STUDIES ON THE INTOXICATION OF VIRALLY TRANSFORMED CELLS BY DIPHTHERIA TOXIN

by

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INTRODUCTION

Statement of the Problem

Iglewski et al. (81) reported that chick embryo fibroblasts (CEF) transformed by the Schmidt-Ruppin strain of Rous sarcoma virus are more sensitive to intoxication by diphtheria toxin than normal, nontransformed CEF. However, since these transformed cells were also producing progeny virus, it was not possible to determine if the increased sensitivity of protein synthesis and cell growth to inhibition by diphtheria toxin was due to the property of cell transformation, the property of viral replication, or both. They suggested that this may be a newly described property of viral transformation. Subsequent reports, however, on the relative sensitivity of protein synthesis of virus transformed or malignant cells to intoxication by diphtheria toxin as compared to normal cells have not yielded consistent results (125,142,175).

The purpose of this study is to extend what is known about the increased sensitivity of transformed cells to diphtheria toxin, to further characterize the properties of viral transformed cells which display an increased sensitivity to intoxication by diphtheria toxin, and to study the cellular level of selective intoxication of transformed cells by diphtheria toxin.

Review of the Literature

A. DIPHTHERIA TOXIN:

The description of diphtheria toxin (DT) as the major substance responsible for the symptoms of diphtheria was first made by Rous and Yersin (138) at the end of the 19th century. Since that time much work has been done on DT and its relationship to diphtheria and on the causative organism of the disease, *Corynebacterium diphtheria*.

Corynebacteriophage β:

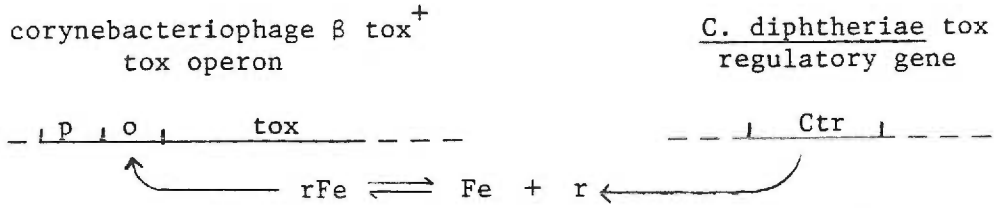
In 1951 Freeman (49) demonstrated that DT was produced in strains of *C. diphtheriae* that had been lysogenized by the bacteriophage β *tox*$^+$ and that non-toxigenic strains could be converted to toxigenic strains as a result of being lysogenized by the phage. The phage responsible for this conversion is a double stranded DNA phage with a genome size of approximately 2.2×10^7 daltons (168).

Holmes and Barksdale (73) were the first to map the *tox* gene within the vegetative β phage genome by using a series of phage mutants. Since that time others, using temperature sensitive (ts) mutants of the bacteriophage, were able to expand upon the genetic map of the phage and demonstrated that *tox* is found near the *c* marker (clear plaque) between the *c* marker and the *h* marker (host range)(102,154,155).

Expression of the tox gene:

Although the data clearly demonstrated that tox was indeed a phage gene, they did not distinguish between the possibilities that the product of the tox gene was in fact the toxin molecule or merely a product that serves as a regulator of a bacterial structural gene. The isolation of phages carrying a mutant tox gene which were able to transmit all the genetic information required for the production of mutated cross reacting material (CRM)(167), gave definitive evidence that the tox gene was indeed the structural gene for the toxin molecule.

In addition to the presence of the tox gene, the physiological state of the host Corynebacterium diphtheriae also plays a role in the production of DT and apparently determines the final toxin yield. Extracellular toxin is not produced until the iron content of the culture medium becomes limiting and the intracellular iron begins to decrease (123,132). A model system was recently suggested by Murphy et al. (111) to explain the molecular mechanism by which tox gene expression is regulated. Using a mutant lysogen of C. diphtheriae that produced toxin in what were normally repressive amounts of iron, they suggested the following model:

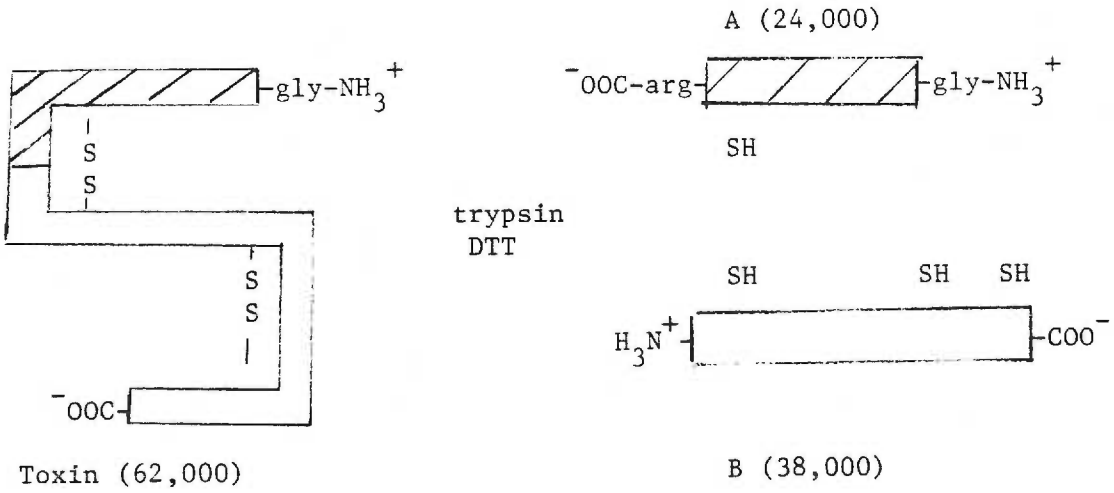


According to this model, Ctr (C. diphtheriae tox regulatory gene) codes for the production of a repressor molecule (r), which,

in the presence of iron, a co-repressor, binds the operator of the tox operon thereby preventing the transcription of the gene product. Under conditions of limiting iron, the equilibrium shifts to the right, the operon becomes derepressed, and toxin is produced.

Diphtheria toxin structure:

DT is produced as a single polypeptide chain with a molecular weight of 62,000 daltons (31,52). Mild trypsinization in a non-reduced state causes the cleavage of an arginine rich area of the toxin molecule. The fragments of the toxin are held together by two disulphide bonds and the resultant molecule is referred to as "nicked toxin". Breakage of the disulfide bonds by reducing agents such as dithiothreital will then lead to the formation of fragment A, with a molecular weight of 24,000 daltons, and fragment B, with a molecular weight of 38,000 daltons. A diagram of the molecule, before and after reduction and trypsinization, is shown below (124).



Effect of DT on eukaryotic protein synthesis:

The first in vitro evidence of the effect of DT on protein synthesis was reported by Strauss and Hender (162). They demonstrated that, upon the addition of a saturating concentration of DT, amino acid incorporation into cellular protein continued at a normal rate for about 1 to 1.5 hours and then rapidly came to a halt. Lower concentrations of DT caused a lengthening of the lag period and a decrease in the decline of protein synthesis. Although other alterations in intoxicated cells, such as the inhibition of DNA and RNA synthesis, have been reported (87,161), these changes show up only after the cessation of protein synthesis and are apparently a secondary result of the inhibition of protein synthesis.

Utilization of cell-free protein synthesizing systems gave a more direct way of testing the effect of DT on protein synthesis. It was demonstrated that HeLa extracts could become insensitive to intoxication after dialysis or passage through a Sephadex G-25 column indicating that inhibition was dependent upon a small component of the cell-free extracts (32). By adding back various cofactors to the dialyzed systems, Collier and Pappenheimer (32) were able to demonstrate that oxidized nicotinamide adenine dinucleotide (NAD) was required for the cell-free protein systems to be sensitive to intoxication by DT.

Subsequently, Collier (29) demonstrated that treatment of cell-free protein synthesizing systems with NAD and DT caused a marked reduction in the activity of cellular elongation factor 2 (EF-2) without affecting the activity of other components of the

cell-free systems. Furthermore, Goor and Pappenheimer (57) presented evidence suggesting that EF-2 was sensitive to inactivation only when it was free in solution with that portion of the EF-2 still bound to ribosomes being protected from inactivation.

It was later suggested that toxin caused the inhibition of cell protein synthesis by catalyzing the transfer of adenosine diphosphate ribose from NAD to EF-2, inactivating EF-2, and terminating the growth of polypeptide chains. The reaction proposed by Honjo et al. (74) was as follows:

$$\text{EF-2} + \text{NAD}^+ \overset{\text{toxin}}{\rightleftharpoons} \text{ADPR-EF-2} + \text{nicotinamide} + \text{H}^+$$

Enzymatic role of fragment A:

About the same time the means by which intoxication of protein synthesis takes place was being elucidated, it was found that the reaction was not catalyzed by the intact toxin molecule but rather by the fragment A of the molecule (30). It was discovered that the intact molecule, although toxic for sensitive animals, is virtually enzymatically inactive in cell-free systems. Furthermore, it was shown that fragment A is highly active in catalyzing the ADP-ribosylation of EF-2 and could account for all the activity previously attributed to the whole toxin. Fragment B, like whole toxin and nicked toxin, does not have any enzymatic activity in cell-free systems. Table 1 summarizes the toxic and enzymatic properties of these various structures (55).

Table 1

Toxicity and Enzymatic Activity of DT and DT Fragments

Structure	Toxicity	Enzymatic activity
Intact toxin	+	-
Nicked toxin	+	-
Fragment A	-	+
Fragment B	-	-

The presence of thiols in the cell-free protein synthesizing systems along with at least small amounts of naturally nicked toxin caused the fortuitous formation of fragment A in the early cell-free studies accounting for the observed results.

Role of fragment B:

Evidence for the role of fragment B has come from a variety of sources. When nicked toxin is incubated with trypsin, fragment B is more rapidly hydrolyzed than fragment A (54). Gill and Pappenheimer (54) reported that the toxicity of trypsin digests of DT to HeLa cells is directly proportional to the amount of fragment B remaining in the preparation even though the activity of the fragmant A remains the same. These data suggest that fragment B is required for the entry of fragment A into the cells.

Various immunologically cross reacting forms of the toxin (CRMs) have been isolated (124,167,168). One of these, CRM 197, has an alteration in the A portion of the molecule which renders

the molecule nontoxic. The B fragment, however, is intact. This CRM is capable of inhibiting the intoxication of cells in tissue culture, although it will not inhibit ADP-ribosylation in cell-free systems. The CRM apparently binds competitively with specific receptors on the surface of the cell. A second CRM, CRM 45, has an enzymatically active fragment A but lacks a large portion of fragment B. Like CRM 197, this molecule is incapable of intoxicating cells. Uchida et al. (169,170) have demonstrated the reconstitution of fully toxic molecules from the fragment A of CRM 45 and the fragment B of CRM 197.

Similar competition experiments have been done using nitrated toxin and purified fragment B maintained in a borate buffer (181) giving further evidence for the role of fragment B in cellular intoxication by DT.

Determination of toxin sensitivity:

Although EF-2 from a variety of eukaryotic organisms tested, including vertebrate, invertebrate, and plants, are equally sensitive to ADP-ribosylation by fragment A in cell-free systems, there is a very large variation in the sensitivities of the organisms to intoxication by DT (57,63,74). For example, although rats and mice are much more resistant to intoxication by DT than guinea pigs, the EF-2 from these resistant cells is as readily ADP-ribosylated by fragment A as the EF-2 of sensitive cells (57). Because of this observation, it has been postulated that sensitivity of cells to DT is determined at the membrane level of the cells either by differences

in the number or availability of specific receptor sites (18,57) or by differences in the cleavage or internalization of the toxin (168).

Endocytosis:

Extracellular material can enter cells by way of plasma limited vacuoles by a process of engulfment referred to as endocytosis. Endocytosis can be divided into two main catagories. Phagocytosis is the uptake of particulate material with a diameter >1 μm. The second catagory is pinocytosis referring to the uptake of soluble material (1). Pinocytosis can be further divided into macropinocytosis, the uptake of material into vacuoles visible by light microscopy, and micropinocytosis, the uptake of material into microvesicles demonstrable only by electron microscopy.

The study of metabolic inhibitors has indicated that glycolytic inhibitors such as sodium fluoride and iodoacetic acid, but not inhibitors of oxidative metabolism, strongly inhibit phagocytosis in most cells (35,60,86,145). It was generally assumed that sodium fluoride and iodoacetic acid inhibited phagocytosis by lowering the concentration of cellular ATP. However, Michl et al. (104) showed that, although sodium floride decreased cellular levels of ATP by 40% and phagocytosis by 80%, 2-deoxyglucose caused a 50% decrease in cellular levels of ATP but did not inhibit phagocytosis. A similar lack of correlation between ATP content of cells treated with metabolic inhibitors and cellular pinocytosis has been reported for L-cells (158). From this it

suggested that endocytosis is dependent upon metabolic energy although ATP may not be the final energy source (153).

The relationship of microtubules and microfilaments to endocytosis has been examined. Compounds such as colchicine, which disrupt microtubules, do not inhibit endocytosis (12,128). On the other hand, cytochalasin B, which inhibits microfilament function (65), inhibits both phagocytosis and pinocytosis (5,176). These results indicate that the cellular microfilaments are responsible for the motion of the cell membrane involved in endocytosis and that microtubules are not.

Edelson and Cohn (42) reported that concanavalin A (Con A) stimulated pinocytosis in mammalian cells and that mannose and glucose were able to block the effect of the lectin. Furthermore, similar effects could be observed with B lymphocytes that had been exposed to antisera against the cell specific immunoglobulins (171). It is of interest that the lectin binding the cell receptor in both the Con A and the antisera cases was a polyvalent lectin. If cells were exposed to monovalent forms of the lectin, capping of the lectin and subsequent stimulation of pinocytosis was not observed (171). The capping of the lectin suggests an increased motility of the lectin binding sites within the fluid membrane of the cell which apparently requires microfilament contraction. These data led to the proposal of the following model for ligand-mediated endocytosis (61):

First, a ligand-receptor interaction generates a signal that initiates the aggregation of microfilaments and leads to the

extension of pseudopods in the area of the material to be engulfed. Next, pseudopod extension leads to further receptor-ligand interactions and this in turn leads to further aggregation of the microfilaments. Finally the process is repeated many times until the plasma membranes meet and fuse with each other, forming an endocytotic vacuole.

Role of endocytosis in the intoxication of cells by DT:

Certain plant lectins have been shown to have differential abilities to intoxicate mammalian cells, and some of them, such as ricin and abrin, have been shown to preferentially intoxicate tumor cells as compared to normal cells (99). Ricin molecules are composed of two nonidentical peptides linked by disulfide bridges, and it has been shown that the toxic action is in one of the two peptide chains (118,119) causing speculation that one chain inhibits protein synthesis while the other is involved in the binding of the toxin molecule to specific receptors on the cell surface (118). Although this molecule has some similarities to DT, ricin acts by modifying the 60S ribosomal subunit thereby impairing the interaction of the EF-2 with the ribosomal subunit and terminating protein synthesis (154) and not with the EF-2 itself as shown with DT (29,57,74).

Recently Nicolson et al. (113,116) presented evidence that differential sensitivity of normal as compared to transformed cells to intoxication by ricin is due to a lectin induced aggregation and endocytosis of the toxin molecule and not due to the

number of toxin receptors on the cell surface.

Moehring and Moehring (106) were among the first to examine the possible role of endocytosis in the determination of sensitivity of cells to DT. They demonstrated that the sensitivity of L-cells, a cell line normally resistant to intoxication by DT, could be increased by exposing the cells to poly-L-ornithine, a substance that increases the endocytotic activity of the cells.

Bonventre et al. (17) examined the role of endocytosis in the intoxication of cells sensitive to DT. Ammonium chloride has been shown to protect normally sensitive cells (Hep-2) from intoxication by DT (84). Although the toxin would still interact with the cell surface, it was maintained at a site accessible to neutralization by diphtheria antitoxin. However, concentrations of ammonium chloride at levels great enough to protect Hep-2 cells from intoxication by DT did not alter the uptake of the toxin by endocytosis. Furthermore, monolayers of L-cells were shown to take up iodinated toxin by endocytosis at a much greater rate than Hep-2 cells. These data led Bonventre et al. (17) to postulate two mechanisms for the uptake of DT by cells. Entry by means of endocytosis is non-specific, occurs in both sensitive and resistant cells, does not require fragment B, and plays only a minor role in the intoxication of cells by DT. Specific entry, on the other hand, involves a surface receptor-fragment B association and is responsible for differential sensitivities of cells to intoxication by DT.

DT receptors:

Although it has often been suggested that fragment B of DT interacts with a specific DT receptor on the surface of the cell (17,18,53,181), direct evidence for the presence of these receptors has not been presented. Other toxins, such as cholera toxin, tetanus toxin, abrin, and ricin, have also been postulated to have specific receptors on the cell surface. Unlike DT, however, receptors for these molecules have been identified. Cholera and tetanus toxins bind to ganglioside sugar residues (72,173) while abrin and ricin bind to galactose containing sugars (120).

Recently, Draper et al. (42) presented the first evidence as to the possible nature of the DT receptor sites. They demonstrated that the intoxication of Chinese hamster V 79 cells by DT could be inhibited by Con A, succinylated Con A, and wheat germ agglutinin (WGA). They further reported that the effects of Con A could be reversed by methyl-α-mannoside and that of WGA by acetylglucosamine. From these data they suggested that DT receptors may be oligosaccharides containing N-acetylglucosamine and mannose.

Recently Boquet and Pappenheimer (18) were able to estimate the number of specific binding sites on the surface of cells by correcting for non-specific internalization of iodinated toxin into endocytotic vesicles. They reported that HeLa cells, a sensitive cell line, specifically bound 4000 toxin molecules on their cell surface while L-cells, a resistant cell line, did not show any specific binding or uptake beyond that expected for

endocytosis. Furthermore, they proposed a three step method by which enzymatically active fragment A enters the cytoplasm of the cells. The first step is a rapid, reversible association of fragment B with toxin specific binding sites on the surface of the cells. The second reaction is a slower irreversible, temperature sensitive reaction during which the receptors become inactivated for reactions with additional toxin molecules. Finally the molecule is processed and fragment A enters the cytoplasm while fragment B remains associated with the cell membrane.

Model for the specific entry of DT:

Boquet et al. (19) demonstrated the hydrophobic property of a portion of the B fragment of DT by quantitating the amount of tritiated Triton X-100 that bound to the whole molecule, CRM 45, and fragments A and B. They found that native CRM 45, which lacks the 17,000 dalton COOH terminal end of the intact molecule (166), readily bound Triton X-100 molecules while the native whole toxin did not. However, if the toxin was first denatured in 0.1% sodium dodecyl sulfate, then it would bind the same amount of Triton X-100 molecules as the CRM 45 (19). They concluded that the toxin molecule contains a hydrophobic region, normally masked, that is located on fragment B near the junction of fragments A and B. A conformational change of the molecule, such as is seen during denaturation with SDS, is necessary to expose the hydrophobic region of the molecule. From these findings they (19) proposed the following model to describe the process by

which the DT fragment A is transported across the plasma membrane:

After initial association of the toxin with specific membrane receptors, the hydrophobic portion of the toxin molecule is exposed either by the removal of a polypeptide from the COOH terminal end of the molecule forming a CRM 45-like molecule or by a major conformational change, as seen under denaturing conditions. The hydrophobic region is then inserted into the lipid bilayer of the cell membrane forming a channel through which fragment A is drawn. Nicking and reduction of the molecule then takes place at the inner membrane surface of the cell releasing the fragment A into the cell cytoplasm.

B. ONCOGENIC VIRUSES:

The role of viruses in the study of transformed and malignant cells began with the discovery that avian leukemia and sarcomas can be transmitted by the inoculation of healthy birds with cell free filtrates from infected birds (45,137). Since that time a number of viruses, both with DNA or RNA genomes, have been shown to produce cell transformation *in vitro* or malignancy *in vivo*.

DNA viruses:

The oncogenic DNA viruses are found in three main groups. These are the papovaviruses, including SV 40 and polyoma, the adenoviruses, and the herpesviruses. Stoker and MacPherson (159) demonstrated that polyoma virus could transform cells *in vitro* and produce tumors when injected into experimental animals.

Since the cells were genetically stable, it was assumed that the virus DNA had either remained in the cell in some unspecified state or had caused a permanant alteration in the genetic makeup of the cell. Subsequent reports did demonstrate the presence of papovavirus DNA in cells that had been transformed by these viruses (4,133,143), and, furthermore, the viral DNA detected was integrated into the cell DNA by means of covalent bonds (143).

When an oncogenic DNA virus infects a cell, one of three events can occur (159). The first is an abortive infection during which the virus is lost without any apparent change in the infected cell.

The second possible event is a lytic infection by the virus. During the lytic cycle the viral genome has been shown to become integrated into the cellular genome (40,131). However, since DNA replication appears to require free DNA, either in a circularized (21,71) or a non-cicularized (127) form, the importance of virus integration in the lytic cycle is unknown. Early mRNA, followed by late mRNA transcription occurs, leading to the translation of viral proteins. Among the early proteins produced in papovavirus infected cells are the T antigen (15,130), the U antigen (98), and the tumor specific transplantation antigen (TSTA)(68,93). Although these early proteins are found in both the lytically infected and the virally transformed cells, their functions in the lytic cycle of the virus is unknown. The late proteins generally consists of the viral structural proteins (48,103,121).

If a cell, for some reason non-permissive for virus replication, is infected with an oncogenic DNA virus, a third event may occur. Although the majority of the cells will remain normal, a small fraction of the cells can become transformed (14,39,151).

As was seen in the lytic infection of cells by these viruses, when cells are transformed by DNA viruses, the viral genome becomes integrated into the cellular genome (71). However, unlike the lytic cycle, during cell transformation much of the early mRNA is transcribed while only a small portion of the late mRNA is transcribed (2,117). Evidence has been presented indicating that transformed cells transcribe 37-75% of the early mRNA while transcribing only 0-8% of the late mRNA (91).

In all stably transformed cells there is evidence for the presence of at least part of the viral genome. Hybridization experiments with SV 40 transformed cells indicate that these cells contain 1-3 equivalents of viral DNA (50,92). Induction of virus particles from transformed cell lines has been done by the fusion of the transformed cells with cells permissive for virus replication (84,178) giving further evidence for the presence of the entire virus genome in at least some virally transformed cells.

RNA oncogenic viruses:

The earliest viruses shown to cause cancer in animals were RNA tumor viruses, also known as retroviruses (45,137). These viruses can be classified into two general types according to their appearance under electron microscopy. These are the type

B and the type C particles (37). A third type, type A, has been described but appears to be an intracellular precursor to mature B particles. Both groups have several similarities.

Retrovirus genome:

The viral genome of retroviruses consists of a large molecular weight 70S single stranded RNA which, when heated, can be disassociated into 35S RNA subunits and an assortment of small 4-7S RNAs (46,47,135,144). Although the number and makeup of the 35S RNA was a thing of speculation for several years, recent evidence indicates that the 70S RNA is made up of two identical 35S RNA fragments linked together by hydrogen bonds at the 5' end of both molecules (11,13,70,94,156).

Reverse transcriptase:

A second common trait of the retroviruses is the presence of a viral RNA-dependent DNA polymerase, the reverse transcriptase (8,163). This polymerase makes a DNA copy of the viral RNA genome using a 4S tRNA as the primer (97). The double-stranded DNA then apparently circularizes and is integrated into the cellular genome (174). The presence of the entire viral genome in transformed cells has been shown by the isolation of infectious DNA from the cellular genome of virus transformed cells (69,87). This DNA is then used to code for the RNA and proteins necessary for viral synthesis and cell transformation (7).

Retrovirus genetics:

Work with conditional and non-conditional mutants of the retroviruses has shown the presence of four genes in the genome of these viruses (9). These four are called gag, pol, env, and onc or src. Gag codes for a 76,000 dalton protein that appears to be the precusor to most of the major internal proteins of the avian RNA tumor viruses (44). These are the non-glycosylated structural proteins of the virion. The env gene codes for the envelope glycoproteins of the virus (9). Viruses that are defective in either of these two gene functions produce non-infectious progeny viruses and are referred to as rd or replication defective mutants (166).

The pol gene is responsible for at least the smaller of the two subunits of the reverse transcriptase (122). This 60,000 dalton subunit may be derived by the cleavage of the larger 90,000 dalton subunit (110). Viruses defective in this function are unable to form a DNA copy of the viral genome, can not integrate into the cellular genome, and, therefore, can neither replicate within nor transform the cells. These mutants are referred to as cd or coordinately defective mutants (166).

The src gene codes for a 60,000 dalton protein apparently responsible for cell transformation (130). This gene product has a protein kinase activity (28) and may act by directly or indirectly causing a chemical modification of the components of the cell (3). Mutants of this class are called td or transformation defective mutants (166). Avian leukosis viruses which do not

transform cells *in vitro* apparently lack the *src* gene (9).

