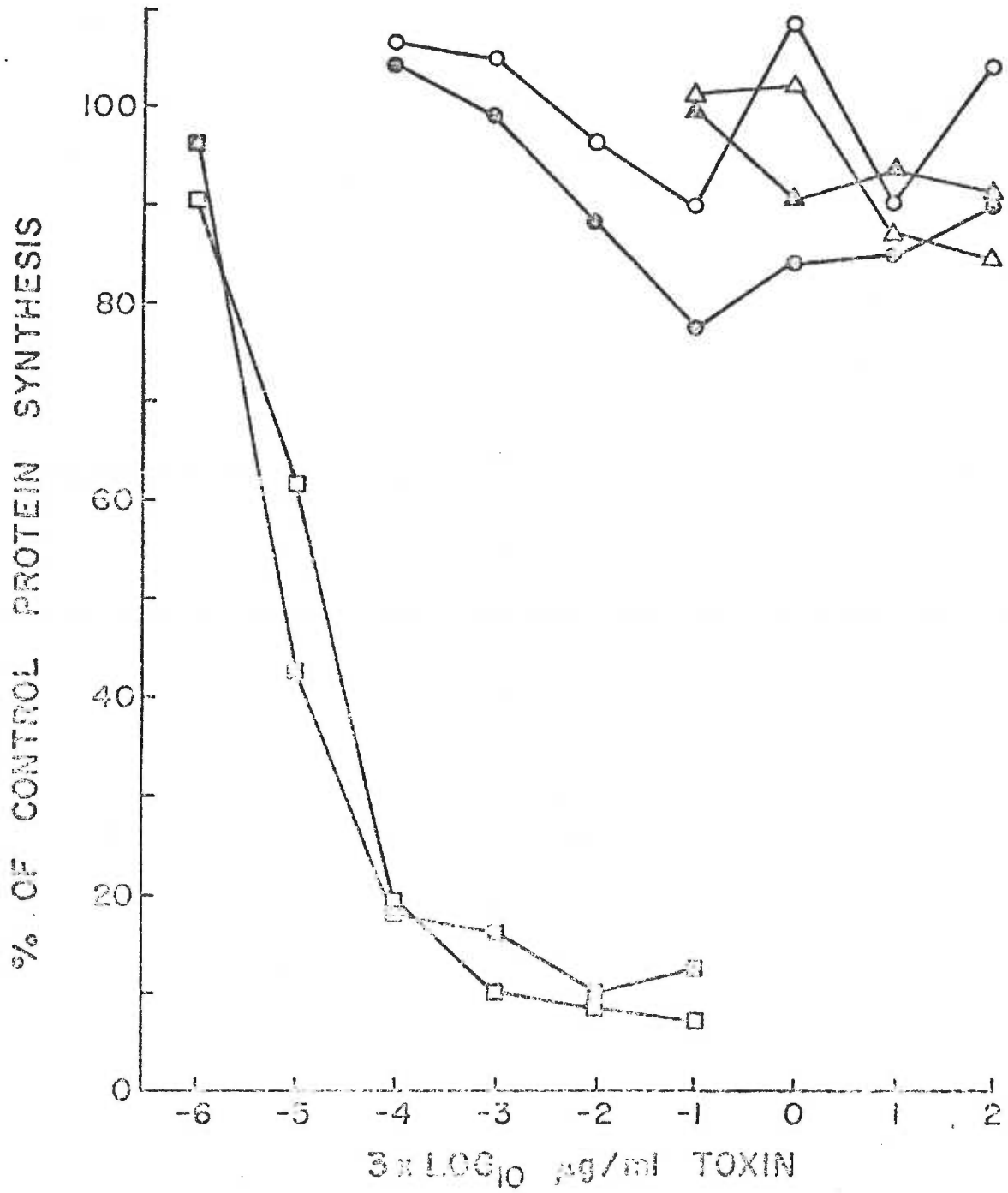

, BHK, 30C


, BHKR, 41C


, BHKR, 30C


, PyBHKR, 41C


, PyBHKR, 30C


% OF CONTROL PROTEIN SYNTHESIS
100
80
60
40
20
0
-6
-5
-4
-3
-2
-1
0
1
2
3 x LOG10 µg/ml TOXIN

site. The normal ADP-ribosylation assay involves a 10 minute incubation of reactants at 37 C. Alterations in the length and temperature of the reaction were attempted, with the results shown in Table 7. There was no significant increase in ADP-ribosylation of either BHKR or PyBHKR cell extracts with increasing time or temperature.

