STRUCTURAL PROTEINS OF A TOXINOGENIC CORYNEBACTERIOPHAGE BETA AND THEIR RELATIONSHIP TO DIPHTHERIA TOXIN

by

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INTRODUCTION

Statement of the Problem

The structural gene for diphtheria toxin resides within the genome of toxinogenic corynebacteriophage (Pappenheimer and Gill, 1973). According to current theory, however, toxin plays no obvious role in phage development or replication (Uchida, Gill and Pappenheimer, 1971). The general question that motivated the research for this thesis was: would an analysis of the structural components of a toxinogenic corynebacteriophage reveal the presence of any polypeptide(s) identical or related to diphtheria toxin?

Preliminary experiments (Elwell and Iglewski, 1972) using rather crude preparations of a lytic β toxinogenic corynebacteriophage (βv tox^+) indicated that the major phage protein co-migrated with fragment B of toxin when electrophoresed in sodium dodecyl sulfate (SDS)-acrylamide gels. This discovery made it possible to define in more specific terms the objectives of this research. They were:

1. To develop methods of purifying large amounts (5-10 mg) of βv tox^+ phage.
2. To analyze and quantitate the protein composition of a purified βv tox^+ phage.
3. To determine if the major phage protein and toxin fragment B are related polypeptides by the following criteria:

(a) functional - can βv tox^+ phage compete with intact toxin for cell binding sites thus inhibiting cellular intoxication, and can a functional toxin molecule be reconstituted using the fragment B-like phage protein and genuine fragment A?

(b) immunological - do phage protein and toxin form precipitin lines when reacted in immunodiffusion slides against diphtheria antitoxin, and does anti-βv tox^+ antibody react with toxin?

(c) biochemical - is the peptide fingerprint of a purified phage protein preparation similar to the fingerprint of toxin fragment B?

Review of the Literature

Diphtheria toxin, a 62,000 molecular weight protein, is responsible for the major symptoms of diphtheria. The causative organism, Corynebacterium diphtheriae, generally lodges in the throat and nasopharynx of susceptible individuals where it multiplies in the superficial layers of the mucuous membranes. Strains of C. diphtheriae gain the capacity to synthesize and release extracellular toxin as a result of being lysogenized by corynebacteriophage β or its close relatives. Released toxin is transported throughout the body producing necrosis in a wide variety of tissues and not

infrequently causing death of the non-immunized host. The lethal dose for the guinea pig is 0.240 µg per kg body weight (Barksdale, Garmise and Horibata, 1960) and a very limited amount of information available suggests that man may be slightly more susceptible (Wilson, 1967).

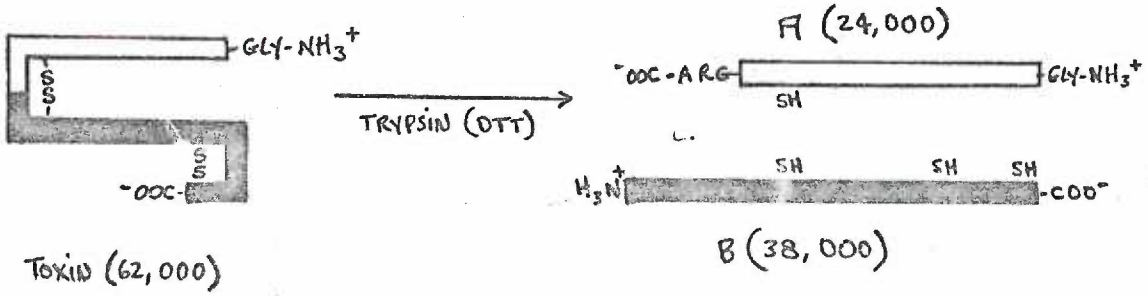
A. NATURE OF DIPHTHERIA TOXIN:

In the intervening eighty-six years since the first description of diphtheria toxin by Rous and Yersin (1888) a large amount of information has been learned about this molecule. Recent and exciting research has dealt with the relationships between toxin structure and its mode of action with such success that the primary events involved in the pathogenicity of diphtheria toxin at the cellular and molecular levels are quite well understood.

Toxin structure:

Diphtheria toxin is released from the bacterial cell as a single 62,000 dalton polypeptide chain (Gill and Dinius, 1971; Collier and Kandel, 1971) and it is easily isolated and purified from supernatants of toxinogenic *C. diphtheriae* strains grown in conditions of limiting iron (Pope and Stevens, 1958; Goor and Pappenheimer, 1967). In the case of Park-Williams 8 (PW8) strain grown in deferrated medium, toxin represents more than 5% of the total bacterial protein

synthesized (Lampidis and Barksdale, 1971). A diagram of the toxin molecule, before and after reduction, followed by mild hydrolysis with trypsin is shown below (Pappenheimer and Gill, 1973).



The intact molecule contains two disulfide bridges, one of which spans an arginine-rich region, highly sensitive to proteolysis. Mild trypsinization of toxin, in the presence of a sulfhydryl reducing agent, yields two large peptides: fragment A (24,000 daltons) and fragment B (38,000 daltons) (Drazin, Kandel and Collier, 1971). When proteolysis is carried out in a non-reduced environment the fragments remain connected by the disulfide bridge and the resultant molecule is referred to as "nicked toxin". Michel, Zanen, Monier, Crispeels and Dirkx (1972) partially characterized diphtheria toxin and its proteolytic fragments. They identified glycine as the N-terminal amino acid both of intact toxin and of the L chain (analogous to fragment A) but not of the H chain (analogous to fragment B) and concluded that fragment A was situated at the N-terminus. Michel et al. (1972)

sequenced the first 17 amino acids from the N-terminus of fragment A.

The isolated fragments of toxin were found to have dissimilar physical and chemical properties (Gill and Dinius, 1971; Collier and Kandel, 1971). Fragment A is highly stable. It is not coagulated by boiling for 2 hr, it is refractory to both high and low pH and it is relatively resistant to proteolysis. The instability of fragment B has made it difficult to characterize. It is reported to contain such a high percentage of hydrophobic residues that it forms aggregates (particularly at acidic pH) in all but denaturing solvents and therefore adheres to glassware, dialysis membranes and other surfaces (Gill and Dinius, 1971). Cukor et al. (1973) managed to keep fragment B in solution using 0.02 M phosphate buffer at pH 7.8. When the pH was lowered to 6.4, fragment B precipitated. It is possible to dialyze B fragment dissolved in 6 M urea, against a 0.01 M phosphate buffer containing 0.5 M glycine and 0.14 M NaCl, pH 7.5 without it precipitating (Elwell and Iglewski, unpublished observations).

Effect of toxin on eucaryotic protein synthesis:

Strauss and Hendee (1959) reported that diphtheria toxin inhibited protein synthesis in cultured HeLa cells. Protein synthesis appeared to be the primary target of toxin since other metabolic parameters remained normal for several hours after amino acid incorporation had ceased (Strauss, 1960). Subsequently, Collier and

Pappenheimer (1964) found that nicotinamide adenine dinucleotide (NAD) was absolutely required for the inhibition of protein synthesis in cell-free systems derived from HeLa cells and rabbit reticulocytes. In further studies involving the reticulocyte *in vitro* system, Collier (1967) showed that in the presence of NAD, toxin inactivated one of the supernatant transfer factors by 85%. This factor was subsequently identified as elongation factor 2 (EF2), the enzyme required for the guanidine triphosphate-dependent translocation of peptidyl-transfer RNA from the "acceptor" to the "donor" site on the mammalian ribosome (Collier and Traugh, 1969; Lucas-Lenard and Lipmann, 1971). The precise interrelationship between NAD and toxin relative to their roles in the inhibition of protein synthesis was first elucidated by Honjo and his associates (Honjo, Nishizuka, Hayaishi and Kato, 1968; Honjo, Nishizuka and Hayaishi, 1969; Honjo, Nishizuka, Kato and Hayaishi, 1971). Using preparations of radioactive NAD, labeled at specific sites within the molecule, they found that toxin enzymatically catalyzed the covalent linkage of the adenosine diphosphate ribose moiety (ADPR) of NAD to EF2. A concommitant of this transfer reaction was the loss of EF2 enzyme activity (Raeburn, Goor, Schneider and Maxwell, 1968). Goor and Maxwell (1969) found that the inactivation of EF2 could be prevented or even reversed in the presence of nicotinamide. Using rat liver EF2 enzyme, Honjo, Nishizuka, Kato and Hayaishi (1971) showed that the ADP-ribosylation of EF2 by toxin is

maximal at pH 8.5 and that the reverse reaction takes place at pH 5.2. In equation form:

$$EF2 + NAD^{+} \xrightleftharpoons{\text{toxin}} \text{ADP-ribosyl-EF2} + \text{Nicotinamide} + H^{+}$$

No eukaryotic protein other than elongation factor 2 has been found capable of accepting ADPR from NAD in the presence of diphtheria toxin. Furthermore, toxin appears to have no effect on polypeptide chain-elongation systems of procaryotic or mitochondrial origin wherein chain translocation is catalyzed not by EF2, but by an unrelated enzyme, EF-G (Richter and Lipmann, 1970). Although inactivation of EF2 enzyme was shown to be the crucial event in diphtheria intoxication, the story was far from complete. The capacity of the toxin molecule to exert toxic or enzymatic activity was found to be dependent upon the configuration of the molecule and/or the flexibility of the fragments within the molecule. For instance: neither intact nor nicked, unreduced toxin showed any in vitro ADP-ribosylation activity but both were toxic to animals and tissue culture cells. Fragment A possessed enzymatic activity whereas fragment B did not and neither fragment alone showed toxicity. Table 1 summarizes the properties of these various structures (Gill, Pappenheimer and Uchida, 1972).

Table 1

	Toxicity	Enz. Activity
Intact toxin	+	-
Nicked toxin	+	-
Fragment A	-	+
Fragment B	-	-

Enzymatic role of fragment A:

Collier and Cole (1969) found that in nicked and reduced toxin the majority of the enzymatic activity was associated with a peptide of much smaller molecular weight than intact toxin. This enzymatically active peptide was identified as fragment A. Hence, ADP-ribosylation activity is an inherent property of the A fragment of toxin, an activity that is in some manner masked when this fragment is an integral part of intact toxin.