Temperature sensitive transformation defective mutants:

Temperature sensitive (ts) mutants of the retroviruses have aided in the study of cell transformation. Martin (101), one of the first to describe ts mutants of the td variety, demonstrated that virus replication continues at the restrictive temperature indicating that transformation is not needed for viral replication. Similar results have been obtained with other ts mutants having similar lesions in their transformation function (6,89).

Although ts mutants have proven invaluable in the study of cell transformation because of their ease of manipulation and the rapidity with which they will convert from one phenotype to the other, it has been shown that, even at the restrictive temperature, some of the properties associated with the transformed state can still occur. For example, cells infected with the virus LA 334, a ts mutant of Rous sarcoma virus, maintain the ability to grow in agar suspension even at the restrictive temperature, a trait normally associated with transformed cells but not with normal cells (180). Becker and Friis (10) have isolated ts mutants which show a level of sugar transport at the restrictive temperature between that of normal and wild type virus transformed cells. Although there is this apparent "leakiness" in at least some of the transformation functions of these viruses, they have still added much to our knowledge of cellular transformation.

C. CELLULAR TRANSFORMATION:

When a cell undergoes transformation by either oncogenic viruses, chemical carcinogens, or radiation, the cell undergoes several modification. Many inconsistancies exist in the literature between transformation systems as to the types or amount of cell components or functions which are altered. There are, however, some distinct differences reported.

LETS protein:

Hynes (77) reported the presence of a large external surface glycoprotein on the surface of normal cells that was not found on the surface of transformed cells. This 250,000dalton glycoprotein, LETS (large external transformation sensitive), has been found on normal, but not transformed, hamster (76), mouse (77), rat (160), and chicken (100) cells. Hynes and Humphreys (78) demonstrated that this component cannot be metabolically labelled in transformed cells, whereas it can in normal cells, indicating that it is probably missing from the cell surface rather than present but somehow masked from iodination on the surface of transformed cells. Metabolic labelling with C^{14}-glucosamine (33) indicated that, although greatly reduced in transformed cells, LETS is produced in Rous sarcoma virus transformed cells and is turned over into the medium. It was suggested that another molecule responsible for its retention in the cell membrane might be missing (33). LETS is sensitive to proteolytic digestion and mild trypsinization will

remove it from the cell surface (77).

A second class of large molecular weight glycoprotein found on the surface of normal cells but not on the surface of transformed cells has been described (141), however, this SF-antigen has since been shown to have many of the same properties of the LETS protein and is now believed to be the same material (90).

Antigenic changes:

Another alteration in the cell occurring during cellular transformation is the appearane of new antigens on the cell surface. One of these, the tumor specific surface antigen (TSSA), has been extensively studied. Transformation of cells by avian sarcoma viruses consistently leads to the expression of TSSA (51,59,95). Yet chick embryo fibroblasts infected with, but not transformed by, leukosis viruses lack this antigen (51). Furthermore, work with ts mutants and transformation defective deletion mutants of avian sarcoma viruses confirms that expression of this antigen is transformation dependent (96).

An increase in the expression of embryonic antigens on the surface of transformed cells has also been demonstrated (22,56,165). As early as 1906 Schone demonstrated that animals could be protected from tumor growth through the immunization of the animals with embryonic tissues (147). These, and similar results, led Coggin and Anderson (27) to postulate that all tumor antigens might be of embryonic origin somehow being derepressed during cellular transfor-

mation.

Glucose uptake:

Hatanaka et al. (66) described an increase in the rate of glucose uptake in murine sarcoma virus transformed cells. This change in glucose uptake was not observed with leukemia virus infected cells even though the sarcoma virus stocks contained helper leukemia virus particles in higher titers than the sarcoma viruses.

Similar results were observed in Rous sarcoma infected cells (67). Furthermore, experiments with transformation defective ts mutants of both the Schmidt-Ruppin strain and the Bryan high titer strain of the virus showed that increased sugar uptake is related to the expression of the transformation function of the virus genome (6,89).

Alteration in lectin agglutinability:

Another property of transformed cells is that they generally agglutinate at a lower concentration of lectins than their normal non-transformed counterparts (23,82,129). The first attempts to show differential agglutination in transformed CEF failed (110) until Burger and Martin (24) demonstrated that transformed cells had to be treated with hyaluronidase prior to exposure to the lectin. Although this increased agglutinability was first thought to be due to an increased number of lectin binding sites, it has been shown that normal and transformed cells possess similar numbers of surface receptors (83,115). It is now believed that

increased agglutinability by lectins is due to an enhanced motility and aggregation of the lectin binding sites (115,135).

Alterations in the level of cAMP:

Virus transformed cells generally have lower levels of cyclic adenosine 3',5'-monophosphate (cAMP) than their normal non-transformed counterparts (126). This intracellular level of cAMP appears to influence such phenotypic characteristics as cell motility, transport of certain materials, synthesis of certain glycosminoglycans, growth in agar, and agglutinability by Con A (126,177) indicating that many of the characteristics of viral transformed cells may be secondary consequences of a decreased level of cellular cAMP.

D. EFFECTS OF PROTEOLYTIC ENZYMES:

Transformed cells have been shown to possess greater proteolytic activity than their non-transformed counterparts (20,144,172), and it has been shown that when normal non-transformed cells are exposed to low concentrations of proteases, they will display certain of the properties normally associated with transformed cells (112,148,149).

Increased lectin agglutinability:

In 1969 Burger (23) first demonstrated that normal cells would agglutinate after proteolytic treatment at concentrations of WGA

equivalent to those necessary for the agglutination of virus transformed cells. This observation has since been expanded to include other plant lectins (82,114,150). It has been suggested that protease treatment increases cellular agglutinability by severing the attachment of lectin receptors to microfilaments and microtubules thereby allowing the increased motility of the receptors within the cell membrane (136).

Plasma membrane proteins and glycoproteins:

Certain of the membrane components have been shown to be altered when normal cells are exposed to mild proteolytic treatment. The most prominent of these alterations is the loss of the LETS protein (77). Besides the LETS protein, there is evidence for the removal of certain sulfated glycosaminoglycans from the cell surface during proteolysis (26). Up to 80% of the cell associated glycosaminoglycans are removed with mild trypsinization.

Cellular multiplication:

Sefton and Rubin (149) originally provided evidence for the stimulation of cell multiplication by protease treatment. Since that time others have shown similar results (25,34), and furthermore, that the stimulatory effect could be prevented by the addition of trypsin inhibitor (34).

Cyclic nucleotide levels:

Trypsin treatment of normal non-transformed murine cells causes

a rapid, transient decrease in the cAMP levels of the cells (16,149). On the other hand, Hovi et al. (75) reported no significant change in the cAMP levels of chick cells although there was an increase in the intracellular cGMP levels. The role these changes in the cyclic nucleotide levels may play in the phenotypic changes observed during proteolytic treatment of normal cells is a matter of speculation, although it has been suggested that the alterations may be secondary consequences of the altered cAMP levels (126, 179).

Materials and Methods

Medium: Chick embryo fibroblasts (CEF) medium (CM-5) consisted of Dulbecco's modified Eagles medium (D-MEM) supplemented with 10% tryptose phosphate broth (TPB), 5% fetal calf serum (FCS), and 50 µg/ml gentamicin.

Primary CEF medium (CK-1) consisted of D-MEM supplemented with 10% TPB, 8% FCS, 1% lactalbumin hydrolysate, and 50 µg/ml gentamicin.

Medium for BHK-21, py-BHK, and BH-CEF was D-MEM supplemented to contain 4.5 g glucose/liter, 10% TPB, 10% FCS, and 50 µg/ml gentamicin.

Medium for 3T3, transformed 3T3, NRK, transformed NRK, and L929 cells was Hanks' minimal essential medium (H-MEM) supplemented with 10% FCS and 50 µg/ml gentamicin.

The diphtheria toxin assay medium consisted of H-MEM modified to contain 1/10 the normal concentration of amino acids, 1% glutamine, 2% FCS, and 50 µg/ml gentamicin.

The primary BHK medium (ETC) consisted of Eagles basal medium modified to contain twice the normal concentration of amino acids and vitamins, 10% FCS, 10% TPB, and 50 µg/ml gentamicin.

Buffers: Tris glucose buffer (TG) consisted of 0.025 M Tris, pH 7.4, 0.14 M NaCl, 0.005 M Na_2HPO_4, and 0.001 M glucose.

Versene consisted of 0.0005 M EDTA, 0.14 M NaCl, 0.003 M KCl, 0.008 M Na_2HPO_4, 0.0015 M KH_2PO_4, and 0.003 M glucose, pH7.4.

Calcium and magnesium free phosphate buffered saline (PBS) consisted of 0.145 M NaCl, 0.008 M Na_2HPO_4, and 0.002 M KH_2PO_4, pH 7.4.

Histamine buffer consisted of 0.11 M histamine, 90 mM Tris-HCL, 70 mM DTT, and 0.017 mM EDTA, pH 8.0.

Cells: Primary CEF were prepared from 9-11 day old cofal negative c/o type chick embryos (Spafas, Inc., Norwich, Conn.) in the manner of Rubin (140) as modified by Hanafusa (64). Chick embryos were aseptically removed from the eggs, decapitated and eviscerated, and minced in sterile TG buffer. The cell clumps were allowed to settle out for three minutes. Those cells remaining in suspension, consisting mostly of erythrocytes, were discarded by decanting the fluid supernatant. Ten ml of 0.25% trypsin in TG buffer were added for each embryo. The mixture was stirred for 5 minutes at room temperature and the cell clumps were allowed to settle out for 3 minutes. The suspended cells were transferred to a small volume of cold CK-1 medium. The trypsinization and settling procedures were repeated three times on the remaining cell clumps. The cells in suspension were then pelleted by centrifugation at 250 x g for 10 minutes, resuspended in CK-1, and seeded at a cell density of 1×10^7 cells/plate in 100 x 15 mm tissue culture dishes (Flow Labs., Inglewood, Calif.). All chick cells were incubated at 41C in a CO_2 incubator unless otherwise indicated.

Primary baby hamster kidney (BHK) cells were prepared as

described by Stoker and MacPherson (159). Kidneys of 1 day old hamsters were left overnight at 4C in 0.25% trypsin in versene. The next day the solution was replaced by fresh trypsin-versene and the kidneys were incubated at 37C for 15 minutes. Strenuous pipetting produced a suspension of single cells and small clumps which were pelleted by centrifugation at 250 x g for 5 minutes. The cells were then suspended in ETC medium and planted in two 250 ml tissue culture flasks (Flow Labs., Inglewood, Calif.)/ kidney. The cells were incubated at 37C.

A continuous line of polyoma transformed baby hamster kidney cells (py-BHK) and their non-transformed counterparts (BHK-21) were obtained from the laboratory of Dr. Eckhardt.

Normal 3T3 cells, a continuous line of mouse cells, were obtained from Dr. J. Hallum, Dr. B. Brockman, and Dr. S. Aaronson. SV 40 transformed 3T3 cells (SV-3T3) were obtained from Dr. J. Hallum and Dr. B. Brockman. Polyoma transformed 3T3 cells (py-3T3) were obtained from Dr. S. Aaronson. Rous sarcoma virus transformed 3T3 cells (SR-3T3) were obtained from Dr. P. Vogt.

L929 cells, a continuous line of mouse cells, were obtained from the laboratory of Dr. B. Iglewski.

Normal rat kidney cells (NRK) were obtained from Dr. J. Dahlberg. Rous sarcoma virus transformed NRK (SR-NRK) were obtained from Dr. S. Aaronson. Murine sarcoma virus transformed NRK (NRK-MSV) were obtained from Dr. J. Bader.

All continuous lines were transferred to new flasks when old monolayers approached 85% confluency.

All primary cells were utilized for experimentation by the fifth passage.

Viruses: Starter stocks of Schmidt-Ruppin type A Rous sarcoma virus (SR-RSV) and ts 68, its transformation defective temperature sensitive mutant (89), were supplied by Dr. H. Hanafusa.

Starter stocks of Bryan high titer Rous sarcoma virus (BH-RSV) and Ta, its transformation defective temperature sensitive mutant (6), were supplied by Dr. J. Bader.

Virus propagation and all cell transformations were done by the method of Hanafusa (64). Primary CEF were harvested by trypsinization and seeded at a density of 5×10^6 cells/100 mm tissue culture dish in CM-5. After 4-6 hours incubation at 41C the culture fluid containing unattached cells was removed. The cells were then exposed to 0.1 ml of the virus suspension having at least 5 FFU/cell in cases leading to rapid transformation or 1 FFU/cell in cases leading to virus propagation. For all viruses except SR-RSV it was necessary to include 0.1 ml DEAE-dextran (250 µg/ml) with the virus to insure infection of the cells. After adsorption of the virus for 1 hour, the cultures were washed and reincubated in CM-5 at 41C. Cells infected with the ts mutants of the viruses, Ta and ts 68, were treated in the same manner except that half the plates of the infected cells were incubated at the permissive temperature of 37C and half at the non-permissive temperature of 41C. Prior to any experimentation with the ts virus infected cells, one plate of cells was shifted from the non-permissive temperature to the

permissive temperature to insure that the cells were infected.

Starter cultures of avian myeloblastosis virus (AMV) were obtained from Dr. G. Beaudreau and the virus propagated by inoculations of newly hatched chicks and the subsequent bleeding of the chicks when they became 4+ leukemics (42).

Toxins and toxin inhibitor: Diphtheria toxin (DT) was obtained from Connaught Medical Research Laboratories (lots D279 and D290 with 1600 flocculating units/mg nitrogen).

CRM 197 was the generous gift of Dr. T. Uchida (167).

Pseudomonas aerugenosa exotoxin A (PA toxin) was the generous gift of Dr. B. Iglewski.

Chemicals: Trypsin was purchased either as a 2.5% solution from Flow Laboratories, Inglewood, Calif., or as a twice recrystalized powder from Sigma, St. Louis, Missouri.

Tritiated amino acids were purchased fromm either New England Nuclear, Gardena, California, or from Amersham Corp., Arlington Heights, Illinois.

Labelled NAD and ^{125}I were purchased from New England Nuclear, Gardena , California.

All other chemicals were purchased from Sigma, St. Louis, Missouri.

Toxin assay: The toxin assay for cell sensitivity to DT and PA toxin were done in two ways. The first was a cell suspension assay done

in the manner described by Iglewski et al. (81). Briefly, cells were harvested by trypsinization, washed three times with assay medium, and seeded at a concentration of 4×10^5 cells in 0.9 ml of assay medium in screw cap tubes in the presence of various concentrations of toxin. Control cells were treated in the same manner without the addition of toxin. The mixture was incubated at 37C for three hours in a roller drum. At that time, 10.0 µCi of ^{3}H-labelled amino acid mixture was added to each tube bringing the final volume to 1.0 ml/ tube. Labelling was allowed to continue for an additional two hours at 37C. The cells were then harvested by centrifugation and lysed by resuspension in 0.002 M EDTA in distilled water and freezing and thawing. Trichloroacetic acid (TCA) was added to a final concentration of 10% and the mixture heated at 90C for 15 minutes. The hot TCA precipitable protein was collected on Millipore filters and the radiation counted in a Beckman LS 200 spectrophotometer.

The second method of assaying the cells for sensitivity to DT and PA toxins was a cell monolayer assay similar to the one described by Venter and Kaplan (175). Cells were harvested and planted on 35 x 10 mm tissue culture plates at a cell density of 5×10^5 cells/ plate. After 16-24 hours, the semi-confluent monolayers were washed three times with assay medium and 0.9 ml medium containing various concentrations of toxin were added to each plate. Control cells were treated in the same manner without the addition of toxin. The plates were incubated in a CO_2 incubator at 37C for three hours. Two µCi of ^{3}H-labelled amino acid mixture was added to each plate

bringing the final volume to 1.0 ml. Labelling was allowed to continue for two hours at 37C. The cells were harvested with 0.25% trypsin in distilled water and lysed by freezing and thawing. TCA was added to a final concentration of 10% and the mixture heated at 90C for 15 minutes. The hot TCA precipitable protein was collected on Millipore filters and the radiation counted in a Beckman LS 200 spectrophotometer.

EF-2 quantitation: Quantitation of EF-2 of normal and SR-RSV transformed CEF was done using the procedure described by Gill and Dinius (53). Cells were washed in PBS and packed by centrifugation at 2000 x g for 10 minutes. Half a gram of cells was lysed in 2.0 ml 0.25 M sucrose by sonication in a Biosonik IV sonicator. Into a tube were placed 1.5 ml of the resulting lysate, 0.25 ml 4 M NaCl with 20 mM dithiothreitol, and 0.4 g washed, activated charcoal. The mixture was shaken at 4C for 15 minutes and centrifuged at 27,000 RPM in a Beckman type 30 rotor for 65 minutes at 4C to remove the cell debris and charcoal from the EF-2 in the supernatant fluid. The supernatant fluid was then stored at -70C until assayed for EF-2 content.

To quantitate the EF-2, 50 µl of the supernatant fluid was added to 300 µl histamine buffer with or without the addition of fragment A (7.5 µg/ml final concentration). After the mixture had been warmed to 37C, 0.125 Ci (0.05 µM) ^{14}C-NAD in a volume of 5 µl was added to each tube. The mixture was vortexed and incubated for 10 minutes at 37C. The reaction was halted by the addition of

several volumes of 5% TCA. The proteins were collected on Millipore filters and the radioactivity counted on a Nuclear-Chicago low background gas flow counter.

Iodination of DT: DT was iodinated utilizing the lactoperoxidase procedure described by David (36). Five grams of activated Sepharose 4B (Pharmacia, Piscataway, New Jersey) were swollen in 30 ml 10^{-3} M HCl and washed with 1.0 l of 10^{-3} M HCl. The beads were then washed rapidly with gentle suction with 0.01 M Na_2HPO_4-0.1M NaCl, pH7.5 (PC buffer) until the pH of the wash fluid was pH 7.5. The beads were mixed with 25 mg lactoperoxidase in 20 ml PC buffer and agitated overnight at 4C. The next day the beads were washed with cold PC buffer. The lactoperoxidase beads (LP-beads) were resuspended in 50 ml cold 0.2 M glycine in PC buffer for a minimum of 5 hours at 4C. They were then thoroughly washed with cold PBS and stored at 4C in PBS with 10^{-5} M merthiolate until used.

Immediately before use the LP-beads were washed with cold PBS to remove the merthiolate. The following were then added to a reaction tube: 100 µl protein (100 µg) in PBS, 10 µl ^{125}I (17 Ci/mg), 20 µl LP-beads (1.5 µg lactoperoxidase), 10 µl 3.3 x 10^{-3} M KI, and 10 µl of a 1 to 2000 dilution of 30% H_2O_2. The mixture was incubated for 30 minutes at 23C and the reaction stopped by the addition of 100 µl 0.25 M NaN_3 in 0.5 M KI and incubation for 15 minutes at 0C. The LP-beads were pelleted by centrifugation at 500 x g for 10 minutes at 5C. Labelled protein was separated from free iodine by passage through a Sephadex G-100 column equilibrated and eluted with

a 0.01 M potassium phosphate buffer, pH 6.9, containing 0.05% BSA.

Assays with glycolytic enzymes: Assays done to determine the effect glycolytic enzymes had on the trypsin effect on DT sensitivity were done as follows: normal and transformed cells were lifted with versene instead of trypsin. The cells were pelleted by centrifugation and washed with PBS. Half the cells were incubated in PBS while the other half were incubated in PBS containing various concentrations of the glycolytic enzyme to be assayed at a cell density of 10^7 cells/ml. After 30 minutes at 37C the cells were washed in PBS and resuspended in either PBS or 0.25% trypsin in PBS and incubated for an additional 30 minutes at 37C. The cells were then washed and assayed by the cell suspension assay as described previously.

RESULTS

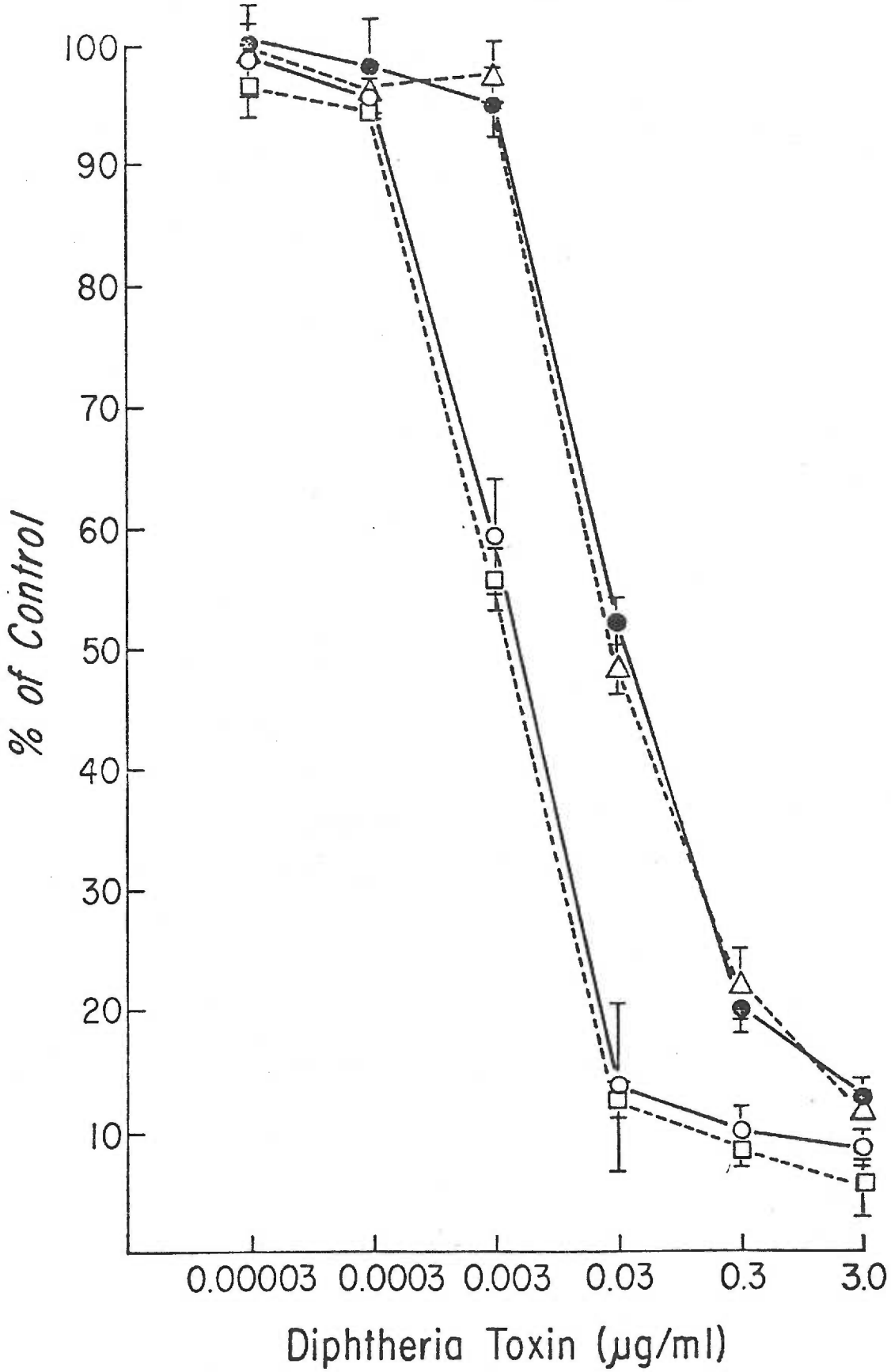
Effect of medium: Iglewski et al. (81) reported that SR-CEF are more sensitive to intoxication by DT than normal, non-transformed CEF derived from the same embryo. However, Pappenheimer and Randall (125), working with Ehrlich-Lettre ascites tumor cells, implied that the assay medium, which contained a modified amino acid concentration and 2% FCS that had been extensively dialyzed, might adversely modify the results of these assays. They reported that dialyzing the serum might remove a serum factor required for the synthesis of proteins in normal cells but not required for the synthesis of proteins in the transformed cells. It was, therefore, necessary to determine what, if any, effect the medium had on the phenomenon of increased sensitivity of transformed cells as compared to normal cells to intoxication by DT. The medium was examined by running parallel assays using the modified H-MEM with 1/100 the normal concentration of amino acids and the dialyzed serum and the normal H-MEM with the normal amino acid concentration and normal undialyzed serum as the assay medium.

Figure 1 shows typical results obtain from this series of experiments. The amount of toxin required to inhibit protein synthesis 50% of the control values is defined as the PID_{50}. There is no significant difference in the PID_{50} of the cells when assayed in either medium. The PID_{50} was approximately 0.003 µg toxin/ml of reaction medium for the SR-CEF while that of the normal CEF was approximately 0.03 µg toxin/ml of reaction medium.