Next, mEF-2 was exposed to varying concentrations of urea or trypsin in the reaction buffer. The results of these attempts to expose a possible ADPR attachment site are presented in Tables 8 and 9. Once again, exposure of mEF-2 to these agents resulted in no appreciable increase in ADP-ribosylation.

Cell-Free Protein Synthesis Assay:

One problem in dealing the mEF-2 was how to detect its presence since the assay used for EF-2, ADP-ribosylation, was ineffective. A cell-free protein synthesizing system inactivated by treatment with diphtheria toxin fragment A could detect mEF-2 if it could substitute for EF-2 in the system and stimulate protein synthesis in the Fragment A inactivated system.

Preliminary experiments with the cell-free protein synthesizing system showed that incorporation of ^{3}H-leucine into an acid-precipitable form was linear for the first 20 minutes, after which it leveled off (Table 10). It was then found that the addition of Fragment A in a final concentration of 20 ug/ml, coupled with the addition of 20 ug/ml NAD^{+} effectively stopped protein synthesis in this system within ten minutes (Table 11).

Knowing that cell-free protein synthesis could be effectively halted by addition of Fragment A, the next experiment was designed to determine if added mEF-2 could reactivate the system. Fragment A and

Table 7

Effect of Time and Temperature

on ADP-Ribosylation of EF-2[1]

Cell Extract	Time			Temp (C)		
	10	60	120	37	45	60
BHK	15,180	16,012	15,700	16,240	3,272	48
BHKR	0	0	0	0	0	0
PyBHK	17,232	15,240	15,071	18,920	7,702	0
PyBHKR	43	0	50	0	36	0

[1] Numbers are expressed in cpm/mg total cell extract protein added above background. Cell extract protein concentrations in the reaction mixture: BHK, 650 ug/ml; BHKR, 570 ug/ml; PyBHK, 520 ug/ml; PyBHKR, 390 ug/ml.

Table 8

Effect of Urea on ADP-Ribosylation of EF-2[1]

Cell Extract	Urea (m) 0	0.5	1	2	4
BHK	7,799	7,348	6,336	681	47
BHKR	93	0	0	0	0
PyBHK	9,461	11,247	9,295	603	0
PyBHKR	44	32	0	44	36

[1] The assay is as described in Materials and Methods with the addition of the indicated concentration of urea to the EF-2 buffer which was incubated with the cell extracts for 10 minutes at 37C prior to the addition of label. Counts are expressed in cpm per mg total cell extract protein added above background. Cell extract protein concentrations in the reaction mixture: BHK, 650 ug/ml; BHKR, 570 ug/ml; PyBHK, 520 ug/ml; PyBHKR, 390 ug/ml.

Table 9

Effect of Trypsin on ADP-Ribosylation of EF-2[1]

Cell Extract	Trypsin (ug/tube) 0	0.5	5	50
BHK	12,424	12,685	12,382	10,744
BHKR	0	0	68	0
PyBHK	11,337	12,078	12,280	9,721
PyBHKR	196	189	203	0

[1] The assay is as described in Materials and Methods with the following alterations: cell extracts were incubated with 100 ul EF-2 buffer containing the indicated amounts of trypsin at 37C for ten minutes, followed by the addition of 100 ul EF-2 buffer containing 100 ug soybean trypsin inhibitor. This was incubated an additional 10 minutes at 37C. At this point either excess Fragment A or H_2O was added in 100 ul EF-2 buffer, and the asay run as usual. Numbers are expressed in cpm/mg total added cell protein above background. Cell extract protein concentrations in the reaction mixture: BHK, 650 ug/ml; BHKR, 570 ug/ml; PyBHK, 520 ug/ml; PyBHKR, 390 ug/ml.

Table 10

Kinetics of Incorporation of ^{3}H-Leucine into an Acid Precipitable Form in a Cell-Free Protein Synthesizing System[1]

Time (Min.)	Acid-Precipitable Radioactivity
0	250
5	8,100
10	17,050
20	20,250
30	18,900
60	18,150

[1]The assay is as described in Materials and Methods.

Table [1]

Inactivation of Cell-Free Protein Synthesis

By Addition of Fragment A and NAD^+ [1]

Additions to the System	Time (min.) 0	2	10	40
Frag A only	402	1,320	4,402	5,120
Frag A + 20 ug/ml NAD^+	2,019	2,240	3,417	2,917
Frag A + 50 ug/ml NAD^+	1,417	1,740	3,002	2,829
Frag A + 100 ug/ml NAD^+	1,222	1,620	2,912	3,160
No Frag A, No NAD^+	1,000	4,505	20,636	22,340

[1] Numbers expressed are cpm ^3H-Leucine incorporated into acid-precipitable form. Time is minutes after addition of label. Fragment A was 20 ug/ml in the reaction mixture.