Role of fragment B in toxicity:

Several pieces of evidence suggested that the role of fragment B was in some way concerned with binding toxin to susceptible cell membranes and facilitated the entry of fragment A into the cytoplasm. The initial experiments studied altered toxin molecules (CRM proteins) released by cells lysogenized by β phage mutants. Uchida, Gill and Pappenheimer (1971) purified a CRM45 protein which contained functional A fragment but incomplete fragment B. This polypeptide was

enzymatically active *in vitro* but was devoid of toxic or necrotizing activity *in vivo*, presumably because the B fragment, required for cell membrane binding, was aberrant. In a similar study Uchida, Pappenheimer and Harper (1972) isolated a CRM197 protein which contained non-functional fragment A but had completely normal fragment B. Although this protein lacked enzymatic activity, it was able to compete effectively with diphtheria toxin for attachment sites on the membranes of cultured HeLa cells. More recently, Cukor, Solotorovsky and Kuchler (1973) succeeded in isolating fragment B by boiling nicked toxin and resuspending the product at pH 6.4. Fragment B precipitated out of solution in a relatively pure form. They were then able to delay the action of toxin on cultured KB cells by pre-incubating the cultures with fragment B. On the basis of the information that had accumulated relative to the structure and function of intact toxin and toxin fragments, Gill and Pappenheimer (1973) proposed a two-step mechanism of cell intoxication:

1. The fragment B portion of the toxin molecule binds to receptor sites on susceptible cell membranes.
2. Once attached to the membrane, toxin is "nicked" by a cellular peptidase(s), the disulfide bonds are reduced and fragment A is transported into the cytoplasm where it inactivates non-ribosomal bound elongation factor 2, thus eventually bringing protein synthesis to a halt.

Several questions remain unanswered: is fragment A split from fragment B on the surface of the membrane or within the cytoplasm?; what is the fate of the B fragment in either case?; are there specific entry sites on plasma membranes?; how do insensitive cells differ from sensitive cells? Moehring and Moehring (1972a and 1972b) have addressed this latter question. In an earlier study they discovered that the toxin resistance of mouse L cells was related to a property of the cell membrane rather than to any intrinsic property associated with protein-synthesizing factors. This was proven by the fact that an L-cell in vitro system proved to be as sensitive to fragment A as those derived from toxin sensitive cells like HeLa or KB. Furthermore, poly-L-ornithine which stimulates cell macromolecular uptake, enhanced mouse L cell intoxication (Moehring and Moehring, 1968). Subsequently Moehring and Moehring (1972a) developed populations of KB cells resistant to diphtheria toxin by exposing normal cells to toxin concentrations which allowed less than 0.001% of the population to survive. Surviving cells were grown in the absence of toxin and then the intoxication procedure was repeated until a toxin resistant line (KB-R) was established. A study of such resistant cells showed that toxin resistance was a stable property of the cell which was retained after many generations in culture. In a related study they found that toxin-resistant KB cells also resisted infection by polio virus, Mengo virus and Newcastle disease virus. Lack of virus

receptors on these cells was ruled out as a mechanism of resistance. This was done by determining the reduction in extracellular virus titer after a 1 hr incubation with normal (toxin sensitive) and toxin-resistant KB cells. Normal and toxin-resistant cell lines absorbed these viruses equally well. Chan and Black (1970) had demonstrated earlier that a labilizing factor on polio-sensitive cell membranes initiated the process of polio virus uncoating, a requisite step in the infective process. Moehring and Moehring concluded that resistance to both viruses and toxin might therefore result from the loss or modification of these hypothesized membrane-associated activating factors. Pappenheimer and Brown (1968) suggested an alternative hypothesis to explain cellular resistance to diphtheria toxin. They incubated toxin-sensitive HeLa cells with toxin labeled with ^{125}I and by autoradiography showed that most of the labeled toxin became associated with the HeLa cell membranes. On the other hand far less label was taken up by toxin insensitive mouse L cells. Pappenheimer and Brown concluded that cells are resistant to toxin because they lack membrane binding sites for the toxin molecule.

B. CORYNEBACTERIOPHAGE β:

The original discovery by Freeman (1951) that a bacteriophage, corynebacteriophage β, was responsible for the conversion of non-toxigenic diphtheria strains to toxigenicity, restimulated research interest in the diphtheria bacillus. Groman (1955) confirmed Freeman's finding and demonstrated, by controlled population studies, that the conversion was indeed phage-induced.

The best studied corynebacteriophages are β tox^+ which are descendants of Freeman's toxigenic phage β. They contain a double-stranded DNA genome of approximately 2.2×10^7 daltons, sufficient to code for 25-30 proteins (Uchida, Pappenheimer and Greany, 1973). The virion of β tox^+ phage has a polyhedral head (45-50 nm on a side) and a long, non-contractile tail (250 nm long) (Nagington and Carne, 1971; Mathews, Miller and Pappenheimer, 1966). The phage is inactivated rapidly by heat and relatively slowly by prolonged storage in the cold. Purified βhv64 tox^+ phage remained at about 50% of its initial titer when stored in 0.1 M phosphate buffer for 2 months in the cold (Matsuda and Barksdale, 1966).

Corynebacteriophage and the tox gene:

Holmes and Barksdale (1969) investigated the genetic basis for the control of toxigenicity by corynebacteriophages using a series of well characterized phage mutants. The following genetic markers were

studied: h (host range), imm (lysogenic immunity), tox, c (clear plaque) and h' (extended host range). It was found that in crosses between β tox^+ and β tox^-, the tox^+ determinant of phage β behaved as a single genetic element and occupied a position between the loci h and imm on the β tox^+ phage genetic map. Their analysis established that tox was a corynebacteriophage gene. As an extension of this work, Holmes and Barksdale (1970) examined morphologically and serologically distinct tox^+ phages for their capacity to recombine genetically as a function of their relatedness. To their surprise many phages carrying the tox^+ marker failed to recombine genetically and further, the tox determinant was found in phages which were serologically and morphologically distinct. They concluded that the tox gene did not appear to endow corynebacteriophages with any special advantage. Singer (1973) and Matsuda, Kanei and Yoneda (1971) independently isolated and genetically analyzed several temperature-sensitive (ts) mutants of toxigenic corynebacteriophage β. Singer mapped temperature ts mutants whereas Matsuda et al. mapped a virulent, non-lysogenizing ts mutant. The maps for both β phage ts mutants were co-linear and the tox gene mapped near the c marker between two clusters of ts loci.

Although it had been established that tox was a discrete phage gene and considered to be part of the phage genome, uncertainty existed as to whether the tox gene actually carried the structural

information for the toxin molecule. This uncertainty was removed in 1971 when Uchida, Gill and Pappenheimer reported on the properties of a nitrosoguanidine-induced temperate β tox^+ mutant, β45. Upon infection of sensitive $C_7(-)$ tox^- cells with β45, a non-toxic protein, serologically related to toxin was released into the medium. This cross-reacting material (CRM45) had a smaller MW (45,000 daltons) than toxin and was found to contain a normal fragment A (including normal enzymatic activity) but an aberrant fragment B in that a 17,000 dalton peptide was missing. These authors felt that a point mutation in the β45 tox gene, leading to premature chain termination, was the most plausible explanation of their results. However, no suppressor strains of C. diphtheriae exist to support this contention and they could not detect phenotypic suppression, i.e. toxin production, by growing the lysogen in the presence of 5-fluorouracil or streptomycin. Other CRM proteins have been subsequently isolated: CRM30, CRM197 and CRM228 (Uchida, Pappenheimer and Greany, 1973) and β-NG2 protein (Matsuda, Kanei and Yoneda, 1972). The properties of these cross-reacting proteins strongly suggested that the β phage tox gene is the structural gene for diphtheria toxin. Additional evidence was provided by Murphy and Pappenheimer (1973) who synthesized toxin in vitro using β tox^+ phage DNA as a template.

Expression of the tox gene:

Although the structural gene for toxin synthesis resides within the phage genome, the expression of tox appears to be under separate control from that which regulates the genes concerned in phage replication. The expression of tox occurs in three states of the phage genome: as a repressed and integrated prophage, as a replicating, obligatory lytic phage, and as a non-replicating, non-integrated intracellular phage.

The classic diphtherial strain PW8 is lysogenized by the tox^+ prophage P which was once thought to have been defective (Barksdale, Garmise and Rivera, 1961). Maximesco (1968) discovered that P phage would plaque on a strain of C. diphtheriae var ulcerans. Toxin is synthesized de novo by PW8 and toxin is released into the extracellular fluid without appreciable bacterial lysis (Hirai et al., 1965). In other lysogenic toxigenic strains, toxin is formed to a very limited extent under normal conditions of exponential cell growth. Toxin synthesis increases when the iron content of the medium becomes limiting. Hatano (1956) could not detect any increased phage production by $C_7(\beta)$ cells under conditions of maximum toxin production. However, Barksdale and his co-workers (Barksdale, Garmise and Horibata, 1960) reported that under their experimental conditions, toxin synthesis increased following the UV-induction of the prophage in $C_7(\beta)$ cells and concluded that phage replication was a prerequisite for

toxin synthesis. Pertaining to the second alternative state of the β tox^+ genome, Matsuda and Barksdale (1966 and 1967) investigated the kinetics of toxin and phage synthesis in $C_7(-)$ cells during the lytic cycle following infection with the highly virulent phage, βhv64 tox^+. Extracellular toxin was detectable approximately 30 min after phage adsorption and free phage was seen approximately 90 min post adsorption. Although toxin synthesis and its release preceded lysis and phage release, in this particular system toxin synthesis and phage multiplication occurred simultaneously in the same cell.

Gill, Uchida and Singer (1972) recently presented additional evidence that the tox gene can be expressed without being replicated. They superinfected $C_7(\beta 45)^{crm^+}$ cells with βch' tox phage. The C gene of β is analogous to those genes of the C I (immunity repressor) gene of coliphage λ. Hence, βch' phage cannot maintain lysogeny and is thought to make no functional repressor but does respect the immunity conferred by the c' marker carried by the β45 lysogens. Following superinfection, the cells were maintained in exponential growth for 5 generations and the extracellular proteins elaborated during each generation time were analyzed on polyacrylamide gels and tested for toxicity. They found that toxin production per cell fell by a factor of 2 for each doubling, indicating that the tox genes were not replicated but merely diluted out during cell growth. However, CRM45 protein production continued at a relatively constant level throughout

the 5 generations. Therefore the tox gene can exist as a non-integrated, non-replicating exogenote.