Figure 1

Effect of media on diphtheria toxin inhibition of normal and SR-RSV-A transformed chick embryo fibrobast protein synthesis.

Triplicate samples were assayed for sensitivity of protein synthesis by diphtheria toxin in the manner described in Materials and Methods using both modified Hanks' MEM (M-MEM) with 1/100 the normal amino acid concentration and dialyzed serum and normal Hanks' MEM (H-MEM) as the assay media. Percentage of control incorporation was determined from the mean of intoxicated samples compared to the mean of control cells not exposed to toxin. Bars indicate the standard error of the mean. ●——●, chick embryo fibroblasts, M-MEM; ▲- -▲, chick embryo fibroblasts, H-MEM; ○——○, SR-RSV-A transformed cells, M-MEM; □- - -□ SR-RSV-A transformed cells, H-MEM.


100
90
80
70
60
50
40
30
20
10
% of Control
0.00003
0.0003
0.003
0.03
0.3
3.0
Diphtheria Toxin (µg/ml)

The only effect of lowering the amino acid concentration and dialyzing the serum was to increase the specific activity of the labelled TCA precipitable protein. In a typical experiment the control CEF incorporated 16,000 CPM with normal H-MEM and 47,160 CPM with the modified H-MEM while the SR-CEF incorporated 14,750 CPM and 42,350 CPM respectively. Therefore, the effect of increased sensitivity to DT of transformed cells as compared to normal cells is not due to the assay medium during the five hour incubation period of the assay. Statistical analysis using analysis of variance and the Student t test indicates that there is no difference in the results obtained using either medium. However, in both media the difference between the cell types was significant with the SR-CEF being more sensitive to intoxication by DT than the normal CEF ($P<0.005$). Subsequent assays were done using the modified H-MEM containing 1/10 the normal amino acid concentration and supplemented with 2% normal, undialyzed FCS.

Toxin sensitivity of ts 68 infected cells: The first step in determining what property of SR-RSV transformed CEF was responsible for the differential sensitivity to intoxication by DT as reported by Iglewski et al. (81) was a study of the effect of DT on cells infected with the temperature sensitive mutant ts 68 of SR-RSV. This mutant transforms cells with virus replication at the permissive temperature of 37C but does not transform cells at the non-permissive temperature of 41C even though similar

amounts of virus are produced at both temperatures (89).

Figure 2a and Table 2 show the results of assays done on ts 68 transformed cells at the permissive temperature. The PID_{50} of the transformed cells is once again less than that of the normal cells. These results are similar to those seen for the wild type SR-CEF (fig. 1). Furthermore, this difference in sensitivities is once again statistically significant at the 0.995 confidence level (Table 2).

Figure 2b and Table 2 demonstrate the results of assays done on ts 68 infected cells cultured from the same embryo but grown and assayed at the non-permissive temperature. There is a decrease in the differential sensitivity of the cells to the toxin. The infected cells appear slightly more sensitive to intoxication by DT than the normal, non-infected CEF; however, this difference was not statistically significant at the 0.99 confidence level. If the confidence level is decreased to 0.90, then the difference becomes significant (Table 2). We conclude that the difference between the toxin sensitivities of the normal CEF and the ts 68 infected CEF is reduced but not eliminated at the non-permissive temperature and is probably due to leakiness in the temperature sensitive expression of the transformation function of the viral genome. Similar results were obtained with assays done on cells infected with the ts mutant Ta of BH-RSV (6)(Table 2).

Effect of temperature: This decrease in the differential sensitivities of these cells to intoxication by DT at the restrictive

Figure 2

Effect of temperature on diphtheria toxin inhibition of protein synthesis in normal and ts 68 infected cells.

Cell suspension assays were done as described in Materials and Methods on cells cultured at the permissive temperature of 37C (a), and at the non-permissive temperature of 41C (b). Percentage of control was determined as described in Fig. 1. ●——● , chick embryo fibroblasts; o——o, ts 68 infected cells.

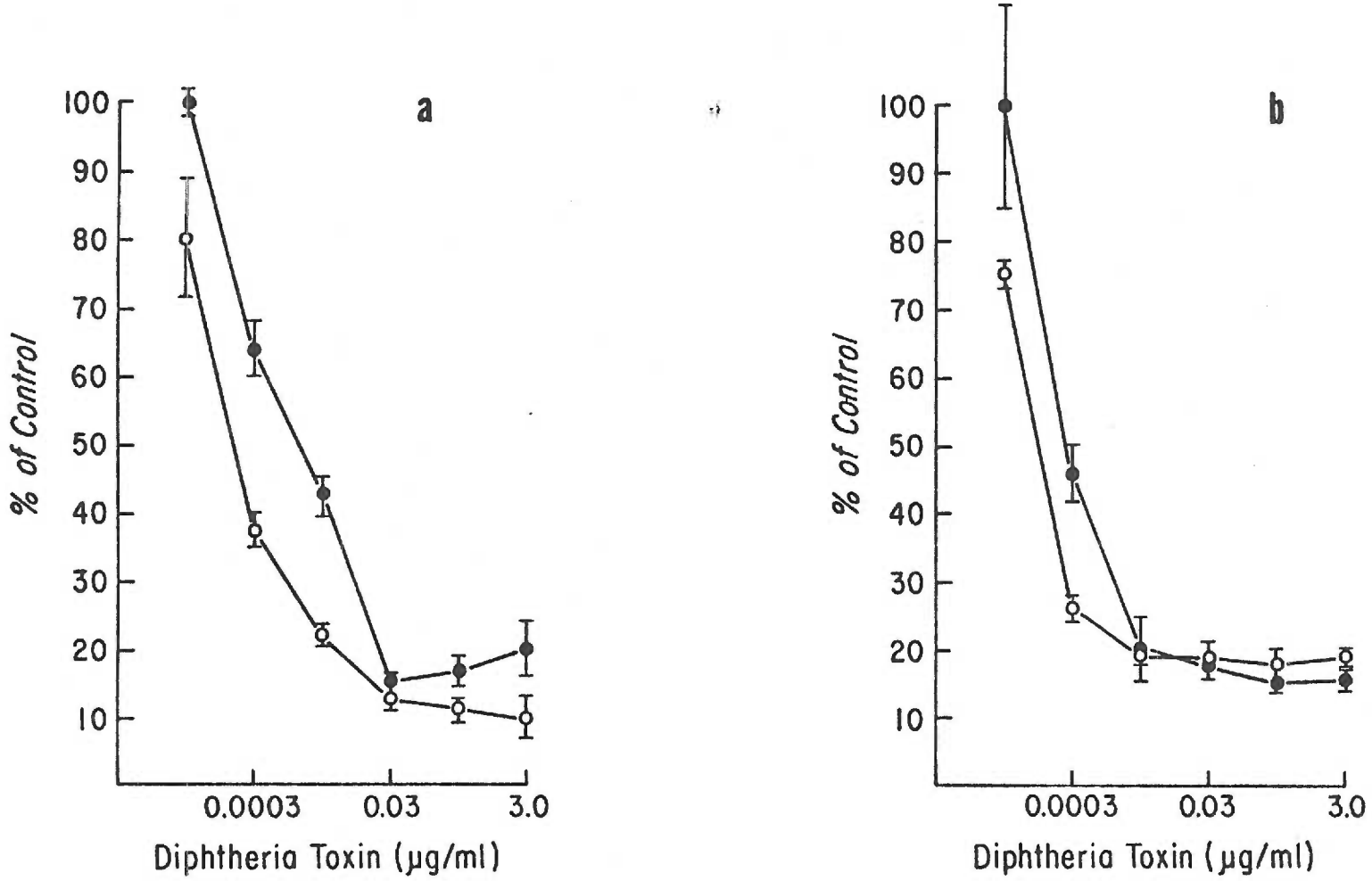

a
b
100
90
80
70
60
50
40
30
20
10
% of Control
0.0003
0.03
3.0
Diphtheria Toxin (µg/ml)

Table 2

PID_{50} OF VARIOUS NORMAL AND TRANSFORMED CELLS TO DT INTOXICATION

Cell type	Temperature	PID_{50} [a]	Probability [b] of difference
CEF	37C	0.06	
SR-CEF		0.006	P<0.001
CEF	41C	0.01	
SR-CEF		0.001	P<0.001
CEF	37C	0.03	
ts 68-CEF		0.003	P<0.001
CEF	41C	0.003	
ts 68-CEF		0.001	P=0.1
CEF	37C	0.02	
BH-RSV		0.003	P<0.001
CEF	41C	0.01	
BH-CEF		0.002	P<0.001
CEF	37C	0.03	
Ta-CEF		0.002	P<0.001
CEF	41C	0.009	
Ta-CEF		0.005	P<0.1
CEF	37C	0.01	----
AMV-CEF		0.01	-----
CEF	37C	0.04	
SR-CEF [c]		0.003	P<0.001

a) in µg/ml

b) determined by analysis of variance and the Student t test

c) using iodinated toxin instead of normal DT

temperature could be due to the reduced expression of the transformation defective function of the viral genome of the ts mutant viruses or to a non-specific effect on the intoxication of the cells caused by the elevated temperature. Therefore, the effect of DT on normal CEF, wild type SR-CEF, and wild type BH-CEF was determined at both 37C and 41C. Figure 3 and Table 2 show that there is still a difference in the sensitivity to intoxication by DT of the normal CEF as compared to the transformed cells at both of the temperatures and that this difference is significant at the 0.995 confidence level. Since the amount of virus produced by the ts 68 and the Ta infected cells is similar at both temperatures (6,89), it appears that the sensitivity to DT intoxication of SR-CEF and BH-CEF as compared to CEF is mainly influenced by the expression of the transformation function of the virus genome and is not associated with the amount of virus produced by the infected cell.

Toxin sensitivity of AMV infected cells: The problem of leakiness in the temperature sensitive transformation function of the ts 68 and Ta genomes was approached by assaying cells that had been infected by the non-transforming retrovirus, AMV. Since AMV lacks the src gene but is still capable of infecting CEF and replicating within them (42), the question of leakiness of the transformation function of the genome was eliminated in assays done on cells infected with this virus.

Figure 3

Effect of temperature on diphtheria toxin inhibition of protein synthesis in normal and SR-RSV-A transformed chick embryo fibroblasts.

Cell suspension assays were done as described in Material and Methods on cells cultured at 37C (a) and at 41C (b). Percentage of control was determined as described in Fig. 1. ●——●, chick embryo cells; o——o, SR-RSV-A transformed cells.

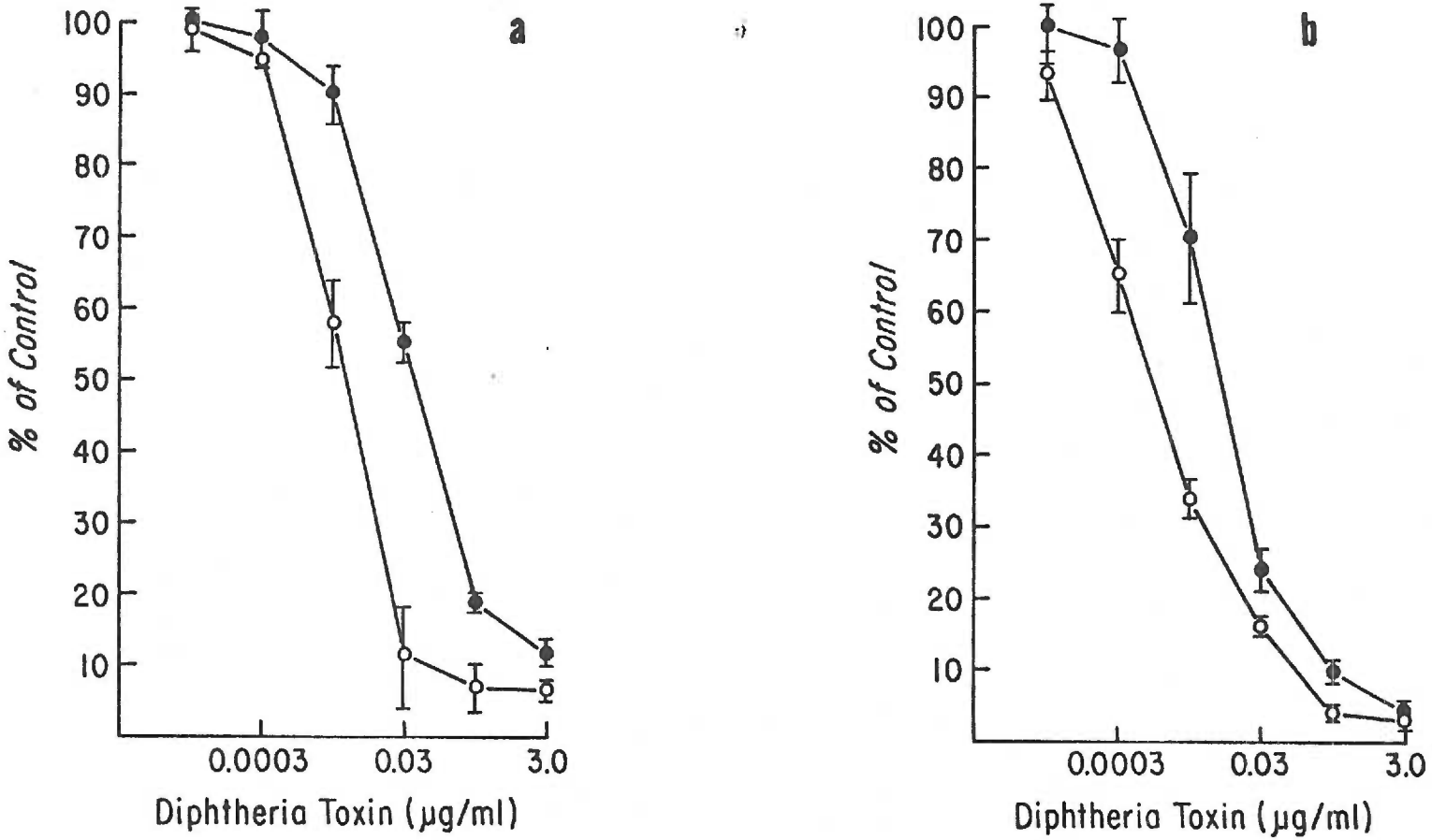

a
b
100
90
80
70
60
50
40
30
20
10
% of Control
0.0003
0.03
3.0
Diphtheria Toxin (µg/ml)

Figure 4 and Table 2 show the results of assays done on normal CEF and CEF infected with AMV. Protein synthesis, as measured by the incorporation of tritiated amino acids into hot TCA precipitable material, is equally sensitive to intoxication by DT in both the normal CEF and the AMV infected CEF. Although these cells are producing virus, because the virus lacks the src gene funtion (9,42), the cells are not transformed. These data give further evidence supporting the contention that transformation, and not virus replication, is the factor associated with the differential sensitivity of CEF as compared to SR-CEF to intoxication by DT.

Toxin sensitivity of normal and transformed BHK cells: It then became of interest to determine if this phenomenon of increased sensitivity to intoxication by DT was a unique characteristic of CEF and/or RSV or could also be found as a characteristic of other cells transformed by other oncogenic viruses. Polyoma virus, like other DNA oncogenic viruses, is capable of transforming appropriate host cells without virus production (159). Figure 5 shows the results of assays done on primary BHK, BKK-21, and py-BHK cells. The py-BHK are significantly ($P<0.005$) more sensitive to intoxication by DT than either the primary BHK or the BHK-21 cells. This indicates that the phenomenon of increased sensitivity to intoxication by DT is not unique to CEF transformed by RSV but is also associated with at least some other cells transformed by some other viruses, and furthermore, that cell trans-

Figure 4

Effect of diphtheria toxin on protein synthesis in cultures of chick embryo fibroblasts and AMV infected chick embryo fibroblasts.

Cell suspension assays were done as described in the Materials and Methods. Percentage of the control was determined as described in Fig. 1. ●——● , Chick embryo fibroblasts; O——O AMV infected chick embryo fibroblasts.

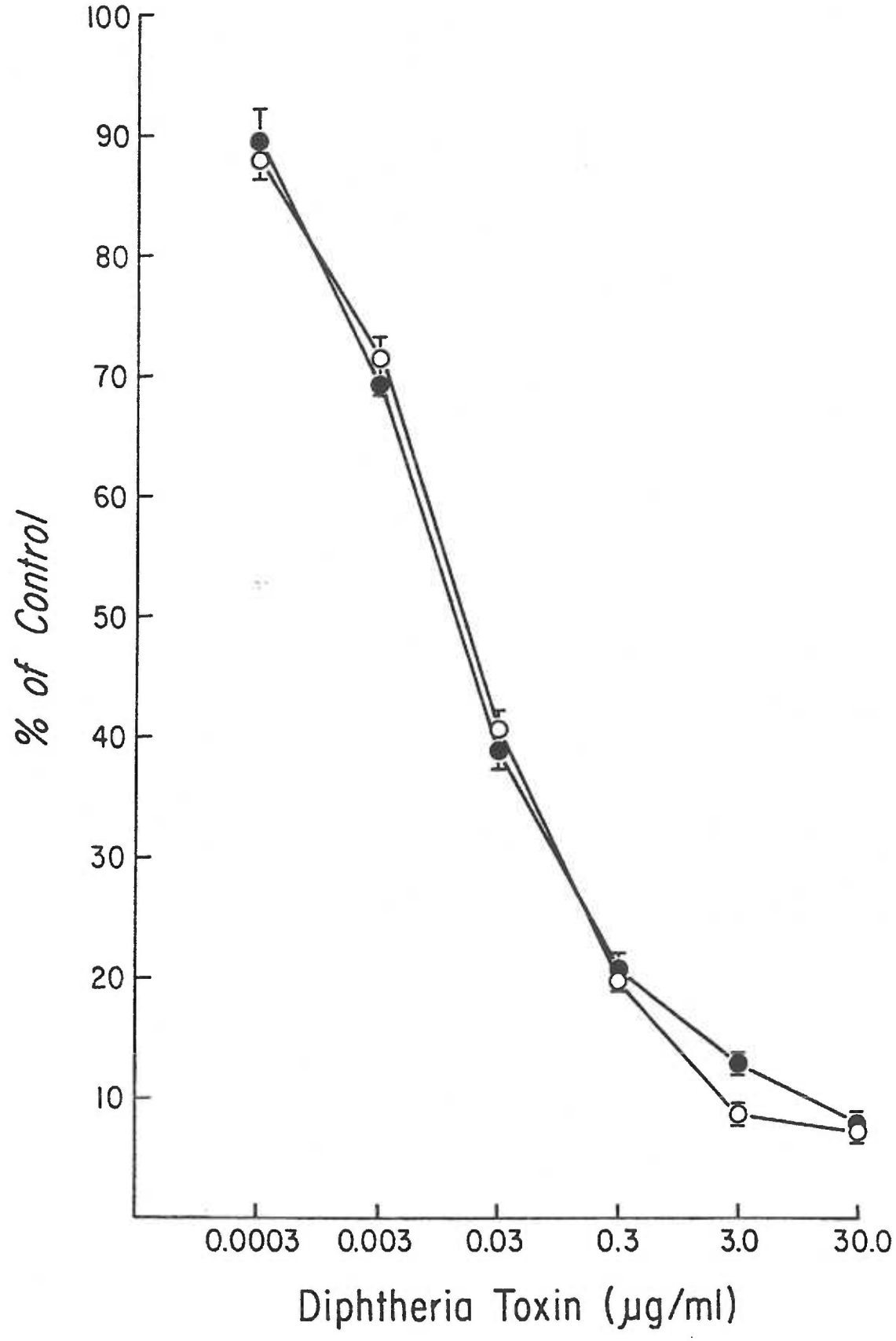
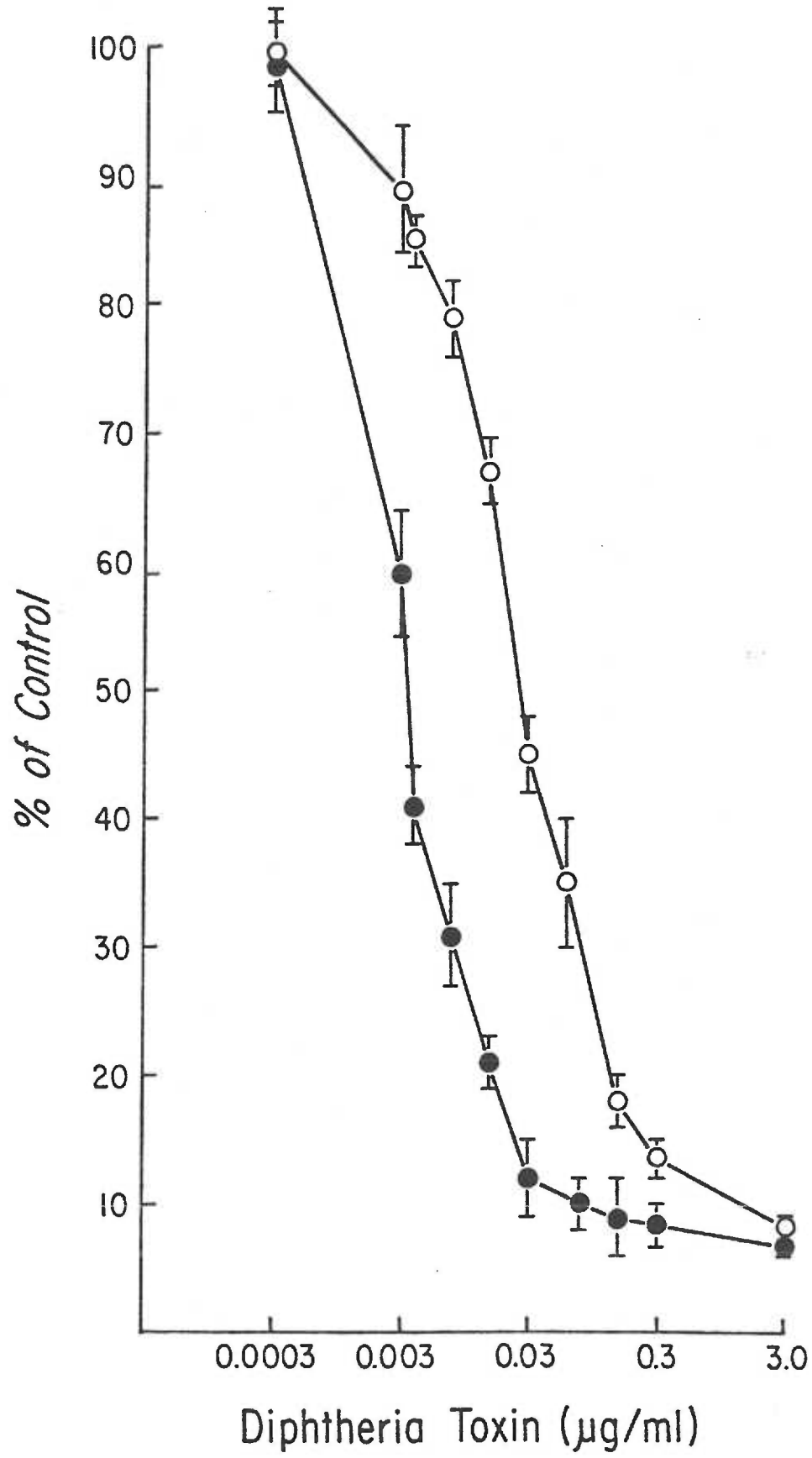

100
90
80
70
60
50
40
30
20
10
% of Control
0.0003
0.003
0.03
0.3
3.0
30.0
Diphtheria Toxin (µg/ml)

Figure 5

Effect of diphtheria toxin on protein synthesis in cultures of BHK-21 and polyoma transformed BHK-21 cells.

Cell suspension assays were done as described in Materials and Methods. Percentage of the control was determined as described in Fig. 1. o—o BHK-21; ●—●, polyoma transformed BHK-21.


100
90
80
70
60
50
40
30
20
10
% of Control
0.0003
0.003
0.03
0.3
3.0
Diphtheria Toxin (µg/ml)

formation, and not virus replication, is necessary for this phenomenon to occur.

Toxin sensitivity of normal and transformed resistant cells: In assays on the sensitivities of normal and transformed rodent cells, Saelinger and Bonventre (142) were unable to show any significant differences in the response of the cells to DT. Since the cells we have previously used were normal and transformed cells from animals that are sensitive to intoxication by DT, it became of interest to determine if the differential sensitivity seen in the CEF/SR-CEF system could by induced from a resistant cell system. The results of assays done on 3T3, a resistant mouse cell line, and SR-3T3, its Rous sarcoma virus transformed counterpart, are shown in figure 6 and Table 3. As can be seen, there is no difference in the PID_{50} of either cell type. Similar results were obtained in assays on 3T3 cells as compared to py-3T3 and SV-3T3cells and in assays on NRK cells as compared to SR-NRK and NRK-MSV cells (Table 3). These data suggest that, although the phenomenon of increased sensitivity to intoxication by DT is not limited to one virus or cell system, it does require a cell system that is relatively sensitive to intoxication by DT.