NAD^+ were added to the cell-free protein synthesis system and incubated at 30 C for ten minutes to halt protein synthesis. At this time mEF-2, in the form of crude cell extracts for the cell-free ADP-ribosylation assay (as described in Materials and Methods), was added in varying amounts, or normal EF-2 prepared similarly, or an equal amount of bovine serum albumin in a buffer identical to that of the EF-2 preparations. ^{3}H-Leucine was added finally, and the mixture incubated. Table 12 summarizes the results of these experiments. It can be seen that in the Fragment A inactivated system, the addition of mEF-2, even in low concentrations in a crude extract, stimulates protein synthesis.

Table 12

Reactivation of a Fragment A Inactivated Cell-Free Protein Synthesis System By mEF-2[1]

Additions to the System	Time (min) 0	2	10	40
180 ug/ml mEF-2 extract	--	--	--	3,050
150 ug/ml mEF-2 extract	360	620	1,322	3,525
120 ug/ml mEF-2 extract	--	--	--	3,556
90 ug/ml mEF-2 extract	--	--	--	3,512
60 ug/ml mEF-2 extract	--	--	--	3,469
None	482	529	740	749
150 ug/ml EF-2 extract	477	450	490	584
150 ug/ml BSA	842	800	810	798

[1] Numbers are expressed in cpm ^{3}H-Leucine incorporated into acid-precipitable form. The system was inactivated in preincubation for 10 min. with 20 ug/ml of each fragment A and NAD^{+}. mEF-2 was from BHKR cells, EF-2 from BHK cells.

DISCUSSION

Reports published prior to this time in which diphtheria toxin resistant progeny cells were selected from sensitive parental cell lines by exposure to toxin (46-51,73) have indicated that there are two general classes of toxin-resistant mutants. (It should be noted that mutation as it is used here refers to a stable, heritable phenotypic change in the cell lines referred to as mutants; this may reflect a genetic change, but an actual alteration in a gene or chromosome has not been demonstrated.)

The first class of mutation conferring toxin resistance appears to be mediated at the level of the cell membrane, an alteration in which the attachment and/or uptake of toxin is reduced. These cells are characterized in whole cells studies, such as the inhibition of protein synthesis assay used in this thesis, by a resistance to toxin which can vary anywhere from 10 to 10^6 times that of the parental cells; no cells of this type have been found which are completely resistant to intoxication. These cells have been termed permeability variants by Moehring and Moehring (46). In their studies they have found the most highly resistant permeability variants that could be isolated from KB and CHO cells required levels of toxin for protein synthesis inhibition approximately the same as those required for mouse L-cells and rat All cells, two naturally occurring diphtheria toxin permeability type resistant cell-lines (47). Further, permeability variant cell extracts are just as sensitive to Fragment A catalyzed protein synthesis inhibition as are sensitive parental extracts (46,47).

The second class of mutation results in cells totally resistant to extremely high toxin concentrations. The only previously reported

mutants of this type were recently isolated by Moehring and Moehring from CHO cells and termed presumptive translational mutants, with the mutation conferring resistance placed at the level of EF-2 (51). It appears that our BHKR and PyBHKR cells fall into this class. They are easily differentiated from permeability variants by their total lack of toxin-mediated protein synthesis inhibition in an inhibition of protein synthesis assay, and by the fact that no ADP-ribosylation of EF-2 occurs in extracts from these cells in the presence of either diphtheria toxin fragment A or activated PAT.

In the isolation procedure leading to BHKR and PyBHKR cell lines, general intermediary levels of resistance were attained by cells (Figs. 4 & 5). The following characteristics of these intermediary cells are similar to permeability variants. The range of resistance varied from 10-fold in the case of BHK cells surviving $1.2x10^{-1}$ ug/ml toxin (Fig. 4) to 10^{5}-fold in PyBHKR cells surviving 12 ug/ml toxin (Fig. 5); this is equal to the range of resistance found in proven permeability variants (47,51). These cells isolated prior to the totally resistant EF-2 mutants were also sensitive to toxin in very high concentrations as measured in an inhibition of protein synthesis assay, and were killed by the toxin in the isolation procedure. Finally, in the three studies published prior to this time, permeability variants were isolated each time (46,51,73), while EF-2 mutants were isolated only once (51). One likely explanation, then, for the increased resistance of cells isolated prior to the isolation of EF-2 mutants in this thesis, is that permeability variants displaying varying resistances were selected for.