The previously mentioned CRM proteins, cross-reactive materials produced following lysogenic conversion of C. diphtheriae by phages carrying mutated tox genes, have a bearing on this problem. Uchida, Pappenheimer and Greany (1973) have isolated five strains of β phage, each carrying a different mutation in its tox gene, as determined by the specific alterations in the extracellularly elaborated CRM proteins. These workers studied the phage released following UV irradiation of $C_7(\beta)$, $C_7(\beta 30)$, $C_7(\beta 45)$ and $C_7(\beta 197)$. They reported that the one-step growth curves and burst sizes of UV-induced lysogenic β phage carrying mutated tox genes were identical with those carrying wild type β tox^+ lysogens and concluded on the weight of this data that the tox gene is not involved in phage replication.

The physiological state of the host bacteria is also a controlling factor in the synthesis of diphtheria toxin. The clearest example of this involves the role of iron. Extracellular toxin is not produced by toxigenic diphtherial strains until the inorganic iron content of the culture medium is largely depleted (Locke and Main, 1931; Pope, 1932; Pappenheimer and Johnson, 1936). Hatano (1956) examined the effect of iron upon the growth of $C_7(-)$ cells, the spontaneous release of phage in these cells and the amount of toxin released extracellularly. Bacterial growth and phage production were not affected, but an

iron concentration of 70 µg per ml inhibited toxin synthesis by 90%. Interestingly, Matsuda and Barksdale (1967) reported that in the $C_7(-)/\beta hv64\ tox^+$ system excess iron did not affect the initial rate of toxin synthesis but did affect final toxin yields. The extracellular toxin yield was suppressed approximately 65-80% and even in cells saturated with iron toxin synthesis was not completely inhibited. Finding significant toxin synthesis in the presence of large amounts of iron led them to suggest that the "iron effect" required further elucidation.

The exact mechanism by which iron regulates toxin synthesis is unknown. Some preliminary evidence indicates that all diphtherial cells, lysogenic or not, contain a factor (regulatory protein) that can reduce the expression of the tox gene in the presence of iron (Murphy, Pappenheimer and Tayart de Borms, 1974). An in vitro system has recently been devised by Lightfoot and Iglewski (1974) which may shed light on this problem. They succeeded in synthesizing diphtheria toxin in an E. coli cell-free protein synthesizing system using m-RNA derived from toxigenic C. diphtheriae. A well characterized, heterologous in vitro system might provide a potent tool for studying the role that regulatory proteins may play in toxin synthesis.

C. CLEAVAGE OF PHAGE STRUCTURAL PROTEINS:

The experimental results of this thesis provide suggestive evidence that diphtheria toxin fragment B is a structural component of the corynebacteriophage βhv64 tox^+. If true, intact diphtheria toxin assumes the role of a structural precursor protein which is processed by being cleaved into two fragments, one of which is inserted into the βhv64 tox^+ virion. The conversion of precursor proteins into lower molecular weight structural peptides has been demonstrated in several unrelated phages.

Coliphage T4 has been the most extensively studied phage in this respect. Laemmli (1970) and Hosada and Cone (1970) independently reported that four major head components (F, A, D and E) of bacteriophage T4 were cleaved during the process of assembly. These four bands on SDS-polyacrylamide gels accounted for more than 90% of the total capsid protein. Protein A, 48,000 dalton MW, accounted for 60% of the total capsid protein. T4-infected cells were pulse-labeled with C^{14} amino acids and the modification of the various phage proteins was followed by analysis in SDS-polyacrylamide gels of samples taken at intervals after infection. Both groups found that a protein coded by gene 23 (P23) was the precursor of A-protein, the major capsid component. The gene 23 product, however, was a peptide 20-25% longer than protein A and a careful analysis of the pulse-chase data clearly showed that the P23-precursor underwent a cleavage from 61,000 to

46,000 MW, the latter peptide being inserted into the phage capsid. They also found three minor components of the head: gene products 22, 24 and a protein called IP, of unknown genetic origin that were also cleaved during the process of assembly. Laemmli failed to find the small fragments of these cleavage reactions on 15% acrylamide gels. He concluded that they were all quickly degraded to undetectable sizes.

Lengyel et al. (1973) examined the head morphogenesis of P2, a temperate coliphage and found the process remarkably similar to that of T4. The protein products of four P2 head genes were identified and precursor-product relationships were determined by pulse-chase experiments followed by SDS-polyacrylamide gel electrophoresis and autoradiography. They found that the product of the N gene was a 40,000 dalton protein which was cleaved to give the major capsid protein, N*, with a MW of 36,000. Coupled to the cleavage of the N protein was the processing of the 0 gene product from a 30,000 dalton protein to 0*, a protein of approximately 17,000 daltons. The fate of 0* was not clear but indirect evidence suggested that it became an acid-soluble capsid peptide. In both T4 and P2 phages, cleavage of the major capsid protein was found to be coupled to cleavages of other proteins: in P2, to the 0 protein and in T4, to the proteins P24, P22 and IP III. Finally, Murialdo and Siminovitch (1972) have reported similar conversion by proteolytic cleavage of a structural capsid protein in bacteriophage λ. This is especially significant in that corynebacteriophage β and coliphage λ are morphologically very similar. Both phage

have icosahedral heads and long non-contractile tails of approximately equal dimensions. They also have approximately the same size double-stranded DNA genomes.

The models that have been proposed to explain the role of cleavage in phage head assembly differ primarily in whether or not they include nucleic acid in the first step of the assembly process. One model proposes the following sequence: empty heads are assembled first and cleavage then occurs as the nucleic acid is incorporated into the pre-formed head (Jacobson and Baltimore, 1968). In a second model: DNA and uncleaved protein initially condense together and here the function of cleavage would be to either destabilize the DNA inside the head so that, upon phage adsorption, it can inject or to stabilize the capsid shell itself, making assembly irreversible (Eiserling and Dickson, 1972).

In any event, proteolytic cleavage of precursor molecules appears to play an important role in the morphogenesis of a variety of bacteriophage.

MATERIALS AND METHODS

BACTERIAL STRAINS

1. Corynebacterium diphtheriae. Nontoxigenic, non-lysogenic C. diphtheriae C_7s(-) tox^- (Barksdale and Pappenheimer, 1954) was obtained from Dr. L. Barksdale, N.Y. University School of Medicine. This strain will be referred to hereafter as C_7(-).

2. C_7(β45) CRM^+. This C_7 strain of C. diphtheriae is lysogenized by phage β45 which directs the synthesis of a 45,000 MW protein, CRM45, which is nontoxic but is serologically related to toxin (Uchida, Gill and Pappenheimer, 1971). CRM45 protein contains a normal fragment A but it has an altered fragment B in that a 17,000 dalton peptide is missing. This strain was provided us by Dr. T. Uchida, Harvard University.

CORYNEBACTERIOPHAGES

Corynebacteriophage βhv64 tox^+ (Matsuda and Barksdale, 1966) is an obligatory lytic bacteriophage which directs the synthesis of diphtheria toxin upon infection of its host bacterial strain C_7(-). This phage was kindly given to us by Dr. L. Barksdale, N.Y. University School of Medicine and will be referred to hereafter as βv tox^+.

MEDIA

1. Heart infusion broth (Groman and Lochart, 1953). This broth was prepared by dissolving 37 g dry powder (Difco) per liter of distilled water.

2. Heart infusion agar (Groman and Lochart, 1953). Forty g of the dry powder (Difco) was dissolved in 1 liter of distilled water.

3. PGT medium (Mueller and Miller, 1941; Barksdale and Pappenheimer, 1954). The complete composition of this complex growth medium and the deferration process have been outlined in detail elsewhere (Lightfoot, 1973). The iron content of the deferrated medium ranged from .050 to .070 μg Fe^{++} per ml.

4. CY medium (Mueller and Miller, 1941; Pappenheimer, Uchida and Avery-Harper, 1972). The procedure for preparing and deferrating this medium is given in complete detail elsewhere (Lightfoot, 1973).

5. Tissue culture medium. To 520 ml distilled water were added: 70 ml Hanks 10X MEM, without $NaHCO_3$ and L-glutamine (Microbiological Associates), 100 ml fetal calf serum (Microbiological Associates) and 0.7 ml penicillin/streptomycin mixture; penicillin G, 100,000 units per liter; streptomycin, 0.1 g per liter

(Pfizer Laboratories). Between 20-25 ml of a 2.8% sterile $NaHCO_3$ solution was added to the medium. The final pH was approximately 7.5.

6. Hanks BSS diluent. To 85 ml of sterile, distilled water were added: 10 ml 10X Hanks BSS, without $NaHCO_3$ (Microbiological Associates) and 1.0 ml calf serum (Microbiological Associates). Between 2.0-2.5 ml of a 2.8% sterile $NaHCO_3$ solution was added. The final pH of the medium was approximately 7.5.

BUFFERS

1. Phosphate buffered saline (PBS). This buffer was prepared by dissolving 3.4 g Na_2HPO_4, 1.3 g KH_2PO_4 and 8.5 g NaCl in 1 liter of distilled, deionized water. The final pH was 7.2.

2. Gel electrophoresis buffer (Weber and Osborn, 1969). This buffer was prepared by dissolving 38.4 g $Na_2HPO_4 \cdot 7H_2O$, 7.72 g $NaH_2PO_4 \cdot H_2O$ and 2.0 g SDS in 2 liters of distilled, deionized water. The final pH was 7.2.

Propagation of βv tox^+ corynebacteriophage (Matsuda and Barksdale, 1967). Corynebacteriophage βv tox^+ was propagated in $C_7(-)$ cells. Deferrated PGT broth, 1 liter in a 4-liter Erlenmeyer flask,

containing 2% deferrated maltose was inoculated with 10 ml of an overnight culture of $C_7(-)$ in the same medium. Flasks were incubated in a 37 C shaking water bath with vigorous aeration. When the cell concentration had reached 2×10^8 cells per ml, as determined spectrophotometrically, the bacteria were pelleted and the medium was saved and kept warm. The pelleted cells were resuspended in approximately 20 ml of PGT and thoroughly dispersed by repeated pipettings using a 5 ml pipette. Pre-warmed βv tox^+ corynebacteriophage was added to the cells at a multiplicity of 3-4 PFU per cell. After 10 min adsorption with very gentle shaking at 37 C the cells were resuspended to their original volume of 1 liter. The infected cells were then incubated for an additional hr with vigorous aeration. Sterile sodium citrate was added in a final concentration of 0.07 M to the cultures to prevent re-adsorption of progeny phage to cells or cell walls (Holmes and Barksdale, 1969). The cultures were incubated 4 more hr and then refrigerated at 4 C overnight to enhance cell lysis and phage release. Cells and cellular debris were removed by centrifugation at 10,000 x g for 20 min.