Cell suspension assay compared to cell monolayer assay: Venter and Kaplan (175), using a variety of human cells, were unable to demonstrate any consistent differences in the sensitivities of normal as compared to transformed cells to intoxication by DT.

Figure 6

Effect of diphtheria toxin on protein synthesis in cultures of 3T3 and SR-RSV transformed 3T3 cells.

Cell suspension assays were done as described in Materials and Methods. Percentage of the control was determined as described in Fig. 1. ●—— ● , normal 3T3; o —— o , SR-RSV transformed 3T3.

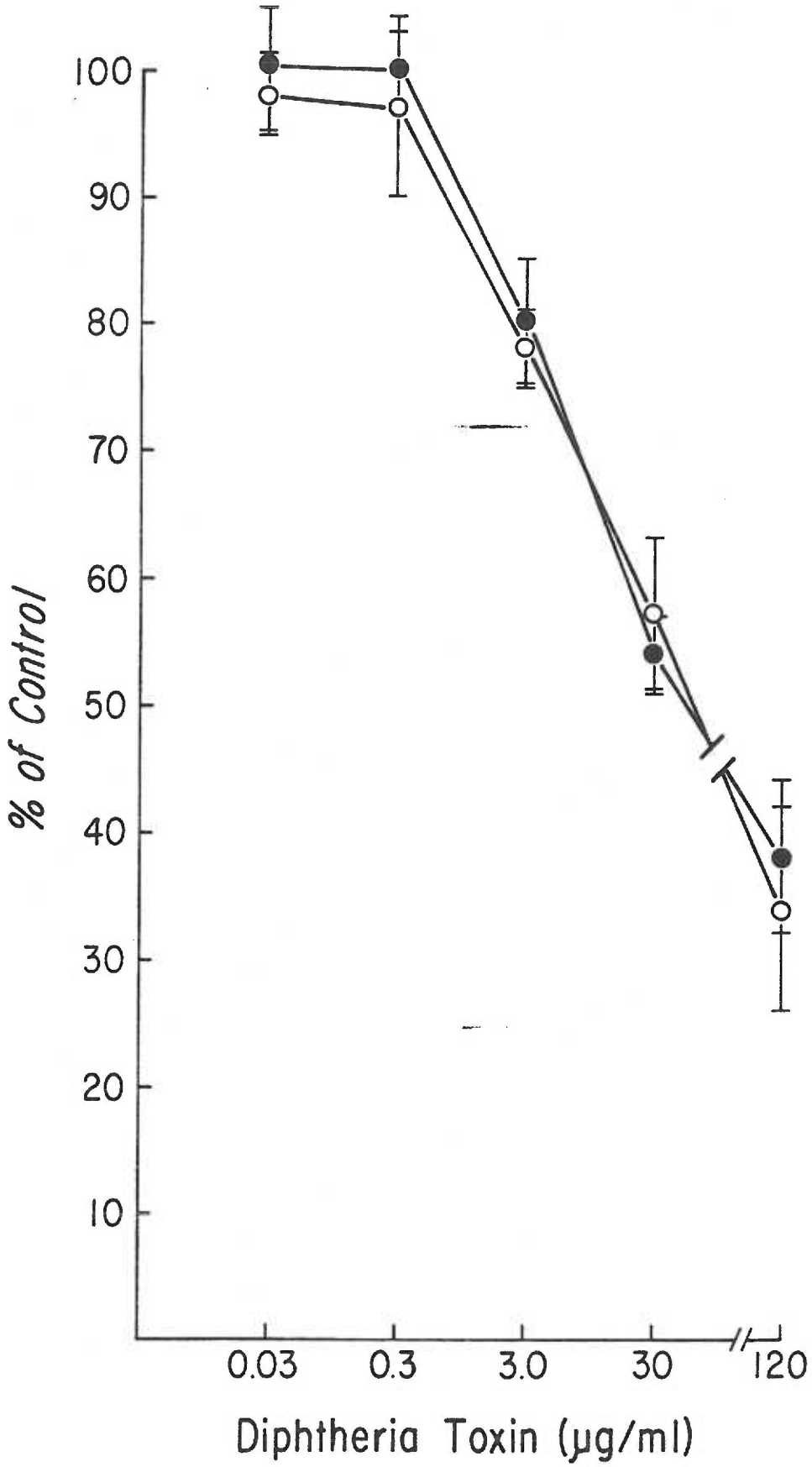

100
90
80
70
60
50
40
30
20
10
% of Control
0.03
0.3
3.0
30
120
Diphtheria Toxin (µg/ml)

Table 3

PID_{50} OF NORMAL AND TRANSFORMED RESISTANT CELLS TO INTOXICATION BY DT

Cell type	PID_{50} [a,b]
3T3	60
SR-3T3	60
py-3T3	60
SV-3T3	60
NRK	80
SR-NRK	80
NRK-MSV	80

a) in µg/ml

b) determined as described in the Materials and Methods

The protein synthesis assay used by them was a cell monolayer assay in which the cells were in a subconfluent monolayer instead of in suspension as originally described by Iglewski and Rittenberg (80). In order to further evaluate our standard assay for the sensitivity of protein synthesis to DT intoxication, we ran parallel assays using the cell suspension assay and the cell monolayer assay systems as described in the Materials and Methods. The suspension assay results were similar to those seen in figure 1. However, figure 7 demonstrates that there was no difference in the PID_{50} of SR-CEF as compared to normal CEF when protein synthesis was assayed for sensitivity to DT when the cells were in monolayers.

Requirement for trypsinization: It then became of interest to determine if the mere physical suspension of the cells would be sufficient to cause the observed differential sensitivity to occur. Cells were harvested for cell suspension assays by two different methods. The first method was by trypsinization as was done in the previous suspension assays. The second was by treatment of the cells with versene. The results of the assays done on the trypsin lifted cells were similar to those shown in figure 1. Figure 8, on the other hand, demonstrates that assays done on versene lifted cells fail to show a differential sensitivity to intoxication by DT, indicating that physical suspension of the cells is not sufficient for the expression of the differential sensitivity to intoxication between normal CEF and SR-CEF.

Figure 7

Effect of diphtheria toxin on protein synthesis in monolayer cultures of normal and SR-RSV-A transformed chick embryo fibroblasts.

Cell monolayer assays were done as described in Materials and Methods. Percentage of the control was determined as described in Fig. 1. ●—●, chick embryo fibroblasts; ○——○ , SR-RSV-A transformed cells.

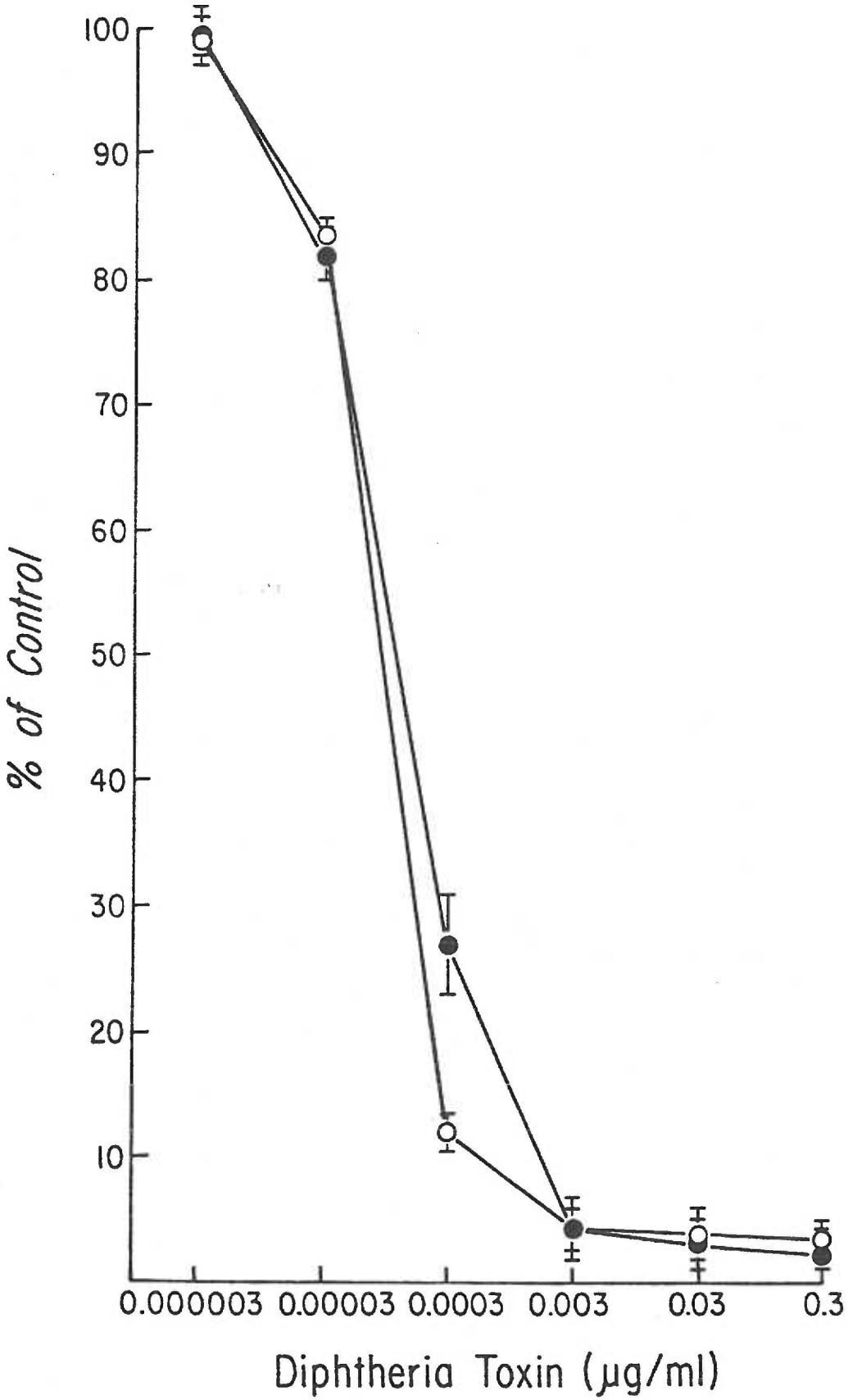
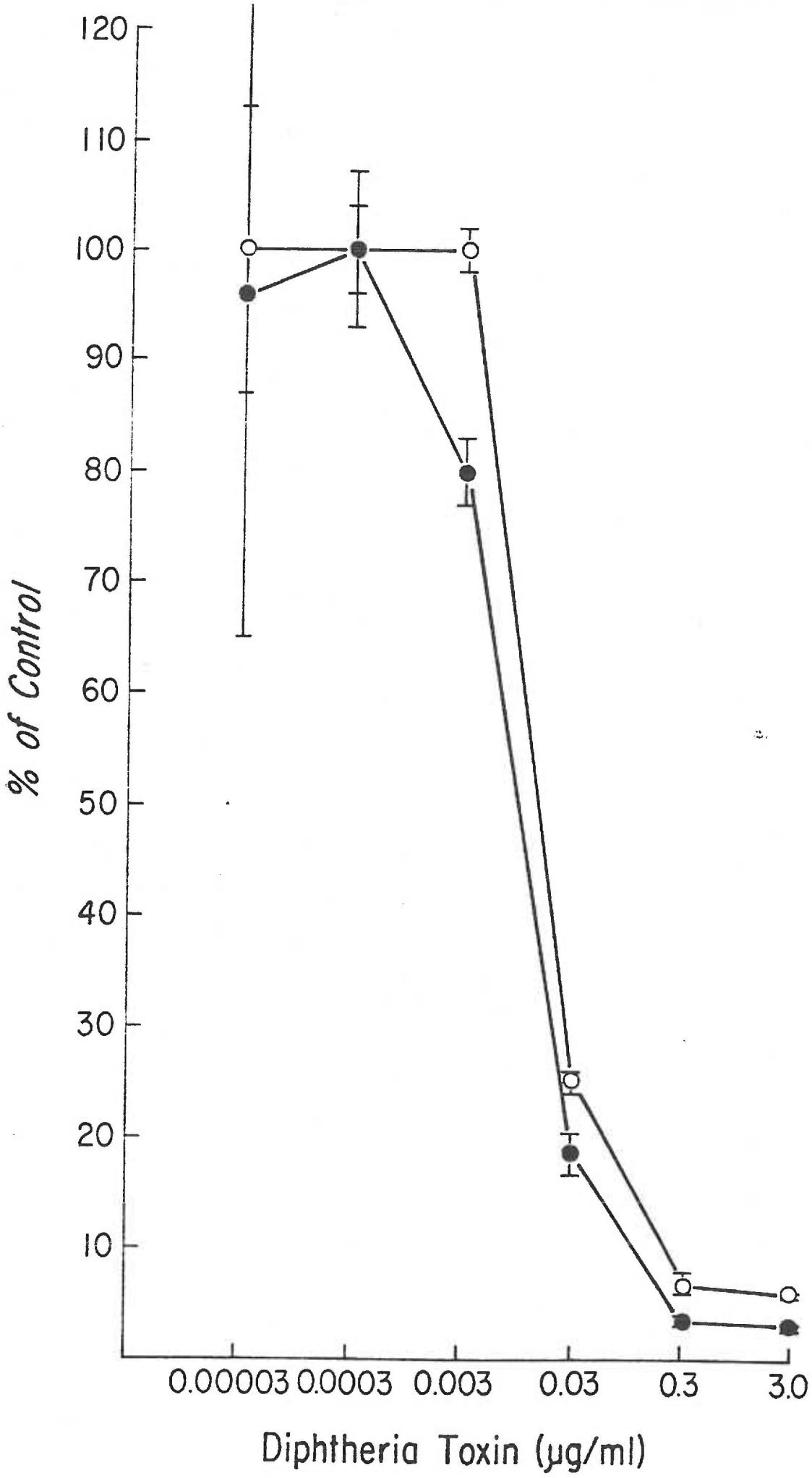

100
90
80
70
60
50
40
30
20
10
% of Control
0.000003
0.00003
0.0003
0.003
0.03
0.3
Diphtheria Toxin (µg/ml)

Figure 8

Effect of diphtheria toxin on protein synthesis in versene lifted cultures of chick embryo fibroblasts and SR-RSV-A transformed chick embryo fibroblasts.

Cell suspension assays were done as described in Materials and Methods except that the cells were lifted with versene instead of trypsin. Percentage of the control was determined as described in Fig. 1. ●——●, chick embryo fibroblasts; ○——○ SR-RSV-A transformed chick embryo fibroblasts.


120
110
100
90
80
70
60
50
40
30
20
10
% of Control
0.00003
0.0003
0.003
0.03
0.3
3.0
Diphtheria Toxin (µg/ml)

Versene lifted cells were then incubated for 30 minutes at 37C in either TG buffer or 0.25% trypsin in TG buffer at a cell density of 10^6 cells/ml. The cells were then washed and assayed by the cell suspension assay method as described previously. Figures 9a and 9b show the results of these assays. As can be seen, trypsinization of versene lifted CEF causes an approximate 10-fold decrease in the sensitivity of the cell protein synthesis to intoxication by DT. On the other hand, identical treatment of SR-CEF has no effect on the sensitivity of these cells to intoxication by DT. Incubation of versene lifted cells with trypsin and trypsin inhibitor causes no shift in the sensitivities of either the CEF or the SR-CEF to intoxication by DT (Table 4). These data indicate that trypsinization of the cells is necessary for the observed differential sensitivity to occur.

Trypsin compared to other proteolytic enzymes: The specificity of the effect of trypsin to cause the differential sensitivity to intoxication was examined. Trypsin hydrolyzes proteins at arginine and lysine residues (38). Chymotrypsin, on the other hand, acts at tyrosine, phenylalanine, and tryptophan residues (38). Chymotrypsin lifted cells were assayed for their sensitivities to intoxication by DT. As shown in figure 10, chymotrypsin causes a decrease in the sensitivities of both the normal CEF and the SR-CEF, and the decrease is similar to that caused by the effect of trypsin on normal cells. Similar results were obtained in assays using protease, pepsin papain, and bromelain to lift the

Figure 9

Effect of trypsinization on sensitivity of versene lifted chick embryo fibroblasts and SR-RSV-A transformed chick embryo fibroblasts.

Monolayer cultures were lifted with versene and washed with TG buffer. The cells were incubated 30 minutes in either TG buffer or 0.25% trypsin in TG buffer at 37C. The cells were then assayed as described in Materials and Methods. Percentage of control was determined as described in Fig. 1. a) ●——●, buffer treated; and o——o, trypsin treated chick embryo fibroblasts; b) ●——●, buffer treated; and o——o, trypsin treated SR-RSV-A transformed chick embryo fibroblasts.

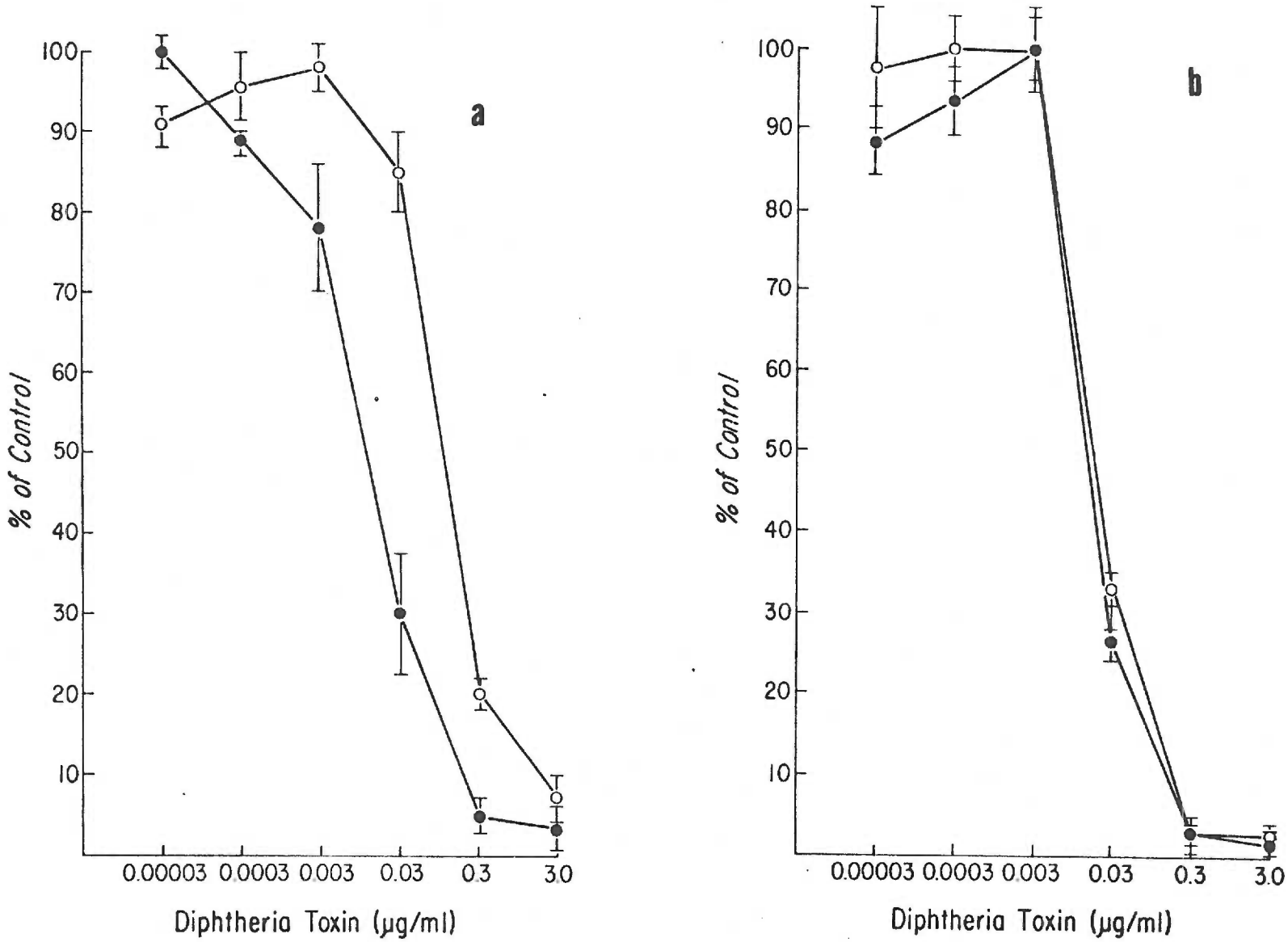

a
b
100
90
80
70
60
50
40
30
20
10
% of Control
0.00003 0.0003 0.003 0.03 0.3 3.0
Diphtheria Toxin (µg/ml)

Figure 10

Effect of chymotrypsin on the differential sensitivity of normal and SR-RSV-A transformed chick embryo fibroblasts to diphtheria toxin.

Cell suspension assays were done as described in Materials and Methods except that cells were lifted with 0.25% chymotrypsin instead of trypsin. Cells lifted with 0.25% trypsin were included as a control. Percentage of control was determined as described in Fig. 1. o——o, chymotrypsin lifted normal cells; •——•, chymotrypsin lifted SR-RSV-A transformed cells; Δ——Δ, trypsin lifted normal cells; X——X, trypsin lifted SR-RSV-A transformed cells.

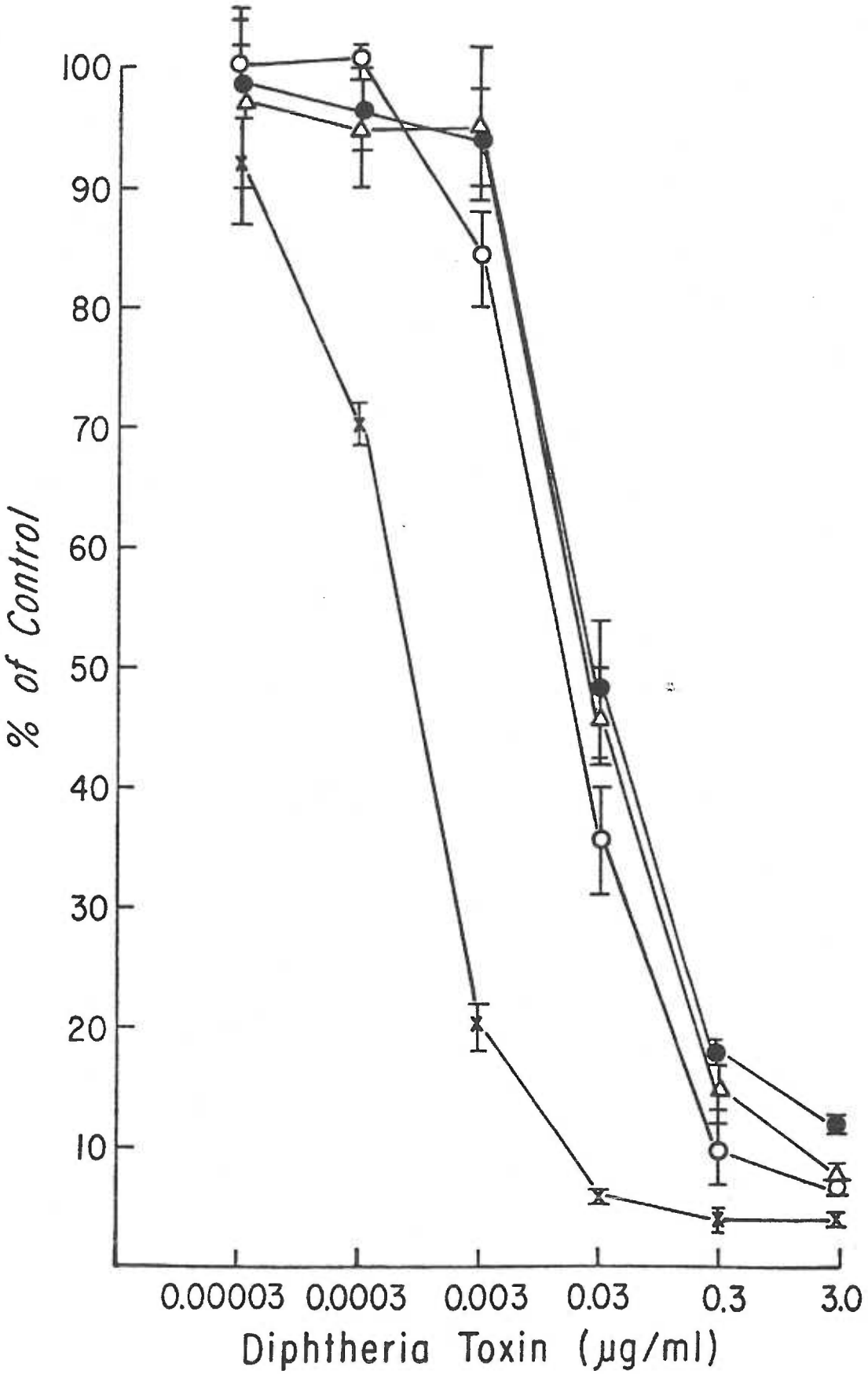

100
90
80
70
60
50
40
30
20
10
% of Control
0.00003
0.0003
0.003
0.03
0.3
3.0
Diphtheria Toxin (µg/ml)

Table 4

EFFECT OF VARIOUS PROTEOLYTIC ENZYMES ON THE PID_{50} OF NORMAL AND TRANSFORMED CEF

Enzyme used	Cell type	PID_{50} [a,b]	Probability of difference [c]
none	CEF	0.002	-----
	SR-CEF	0.002	-----
trypsin 10 u/ml	CEF	0.03	
	SR-CEF	0.001	P 0.001
chymotrypsin 10 u/ml	CEF	0.03	-----
	SR-CEF	0.03	-----
Protease 10 u/ml	CEF	0.02	-----
	SR-CEF	0.02	-----
pepsin 10 u/ml	CEF	0.04	-----
	SR-CEF	0.04	-----
papain 10 u/ml	CEF	0.03	-----
	SR-CEF	0.03	-----
bromelain 10 u/ml	CEF	0.02	-----
	SR-CEF	0.02	-----
trypsin 10 u/ml trypsin inhibitor 0.5 mg/ml	CEF	0.003	-----
	SR-CEF	0.003	-----

a) in µg/ml

b) determined as described in the Materials and Methods

c) determined by analysis of variance and the Student t test

cells (Table 4). Transformation of CEF apparently causes an alteration of the cell that renders the cells insensitive to the effect of trypsin on DT sensitivity but not insensitive to the effect of these other enzymes on toxin sensitivity.