Alternative explanations for the intermediate sensitivities expressed in cells isolated during the selection procedure cannot, how-

ever, be rigidly excluded. There could conceivably exist different types of mutant EF-2s with various conformational changes which might only allow them to interact with Fragment A weakly, therefore requiring higher levels of the toxin to ADP-ribosylate the EF-2 and result in an inhibition of protein synthesis. It must also be kept in mind that only the final passaged cells of each type were cloned for single-cell colonies, the intermediate levels of resistance could therefore be due to mixtures of cell variants expressing different types of resistance. The data presented in Figure 6 shows clearly that the PyBHK cells surviving exposure to 120 ug/ml toxin were indeed a heterogeneous group, with various single cell clones displaying at least three different responses to high levels of toxin. In that figure, Clone E11, termed PyBHKR, displays a complete resistance to high levels of toxin in an inhibition of protein synthesis assay, similar to Moehring and Moehring's EF-2 mutants. Clone F2 responds like known permeability variants, with a sharp decrease in protein synthesis over a short range of high toxin levels. Clones C3 and G6 display a response to toxin unlike any previously reported cells, with a gradual decrease in protein synthesis over a wide range of toxin concentrations. It is interesting to postulate that this type of response may be due to a different form of mutant EF-2. The resolution of the question of how these intermediate cells express their resistance must, however, await further studies utilizing these monoclonal cell types.

This thesis is concerned with an exploration of the properties of the BHKR and PyBHKR cell lines. The first question explored was whether or not these highly resistant cells were actually descendants of the original sensitive lines. The series of experiments described in part

2 of the Results Section indicate that in terms of morphology, ability to clone in soft agar, growth rates, and rates of protein synthesis, the progeny cell lines were nearly identical to their respective parental lines. Additionally, PyBHK and PyBHKR cells each were found to have 84 chromosomes in a similar distribution, and to be able to induce tumors in hamsters. Although the possibility that contaminating cell lines were selected for cannot be rigidly excluded, it appears that, based on the above criteria, the resistant cell lines are derived from the parental lines.

It is interesting to note here that the data presented in Figure 9, showing the response of cell lines derived from the hamster grown tumors indicates that in the case of the two toxin-resistant cell lines, PRT1 and PRT2, protein synthesis remained at 100% of control at all toxin levels. This implies that these cell lines were composed essentially 100% of resistant tumor cells, with little if any normal hamster cell contamination. The care with which only tumor tissue from the center of the mass was used for adaptation to cell culture, coupled with the ease and rapidity with which these PRT cells adapted to culture could account for this result.

Next, the question of the level at which the mutant cells achieved their toxin resistance was explored. The finding of a lack of an inhibition of protein synthesis in BHKR and PyBHKR cells exposed to PAT in concentrations more than 10^3 higher than those sufficient to inhibit protein synthesis in the parental lines by 50% (Fig. 10) indicated a dual resistance to both PAT and diphtheria toxin. Since the modes of uptake and/or internalization of the two toxins differ, this experiment

was an indication that the mutation conferring resistance was not acting at the membrane level. This was confirmed when cell extracts containing EF-2 from BHKR and PyBHKR cells were found not to be ADP-ribosylated by the enzymatically active forms of either PAT or diphtheria toxin. There are two theoretical explanations of the inability of these toxins to covalently attach ADPR to EF-2 in the resistant extracts. One postulates a new factor or factors in the resistant cell which interfere with the ADP-ribosylation reaction by inactivating the enzymatically active toxins in some way; the other possibility is that the EF-2 in the resistant cells is altered to a form which does not accept ADPR in the toxin-catalyzed reaction. Mixing the extracts from resistant and sensitive cells, and subsequently measuring the ADP-ribosylation of the total EF-2 showed that the resistant extracts did not interfere appreciably with the interaction of toxin with EF-2 from normal cells (Table 6). It appeared at this point that resistance to intoxication was achieved in the BHKR and PyBHKR cells through an altered EF-2 incapable of accepting ADPR in the presence of either diphtheria toxin Fragment A, or activated PAT. This altered molecule was termed mutant EF-2 (mEF-2). Further studies on the resistant cell extracts showed that the mEF-2 could not be ADP-ribosylated by varying the time or temperature of the reaction (Table 7). Attempts to ADP-ribosylate mEF-2 which had been exposed to various amounts of trypsin or urea in order to alter its conformation in hopes of exposing the ADPR attachment point were also unsuccessful (Tables 8,9).