Purification of corynebacteriophage βv tox^+.

A. Differential centrifugation followed by CsCl equilibrium centrifugation (Elwell and Iglewski, 1972). Crude lysates

containing approximately 2 x 10^{10} PFU/ml were centrifuged at 12,000 x g (low speed) for 20 min to remove bacterial debris. The phage was then centrifuged out of suspension at 105,000 x g for 2 hr. The phage pellet was resuspended in sterile 0.1 M sodium phosphate buffer, pH 7.2, and subjected to 3 more cycles of low and high speed centrifugations. The final phage pellet was resuspended in 4.5 ml of an aqueous CsCl solution, specific density of 1.30, and the phage spun to equilibrium in a Beckman SW 50-L swinging bucket head for 48 hr at 100,000 x g. The CsCl gradients were fractionated by collecting drop fractions from the bottom of the tubes. All fractions were assayed for infectivity and only the fraction containing peak infectivity was used. The phage was dialyzed against 0.1 M sodium phosphate buffer, pH 7.2.

B. Two phase separation followed by CsCl equilibrium centrifugation (Albertsson, 1967). To 1 liter of crude cell lysate was added: 70 g carbowax polyethylene glycol 6000 flakes (Schwarz/Mann), 2.15 g dextran sulfate 500 (MW 500,000; Sigma) and 19.0 g NaCl. After thorough mixing, the flask was allowed to stand at 4 C overnight. A turbid sediment, approximately 10 ml, collected on the bottom of the flask. The clear top layer was aspirated off and the turbid bottom layer containing the phage, and a mixture of both solvent phases, was transferred to a centrifuge tube and

centrifuged at 700 x g for 15 min. The bacteriophage was concentrated in a "cake" which occurred between the two solvent phases. This interface was resuspended in 30 to 40 ml of a 1% (w/w) dextran sulfate solution (MW 500,000; Sigma). Then 0.15 ml of a 3 M KCl solution was added per ml of suspension which precipitated the dextran sulfate. The resultant mixture was allowed to stand at 4 C for 2 more hr and then centrifuged 10 min at 700 x g. The supernatant contained the partially purified phage plus some cellular debris. The supernatant from this step was subjected to repeated centrifugations at 12,000 x g until no more pellets of cellular debris were observed. This final supernatant, containing the phage, was spun for 3 hr at 78,000 x g. The pellet was resuspended in 9 ml of an aqueous CsCl solution, specific density of 1.30. The phage suspension was then aliquoted into cellulose nitrate tubes, 4.5 ml each into two tubes. The phage suspensions were then overlayed with mineral oil and spun to equilibrium in a Beckman SW 50-L swinging bucket head for 48 hr at 100,000 x g.

Titration of corynebacteriophage βv tox^+ (Groman, 1955). The CsCl gradients were fractionated by collecting 5 drop fractions from the bottom of the tubes. Each fraction so collected was refrigerated until titered. The indicator strain $C_7(-)$ was grown overnight in heart infusion (HI) broth at 37 C with aeration.

HI broth, 20 ml, was then inoculated with the overnight cultures of $C_7(-)$ and incubated at 37 C with aeration. The culture was grown to a cell concentration of 5×10^8 cells per ml. Serial one to ten dilutions of the gradient fractions were made in HI broth. The phage dilutions, 1 ml, were mixed with 0.15 ml of the indicator strain, 2.5 ml of a 1% heart infusion agar overlay and the mixture was layered onto a base of HI agar in a petri dish. The agar overlay was allowed to harden at room temperature and the plates were incubated at 34 C overnight. βv tox^+ bacteriophage produced clear, discreet plaques which were counted using a Quebec colony counter. In every CsCl gradient run, the fractions containing the majority of the infective particles corresponded to an amber band which was easily visible within the gradient. The fractions containing peak phage infectivity were dialyzed overnight against PBS and the phage stored at 4 C.

Protein determination (Reiner and Chung, 1961). The protein contents of the various preparations were determined using a modified Folin-Ciocalteau method. Bovine serum albumin was used as a "standard" protein.

Extraction of phage protein (Forrest and Cummings, 1970). A 3 ml aliquant of purified βv tox^+ corynebacteriophage (3.5 to 4.0 mg protein per ml) was adjusted to a volume of 30 ml using sterile PBS. The phage was then pelleted by a 3 hr centrifugation at 78,000 x g. Seven ml of a solution containing 6 M guanidine hydrochloride (Schwarz/Mann; ultrapure), 0.05 M LiCl, 0.01 M ethylenediaminetetraacetate (EDTA) and 5 mM dithiothreitol (DTT) was added to the pellet and the phage were suspended in this solution. The phage suspension was incubated at 40 C in a shaking water bath for various lengths of time (2 to 6 hr). Phage remnants were pelleted by one 2 hr centrifugation at 78,000 x g and the resultant supernatant, "extracted phage protein", was exhaustively dialyzed against PBS at 4 C. In most preparations an insoluble "precipitate" appeared during the course of the dialysis which was removed by a 10 min centrifugation at 3,000 x g. The phage protein solution was then concentrated within dialysis tubing embedded in Ficoll, MW 400,000 (Pharmacia Fine Chemicals), to a volume of 1 to 3 ml and stored at -20 C.

SDS-polyacrylamide gel electrophoresis (Davis, 1964; Ornstein, 1964). Polyacrylamide gels containing 10% recrystallized acrylamide (Eastman) in 0.1 M sodium phosphate buffer pH 7.2, 0.1% in SDS were prepared according to the method of Weber and Osborn

(1969). The gel dimensions were 17 x 0.8 cm. Protein samples, 20-100 µg, were dissolved in 10 µl of 0.1 M sodium phosphate buffer containing 1% SDS and 10% sucrose and in some cases 1% (w/v) DTT. The samples were heated at 100 C for 2 min prior to loading. The gels were electrophoresed at room temperature for 12 to 14 hr at a constant current of 4.5 ma per gel in the buffer system described by Weber and Osborn (1969). Peptides larger than about 10,000 daltons ran more slowly than the tracking dye, 0.002% bromophenol blue. Gels were stained in 0.7% Coomassie brilliant blue for 6 to 8 hr and destained by diffusion in a solution of 20% methanol and 10% acetic acid.

Scanning of SDS-polyacrylamide gels (Gill and Dinius, 1971). The relative protein contents of the resultant bands were estimated from the areas of the peaks obtained by scanning the stained gels at 610 nm in a Densicord Model S42A scanner (Photovolt Corp.). The peaks thus obtained were cut out and weighed, to an accuracy of .001 gm, on a Mettler (Model H10) balance.

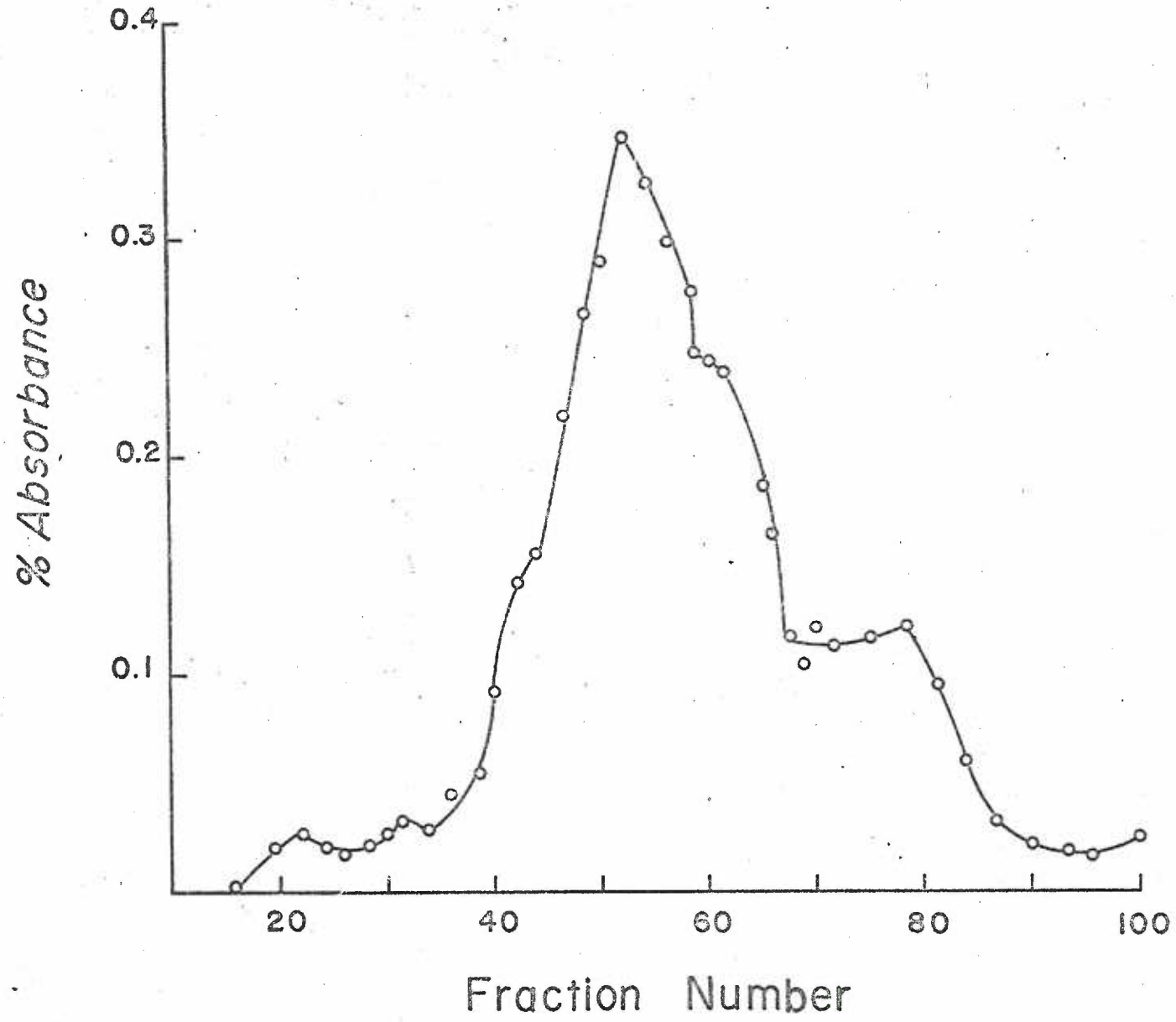
Purification of diphtheria toxin (Collier and Cole, 1969; Cukor, Solotorovsky and Kuchler, 1973). In the first stages of this research I used electrophoretically pure toxin provided by Dr. R. J. Collier, Univ. of California, Los Angeles. Subsequently