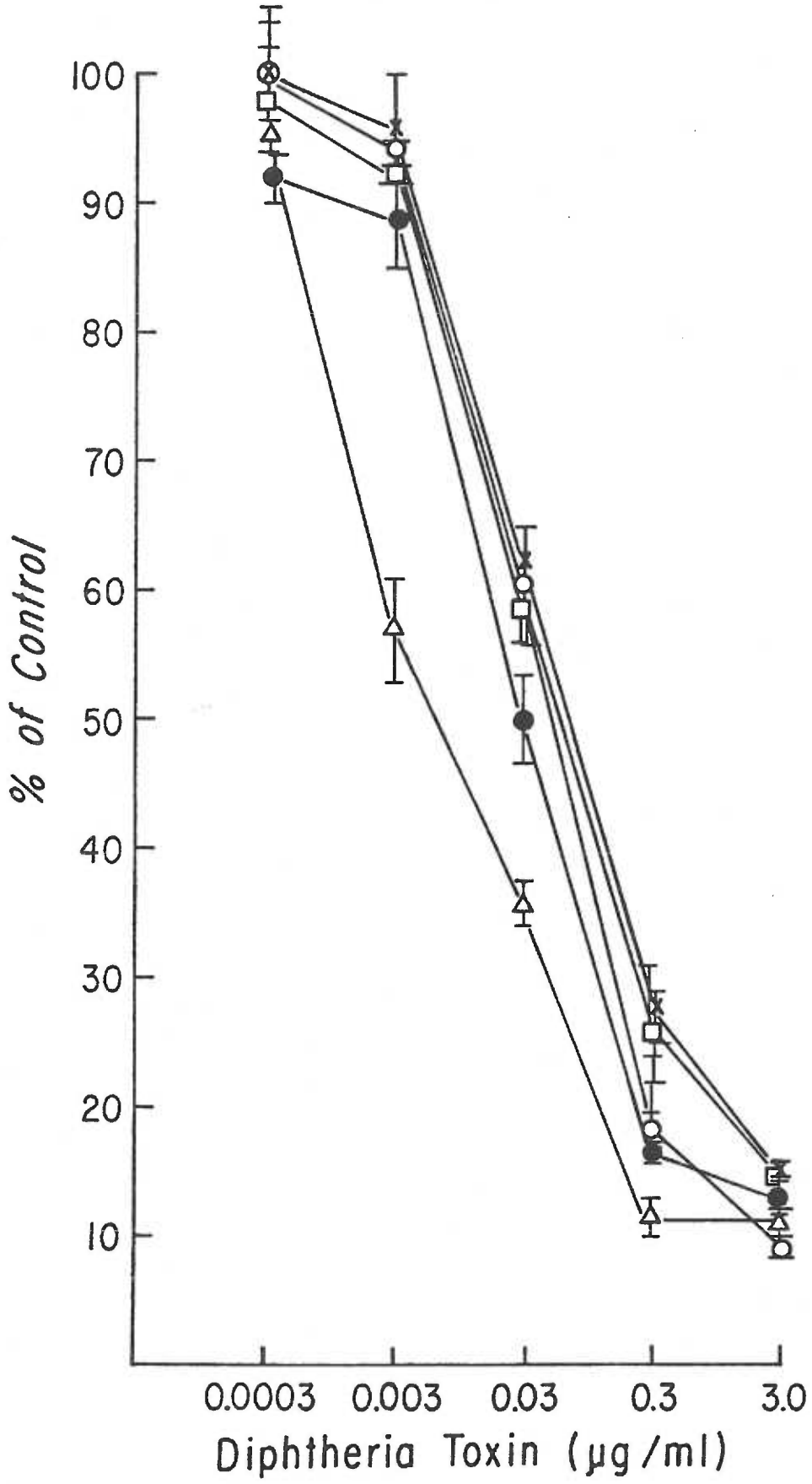
Effect of trypsin concentration: Since the effect of trypsin mediates the differential sensitivity between normal and transformed cells to DT intoxication, it was of interest to determine what conditions of trypsinization were required to minimize the sensitivity of normal CEF to DT intoxication. Versene lifted CEF were exposed to various concentrations of trypsin as previously described, washed, and then assayed for their sensitivity to intoxication by DT. Figure 11 shows that treatment of CEF with at least 0.1% trypsin at 37C for 30 minutes is required to provide the maximum protection of these cells against intoxication. Therefore, our comparative assays of the DT sensitivities of CEF and SR-CEF treated with 0.25% trypsin and those previously reported (81) have shown the maximum differential sensitivity to be expected between these normal and transformed cells.

Reversibility of the trypsin effect: Moehring and Crispell (107) reported that the effect of trypsin on the sensitivity of KB cells to intoxication by DT is a transient effect. In order to determine how transient this effect is in our system, versene lifted CEF were trypsinized as previously described and assayed for their sensitivity to intoxication by DT at various intervals of time after

Figure 11

Effect of trypsin concentration on the sensitivity of normal chick embryo fibroblasts to intoxication by diphtheria toxin.

Versene lifted normal CEF were incubated for 30 minutes in either TG buffer or in various concentrations of trypsin at 37C. The cells were assayed using the cell suspension assay as described in Materials and Methods. Percentage of control was determined as described in Fig. 1. Δ—Δ, buffer treated cells; ●—● , 0.05% trypsin; □—□ , 0.10% trypsin; o—o, 0.25% trypsin; x—x, 0.50% trypsin.


100
90
80
70
60
50
40
30
20
10
% of Control
0.0003
0.003
0.03
0.3
3.0
Diphtheria Toxin (µg/ml)

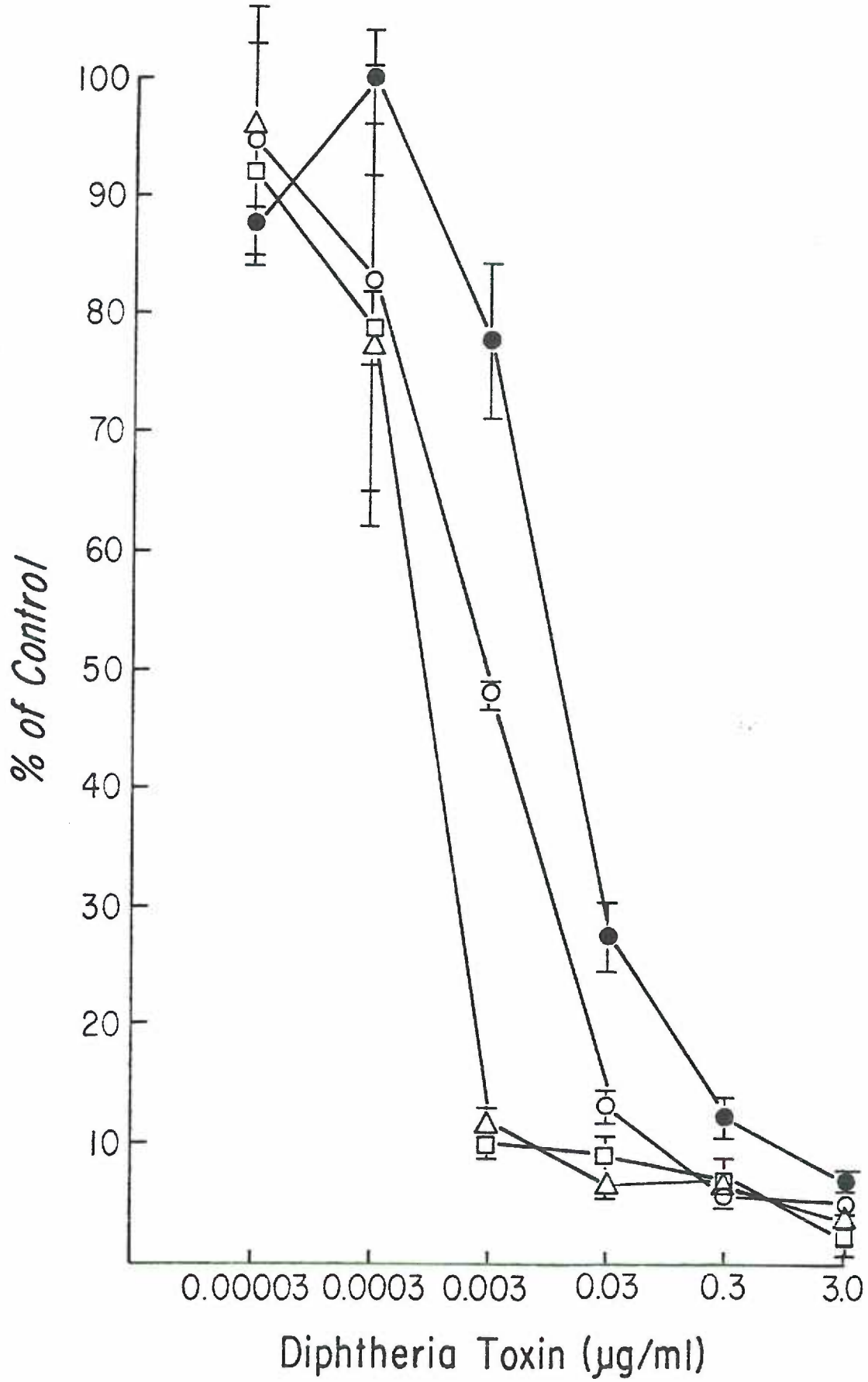
trypsinization. As can be seen in figure 12, by four to eight hours after trypsinization the sensitivity of CEF to intoxication by DT has returned to the pre-trypsinization level. Since the trypsin effect would be completely eliminated during the 16-24 hour incubation period used in the cell monolayer assay, it is not surprising that these later assays show no difference in the sensitivity of the normal and transformed cells to intoxication by DT.

Kinetics of intoxication: In order to determine the kinetics of intoxication by DT of trypsinized and non-trypsinized CEF and SR-CEF, cells were harvested in the usual manner using either trypsin or versene, washed, and suspended in the presence of various concentrations of DT and 2 μCi/ml tritiated amino acids. The cells were then processed to determine the incorporation of labelled amino acids into synthesized proteins at various times after the addition of the toxin. Figure 13 shows that there is no difference in the intoxication profiles of versene lifted normal and transformed cells. Trypsinization of the cells, on the other hand, markedly alters the profile of the normal CEF without altering that of the SR-CEF (fig. 14). Following trypsinization there is a dramatic difference between the CEF and the SR-CEF in the kinetics of incoporation of amino acids into cellular protein at all toxin concentrations tested. The figures also show a linear incorporation of the labelled amino acids in the non-intoxicated cells indicating that the assay medium is not adversely

Figure 12

Effect of time after trypsinization on the sensitivity of CEF protein synthesis to intoxication by DT.

Normal chick embryo fibroblasts were lifted with versene and trypsinized as described in Fig. 9. Cell suspension assays were done at various times after trypsinization in the manner described in the Materials and Methods. Percentage of control was determined as described in Fig. 1. ● —— ●, 0 hour; ○ —— ○ , 2 hours; □ —— □ , 4 hours; Δ —— Δ , 8 hours.


100
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% of Control
0.00003
0.0003
0.003
0.03
0.3
3.0
Diphtheria Toxin (µg/ml)

Figure 13

Kinetics of intoxication of versene lifted normal CEF and SR-CEF.

Normal CEF and transformed SR-CEF were lifted with versene and processed to determine the kinetics of intoxication in the manner described in Materials and Methods. - - -, SR-CEF; ———, CEF; ● = no DT; X = 0.003 µg/ml DT; △ = 0.03 µg/ml DT; O = 0.3 µg/ml DT; □ = 3.0 µg/ml DT.

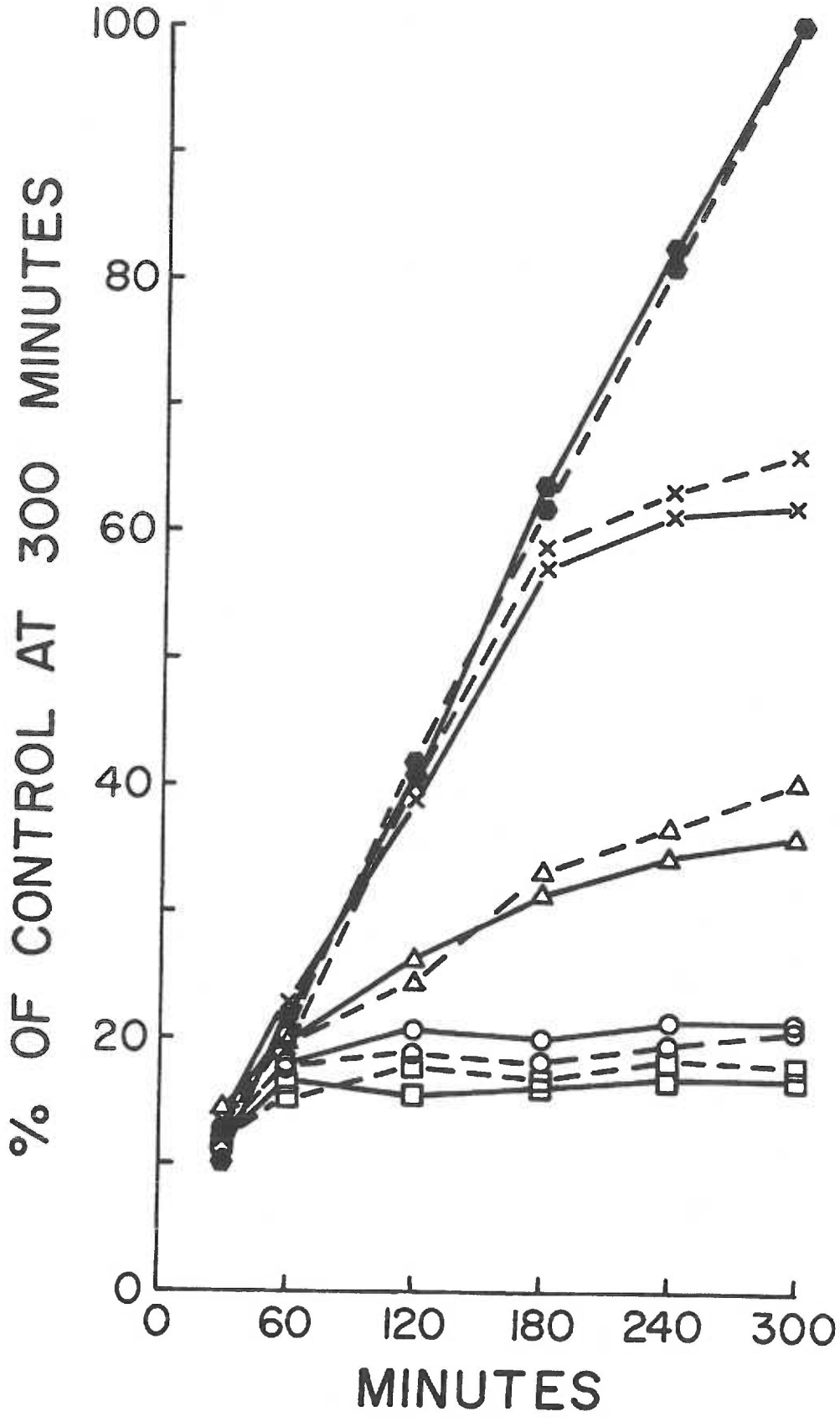
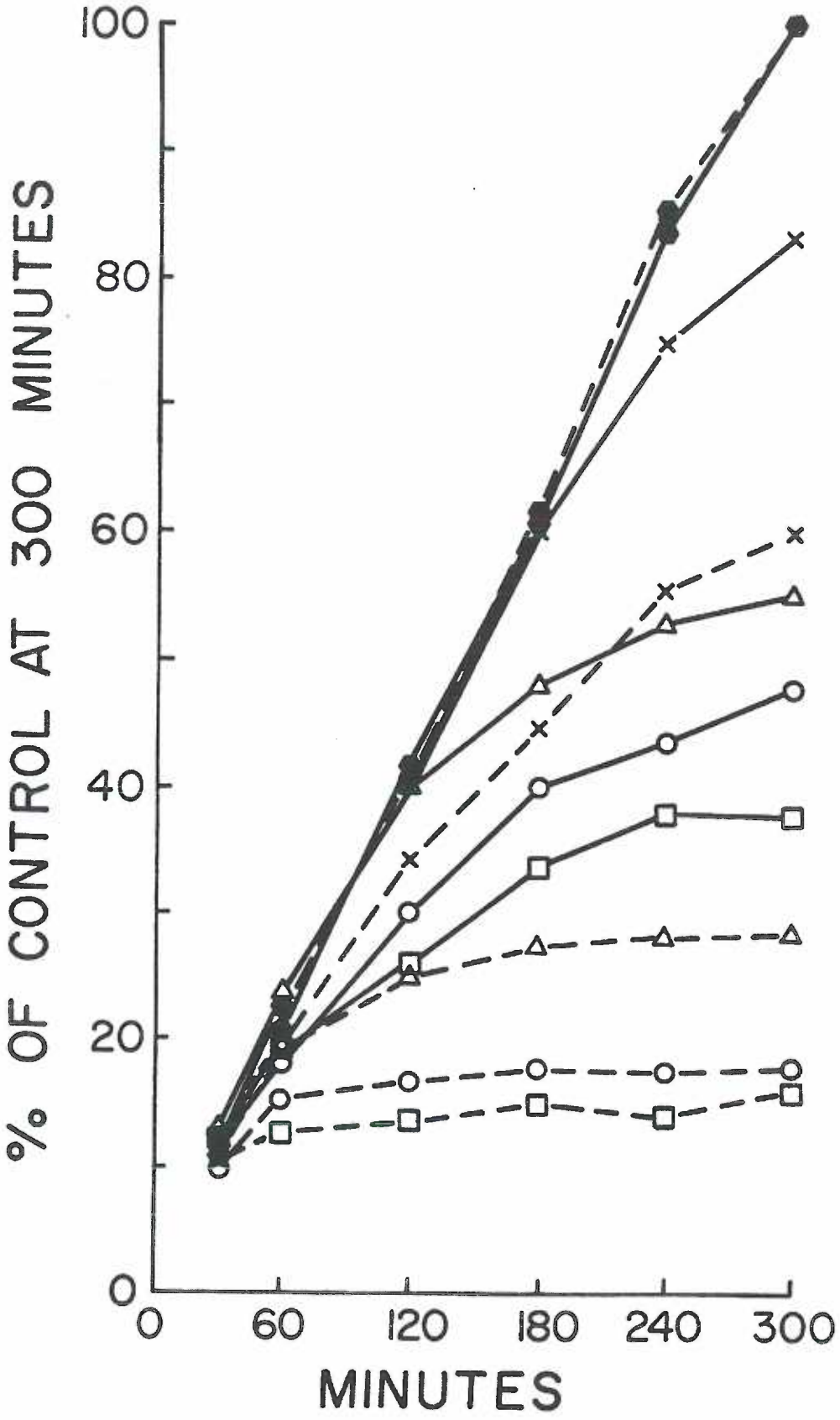

100
80
60
40
20
0
% OF CONTROL AT 300 MINUTES
0
60
120
180
240
300
MINUTES

Figure 14

Kinetics of intoxication of trypsinized normal CEF and SR-CEF.

Normal CEF and transformed SR-CEF were lifted by trypsinization and processed to determine the kinetics of intoxication in the manner previously described. - - -, SR-CEF; ——, CEF, ● = no DT; ✗ = 0.003 µg/ml DT; Δ = 0.03 µg/ml DT; O = 0.3 µg/ml DT; □ = 3.0 µg/ml DT.


100
80
60
40
20
0
% OF CONTROL AT 300 MINUTES
0
60
120
180
240
300
MINUTES

affecting the protein synthesis of either cell type during the period of the assays.

Pseudomonas aeruginosa exotoxin A intoxication: In order to determine the specificity of DT for the differential sensitivity to occur, cells were assayed for their sensitivities to PA toxin. PA toxin inhibits protein synthesis by ADP-ribosylating EF-2 in a manner similar to that of DT (79) but is thought to have different binding and/or internalization mechanisms (105). Figures 15 and 16 demonstrate that even after trypsinization neither the CEF/SR-CEF system nor the BHK-21/py-BHK system show the differential sensitivity to intoxication with PA toxin as is seen with cells intoxicated with DT. These data give evidence that selective intoxication is not because of a non-specific uptake of the toxin molecules.

EF-2 levels of the cells: Since DT intoxicates cells by ADP-ribosylating the cellular EF-2, it became of interest to quantitate the EF-2 of normal CEF and SR-CEF. The differential sensitivity could be due to a difference in the EF-2 levels of the cells. Table 5, however, shows that the EF-2 levels of the cells are similar. These data plus those shown for PA toxin (fig. 15 and 16) support the contention that selective intoxication of SR-CEF as compared to CEF is not at the intracellular level.

Toxin uptake: In order to determine if toxin uptake due to endocytosis might be different between the CEF and the SR-CEF, cells

Table 5

EF-2 CONTENT OF CHICK EMBRYO FIBROBLASTS AND SR-RSV-A TRANSFORMED CEF

Cells	EF-2 content [a]	
	nM/g	nM/cell [b]
CEF	0.51	1.8×10^{-7}
SR-CEF	0.23	1.2×10^{-7}

a) EF-2 content was determined as described in Materials and Methods

b) there were 2.8×10^{8} CEF/g and 1.9×10^{8} SR-CEF/g.

Figure 15

Effect of PA toxin on protein synthesis in normal CEF and SR-CEF.

Cell suspension assays were done as described in Materials and Methods except that PA toxin was used instead of diphtheria toxin. Percentage of control was determined as described in Fig. 1. ●—● , CEF; ○—○ , SR-CEF.