A final experiment tested the ability of mEF-2 to substitute for EF-2 in a cell-free protein synthesis system which had been inactivated by the addition of Fragment A and NAD^+ (Table 11). The results in

Table 12 show that while as much as 150 ug/ml added EF-2 extract from normal cells did not stimulate protein synthesis, 60 ug/ml added mEF-2 extract did stimulate protein synthesis five fold within a 40 minute period. Varying the amount of mEF-2 extract added from 60 to 180 ug/ml did not alter the level of this stimulation. This indicated that either the system was saturated with mEF-2 at 60 ug/ml added extract; or that if the mEF-2 was not saturating, increasing the amount of extract added also increased the concentration of some factor, such as salt present in large amounts in the EF-2 extract, which interfered with protein synthesis. This experiment demonstrates that mEF-2 can substitute for EF-2 in the protein synthesis machinery of rabbit reticulocytes. Additionally, this experiment shows clearly that in a cell-free system containing enough Fragment A and NAD^+ to inactivate added normal EF-2, mEF-2 is enzymatically active; thus it is demonstrated directly that mEF-2 is resistant to enzymatic inactivation by diphtheria toxin Fragment A. This confirms the theory that toxin resistance on the cellular level in BHKR and PyBHKR cells is due to an alteration in the EF-2 molecule.

As mentioned in the Results Section, there are two theoretical means by which mEF-2 could be rendered toxin resistant; either a conformational change occurs which masks the specific ADPR attachment site, amino acid X, or otherwise prevents the interaction of Fragment A and mEF-2; or amino acid X is lost or altered through substitution, deletion, or possibly the lack of a suitable modification system. It is impossible to determine absolutely which of these mechanisms is acting with the data presented in this thesis, however, theoretically, it is more likely that amino acid X has been lost or altered rather

than hidden for the following reasons.

First, there is a total lack of ADP-ribosylation of mEF-2 despite increases in the time and temperature of the reaction (Table 7), as well as exposure of the mEF-2 to trypsin and urea (Tables 8,9). If the resistance of mEF-2 is due to a conformational change, it is unlikely that a random reordering of the mEF-2 molecule through these means would result in the correct conformation necessary for ADP-ribosylation to take place. However, while a conformational change in the molecule might conceivably allow a low level of ADP-ribosylation, the loss or alteration of amino acid X would necessarily prevent the reaction entirely.

Second, and more importantly, mEF-2 is capable of substituting, at least to a limited extent, for EF-2 in a protein synthesis system derived from normal cells. This indicates that mEF-2 has not been altered significantly in terms of its ribosomal binding site, or any of the other properties involved in its enzymatic role in protein synthesis. Once again the simplest explanation of a change conferring resistance to toxin while leaving the rest of the enzyme unaltered is one which leads to the least overall conformational change: the change of a single specific amino acid, especially if it occurs via the conservative substitution of a similar amino acid, is the most effective theoretical means of preventing ADP-ribosylation while preserving the enzymatic activity of the mEF-2 molecule. Final proof of the presence or absence of amino acid X will have to await tryptic peptide analysis and amino acid sequencing of mEF-2.

Putting aside for the moment the precise means by which the mEF-2 is altered, it is interesting that a resistance to ADP-ribosylation

should occur at all. Every naturally occurring EF-2 studied so far, from sources as evolutionarilly diverse as yeast and rats, is readily ADP-ribosylated by D.T. Fragment A. Although the presence of amino acid X has been demonstrated specifically only in rat and rabbit EF-2, there is no counter-indication to its existence in all eucaryotic EF-2's. For the purpose of discussion, therefore, it may be assumed that similar or identical sites on EF-2 which interact with Fragment A leading to the covalent attachment of ADPR have been strictly conserved through the evolution of all eucaryotes. Such strict conservation implies a function for these sites. Since these sites are clearly altered in mEF-2, the enzyme may be expected to have an altered functional ability; however, protein synthesis and cell division are essentially the same in cells utilizing both normal and mEF-2 (Fig. 8, Table 2).