I purified my own toxin. Partially purified toxin, 2,000 LF per ml, was obtained from Connaught Laboratories (Ontario, Canada). An initial volume of 1,800 ml was concentrated to a final volume of 25 ml in an Amicon ultrafiltration cell (Amicon Corp.) using a 30,000 MW (PM-30) membrane filter. The viscosity of the concentrate (using a glass tube viscometer) was adjusted to 2.5 times that of water by diluting it to a final volume of 65 ml. Toxin was then chromatographed, 8 ml loaded per column, on Sephadex G-100 columns (Pharmacia; 2.5 x 48 cm) equilibrated in a buffer, 50 mM in Tris-HCl, pH 7.5 and 0.5 mM in EDTA. Three ml fractions were collected using a Gilson fraction collector (Model MF) and the absorbancy at 280 nm was determined on a Beckman Acta III spectrophotometer. A representative elution profile is shown in Fig. 1. The fractions (#70 to #85) comprising the "shoulder" of the elution profiles were discarded. The peaks from each of the 8 columns were pooled and concentrated to a volume of 20 ml in an Amicon ultrafilter using a 50,000 MW (XM-50) membrane filter. The toxin was electrophoresed in SDS-polyacrylamide gels and was found to contain several contaminating proteins. Further purification was required. The toxin was dialyzed overnight against 0.02 M sodium phosphate buffer, pH 6.9 at 4 C. It was then chromatographed on a diethylaminoethyl-cellulose DE-52 (Whatman) column (1.7 x 27 cm; Pharmacia) equilibrated in 0.02 M phosphate buffer,

Figure 1

Fractionation of crude diphtheria toxin on Sephadex G-100.

Approximately 300 mg of protein was dissolved in 8 ml of buffer (50 mM in tris-HCl, pH 7.5 and 0.5 mM in EDTA) and fractionated on a column of Sephadex G-100 (2.5 x 48 cm) equilibrated with the same buffer. Fractions (3.5 ml) were collected and the absorbance at 280 nm was determined on a Beckman Acta III spectrophotometer.


0.4
0.3
0.2
0.1
% Absorbance
20
40
60
80
100
Fraction Number

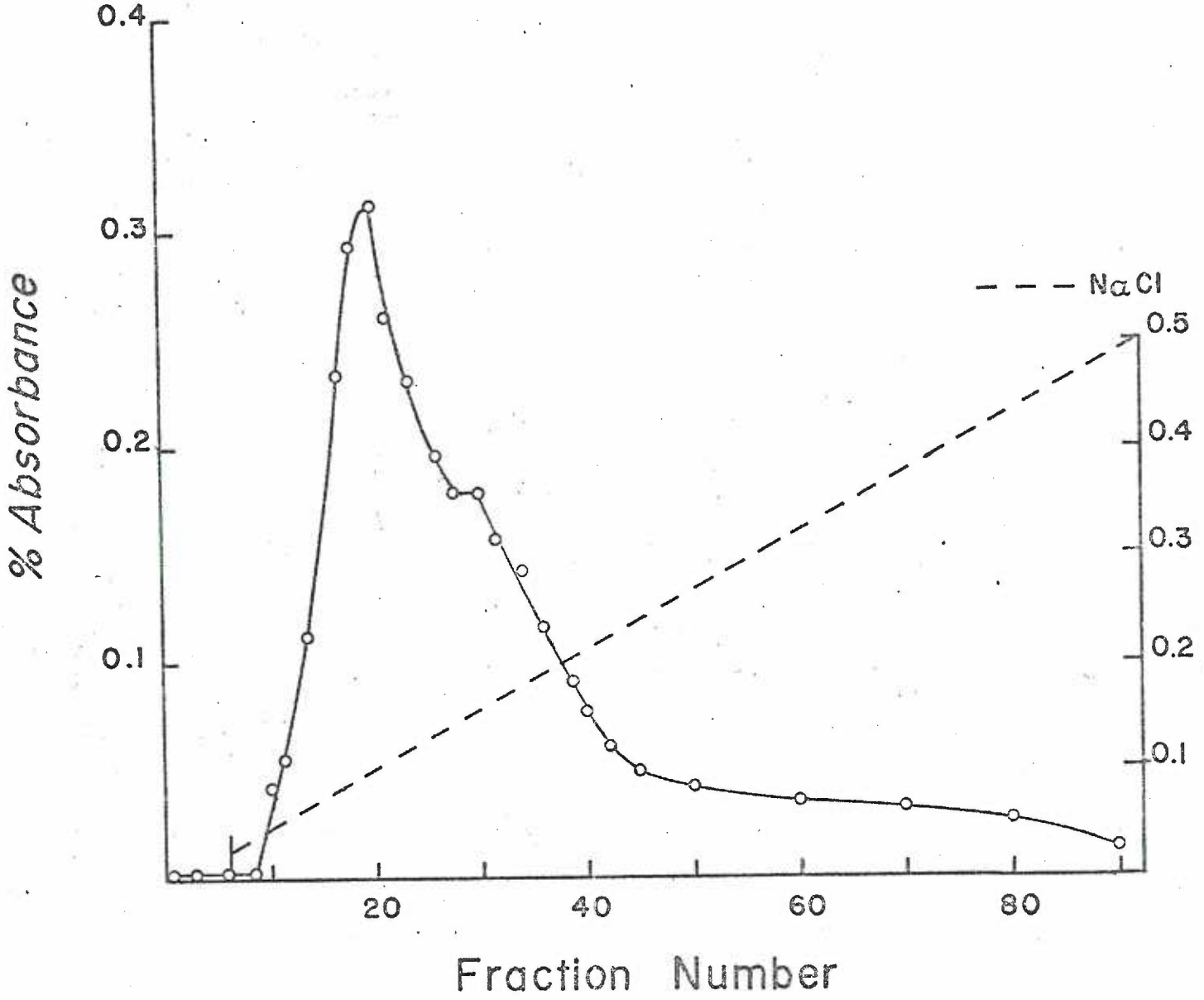
pH 6.9, using a 0.05 to 0.50 M stepwise NaCl gradient (Johnson, Kuchler and Solotorovsky, 1968). Fractions (3 ml) were collected and their absorbance at 280 nm determined. Toxin eluted at 0.10 M NaCl. The material eluting at 0.10 M NaCl from 3 such DE-52 columns was pooled, concentrated using a 50,000 MW membrane filter and re-chromatographed in a 0.05 to 0.50 stepwise NaCl gradient. The final DE-52 column profile is shown in Fig. 2. The peak eluting at 0.10 M NaCl off the final column banded in SDS-polyacrylamide gels at a position corresponding to a 62,000 MW protein (using purified toxin supplied by Dr. R. J. Collier as a marker) and showed a single line when reacted against antitoxin in an immunodiffusion slide. The purified toxin, 26 mg protein per ml, was stored at -20 C in small volume aliquots.

Trypsin digestion of diphtheria toxin (Gill and Dinius, 1972). Purified toxin, diluted to 1 mg per ml in 0.05 M Tris-HCl, pH 8, 5% glycerol and 0.05 M DTT was pre-warmed to 37 C. Freshly prepared trypsin (Sigma; chymotrypsin-free) was added to a final concentration of 0.01 mg per ml. The tube was incubated at 37 C for 10 min and the digestion was stopped by the addition of soybean trypsin inhibition (Sigma) in a final concentration twice that of trypsin. The mixture was incubated 2 additional hr at 37 C and the digested toxin was used immediately or was frozen at -20 C.

Figure 2

Chromatography of diphtheria toxin on diethylaminoethyl-cellulose.

Approximately 200 mg of protein (from the peak in Fig. 1) was dialyzed against 0.02 M sodium phosphate buffer, pH 6.9 at 4 C. The protein (4.5 ml) was chromatographed on a diethylaminoethyl-cellulose (DE-52) column (1.7 x 27 cm) equilibrated with the same buffer using a 0.05 to 0.50 M stepwise NaCl gradient. Fractions (2.5 ml) were collected and the absorbance at 280 nm was determined. Toxin eluted with 0.10 M NaCl.


0.4
0.3
0.2
0.1
% Absorbance
NaCl
0.5
0.4
0.3
0.2
0.1
20
40
60
80
Fraction Number

Separation of toxin into A and B fragments (Pappenheimer, Uchida and Harper, 1972). To 5.1 ml of purified toxin (130 mg) containing 15,154 LF per ml, was added 1.0 ml of 0.1 M EDTA and 100 µl of 1.0 M DTT. The solution was adjusted to pH 8 with 1 M Tris, pH 9 and then 50 µl of a 1% crystalline trypsin solution, in 0.001 N HCl, was added. After 10 min at 37 C the reaction was stopped by the addition of 100 µl of a 1% soybean trypsin inhibition solution and 200 µl of a 0.7% alcoholic solution of phenylmethylsulfonyl-fluoride (Sigma). Then 1.8 g urea (Schwarz/Mann; ultrapure) was added and the mixture was chromatographed through a Sephadex G-150 column (2.5 x 24 cm) equilibrated in 0.02 M phosphate buffer, pH 6.8 containing 6 M urea and 1 mM DTT. Fractions of 2.5 ml each were collected and 3 peaks absorbing at 276 nm were observed (see Fig. 3). These peaks were precipitated by dialysis against 80% saturated ammonium sulfate. The precipitates in a volume of about 5 ml were dialyzed against the 6 M urea, 1 mM DTT buffer and re-chromatographed through Sephadex G-150. Peak #3, presumably fragment A, emerged as a single peak when re-chromatographed. Peak #2, when re-chromatographed, emerged as two distinct peaks as shown in Fig. 4. Peak #2b migrated as a single band on SDS-acrylamide gels in a position corresponding to fragment B of toxin. Peak #2a consisted of a mixture of undigested toxin and fragment B when analyzed on SDS-acrylamide gels. Peak #3 and Peak #2b were tested

Figure 3

Fractionation of trypsinized diphtheria toxin on Sephadex G-150.