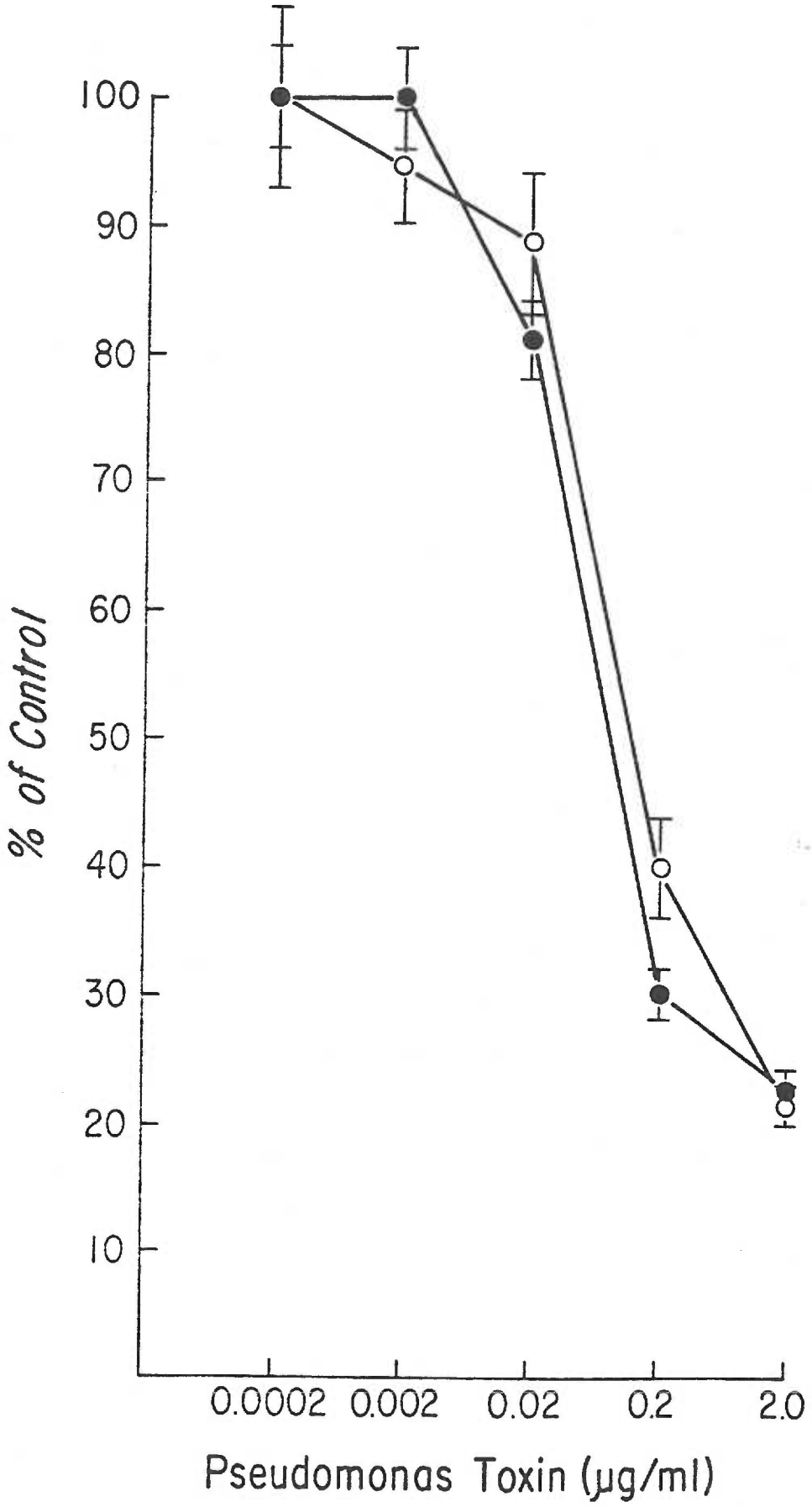
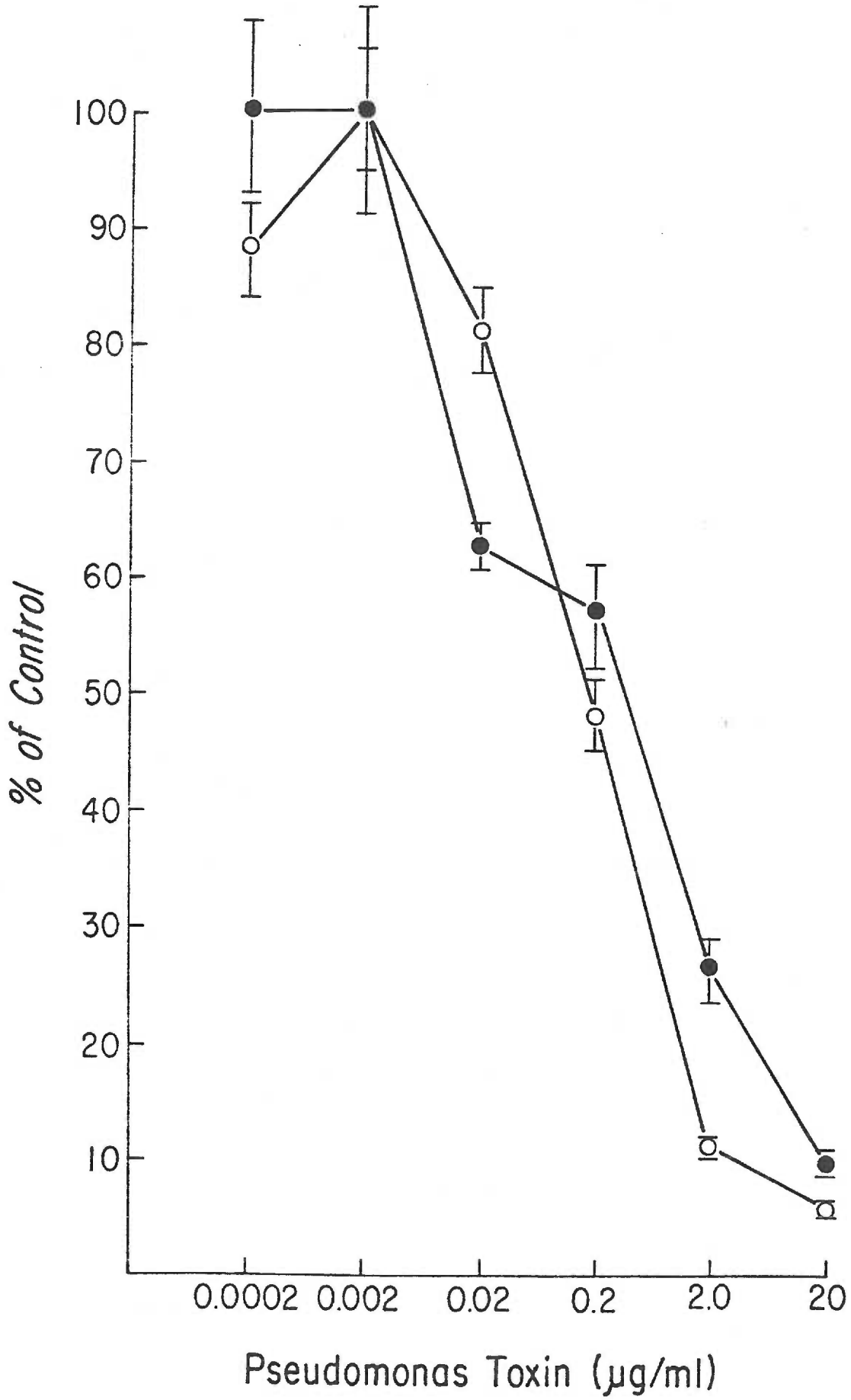

100
90
80
70
60
50
40
30
20
10
% of Control
0.0002
0.002
0.02
0.2
2.0
Pseudomonas Toxin (µg/ml)

Figure 16

Effect of PA toxin on protein synthesis in BHK-21 and py-BHK cells.

Cell suspension assays were done as described in Materials and Methods except that PA toxin was used instead of diphtheria toxin. Percentage of control was determined as described in Fig. 1.

●——●, BHK-21 cells; o——o, py-BHK cells.


100
90
80
70
60
50
40
30
20
10
% of Control
0.0002
0.002
0.02
0.2
2.0
20
Pseudomonas Toxin (µg/ml)

were exposed to ^{125}I labelled DT for various time periods. Figure 17 shows that the SR-CEF takes up toxin at a slightly elevated rate as compared to the normal CEF in both cell suspensions and monolayer cultures. This difference in endocytotic uptake is probably not responsible for the differential sensitivity of the cells to intoxication by DT since both the cell suspension cultures and the monolayer cultures give the same results, yet only the cell suspension assay shows the differential sensitivity to intoxication by DT.

Effect of thrombin on toxin sensitivity: Besides acting as a proteolytic enzyme, trypsin has been shown to have a mitogenic effect on cells (148). In order to determine what effect this property of trypsin has on the differential sensitivity of the cells to intoxication by DT, it was necessary to treat versene lifted cells with a substance that has a similar mitogenic effect to that of trypsin but does not have the extensive proteolytic activity exhibited by trypsin. Thrombin has been shown to fulfill these requirements (164). It is able to serve as a mitogenic agent on CEF but does not proteolytically strip the cell surface the way trypsin does. For example, thrombin will not remove the LETS protein (164) while trypsin will (77). Treatment of the CEF and SR-CEF cultures with thrombin does not cause the differential sensitivity to intoxication by DT observed with cells treated with trypsin (fig. 18). These data suggest that the differential sensitivity to DT intoxication seen in

Figure 17

Effect of trypsinization on the uptake of ^{125}I labelled DT.

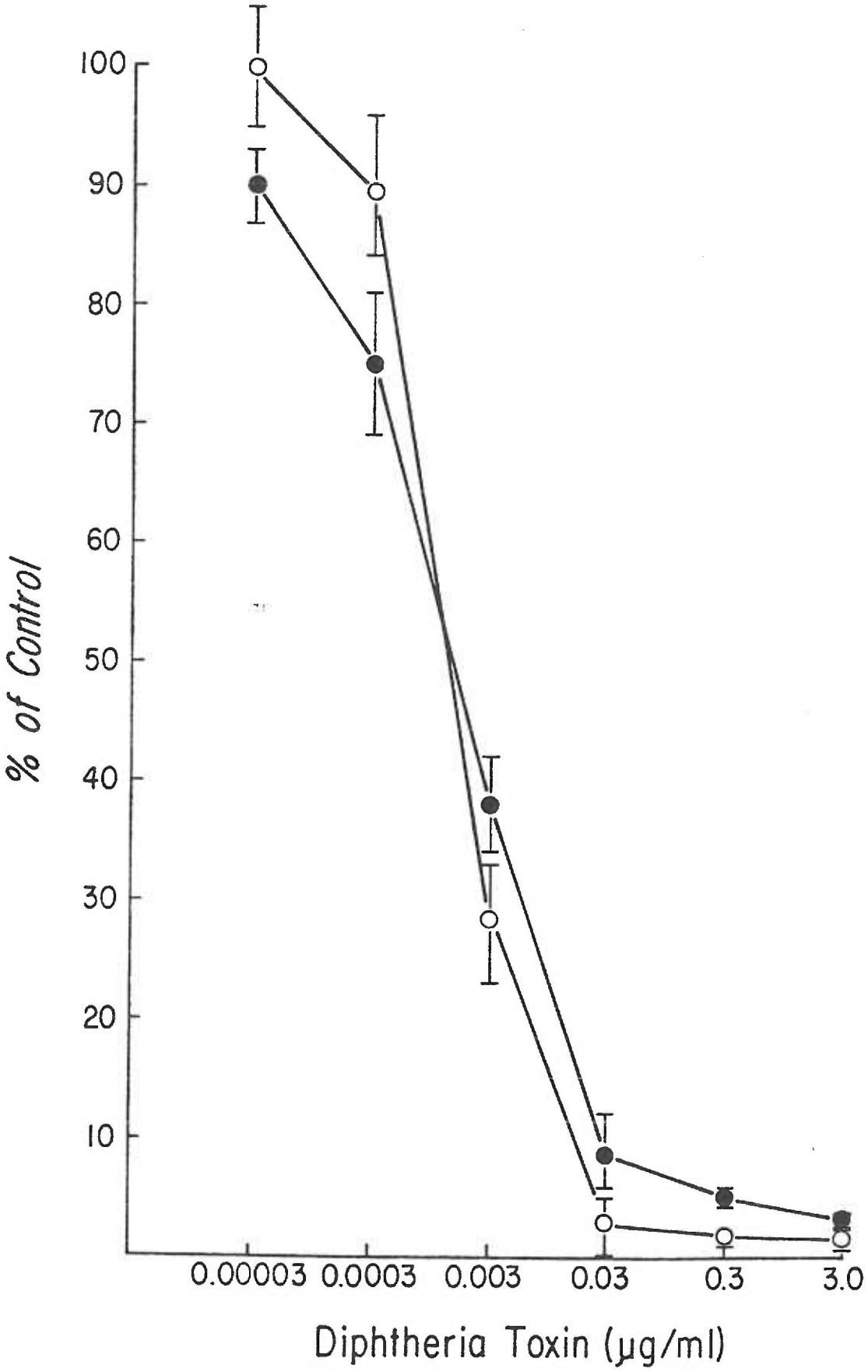
Normal CEF and transformed SR-CEF were exposed to ^{125}I-DT in either (a) cell suspension after trypsinization, or (b) cell monolayer cultures. Incorporated label was determined at various times after exposure to the labelled toxin. ●—●, CEF; ✕—✕, SR-CEF.

TIME (hrs)
0
1
2
3
4
5
1
3
5
7
a
b
CPM x 10^{-4}

Figure 18

Effect of thrombin treatment on the sensitivity to DT of CEF and SR-CEF

Versens lifted cells were incubated 30 minutes in TG buffer containing 250 u/ml thrombin at 37C. The cells were then assayed as described in Materials and Methods. Percentage of control was determined as described in Fig. 1. o—o , CEF; ●—● SR-CEF.


100
90
80
70
60
50
40
30
20
10
% of Control
0.00003
0.0003
0.003
0.03
0.3
3.0
Diphtheria Toxin (µg/ml)

trypsinized cells is not due to the mitogenic activity of the enzyme.

Effect of glycolytic enzymes on trypsinization: It has been shown that some of the membrane alterations occurring during cell transformation are able to mask some of the properties normally associated with transformed cells. For example, Burger and Martin demonstrated that SR-CEF must be treated with hyaluronidase before they will show increased agglutinability to plant lectins, a property normally associated with transformation (24). It is possible that a similar alteration has affected the accessibility of the toxin receptor sites to the action of trypsin and that treatment of the SR-CEF with a glycolytic enzyme prior to trypsinization might render these cells equally as sensitive to the effect of trypsin on cell sensitivity to intoxication by DT as seen with the normal CEF.

Versene lifted CEF and SR-CEF were incubated with various glycolytic enzymes prior to trypsinization as described in the Materials and Methods. The glycolytic enzymes tested were β-galactosidase, hyaluronidase, lysozyme, and α-glucosidase. As can be seen in Table 6, there are no differences in the sensitivities of the cellular toxin sensitivity to trypsinization with any of these enzymes. These data suggest that alterations in the makeup of membrane associated polysaccharides sensitive to these enzymes are probably not responsible for the differential

Table 6

EFFECT OF GLYCOLYTIC ENZYMES ON TRYPSINIZATION EFFECT ON PID_{50} OF CEF TO INTOXICATION BY DT

Enzyme used	Cell type	PID_{50} [a,b]	Probability of difference [c]
none	CEF	0.09	
	SR-CEF	0.005	P<0.001
β-galactosidase 100 u/ml	CEF	0.07	
	SR-CEF	0.003	P<0.001
Hyaluronidase 250 u/ml	CEF	0.03	
	SR-CEF	0.002	P<0.001
Lysozyme 250 u/ml	CEF	0.06	
	SR-CEF	0.003	P<0.001
α-glucosidase 200 u/ml	CEF	0.05	
	SR-CEF	0.005	P<0.001

a) in µg/ml

b) determined as described in the Material and Methods

c) determined by analysis of variance and the Student t test

sensitivity of these cells to intoxication by DT.

Inhibition of intoxication by CRM 197: CRM 197 is a non-toxic form of DT which is capable of inhibiting cellular intoxication presumably by competing with the toxin for specific binding sites on the cell surface (167). In order to determine the effect trypsinization of the cells had on the competition between DT and CRM 197 for receptor sites, experiments were done as follows: Cells were lifted with versene and incubated in either PBS or trypsin in PBS as previously described. The cells were then processed for the cell suspension assay with the following alteration: Immediately prior to the addition of the various dilutions of DT to the tubes, either 0.5 µg or 5.0 µg CRM 197 was added to each tube. Control tubes were treated in the same manner without the addition of the CRM. Figure 19 demonstrates that trypsinization of CEF causes a decrease in the amount of CRM 197 necessary to inhibit intoxication by DT. That is, 0.5 µg/ml CRM 197 will cause an inhibition of intoxication in the trypsinized CEF but not in the non-trypsinized CEF. Figure 20 shows that both trypsinized SR-CEF and non-trypsinized SR-CEF respond to CRM 197 in the same manner as the non-trypsinized CEF. These data give further evidence for the hypothesis that transformation of the CEF causes an alteration in the sensitivity of toxin binding sites to the effect of trypsinization.

Figure 19

Effect of trypsinization on the inhibition by CRM 197 of intoxication by DT of normal CEF.

Normal CEF were lifted with versene and incubated in either (a) TG buffer, or (b) 0.25% trypsin as described in Fig. 9. Cells were then exposed to various concentrations of CRM 197 and assayed for sensitivity to DT as described in Materials and Methods. Percentage of control was determined as described in Fig. 1. ●—●, no CRM 197; o—o, 0.5 µg CRM 197/ml; ×—×, 5.0 µg CRM 197/ml.

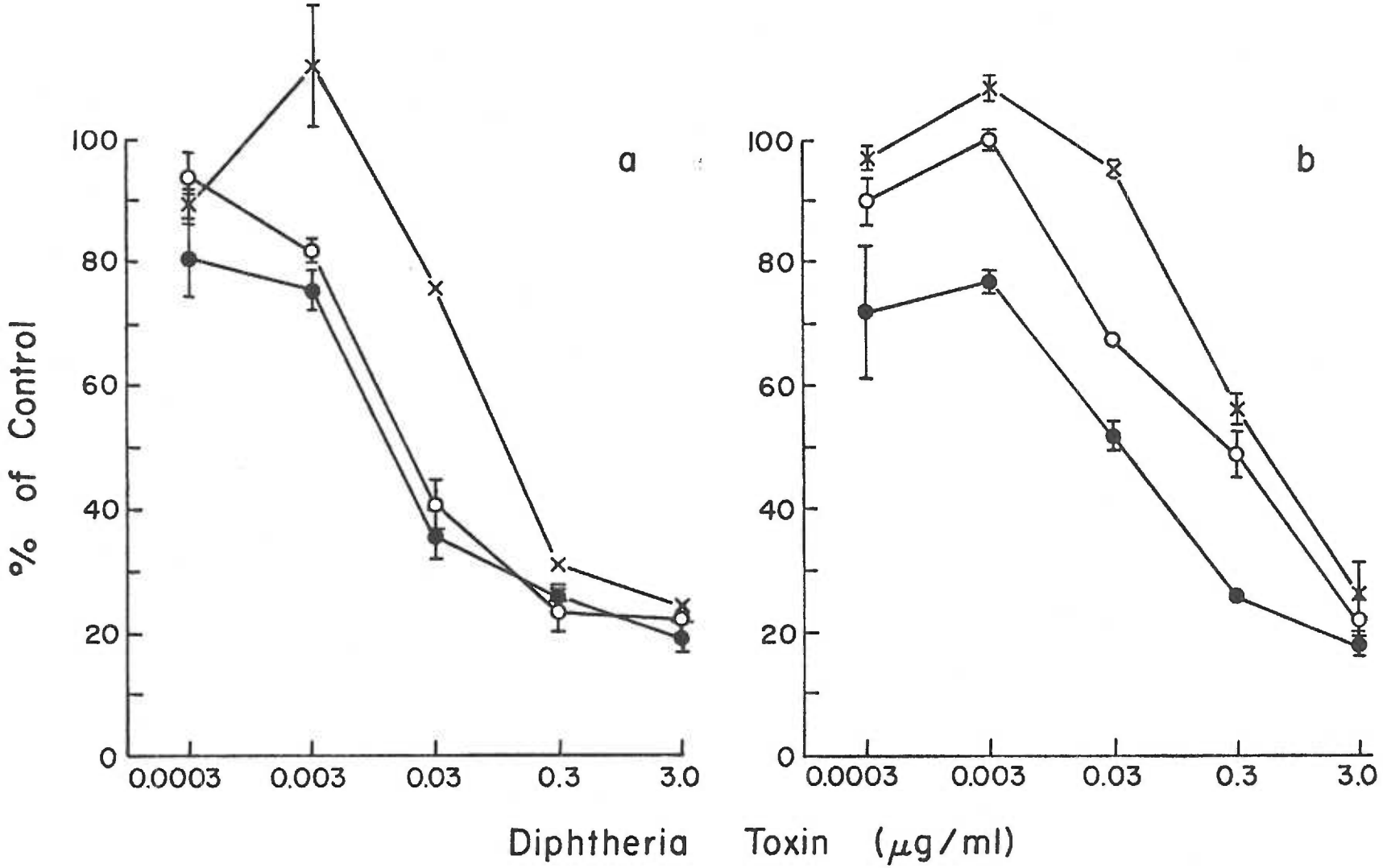
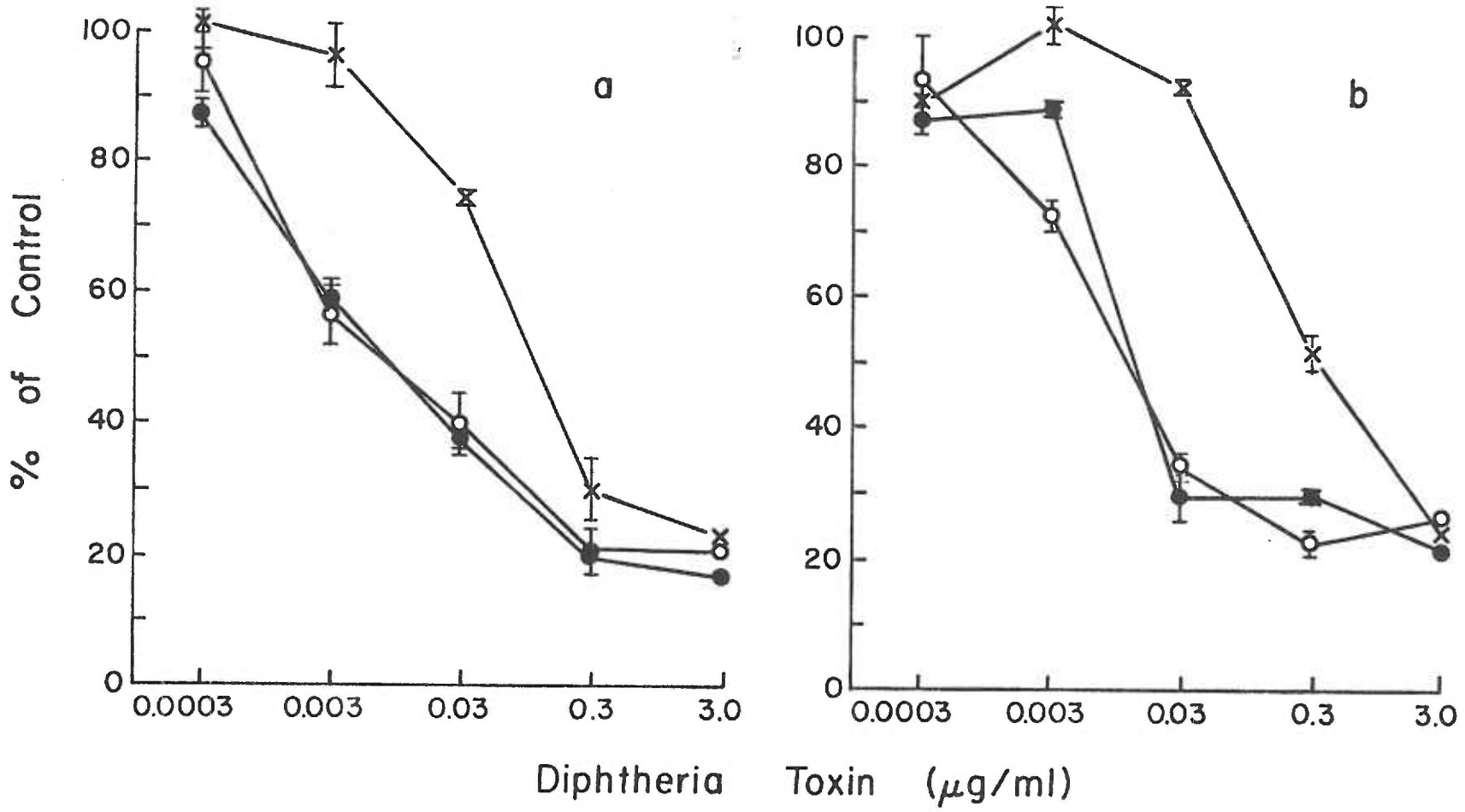

a
b
100
80
60
40
20
0
% of Control
0.0003
0.003
0.03
0.3
3.0
Diphtheria Toxin (μg/ml)

Figure 20

Effect of trypsinization on the inhibition by CRM 197 of intoxication by DT of SR-CEF.

SR-CEF were treated the same way described for cells in Fig. 19. ●—●, no CRM 197; o—o, 0.5 µg CRM 197/ml; ✗—✗, 5.0 µg CRM 197/ml.


a
b
% of Control
100
80
60
40
20
0
0.0003
0.003
0.03
0.3
3.0
Diphtheria Toxin (μg/ml)

DISCUSSION

Iglewski et al. (81) reported that SR-CEF are more sensitive to intoxication by DT than normal non-transformed CEF and suggested that this difference was due to alteration in the cell membrane occurring during viral transformation. Pappenheimer and Randall (123) implied that the assay medium used in these assays would adversely affect the results obtained. They claimed that amino acid starvation and the absence of serum factors could preferentially inhibit protein synthesis in transformed cells over normal cells rather than the action of DT. Here we have shown, at least in the case of CEF and SR-CEF, altering the amino acid concentration and dialyzing the serum used in the assay medium has no discernable effect on the relative sensitivities of these cell types (fig. 1). Furthermore, both the CEF and the SR-CEF show linear incorporation of amino acids throughout the assay period (fig. 13 and 14) indicating that the assay medium did not lack a serum factor required for the protein synthesis of one of the cell types but not required for the protein synthesis of the other type as suggested by Pappenheimer and Randall(125).