It is intriguing to speculate how this mutational loss of evolutionarilly conserved sites on an important enzyme could result in no readily apparent change in the overall physiology of the mutant cells, and especially in protein synthesis, the process in which EF-2 is most involved. Possibly the mEF-2 has lost some functional ability, and is less active enzymatically than EF-2, but this lowered efficiency is balanced by an overall increase in the cell's protein synthesizing machinery resulting in the same overall rate of protein production. Alternatively, these EF-2 sites involved in the ADP-ribosylation reaction may have a function which is not directly related to protein synthesis; their loss in mEF-2 would have an effect, but in an as yet undiscovered area. A final possibility is that hamster cells specifically have in some way lost their necessity for these sites on EF-2. This hypothesis, although seemingly farfetched, does fit with the fact that EF-2 type

mutations have been found only in CHO, BHK, and PyBHK cells, all hamster lines, while Moehring and Moehring's extensive work with the isolation of D.T. resistant mutants of human KB cells, and Venter's work with HeLa cells, resulted only in permeability type mutants (47-51,73).

Further work with mEF-2 should begin with the purification of the enzyme; this will allow further inquiries into the means by which it achieves its resistance to ADP-ribosylation. Purified mEF-2 could be subjected to tryptic peptide analysis to verify the presence or absence of amino acid X. Purified mEF-2 could also be compared to purified EF-2 in a stimulation of protein synthesis assay to determine if the two enzymes are, in a molar basis, equally effective catalytically. Comparisons of EF-2 to mEF-2 will then, hopefully, help elucidate the means at the molecular level by which ADP-ribosylation takes place, as well as clarify the functional importance of the sites involved in this reaction to the EF-2 molecule.

SUMMARY AND CONCLUSIONS

PyBHK and BHK cells in culture were exposed to sequentially increasing levels of diphtheria toxin, and cells showing resistance to the toxin were isolated. Cells were isolated by this procedure which were totally unaffected by levels of toxin 10^5 times those sufficient to inhibit protein synthesis in the parental cell lines. These were termed BHKR and PyBHKR. These cells were found to be simultaneously resistant to Pseudomonas aeruginosa exotoxin A (PAT). Cell-free EF-2 extracts from BHKR and PyBHKR cells would not accept ADPR in the presence of either diphtheria toxin Fragment A or activated PAT under a variety of reaction conditions. The resistance to toxin was found to be stable both in vivo and in vitro. Addition of extracts from resistant cells to a cell-free protein synthesizing system inactivated by treatment with Fragment A resulted in a stimulation of protein synthesis not observed upon addition of similar extracts from sensitive cells.

It appears that the BHKR and PyBHKR cells are resistant to diphtheria toxin because of an alteration in their EF-2. The form of this alteration is unknown, however the simplest explanation is that the specific ADPR attachment point in EF-2, amino acid X, has been lost. This alteration does not affect the rates of protein synthesis or cell division in the mutant cells.

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Appendix A: The Effect of *Pseudomonas aeruginosa* Exoenzyme S on Sensitive and Resistant Cells

Iglewski, et al (1) have reported that an exoenzyme of *Pseudomonas aeruginosa*, termed Exoenzyme S, is an ADP-ribosyltransferase distinct by several criteria from Toxin A (PAT). While exoenzyme S does catalyze the transfer of ADPR from NAD^+ to one or more protein acceptors in eucaryotic cell extracts, it is known that EF-2 is not an acceptor.

In order to verify that diphtheria toxin resistant BHKR cell extracts did not contain some factor which interferes with all ADP-ribosylating enzymes, exoenzyme S was added to BHK and BHKR extracts in a cell-free ADP-ribosylation assay. The results in Table 1 demonstrate that exoenzyme S is equally effective as an ADP-ribosyltransferase in either diphtheria toxin sensitive or resistant extracts. This indicates that the BHKR cells do not contain a factor which inhibits all ADP-ribosyltransferase reactions.

Table 1

The Effect of *Pseudomonas aeruginosa* Exoenzyme S on BHK and BHKR Cell Extracts[1]

Cell Extract	Toxin		
	D.T. Fragment A	Exoenzyme S[2]	Fragment A + Exoenzyme S
BHK	20,605	85,655	100,515
BHKR	30	83,230	79,335

[1]The assay is as described in Materials and Methods, exepting that 50 ul of either exoenzyme S or distilled H_2O was added to 250 ul histamine buffer prior to the addition of 0.25 uCi ^{14}C-NAD^+. Numbers are expressed in cpm per mg added EF-2 extract above background. Final cell extract protein concentrations: BHK, 580 ug/ml, BHKR, 440 ug/ml.

[2]Exoenzyme S, prepared as previously described (1), was the gift of Dr. Barbara H. Iglewski.