Approximately 130 mg of trypsinized diphtheria toxin (see Methods) in 5.1 ml was fractionated on a Sephadex G-150 column (2.5 x 24 cm) equilibrated in 0.02 M phosphate buffer, pH 6.8 containing 6 M urea and 1 mM DTT. Fractions (2.5 ml) were collected and the absorbance at 276 nm was determined.

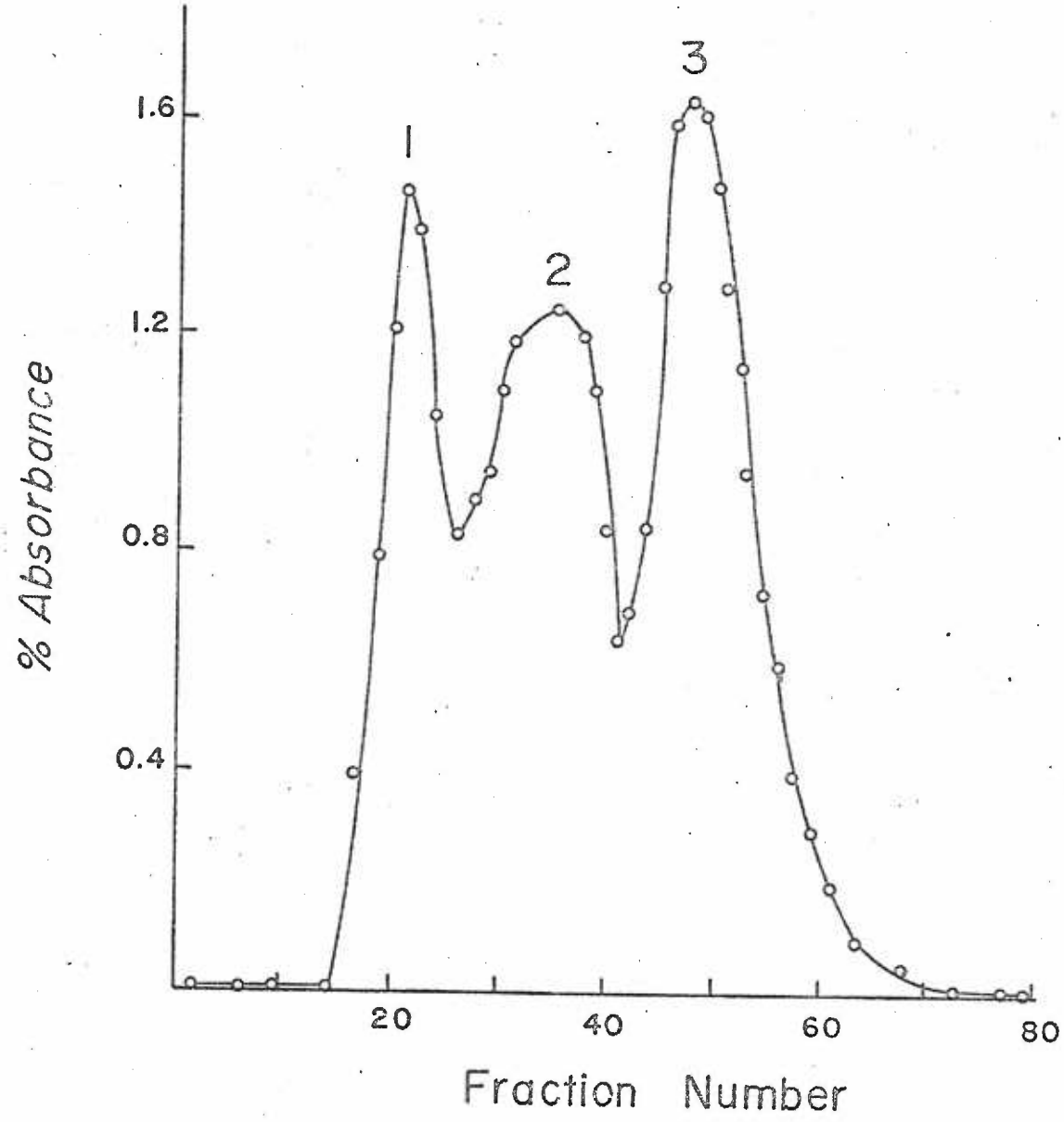
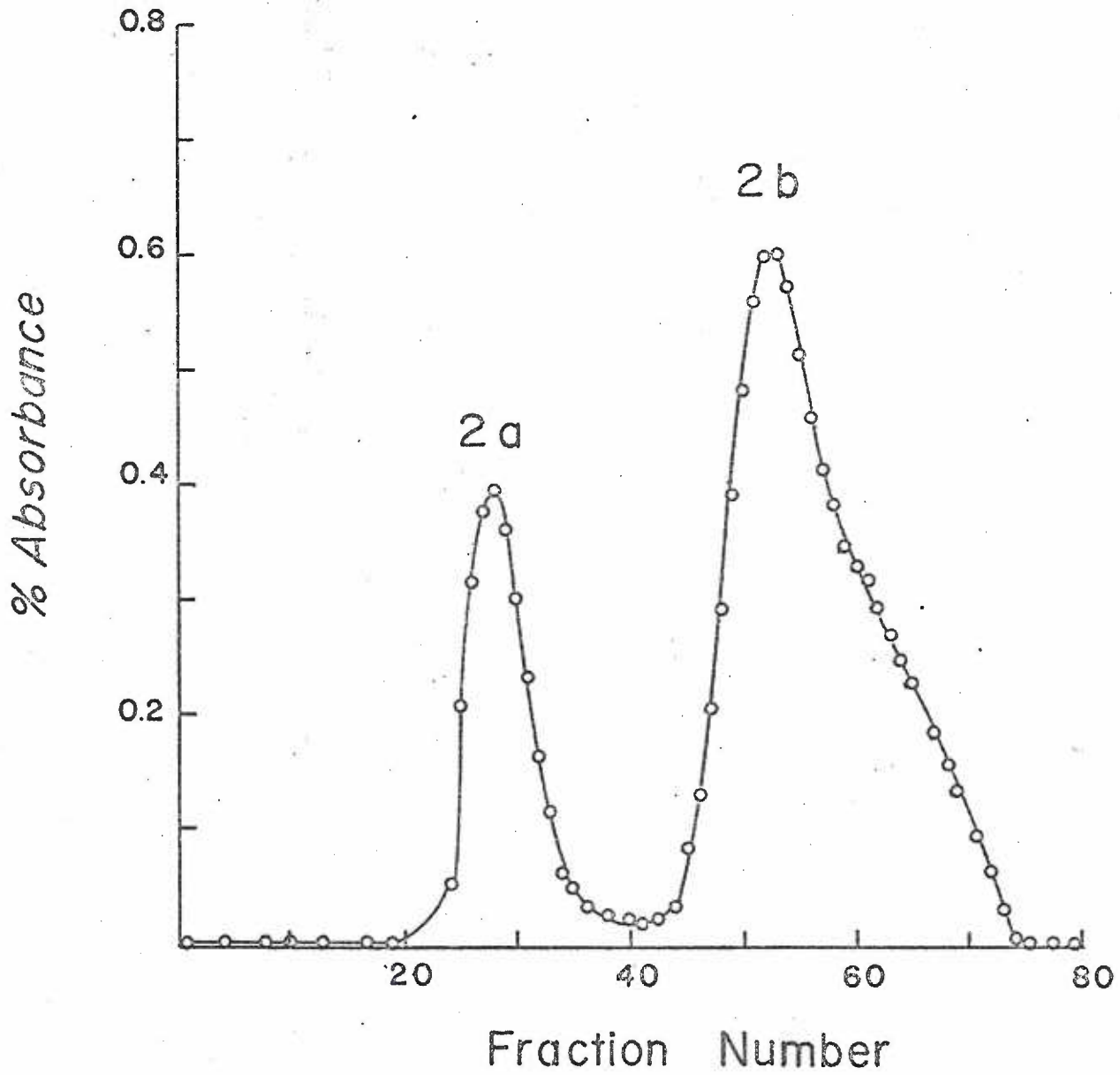

1.6
1.2
0.8
0.4
% Absorbance
1
2
3
20
40
60
80
Fraction Number

Figure 4

Re-chromatography of peak #2 (Fig. 3) on Sephadex G-150.

The protein represented by peak #2 (Fig. 3) was precipitated by dialysis against 80% saturated ammonium sulfate. The precipitate was resuspended in 5 ml of 0.02 M phosphate buffer, pH 6.8 containing 6 M urea and 1 mM DTT and chromatographed on a Sephadex G-150 column (2.5 x 24 cm) equilibrated with the same buffer. Fractions (3.0 ml) were collected and the absorbance at 276 nm was determined.


0.8
0.6
0.4
0.2
% Absorbance
2a
2b
20
40
60
80
Fraction Number

for their ability to catalyze enzymatically the transfer of ADP-ribose from NAD to mammalian elongation factor 2 (EF2). Peak #3, presumably fragment A, was enzymatically active whereas peak #2b contained less than 10% of the activity associated with peak #3.

Preparation of mammalian elongation factor 2 (EF2) (Allen and Schweet, 1962; Collier and Kandel, 1971). The method for obtaining partially purified EF2 enzyme from rabbit reticulocytes is presented in complete detail by Lightfoot (1973).

Assay of ADP-ribosylation activity (Collier and Kandel, 1971). The assay mixtures in total volumes of 250 µl contained 50 mM Tris-HCl pH 8.2, 0.1 mM EDTA, 40 mM DTT, 100 µl EF2 enzyme and 0.735 µM C^{14}-NAD^+ (adenine-C^{14}-(U), 136 mCi/mM; Amersham/Searle). The assay mixtures also contained one of the following: 10 µl of the Sephadex G-150 peaks presumed to correspond to fragment A and B, or 10 µl of various dilutions of purified fragment A provided by Dr. R. J. Collier, Univ. of California, Los Angeles. Controls contained everything except fragment A or the test samples. All the components except NAD^+ were mixed at 0 C. Following 2 min incubation at 25 C the reaction was initiated by the addition of 10 µl NAD^+. The tubes were incubated for 30 min at 25 C. At the end of incubation, 250 µl of 10% CCl_3 COOH was added to each

reaction mixture and the precipitate was collected on type HA filters (Millipore), washed 3x with 5% CCl_3 COOH, then 1x with 95% ethanol and dried. The filters were placed in glass scintillation vials and 10 ml Omnifluor-toluene scintillation fluid was added and the radioactivity of each sample was determined using the C^{14}-window of a Beckman LS-200B liquid scintillation counter.