The evidence presented here indicates that this alteration in cell sensitivity to intoxication by DT correlates with the transformation function of the viral genome. The experiments done with the ts 68 and Ta mutants (fig. 2 and Table 2) suggest that transformation of CEF is required for the differential

sensitivity to occur. The slight alteration in the sensitivity to intoxication by DT observed in the experiments done at the restrictive temperature of 41C (fig. 2b and Table 2) is probably due to a slight degree of leakiness in the temperature sensitive function of the viral genome (10,180).

Since AMV is a leukosisvirus lacking the src gene but still being capable of infecting CEF and replicating within them (42), the problem of leakiness was eliminated in assays with CEF infected with this virus. These assys (fig. 4) indicated that mere infection of CEF with an avian retrovirus is not enough to cause the differential sensitivity to intoxication by DT to occur.

The assays on the BHK, py-BHK system (fig. 6) give further evidence that transformation of the cells is associated with the differential sensitivity and not viral replication. Polyoma virus, like other DNA tumor viruses, can transform certain host cells without viral replication (159).

However, this property of differential sensitivity to intoxication by DT is not a general property of all normal and transformed cell systems. In order for the difference to appear, it is necessary to use a cell system involving cells that are sensitive to intoxication by DT, such as chicken and hamster cells, and not systems involving cells resistant to intoxication by DT, such as mouse or rat cells (fig. 6 and Table 3).

Some alteration in the cell occurring during transformation apparently is responsible for the differential sensitivity to intoxication by DT observed in the toxin sensitive cell system.

This alteration could be either at the intracellular level or at the cell surface. Although the EF-2 from normal cells are equally sensitive to ADP-ribosylation by fragment A in cell-free systems (57,63,74), mutant cells have been isolated that contain an EF-2 resistant to the effect of fragment A (62, 108). The possibility that the differential sensitivity to intoxication by DT is caused by an alteration in the EF-2, either in the sensitivity of the EF-2 to ADP-ribosylation or in the amount of EF-2 within the cell was discounted by comparing the sensitivities of the cells to PA toxin (fig. 15 and 16) and by quantitating the EF-2 levels of normal CEF and SR-CEF (Table 5). Since PA toxin is capable of ADP-ribosylating EF-2 in the same manner as DT(26b,79), if differential sensitivity to intoxication was due to an alteration in the EF-2, then the differential sensitivity should also show up in assays done with PA toxin. If, on the other hand, differential sensitivity was due to an alteration in the binding or the uptake of the toxin molecule, then there may or may not be the appearance of the differential sensitivity in cells intoxicated with PA toxin. Since DT and PA toxin apparently have different mechanisms of getting the active form of the toxin past the cell membrane and into the cell cytoplasm (105), if no differential sensitivity in intoxication appears in cells intoxicated with PA toxin, then the alteration is most likely to be at the cell membrane. No difference was seen in either the cell sensitivities to PA toxin (fig.15 and 16) or the cellular levels of EF-2 in the

normal as compared to the transformed CEF (Table 5). These data support the hypothesis that differential sensitivity of CEF as compared to SR-CEF to intoxication by DT is determined at the membrane level of the cell and not at the level of the intracellular EF-2.

Venter and Kaplan (175), using a cell monolayer assay system instead of the cell suspension assay system of Iglewski and Rittenberg (80), were unable to show any difference in the sensitivities of normal as compared to malignant cells to intoxication by DT. Figures 9-12 and Table 4 indicate that it is necessary for the cells to be trypsinized before the differential sensitivity to intoxication can be seen and that the effect of this trypsinization is a transient effect lasting only a few hours. Since Venter and Kaplan (175) assayed their cells the day after they had been plated, any effect that may have occurred because of the trypsinization of the cells would have dissappeared. Therefore, they would not have observed any differential sensitivity to intoxication by DT.

The studies on the kinetics of intoxication (fig. 13 and 14) give further evidence supporting the hypothesis that trypsinization affects the sensitivity of CEF to intoxication by DT without affecting the sensitivity of SR-CEF. The net result of this effect is that the trypsinized SR-CEF are intoxicated by DT at lower concentrations of the toxin and in less time than the trypsinized CEF, yet the intoxication curves for the non-trypsinized cells are the same.

These data, along with that shown for the assays on the toxin resistant cell lines (fig. 6 and Table 3), argue against the appearance of additional binding sites during transformation being responsible for the observed differential sensitivity of the cells to intoxication by DT. If transformation of normal cells caused an increase in the number of toxin specific receptors and if this increase was responsible for the differential sensitivity, then both the resistant cell system and the cell monolayer assay system should show the differential sensitivity. Neither of these systems show the differential sensitivity to intoxication, thereby discounting the hypothesis that the differential sensitivity to intoxication by DT is the result of an increase in the number of toxin receptor sites on the surface of the transformed cells.

In order to determine which of the properties of cell trypsinization might be responsible for the observed differential sensitivity to intoxication by DT between normal CEF and transformed SR-CEF, it was necessary to examine some of the properties of trypsin. When cells are trypsinized many alterations occur. Some of these will cause the cells to display certain of the properties normally associated with transformed cells (112,148,149). Trypsinized cells demonstrate an increased agglutinability by certain plant lectins. This increased agglutinability appears to be associated with an increased mobility of the lectin binding sites and an enhanced endocytosis of the lectin molecule by the cell (82,112,115). Since DT appears to have some properties similar to certain plant lectins (41), it

can be assumed that trypsinization of normal cells causes an enhancement of membrane mobility and an increase in the endocytosis of DT by the cell. The role of endocytosis was first examined by Moehring and Moehring in 1968 (106) when they showed that the sensitivity of resistant cells to intoxication by DT could be increased by exposing the cells to poly-L-ornithine, a substance that increases the endocytosis of the toxin molecule by the cells. Yet Ivins et al. (84) demonstrated that poly-L-ornithine actually provided a partial protection of Hep-2 cells, a toxin sensitive cell line. It was this work that led Bonventre et al. (17) to postulate two mechanisms for the uptake of DT by the cells. A non-specific uptake by means of endocytosis and a specific uptake involving an association of the toxin molecule with a toxin receptor of the cell surface. To examine the role that endocytosis might play in the differential sensitivity of the cells to intoxication by DT, cells were exposed to ^{125}I labelled DT for a five hour period of time. Figure 17 indicates that endocytosis of DT is probably not responsible for the observed differential sensitivity of the cells to intoxication by DT. The possibility that the iodinated DT might not be biologically active, thereby making this series of experiments invalid, was discounted as shown in Table 2.

Treatment of normal cells with a protease such as trypsin will cause the cells to undergo an increased rate of cell division (148). It is unlikely that this property of trypsin is responsible for the differential sensitivity of CEF as compared

to SR-CEF to intoxication by DT. Both chymotrypsin and thrombin have mitogenic activities similar to that of trypsin (148,164) yet neither of these enzymes are able to cause the differential sensitivity to intoxication by DT as is seen with trypsin treated cells (fig. 10, and 18, and Table 4). They differ from the effect of trypsin in two different ways. Chymotrypsin causes a decrease in the sensitivity to intoxication of both the normal CEF and the transformed CEF. Thrombin, on the other hand, does not affect the sensitivity of either of the cells to intoxication by DT.

Besides these properties, trypsin has also been shown to remove certain cell surface markers due to its proteolytic activity. For example, mild trypsinization of normal cells will cause the removal of the LETS protein from the cell surface (77) as does chymotrypsin. Thrombin, on the other hand, does not (164), indicating that thrombin has a much milder proteolytic activity than trypsin. In 1974 Moehring and Crispell (107) reported that the sensitivity of KB cells to intoxication by DT could be decreased by the action of certain proteolytic enzymes, including trypsin, chymotrypsin and protease, on the cell surface. The effect of these proteolytic enzymes was reversible after about eight hours. They postulated that the activity of these enzymes on cell surface toxin receptors was the cause of this decrease in the sensitivity of these cells to intoxication by DT. The data presented here agree with this hypothesis. Trypsinization of normal CEF causes a decrease in the sensitivity of these cells to intoxication by DT. Yet trypsinization of SR-CEF causes no alteration in the

sensitivity of these cells to intoxication. Apparently transformation of the CEF causes an alteration in the cell membrane that renders the DT receptors insensitive to the effect of trypsin. However, this alteration does not protect the toxin binding sites from the effect of chymotrypsin or the other proteolytic enzymes tested.

Cells that are resistant to intoxication by DT, such as mouse and rat cells, have been postulated to be resistant because of a lack of toxin specific receptor sites (17,57,181). If trypsinization of sensitive cells causes a decrease in the sensitivity of the cells to intoxication by DT because of an effect of the enzyme on toxin receptor sites, then trypsinization of resistant cells should have no effect on the sensitivity of these cells to intoxication by DT. Trypsinization of resistant cells does not cause a shift relative to transformed cells in the sensitivity of the cells to DT intoxication (fig. 6 and Table 3) These data give further evidence that the differential sensitivity to intoxication by DT of CEF as compared to SR-CEF is due to the effect of trypsin on toxin specific binding sites on the cell surface. They, furthermore, explain the results reported by Saelinger and Bonventre (142) when they were unable to find any difference in the relative sensitivity of normal as compared to malignant rodent cells.

Probably the most definitive evidence for the role of toxin receptors in the differential sensitivity of cells to intoxication by DT is that shown in the experiments utilizing CRM 197 (fig. 19 and 20). CRM 197 is a non-toxic mutant form of DT which has an intact fragment B but an inactive fragment A and is capable of

competing with DT for toxin receptor sites (168). Boquet and Pappenheimer (18) have presented evidence indicating that when a receptor interacts with a toxin molecule an irreversible reaction occurs inactivating that receptor for reactions with other toxin molecules. CRM 197, with its intact fragment B, will respond with the receptors in the same manner thereby blocking the receptors from interactions with toxin molecules. The data showing that less CRM 197 was necessary for the inhibition of intoxication of trypsinized CEF than was necessary for the inhibition of intoxication of either the non-trypsinized CEF or the trypsinized and non-trypsinized SR-CEF suggests that the trypsinization of the CEF causes a reduction in the number of toxin receptors relative to the number found on the other cells.

The following model is proposed to explain the observed data: In order for differential sensitivity to occur, the cell system must have DT receptors on the cell surfaces. These receptors have sites that are sensitive to hydrolysis by trypsin and separate sites that are sensitive to hydrolysis by chymotrypsin and the other proteolytic enzymes tested. Hydrolysis of these sites causes a decrease in the sensitivity of the cells to intoxication by DT. The failure of proteolytic enzymes to cause a complete reduction in the sensitivity of the cells to intoxication by DT to a level similar to that seen for toxin resistant cells can be explained in two ways. It is possible that the proteolytic enzymes do not completely destroy the entire toxin receptor but do have enough of an effect to reduce the affinity of the receptor to the

toxin molecule. Therefore, the specifi c uptake of the toxin molecule by means of these receptors is less efficient causing a decrease in the relative sensitivity of the cells to intoxication by DT. The possibility that not all of the receptors are equally sensitive to hydrolysis by the enzymes also exists. This could be caused by either different subpopulations of receptors on the cell surface or by the presence of membrane components that prevent the action of the proteolytic enzymes on the toxin receptors. Transformation causes an increase in the amount of certain cell surface components (24,177). It is proposed that one, or more, of these components blocks the action of trypsin on the toxin receptor sites without blocking the action of the other proteolytic enzymes on the receptor sites. Cells resistant to intoxication, such as mouse cells, which lack receptor sites, would not show this differential sensitivity to intoxication because they lack the necessary receptors for the enzymes to act upon.

SUMMARY AND CONCLUSIONS

In order for the appearance of differential sensitivity of normal as compared to transformed cells to intoxication by DT to occur, two conditions must be met. First, the cell system being examined must consist of cells that are relatively sensitive to intoxication by DT, such as chicken or hamster cells. Cells that are resistant to intoxication by DT will not demonstrate differential sensitivity to intoxication of normal as compared to transformed cells. The second condition that must be met is that the cells must be pretreated with trypsin prior to assaying them for their relative sensitivities to intoxication by DT.

Trypsinization of the normal sensitive cell causes a decrease in the sensitivity of the cell to intoxication by DT. Similar treatment of transformed sensitive cells causes no such alteration. This alteration in sensitivity is specific for DT as PA toxin does not demonstrate a similar alteration in sensitivity or the appearance of differential sensitivities to intoxication.

Similarly, the differential alteration in the sensitivity of intoxication by DT is specific for trypsin treatment since treatment of the normal and transformed cells with other proteolytic enzymes, such as chymotrypsin and protease, causes a decrease in the sensitivities of both cell types.

The alteration occurring during trypsinization responsible for the differential sensitivity to intoxication of normal as

compared to transformed cells is probably not at the intracellular level. Both cell types are equally sensitive to intoxication by PA toxin and both have similar amounts of EF-2. Furthermore, since cells that lack membrane receptor sites for DT, such as mouse and rat cells, do not demonstrate an alteration in sensitivity to intoxication by DT when they are trypsinized, then the effect of trypsinization is probably on the toxin binding sites found on the cell surface.

In summary, the toxin sensitivity of virally transformed cells that are sensitive to DT intoxication was found to be unaffected by the action of trypsin while that of their normal counterparts decreased following trypsinization. Transformation of the cells apparently causes an alteration of the cell membrane that renders the toxin binding sites of that cell insensitive to the effect of trypsin treatment.

REFERENCES

1. Allison, A.C., and Davies, P. 1974. Interactions of membranes, microfilaments, and microtubules in endocytosis and exocytosis. Adv. in Cytopharmacol. 2,237-248.

2. Aloni, Y., Wincour, E., and Sachs, L. 1968. Characterization of the simian virus 40-specific RNA in virus-yielding and transformed cells. J. Mol. Biol. 31,415-429.

3. Ash, J.F., Vogt, P.K., and Singer, S.J. 1976. Reversion from transformed to normal phenotype by inhibition of protein synthesis in rat kidney cells infected with a temperature sensitive mutant of Rous sarcoma virus. Proc. Natl. Acad. Sci. 73,3603-3607.

4. Axelrod, D., Habel, K., and Bolton, E.T. 1964. Polyoma virus genetic material in a virus-free polyoma-induced tumor. Science 146,1466-1469.

5. Axline, S.G., and Reaven, E.P. 1974. Inhibition of phagocytosis and plasma membrane motility of the cultivated macrophage by cytoclasin B. Role of subplasmalemmal microfilaments. J. Cell Biol. 62,647-659.

6. Bader, J.P. 1972. Temperature dependent transformation of cells infected with a mutant of Bryan Rous sarcoma virus. J. Virol. 10,267-276.

7. Bader, J.P. 1975. Reproduction of RNA tumor viruses. Comprehensive Virology 4,253-332.

8. Baltimore, D. 1970. RNA-dependent DNA polymerase in virions of RNA tumour viruses. Nature 226,1209-1211.

9. Baltimore, D. 1974. Tumor viruses. Cold Spring Harbor Symp. Quant. Biol. 39,1187-1200.

10. Becker, F., and Friis, R.R., Personal Communication cited in Wyke, J.A. 1975. Temperature sensitive mutants of avian sarcoma viruses. Biochim. Biophys. Acta 417,91-121.

11. Beemer, K., Deusberg, P., and Vogt, P. 1974. Evidence for crossing-over between avian tumor viruses based on analysis of viral RNAs. Proc. Natl. Acad. Sci. 71, 4254-4258.

12. Bhisey, A.N., and Freed, J.J. 1971. Altered movement of endosomes in colchicine-treated cultured macrophages. Exp. Cell Res. 64,430-438.

13. Billeter, M.A., Parson, J.T., and Coffin, J.M. 1974. The nucleotide sequence complexity of avian tumor virus RNA. Proc. Natl. Acad. Sci. 71,3560-3564.

14. Black, P.H., and Lowe, W.P. 1963. SV-40 induced proliferation of tissue culture cells of rabbit, mouse, and porcine origin. Proc. Soc. Exp. Biol. Med. 114,721-727.

15. Black, P.H., Lowe, W.P., Turner, H.C., and Heubner, R.J. 1963. A specific complement-fixing antigen present in SV40 tumor and transformed cells. Proc. Natl Acad. Sci. 50,1148-1156.

16. Bombik, B.M., and Burger, M.M. 1973. C-AMP and the cell cycle: inhibition of growth stimulation. Exp. Cell Res. 80,88-94.

17. Bonventre, P.F., Saelinger, C.F., Ivins, B., Woscinski, C., and Amorini, M. 1975. Interaction of cultured mammalian cells with (125 I) diphtheria toxin. Infect. Immun. 11,675-684.

18. Boquet,P., and Pappenheimer, A.M.Jr. 1976. Interaction of diphtheria toxin with mammalian cell membranes. J. Biol. Chem. 251,5770-5778.

19. Boquet, P, Silverman, M.S., Pappenheimer, A.M.Jr., and Vernon, W.B. 1976. Binding of Triton X-100 to diphtheria toxin, cross-reacting material 45, and their fragments. Proc. Natl. Acad. Sci. 73,4449-4453.

20. Bosmann, H.B. 1972. Elevated glycosidases and proteolytic enzymes in cells transformed by RNA tumor virus. Biochim. Biophys. Acta 264,339-343.

21. Bourgaux, P., Bourgaux-Ramoisg, D., and Dulbecco, R. 1969. The replication of the ring-shaped DNA of polyoma virus, I. Identification of the replicative intermediate. Proc. Natl. Acad. Sci. 64,701-708.

22. Brawn, J. 1970. Possible association of embryonal anitgen(s) with several primary 3-methylclolanthrene-induced murine sarcomas. Int. J. Cancer 6,245-249.

23. Burger, M.M. 1969. Adifference in the architecture of the surface membrane of normal and virally transformed cells. Proc. Natl. Acad. Sci. 62,994-1001.

24. Burger, M.M., and Martin, G.S. 1972. Agglutination of cells tranformed by Rous sarcoma virus by wheat germ agglutinin and concanavalin A. Nature 237,9-12.

25. Chen, L.B., and Buchanan, J.M. 1975. Mitogenic activity of blood components. I. Thrombin and prothrombin. Proc. Natl. Acad. Sci. 72,131-135.

26. Chiarugi, V.P., Vanucci, S., and Urbano, P. 1974. Exposure of trypsin-removable sulphated polyanions on the surface of normal and virally transformed BHK-21/C13 cells. Biochim. Biophys. Acta 345,283-293.

26b. Chung, D.W., and Collier, R.J. 1977. Enzymatically active peptide from the adenosine diphosphate-ribosylating toxin of Pseudomonas aerugenosa. Infect. Immun. 16,832-841.

27. Coggin, J.H., and Anderson, N.G. 1974. Cancer, differentiation and embryonic antigens: some central problems. Adv. Cancer Res. 19, 105-165.

28. Collett, M.S., and Erikson, R.L. 1978. Protein kinase activity associated with avian sarcoma virus src gene product. Proc. Natl. Acad. Sci. 75,2021-2024.

29. Collier, R.J. 1967. Effect of diphtheria toxin on protein synthesis: Interaction of one of the transfer factors. J. Mol. Biol. 25,83-98.

30. Collier,R.J., and Cole, H.A. 1969. Diphtheria toxin subunit active in vitro. Science 164,1179-1182.

31. Collier, R.J., and Kandel, J. 1971. Structure and activity of diphtheria toxin. I. Thiol-dependent dissociation of a fraction of toxin into enzymatically active and inactive fragments. J. Biol. Chem. 246,1496-1503.

32. Collier, R.J., and Pappenheimer, A.M. Jr. 1964. Studies on the mode of action of diphtheria toxin. II. Effect of toxin on amino acid incorporation in cell-free systems. J. Exp. Med. 120,1019-1039.

33. Critchley, D.R., Wyke, J.A., and Hynes, R.O. 1976. Cell surface and metabolic labelling of the proteins of normal and transformed chicken cells. Biochim. Biophys. Acta 436,335-352.

34. Cunningham, D.D. and Ho, T.S. 1975. Effects of added proteases on concanavalin A-specific agglutinability and proliferation of quiescent fibroblasts. In: E. Reich, D. Rifkin, and E. Shaw (Eds.) Proteases and biological control. Cold Spring Harbor Lab., Cold Spring Harbor, New York. pp 795-806.

35. Dales, S., and Kajioka, R. 1964. The cycle of multiplication of vaccinia virus in Earles strain L cells I. Uptake and penetration. Virology 24,278-294.

36. David, G.S. 1972. Solid state lactoperoxidase: a highly stable enzyme for simple, gentle iodination of proteins. Biochem. Biophys. Res. Comm. 48, 464-471.

37. de Harven, E. 1974. Remarks on the ultrastructure of type A, B, and C virus particles. Adv. Virus Res. 19,221-264.

38. Desnuelle, P., and Rovery, M. 1961. The proteins of the exocrine pancreas. Adv. Prot. Chem. 16,139-195.

39. Diderholm, H., Berg, R., and Wesslen, T. 1966. Transformation of rat and guinea pig cells in vitro by SV 40, and the transplantability of the transformed cells. Int. J. Cancer 1,139-148.

40. Doerfler, W. 1968. The fate of the DNA of adenovirus type 12 in baby hamster kidney cells. Proc. Natl. Acad. Sci. 60,636-643.

41. Draper, R.K., Chin, D., and Simon, M.I. 1978. Diphtheria toxin has the properties of a lectin. Proc. Natl. Acad. Sci. 75,261-265.

42. Eckert, E.A., Beard, D., and Beard, J.W. 1951. Dose-response relations in experimental transmission of avian erythromyeloblastostic leukosis. I. Host response to the virus. J. Natl. Cancer Inst. 12,447-463.

42. Edelson, P.J., and Cohn, Z.A. 1974. Effects of concanavalin A on mouse peritoneal macrophages. I. Stimulation of endocytic activity and inhibition of phago-lysosome formation. J. Exp. Med. 140,1364-1388.

44. Eisenman, R., Vogt, V.M., and Diggelmann, H. 1974. Synthesis of avian RNA tumor virus structural proteins. Cold Spring Harbor Symp. Quant. Biol. 39,1067-1076.

45. Ellerman, V., and Bang, O. 1909. Experimentelle Leukamie bei Huhnern. Z. Hyg. Infektionskr. 63,231-243.

46. Erikson, R.L. 1969. Studies on the RNA from avian myelobastosis virus. Virology 37,124-131.

47. Faras, A.J., Garapin, A.C., Levinson, W.E., Bishop, J.M., and Goodman, H.M. 1973. Characterization of the low-molecular weight RNAs associated with the 70S RNA of Rous sarcoma virus. J. Virol. 12,334-342.

48. Fischer, H., and Sauer, G. 1972. Identification of virus induced proteins in cells productively infected with simian virus 40. J. Virol. 9,1-9.

49. Freeman, V.J. 1951. Studies on the virulence of bacteriophage infected strains of Corynebacterium diphtheriae. J. Bacteriol. 61,675-688.

50. Gelb, L.D., Kahne, D.E., and Martin, M.A. 1971. Quantitation of simian virus 40 sequences in African green monkey, mouse and virus-transformed cell genomes. J. Mol. Biol. 57,129-145.

51. Gelderblom, H., Bauer, H., and Graf, T. 1972. Cell-surface antigens induced by avian RNA tumor viruses:

detection by immunoferritin technique. Virology 47,416-425.

52. Gill, D.M. and Dinius, L.D. 1971. Observation on the stucture of diphtheria toxin. J. Biol. Chem. 246,1485-1491.

53. Gill, D.M., and Dinius, L.D. 1973. The elongation factor 2 content of mammalian cells. J. Biol. Chem. 248,654-658.

54. Gill, D.M., and Pappenheimer, A.M.Jr. 1971. Structure-activity relationships in diphtheria toxin. J. Biol. Chem. 246,1492-1495.

55. Gill, D.M., Pappenheimer, A.M.Jr., and Uchida, T. 1973. Diphtheria toxin protein synthesis and the cell. Fed. Proc. 32,1508-1515.

56. Girardi, A.J., Rappucci, P., Dierlam, P., Rutala, W., and Coggin, J.H. 1973. Prevention of simian virus 40 tumors by hamster fetal tissue: Influence of parity status of donor females on immunogenicity of fetal tissue and on immune cell cytotoxicity. Proc. Natl. Acad. Sci. 70,183-186.

57. Goor, R.S., and Pappenheimer, A.M.Jr. 1967. Studies on the mode of action of diphtheria toxin. III. Site of toxin action in cell free extracts. J. Exp. Med. 126,899-912.