Toxin assay in cultured mammalian cells (Moehring and Moehring, 1968). Stationary tube cultures of KB cells (human epidermoid carcinoma cells, kindly given to us by Drs. T. J. and J. M. Moehring; Univ. of Vermont Medical School, Burlington, Vt.) were grown to confluency (1-3 x 10^5 cells per tube) in Hanks MEM medium containing 12% fetal calf serum and 1% glutamine. The medium was poured off and 0.9 ml of Hanks BSS diluent was added to each tube. Serial dilutions of diphtheria toxin were prepared in Hanks BSS plus serum and 0.1 ml of each toxin dilution was added to the control cultures. After 3 hr of incubation at 37 C, 0.1 ml of a C^{14} amino acid mixture (New England Nuclear; L-amino acid-C^{14} mixture) was added to each tube at a final specific activity of 0.1 μCi/tube. After 2 additional hr incubation the cells were washed twice with PBS and a 0.1% trypsin solution was added for 2 min to remove the cells from the glass. This trypsinization procedure was not sufficient to cause cell lysis. The

trypsinized cells were vigorously vortexed and trichloroacetic acid (TCA) was added to a final concentration of 5%. The precipitates were collected on Millipore filters, washed 3x with 5% TCA, once with 95% ethanol, dried and counted in a liquid scintillation counter.

KB cell protection experiments (Elwell and Iglewski, 1972; Cukor, Solotorovsky and Kuchler, 1973). Tube cultures of KB cells were grown to confluency (1-3 x 10^5 cells per tube). The tissue culture medium was poured off and 0.1 ml of various concentrations of βv tox^+ phage, bovine serum albumin, or T4 coliphage diluted in 10% Hanks BSS containing 1% calf serum were added to the cells. The cultures were incubated for 10 min with occasional rocking. Then, 0.9 ml of Hanks BSS diluent was added to each tube and following 1 hr incubation at 37 C, 0.1 ml of diphtheria toxin was added to the cultures in sufficient concentration to inhibit protein synthesis by 90-95% relative to control cells that received no toxin. The balance of the procedure is identical to that outlined in the preceding section.

Preparation of CRM45 protein (Pappenheimer, Uchida and Harper, 1972). C_7(β45) was inoculated into deferrated CY medium, pH 7.2 containing 1% maltose (Difco), to an initial OD of 0.05. Six

Fernbach flasks, each containing 300 ml of culture, were shaken at 37 C at 200 rpm on a Gyrotory Shaker (Precision Instruments) for 47 hr. The pH and OD of the cultures were checked periodically. At the end of the incubation the cultures were centrifuged at 10,000 x g for 15 min. The supernatants were brought to 75% saturation with ammonium sulfate and allowed to stand 48 hr at 4 C. The precipitates were collected, dissolved in approximately 10 ml of 0.01 M sodium phosphate, pH 7.2 and dialyzed against 5 changes of the same buffer. A small amount of insoluble material was removed by centrifugation and the final solution was concentrated in an Amicon ultrafilter using a 10,000 MW (PM-10) membrane. The final volume was approximately 3 ml. The solution was then passed through a DE-52 column (1.7 x 27 cm) equilibrated in 0.01 M phosphate buffer and eluted using a 0.01 to 0.20 M stepwise NaCl gradient. CRM45 protein emerged as a single, distinct peak over a NaCl concentration range of 0.125 and 0.147 M. The fractions containing the peak were concentrated to a volume of 2.5 ml in an Amicon ultrafilter using a 30,000 MW membrane filter. CRM45 protein, 1.68 mg per ml, gave one distinct band at a position corresponding to a molecular weight of 45,000 daltons when analyzed on an SDS-polyacrylamide gel using intact diphtheria toxin (supplied by Dr. R. J. Collier) as a marker.

Reconstitution of CRM45 protein and phage protein (Uchida, Pappenheimer and Harper, 1972; Uchida, Pappenheimer and Harper, 1973). A solution containing 400 µg of CRM45 protein was adjusted to 0.05 M Tris and 10 mM DTT, pH 8.0. CRM45 was then treated with 4 µg of trypsin (chymotrypsin-free) at 37 C for 10 min. After 10 min incubation the reaction was stopped by the addition of 8 µg soybean-trypsin inhibitor. A phage protein solution containing 300 µg protein in 0.4 ml was also adjusted to 0.05 M Tris and 10 mM DTT. This solution was added to the trypsin-treated CRM45 protein and the mixture was incubated at 37 C for 15 min. This reaction mixture was then exhaustively dialyzed in the cold against 0.01 M phosphate buffer, pH 7.2 to remove the DTT and permit the disulfide bonds to reform. The hybrid molecules thus formed were tested for their ability to inhibit protein synthesis in cultured KB cells. Phage protein, CRM45 protein and a non-trypsinized mixture of the two were included as controls.

Preparation of rabbit diphtheria antitoxin. Toxoid was prepared from electrophoretically pure diphtheria toxin by the method of Linggood, Stevens, Fulthorpe, Woiwood and Pope (1963). The procedure is presented in detail by Lightfoot (1973).

Preparation of rabbit anti-fragment B antibody. Three ml of electrophoretically pure fragment B, containing 280 µg protein per ml, was vigorously mixed with 3 ml of complete Freund's adjuvant. The emulsified antigen was injected into the rear foot pads and in the backs of 3 adult New Zealand rabbits (6 injection sites per rabbit). Each animal received 200 µg of antigen in the initial injection. Fourteen days after the primary injection the animals were boosted by an injection of 93 µg fragment B in the rear foot pads. Serum was collected at weekly intervals and tested for the presence of anti-fragment B antibody by double gel diffusion in agar.

Preparation of anti-βv tox^+ antibody. Corynebacteriophage βhv64 tox^+ was purified by the methods outlined earlier. A 1.5 ml aliquot of purified phage (6 mg total protein) was emulsified with 1.5 ml complete Freund's adjuvant. Two rabbits were injected with 3 ml (3 mg antigen per animal) of the emulsified antigen in the rear foot pads and in the back. Sixteen days after the primary injection each animal was boosted by an injection of 1.4 mg of purified phage, in complete Freund's adjuvant, in both rear foot pads. Serum was collected at weekly intervals and tested for antibody using double diffusion agar slides.

Immunodiffusion analysis (Matsuda, Kanei and Yoneda, 1972). Immunodiffusion was carried out at 4 C in Ouchterlony slides employing 0.75% agarose (Seakem; Dist. by Bausch and Lomb) in 0.01 M phosphate buffer, pH 7.5 containing 0.5 M glycine and 0.14 M NaCl. Standard microscope slides were thoroughly cleaned and coated with 1% ionagar. Approximately 2.4 ml of the agarose solution were layered onto each slide, allowed to harden and the appropriate wells were cut out of the agar. The slides were stored at 4 C in a moist chamber to retard drying.

Peptide fingerprint analysis of fragment B and phage protein (Zanetta, Vincendon, Mandel and Gombos, 1970). Electrophoretically pure fragment B and extracted phage protein (see Fig. 17 for the acrylamide gels of these preparations) were analyzed by the dansylation method developed by Zanetta et al. (1970). Approximately 310 µg of extracted phage protein (total volume of 0.65 ml dissolved in 6 M urea) and 280 µg of fragment B (total volume of 1.0 ml dissolved in 6 M urea) were made alkaline by the addition of 2 volumes of 0.3 M $NaHCO_3$. The samples were hydrolyzed by the addition of 6 µg of trypsin (Worthington; 3X crystallized, chymotrypsin-free) and incubated 2 hr at 37 C. The trypsinization procedure was repeated and the samples incubated an additional hr at 37 C. For the dansylation procedure, the pH of the samples

was adjusted to 9.5 by the addition of 1 N NaOH. 1-dimethyl-aminonaphthalene-5-sulphonyl chloride (dans-Cl; Pierce Chemical Co.) dissolved in 4 ml of acetone was added to the phage protein in a final concentration of 300 µg dans-Cl per nM protein. Dans-Cl dissolved in 6 ml of acetone was added to fragment B in a final concentration of 400 µg per nM protein and both solutions were allowed to stand overnight at room temperature. A short column, approximately 3.5 cm, of AG-50 cation exchange resin (Bio Rad Laboratories) was prepared and equilibrated using 0.1 M acetic acid. The samples were acidified to pH 2 with concentrated HCl and each sample was loaded onto separate AG-50 columns and washed with 0.1 M acetic acid until the effluent ceased to fluoresce. The dans-peptides were then eluted off the columns with water-acetone-25% ammonia (80:20:4) until the yellow fluorescent band had disappeared from the column. Fluorescence was monitored using a Mineral-Light (UV Products; San Gabriel, California). Some dans-peptides in both the extracted phage protein sample and the fragment B sample stuck to the top of the AG-50 column. This probably accounted for the fact that the number of peptides seen on the fragment B chromotogram was less than the theoretical yield according to amino acid composition. The fluorescent eluates were evaporated to dryness in a rotary evaporator and dissolved in 500 µl of 90% acetone for chromatography. Samples of 100 µl were

placed on silica gel plates (Brinkman). Chromatograms were developed in the first dimension with methyl acetate-isopropanol-ammonia (90:60:40). The plates were dried until the solvent odor had disappeared and then developed in the second dimension using isobutanol-acetic acid-water (150:40:20). After drying overnight at room temperature the chromatograms were examined under UV light and the dans-peptide spots were outlined in pencil.