58. Goor, R.S., Pappenheimer, A.M.Jr., and Ames, E. 1967. Studies on the mode of action of diphtheria toxin. V. Inhibition of peptide bond formation by toxin and NAD in cell free systems and its reversal by nicotinamide. J. Exp. Med. 126,923-939.

59. Graf, T., and Friis, R.R. 1973. Differential expression of trasformation in rat and chicken cells infected with an avian sarcoma ts mutant. Virology 56,369-374.

60. Griffin, F.M.Jr., Griffin, J.A., Leider, J.E., and Silverstein, S.C. 1975. Studies on the mechanism of phagocytosis. I. Requirements for circumferential attachment of particle-bound ligands to specific receptors on the macrophage plasma membrane. J. Exp. Med. 142,1263-1282.

61. Griffin, F.M.Jr., Griffin, J.A., and Silverstein, S.C. 1976. Studies on the mechanism of phagocytosis. II. The interaction of macrophages with anti-immunoglobulin IgG-coated bone marrow-derived lymphocytes. J. Exp. Med. 144,788-809.

62. Hager, T. 1978. The isolation and characterization of BHK and pyBHK cells highly resistant to diphtheria toxin: EF-2 mutants. Master's thesis, Dept. Micro. and Immun., Univ. Ore. Health Sci. Ctr., Portland, Ore.

63. Hameister, H., and Richter, D. 1970. Inhibition of peptide chain elongation in cell-free system from yeast by pre-incubation of the yeast peptidyl-translocase with diphtheria toxin and NAD. Hoppe Seyler Z. Physiol. Chem. 351,532-536.

64. Hanafusa, H. 1969. Rapid transformation of cells by Rous sarcoma virus. Proc. Natl. Acad. Sci. 63,318-325.

65. Hartwig, J.H., and Stossel, T.P. 1976. Interactions of actin, myosin, and an actin-binding protein of rabbit pulmonary macrophages. III. Effects of cytochalasin B. J. Cell Biol. 71,295-303.

66. Hatanaka, M., Gilden, R.V., and Heubner,, R.J. 1969. Alterations in the characteristics of sugar uptake by mouse cells transformed by murine sarcoma viruses. J. Natl. Cancer Inst. 43,1091-1096.

67. Hatanaka, M., and Hanafusa, H. 1970. Analysis of a functional change in membrane in the process of cell transformation by Rous sarcoma virus: Alteration in the characteristic of sugar transport. Virology 41,647-652.

68. Hebel, K. 1961. Resistance of polyoma virus immune animals to transplanted polyoms tumors. Proc. Soc. Exp. Biol. Med. 106,722-725.

69. Hill, M., and Hillova, J. 1972. Rous sarcoma virus recovered from chicken fibroblasts treated with DNA from non-producing Rous sarcoma cells. Nature 237,35-39.

70. Hill, M., and Hillova, J. 1974. RNA and DNA forms of the genetic material of c-type viruses and the integrated state of the DNA form in the cellular chromosome. Biochim. Biophys. Acta 355,7-48.

71. Hirt, B. 1969. Replicating molecules of polyoma virus DNA. J. Mol. Biol. 40,141-144.

72. Hollenberg, M.D., Fishman, P.H., Bennett, V., and Cuatrecasas, P. 1974. Cholera toxin and cell growth: role of membrane gangliosides. Proc. Natl. Acad. Sci. 71,4224-4228.

73. Holmes, R.K., and Barksdale, L. 1969. Genetic analysis of tox^+ and tox^- bacteriophages of Corynebacterium diphtheriae. J. Virol. 3,586-598.

74. Honjo, T., Nishizuka, Y., Hayaishi, O., and Kato, I. 1971. Adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis by diphtheria toxin. J. Biol. Chem. 246,4251-4259.

75. Hovi, T., Keski-Oja, J., and Valeri, A. 1974. Growth control in chick embryo fibroblasts; no evidence for a specific role for cyclic purine nucleotides. Cell 2,235-240.

76. Hynes, R.O. 1973. Alteration of cell-surface proteins by viral transformation and proteolysis. Proc. Natl. Acad. Sci. 70,3170-3174.

77. Hynes, R.O. 1974. Role of surface alterations in cell transformation: the importance of proteases and surface proteins. Cell 1147-156.

78. Hynes, R.O., and Humphreys, K.C. 1974. Characterization of the external proteins of hamster fibroblasts. J. Cell Biol. 62,438-448.

79. Iglewski, B.H., and Kabat, D. 1975. NAD dependent inhibition of protein synthesis by Pseudomonas aeruginosa toxin. Proc. Natl. Acad. Sci. 72,2284-2288.

80. Iglewski, B.H., and Rittenberg, M.B. 1974. Selective toxicity of diphtheria toxin for malignant cells. Proc. Natl. Acad. Sci. 71,2707-2710.

81. Iglewski, B.H., Rittenberg, M.B., and Iglewski, W.J. 1975. Preferential inhibition of growth and protein synthesis in Rous sarcoma virus transformed cells by diphtheria toxin. Virology 65,272-275.

82. Inbar, M., and Sachs,L. 1969. Interaction of the carbohydrate-binding protein concanavalin A with normal and transformed cells. Proc. Natl. Acad. Sci. 63,1418-1425.

83. Inbar, M., Ben-Basset, H., and Sachs, L. 1971. A specific metabolic activity on the surface membrane in malignant cell-transformation. Proc. Natl. Acad. Sci. 68,2748-2751.

84. Ivins, B., Saelinger, C.B., Bonventre, P.F., and Woskinski, C. 1975. Chemical modulation of diphtheria toxin on cutured mammalian cells. Infect. Immun. 11,665-674.

85. Kaprowski, H., Genson, F.C., and Steplewski, Z. 1967. Activation of production of infectious tumor virus SV 40 in heterokaryon cultures. Proc. Natl. Acad. Sci. 58,127-133.

86. Karnovsky, M.L. 1962. Metabolic basis of phagocytic activity. Physiol. Rev. 42,143-168.

87. Karpas, A., and Milstein, C. 1973. Recovery of the genome of murine sarcoma virus (MSV) after infection of cells with nuclear DNA from MSV transformed non-producing cells. Eup. J. Cancer 9,295-301.

88. Kato, I., and Pappenheimer, A.M.Jr. 1960. An early effect of diphtheria toxin on the metabolism of mammalian cells growing in culture. J. Exp. Med. 112,329-349.

89. Kawai, S., and Hanafusa, H. 1971. The effects of reciprocal changes in temperature on the transformed state of cells infected with a Rous sarcoma virus mutant. Virology 46,470-479.

90. Keski-Oja, J., Valheri, A., and Ruoslahti, E. 1976. Fibroblast surface antigen (SF): the external glycoprotein lost in proteolytic stimulation and malignant transformation. Int. J. Cancer 17,261-269.

91. Khoury, G, Byrne, J.C., Takenoto, K.K., and Martin, M.A. 1973. Patterns of simian virus 40 deoxyribonucleic acid transcription II. In tranformed cells. J. Virol. 11,54-60.

92. Khoury, G., Howley, P., Brown, M., and Martin, M. 1974. The detection and quantitation of SV 40 nucleic acid sequences using single-stranded SV 40 DNA probes. Cold Spring Harbor Symp. Quant. Biol. 39,147-152.

93. Koch, M.A., and Sabin, A.B. 1963. Specificity of virus-induced resistance to transplantation of polyoma and SV 40 tumors in adult hamsters. Proc. Soc. Exp. Biol. Med. 113, 4-12.

94. Kung, H.J., Bailey, J.M., Davidson, N., Nicolson, M.O., and McAllister, R.M. 1975. Struture, subunit composition, and molecular weight of RD-114 RNA. J. Virol. 16,397-411.

95. Kurth, R., and Bauer, H. 1972. Common tumor-specific surface antigens on cells of different species transformed by avian RNA tumor viruses. Virology 49,145-159.

96. Kurth, R., Wyke, J.A., and Friis, R.R. 1976. The effect of transformation-defective avian oncornavirus mutants on tumor antigen expression. J. Supramol. Struct. 4,133-139.

97. Leis, J.P., Smith, R.E., Dierks, P. Parsons, J.T., Collett, M.S., and Faras, A.J. 1978. In vitro transcription of reconstituted 35S RNA-tRNA trp template primer complexes by the avian oncornavirus DNA polymerase. Effect of temperature on the size of the DNA transcripts. Virology 85,28-42.

98. Lewis, A.M., and Rowe, W.P. 1971. Studies on nondefective adenovirus-simian virus 40 hybrid viruses. I. A newly characterized simian virus 40 antigen induced by the $Ad2^{+}ND_{1}$ virus. J. Virol. 7,189-197.

99. Lin, J.Y., Tserng, K.Y., Chi-Ching, C., Lin, L.T., and Tung, T.C. 1970. Abrin and ricin : New anti-tumour substances. Nature 227,292-293.

100. Marciani, D.J., and Bader, J. 1975. Polypeptide composition of cell membranes from chick embryo fibroblasts transformed by Rous sarcoma virus. Biochim. Biophys. Acta 401,386-392.

101. Martin, G.S. 1970. Rous sarcoma virus: A function required for maintenance of the transformed state. Nature 227,1021-1023.

102. Matsuda, M., Kanei, C_+, and Yoneda, M. 1971. Degree of expression of the tox gene introduced into corynebacteria by phages derived from diphtheria bacilli having different capacities to produce toxin. Bilken J. 14,365-368.

103. Mayor, H.D., Stinebaugh, S.E., Jamison, R.M., Jordan, L.E., and Melnick, J.L. 1962. Immunofluorescent, cytochemical, and microcytological studies on the growth of simian vacuolating virus (SV-40) in tissue culture. Exp. Mol. Pathol. 1,397-416.

104. Michl, J., Ohlbaum, D.J., and Silverstein, S.C. 1976. 2-Deoxyglucose selectively inhibits Fc and complement receptor-mediated phagocytosis in mouse peritoneal cells. II. Dissociation of the inhibitory effects of 2-deoxy-glucose on phagocytosis and ATP generation. J. Exp. Med. 144,1484-1493.

105. Middlebrook, J.L., and Dorland, R.B. 1977. Response of cultured mammalian cells to the exotoxins of Pseudomonas aeruginosa and Corynebacterium diphtheriae: differential cytotoxicity. Can. J. Microbiol. 23,183-189.

106. Moehring, J.M., and Moehring, T.J. 1968. The response of cultured mammalian cells to diphtheria toxin. II. The resistant cell: Enhancement of toxin action by poly-L-ornithine. J. Exp. Med. 127,541-554.

107. Mohring, T.J., and Crispell, J.P. 1974. Enzyme treatment of KB cells: the altered effect of diphtheria toxin. Biochem. Biophys. Res. Commun. 60,1446-1452.

108. Moehring, T.J., and Moehring, J.M. 1977. Selection and characterization of cells resistant to diphtheria toxin and pseudomonas exotoxin A: presumptive translational mutants. Cell 11,447-454.

109. Moelling, K. 1974. Reverse transcriptase and RNase H: Present in a muring virus and in both subunits of an avian

virus. Cold Sprin Harbor Symp. Quant. Biol. 39,969-974.

110. Moore, E.G., and Temin, H.M. 1971. The lack of correlation between conversion by RNA tumour viruses and increased agglutinability of cells by concanavalin A and wheat germ agglutinin. Nature 231,117-121.

111. Murphy, J.R., Skiver, J. and McBride, G. 1976. Isolation and partial characterization of a corynebacterophage β, tox operator constitutive-like mutant lysogen of Corynebacterium diphtheriae. J. Virol. 18,235-244.

112. Nicolson, G.L. 1972. Topography of membrane concanavalin A sites modified by proteolysis. Nature 239,193-197.

113. Nicolson, G.L. 1974. Ultrastructurao analysis of toxin binding and entry into mammalian cells. Nature 251,628-630.

114. Nicolson, G.L., and Blaustein, J. 1972. The interaction of Ricinus communis agglutinin with normal and tumor cell surfaces. Biochim. Biophys. Acta 266,543-547.

115. Nicolson, G.L., Lacorbiere, M., and Eckhart, W. 1975. Qualitative and quantitative interactions of lectins with untreated and neuraminidase-treated normal, wild-type, and temperature-sensitive polyoma-transformed fibroblasts. Biochemistry 14,172-179.

116. Nicolson, G.L., Lacorbiere, M., and Hunter, T.R. 1975. Mechanism of cell entry and toxicity on an affinity-purified lectin from Rininus communis and its differential effects on normal and virus transformed fibroblasts. Cancer Res. 35,144-155.

117. Oda, K. and Dulbecco, R. 1968. Regulation of transcription of the SV 40 DNA in productively infected and transformed cells. Proc. Natl. Acad. Sci. 60,525-532.

118. Olsnes, S., Heiburg, H., and Pihl, A. 1973. Inactivation of eucaryotic ribosomes by the toxic plant proteins abrin and ricin. Mol. Biol. Rept. 1,15-20.

119. Olsnes, S., and Pihl, A. 1973. Different biological properties of the two constituent peptide chains of ricin, a toxic protein inhibiting protein synthesis. Biochemistry 12,3121-3126.

120. Olsnes, S., Refsnes, K., and Pihl, A. 1974. Mechanism of action of the toxic lectins abrin and ricin. Nature 249,627-631.

121. Ozer, H.L. 1972. Synthesis and assembly of simian virus 40. I. Differential synthesis of intact virions and empty shells. J. Virol. 9,41-51.

122. Panet, A., Verma, I.M., and Baltimore, D. 1974. Role of the subunits of the avian RNA tumor virus reverse transcriptase. Cold Spring Harbor Symp. Quant. Biol. 39,919-924.

123. Pappenheimer, A.M.Jr. 1955. The pathogenesis of diphtheria. Symp. Soc. Gen. Microbiol. 5,40-56.

124. Pappenheimer, A.M.Jr., and Gill, D.M. 1973 Diphtheria. Science 182,353-358.

125. Pappenheimer, A.M.Jr., and Randall, V. 1975. On the alleged high sensitivity of mouse Ehrlich-Lettre ascites tumor cells to diphtheria toxin. Proc. Natl. Acad. Sci. 72,3149-3152.

126. Pastan, I., and Johnson, G.S. 1974. Cyclic AMP and the transformation of fibroblasts. Adv. Cancer Res. 19,303-370.

127. Pearson, G.D., and Hanawalt, P.C. 1971. Isolation of DNA replication complexes from uninfected and adenovirus-infected HeLa cells. J. Mol. Biol. 62,65-80.

128. Pesanti, E.L., and Axline, S.G. 1975. Colchcine effects of lysosomal enzyme induction and intracellular degradation in the cultivated macrophage. J. Exp. Med. 141,1030-1046.

129. Pollack, R.E., and Burger, M.M. 1969. Surface-specific characteristics of a contact-inhibited cell line containing the SV-40 genome. Proc. Natl. Acad. Sci. 62,1074-1083.

130. Purchio, A.F., Erikson, E., Brugge, J.S., and Erikson, R.L. 1978. Identification of a polypeptide encoded by avian sarcoma virus src gene. Proc. Natl. Acad. Sci. 75,1567-1571.

131. Ralph, R.K., and Colter, J.S. 1972. Evidence for the integration of polyoma virus DNA in a lytic system. Virology 48,49-58.

132. Rapp, F., Kitahara, T., Butel, J.S., and Melnick, J.L. 1964. Synthesis of SV 40 tumor antigens during replication of simian papovavirus (SV 40). Proc. Natl. Acad. Sci. 52, 1138-1142.

133. Reich, P.R., Black, P.H., and Weissman, S.M. 1966. Nucleic acid homology stucies of SV 40 virus-transformed and normal hamster cells. Proc. Natl. Acad. Sci. 56,78-85.

134. Righelato, R.C., and vanHemert, P.A. 1969. Growth and toxin synthesis in batch and chemostat cultures of Corynebacterium diphtheriae. J. Gen. Microbiol. 58,403-410.

135. Robinson, W.S., Piktanen, A., and Rubin, H. 1965. The nucleic acid of the Bryan strain of Rous sarcoma virus: Purification of the virus and isolation of the nucleic acid. Proc. Natl. Acad. Sci. 54,137-144.

136. Roblin, R., Chen, I., and Black, P.H. 1975. Proteolytic enzymes, cell surface changes and viral transformation. Adv. Cancer Res. 22,203-260.

137. Rous, P. 1911. A sarcoma of the fowl transmissible by an agent from the tumor cells. J. Exp. Med. 13,397-404.

138. Rous, E., and Yersin, A. 1888. Contribution a d'etude de la diphtherie. Ann. Inst. Pasteur 2,629-661.

139. Rowlatt, C., Wicker, R., and Bernhard, W. 1973. Ultra-structural distribution of concanavalin A receptors on hamster embryo and adenovirus tumour cell cultures. Int. J. Cancer 11,314-326.

140. Rubin, H. 1960. A virus in chick embryos which induces resistance in vitro to infection with Rous sarcoma virus. Proc. Natl. Acad. Sci. 46,1105-1119.

141. Ruoslahti, E., Vaheri, A., Kuusela, P., and Linder, W. 1973. Fibrblast surface antigen: a new serum protein. Biochim. Biophys. Acta 322,352-358.

142. Saelinger, C.B., and Bonventre, P.F. 1977. Comparable resistance of normal and malignant rodent cells to diphtheria toxin. J. Natl. Cancer Inst. 59,451-454.

143. Sambrook, J., Westphal, H., Srinivasan, P.R., and Dulbecco, R. 1968. The integrated state of viral DNA in SV 40 transformed cells. Proc. Natl. Acad. Sci. 60,1288-1296.

144. Sawyer, R.C., and Dahlberg, J.E. 1973. Small RNAs of Rous sarcoma virus: characterization by two-dimensional poly-acrilamide gel electrophoresis and fingerprint analysis. J. Virol. 12,1226-1237.

145. Sbarra, A.J., and Shirley, W. 1963. Phagocytic inhibition and reversal. I. Effect of glycolytic intermediates and nucleotides on particle uptake. J. Bacteriol. 86, 259-265.

146. Schnebli, H.P., and Burger, M.B. 1972. Selective inhibition of growth of transformed cells by protease inhibitors. Proc. Natl. Acad. Sci. 69,3825-3827.

147. Schone, G. 1906. Untersuchungen über die Karzinomimmunität bei Maüsen. Muench. Med. Wochenschr. 53,2517-2528.

148. Sefton, B.M., and Rubin, H. 1970. Release from density dependent growth inhibition by proteolytic enzymes. Nature 227,843-845.

149. Sefton, B.M., and Rubin, H. 1971. Stimulation of glucose transport in culture of density-inhibited chick embryo cells. Proc. Natl. Acad. Sci. 68,3154-3157.

150. Sela, B.A., Lis, H., Sharon, N., and Sachs, L. 1971. Quantitation of N-acetyl-D-galactosamine-like sites of the surface membrane of normal and transformed mammalian cells. Biochim. Biophys Acta 249,564-570.

151. Shein, H.M., and Enders, J.F. 1962. Transformation induced by simian virus 40 in human renal cell cultures. I. Morphology and growth characteristics. Proc. Natl. Acad. Sci. 48,1164-1172.

152. Sheppard, J.R. 1972. Difference in the cyclic adenosine 3'5'-monophosphate levels in normal and transformed cells. Nature 236,14-16.

153. Silverstein, S.C., Steinman, R.M., and Cohn, Z.A. 1977. Endocytosis. Ann. Rev. Biochem. 46,669-722.

154. Singer, R.A. 1973. Temperature-sensitive mutant of toxinogenic corynebacteriophage beta. I. Genetics. Virology 55, 347-356.

155. Singer, R.A. 1973. Temperature-sensitive mutants of toxinogenic corynebacteriophage beta. II. Properties of mutant phages. Virology 55,357-362.

156. Smotkin, D., Gianni, A.M., Rozenblatt, S., and Weinberg, R.A. 1975. Infectious viral DNA of murine leukemia virus. Proc. Natl. Acad. Sci. 72,4910-4913.

157. Sperti,S., Montanero, L., Mattioli, A., and Stirpe, F. 1973. Inhibition by ricin of protein synthesis in vitro: 60S ribosomal subunit as the target of the toxin. Biochem. J. 136,813-815.

158. Steinman, R.M., Silver, J.M., and Cohn, Z.A. 1974. Pinocytosis in fibroblasts. Quantitative studies in vitro. J. Cell Biol. 63,949-969.

159. Stoker, M., and MacPherson, I. 1961. Studies on the transformation of hamster cells by polyoma virus in vitro. Virology 14,459-470.

160. Stone, K.R., Smith, R.E., and Joklik, W.K. 1974. Changes in membrane polypeptides that occur when chick embryo fibroblasts and NRK cells are transformed with avian sarcoma viruses. Virology 58,86-100.

161. Strauss, N. 1960. The effect of diphtheria toxin on the metabolism of HeLa cells. II. Effect on nucleic acid metabolism. J. Exp. Med. 112,351-359.

162. Strauss, N., and Hender, E. 1959. The effect of diphtheria toxin on the metabolism of HeLa cells. J. Exp. Med. 109, 145-163.

163. Temin, H.M., and Mizutani, S. 1970. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature 226,1211-1214.

164. Teng, N.N.H., and Chen, L.B. 1975. The role of surface proteins in cell proliferation as studied with thrombin and other proteases. Proc. Natl. Acad. Sci. 72,413-417.

165. Ting, C.C., Lavrin, D.H., Shiu, G., and Heberman, R.B. 1972. Expression of fetal antigens in tumor cells. Proc. Natl. Acad. Sci. 69,1664-1679.

166. Tooze, J. 1973. The Molecular Biology of Tumor Viruses. Cold Spring Harbor Lab., Cold Spring Harbor, New York.

167. Uchida, T., Gill, D.M., and Pappenheimer, A.M.Jr. 1971. Mutation in the structural gene for diphtheria toxin carried by temperate phage β. Nature 233,8-11.

168. Uchida, T., Pappenheimer, A.M.Jr., and Greany, R. 1973. Diphtheria toxin and related proteins. I. Isolation and properties of mutant protein serologically related to diphtheria toxin. J. Biol. Chem. 248,3838-3844.

169. Uchida, T., Pappenheimer, A.M.Jr., and Harper, A.A. 1972. Reconstruction of diphtheria toxin from the nontoxic crossreacting proteins. Science 175,901-903.

170. Uchida, T., Pappenheimer, A.M.Jr., and Harper, A.A. 1973. Diphtheria toxin and related proteins. III. Reconstitution of hybrid "diphtheria toxin" from nontoxic mutant proteins. J. Biol. Chem. 248, 3851-3854.

171. Unanue, E.R., Perkins, W.D., and Karnovsky, M.J. 1972. Ligand-induced movement of lymphocyte membrane macromolecules.I. Analysis by immunofluorescence and ultrastructural radioautography. J. Exp. Med. 136,885-906.

172. Unkeless, J.C., Tobia, A., Ossowski, L., Quigley, J.P.,

Rifkin, D.B., and Reich, E. 1973. An enzymatic function associated with transformation of fibroblasts by oncogenic viruses. I. Chick embryo fibroblast cultures transformed by avian RNA tumor viruses. J. Exp. Med. 137,85-93.

173. Van Heyningen, W.E. 1974. Gangliosides as membrane receptors for tetanus toxin, cholera toxin and serotonin. Nature 249, 415-417.

174. Varmus, H.E., Guntaka, R.V., Deng, C.T., and Bishop, J.M. 1975. Synthesis, structure, and function of avian sarcoma virus-specific DNA in permissive and nonpermissive cells. Cold Spring Harbor Symp. Quant. Biol. 39,987-996.

175. Venter, B.R., and Kaplan, N.O. 1976. Diphtheria toxin effects on human cells in tissue culture. Cancer Res. 36,4590-4594.

176. Wagner, R., Rosenberg, M., and Estensen, R. 1971. Endocytosis in Chang liver cells. Quantitation by sucrose-^{3}H uptake and inhibition by cytochalasin B. J. Cell Biol. 50,804-817.

177. Warren, L., Critchley, D., and MacPherson, I. 1972. Surface glycoproteins and glycolipids of chicken embryo cells transformed by a temperature-sensitive mutant of Rous sarcoma virus. Nature 235,275-278.

178. Watkins, J.F., and Dulbecco, R. 1967. Production of SV 40 virus in heterokaryons of transformed and susceptible cells. Proc. Natl. Acad. Sci. 58,1396-1403.

179. Willingham, M., and Pasten, I. 1974. Cyclic AMP mediates the concanavalin A agglutinability of mouse fibroblasts. J. Cell Biol. 63,288-294.

180. Wyke, J.A., and Linial, M. 1973. Temperature-sensitive avian sarcoma viruses: A physiological comparison of twenty mutants. Virology 53,152-161.

181. Zanen, J., Muyldermans, G., and Beugnier, N. 1976. Competitive antagonists of the action of diphtheria toxin in HeLa cells. FEBS Letters 66,261-263.