RESULTS

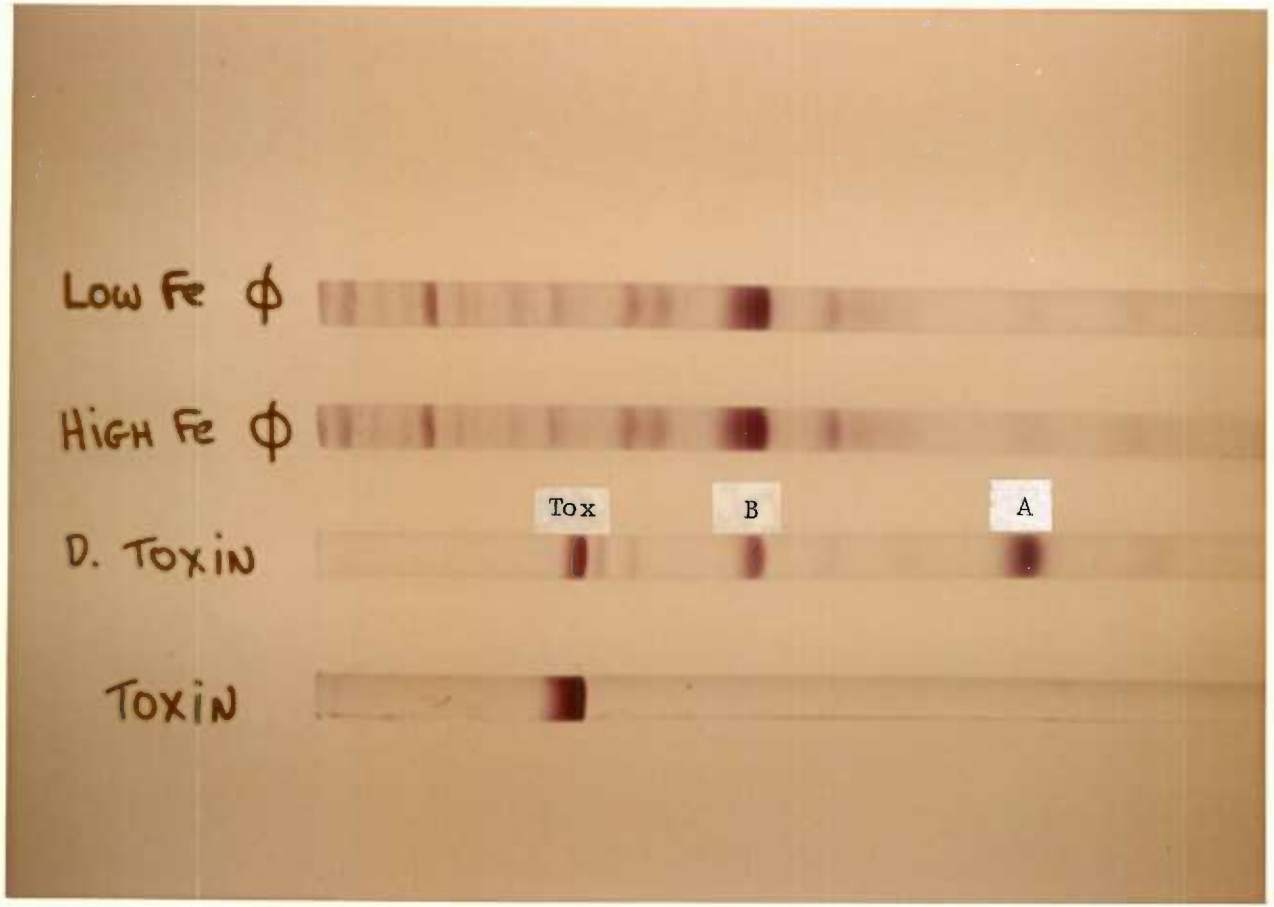
1. SDS-polyacrylamide gel profiles of purified βv tox$^+$ phage:

In the first stages of this research βv tox$^+$ phage were partially "purified" by differential centrifugation followed by equilibrium centrifugation in CsCl (see Methods). Fig. 5 is a photograph of polyacrylamide gels of two SDS-treated phage preparations purified by differential/CsCl equilibrium centrifugation. Phage were propagated in low iron medium (.050 μg Fe^{++}/ml) in one preparation and in high iron medium (4.0 μg Fe^{++}/ml), sufficient to inhibit toxin synthesis approximately 70%, in the other preparation. An SDS-polyacrylamide gel of trypsinized toxin containing intact toxin (62,000 daltons), fragment B (39,200 ± 800 daltons) and fragment A (24,200 ± 500 daltons) was run concomitantly with the phage gels. The toxin used here was a gift of Dr. R. J. Collier and the molecular weights of the fragments were reported by Collier and Kandel (1971). These proteins of known molecular weight provided useful standards from which the molecular weights of other proteins e.g., phage components could be determined. The gels of SDS-treated phage, both the high and low iron preparations, contained approximately 20 bands. This represented an excessive number of structural proteins, considering the size and simplicity of βv tox$^+$ phage structure. Eventually it was discovered that these phage preparations were not pure, and that most of the 20

Figure 5

SDS-polyacrylamide gels of SDS-treated βv tox^+ phage propagated in high and low iron media and purified by differential centrifugation/CsCl equilibrium centrifugation.

"Purified" phage samples, 100 µg, were dissolved in 30 µl of 0.1 M sodium phosphate buffer containing 1% SDS and 10% sucrose. Samples were heated at 100 C for 2 min prior to loading onto polyacrylamide gels containing 10% recrystallized acrylamide. Gels were electrophoresed at room temperature for 12-14 hr at a constant current of 4.5 ma per gel in the buffer system described in Methods. Gels were stained in 0.7% Coomassie brilliant blue for 8 hr and destained by diffusion in a solution of 20% methanol and 10% acetic acid. Toxin was digested by the procedure described in Methods.


Low Fe ϕ
High Fe ϕ
Tox
B
A
D. Toxin
Toxin

bands were contaminating proteins. It is apparent (Fig. 5) that the major protein in both phage preparations co-migrated with fragment B of toxin. Minor proteins were observed in both phage preparations which migrated in a position corresponding to fragment A of diphtheria toxin. These gels are representative of five phage preparations "purified" by differential/CsCl equilibrium centrifugation.

In an effort to obtain preparations of greater purity, βv tox^+ was purified employing a polyethylene glycol-dextran sulfate two-phase method followed by CsCl equilibrium centrifugation (see Methods). The protein profiles of three separate phage preparations (gels B, C and D) purified by this method and analyzed on SDS-polyacrylamide gels are shown in Fig. 6. Gel A contains trypsinized toxin. There were significantly fewer protein bands in these phage preparations, between six and eight were clearly visible. Electron micrographs of these preparations, done by Dr. Robert Brooks, showed them to be relatively free of cellular debris. These highly purified phage preparations shared one characteristic with the phage in Fig. 5. The major protein band co-migrated with the B fragment of toxin. A second protein band which migrated in a position corresponding to a molecular weight of 77,000 daltons was evident in the phage preparations seen in gels B and C. In the third preparation, gel D, a relatively large amount of protein migrating in a position corresponding to 31,000 daltons appeared (Weber and Osborn, 1969). Six different phage preparations

Figure 6

SDS-polyacrylamide gels of SDS-treated βv tox^+ phage purified by a preliminary two-phase separation followed by CsCl equilibrium centrifugation.

Purified phage samples, 75 µg, were dissolved in 30 µl of 0.1 M sodium phosphate buffer containing 1% SDS and electrophoresed as described in Fig. 5.

Gel A: 40 µg of trypsinized diphtheria toxin

Gels B, C and D: three separate purified βv tox^+ phage preparations


Tox
Frag. B
Frag. A
A
B
C
D

purified by the two-phase/CsCl equilibrium method were analyzed on SDS-polyacrylamide gels. Four preparations resembled the profiles of gels B and C; however, the relative amounts of the large polypeptides (MW of 77,000 daltons) was variable among the four, being present in low amounts in some preparations. The two remaining phage preparations were similar to gel D. Evidence was subsequently obtained indicating that the large molecular weight protein seen in gels B and C was the result of aggregation (more than likely a dimer) of the 38,000 dalton protein and the smaller polypeptide (31,000 MW) seen in gel D represented a degradation product of this protein. This evidence will be discussed later.

2. Densitometry scans of βv tox^+ phage gels:

To determine what percentage of the total phage protein was represented by the various protein bands, stained gels of SDS-treated, purified βv tox^+ phage, representing three separate preparations were scanned in a recording densitometer. The densitometry was not done on the gels shown in Fig. 6 but on three additional and separate βv tox^+ preparations. The results of these scans are summarized in Table 2. Eight common protein bands were discernible in two of the phage preparations (preparations #1 and #2). The third preparation contained only six bands; band VI and VIII were missing in this phage. The major protein in all of the phage preparations analyzed corresponded

Table 2

Densitometry scans of the stained SDS-acrylamide gels of three purified βv tox^+ phage preparations.

Stained SDS-gels of three separate βv tox^+ phage preparations were scanned in a Densicord 542A scanner (wavelength, 610 nm). Peaks from the tracings were cut out and weighed on a Mettler (Model H10) balance and the percentage of the total tracing represented by individual peaks was computed.

Table 2

	PHAGE PREPARATION		
	#1	#2	#3
BAND	% OF TOTAL	% OF TOTAL	% OF TOTAL
I	4.2	3.2	7.3
II	5.3	7.0	8.2
III	7.4	1.5	3.1
IV	4.2	1.8	12.8
V	8.8	4.8	3.7
VI	7.2	4.1	-
VII	50.1	66.8	64.8
VIII	12.4	10.6	-
	99.6	99.6	99.9

to band VII. The average value of band VII relative to the total phage protein in these three preparations was 60.6% ± 10.0. As mentioned earlier, band VIII was thought to be derived by cleavage from the protein represented in band VII. Band VIII accounted for 12% of the total protein in βv tox^+ preparation #1, 11% of phage preparation #2 and was absent in the third preparation.

3. Extraction of band VII protein from βv tox^+ phage:

Forrest and Cummings (1970) devised a method whereby they could extract the major capsid protein components from T4 coliphage. Their procedure entailed treating T4 phage with a solution of 6 M guanidine hydrochloride, LiCl, EDTA and DTT. Purified βv tox^+ phage was pelleted and extracted using 6 M guanidine HCl at 40 C for various lengths of time. The phage remnants were removed by centrifugation and the resultant supernatant fluid was dialyzed and concentrated approximately twelvefold. Fig. 7 shows an SDS-polyacrylamide gel of 50 μg of this extracted phage protein preparation (gel D) compared to trypsinized diphtheria toxin (gel A). The phage preparation from which this protein was extracted was electrophoresed in gel B. Clearly, the guanidine hydrochloride reagent almost exclusively extracted the major protein seen in the SDS-treated intact βv tox^+ phage preparations. Furthermore, this "isolated" protein continued to co-migrate with toxin fragment B in SDS-polyacrylamide gels. The

Figure 7

SDS-polyacrylamide gels of SDS-treated βv tox$^+$ phage and the guanidine hydrochloride-extracted phage protein.

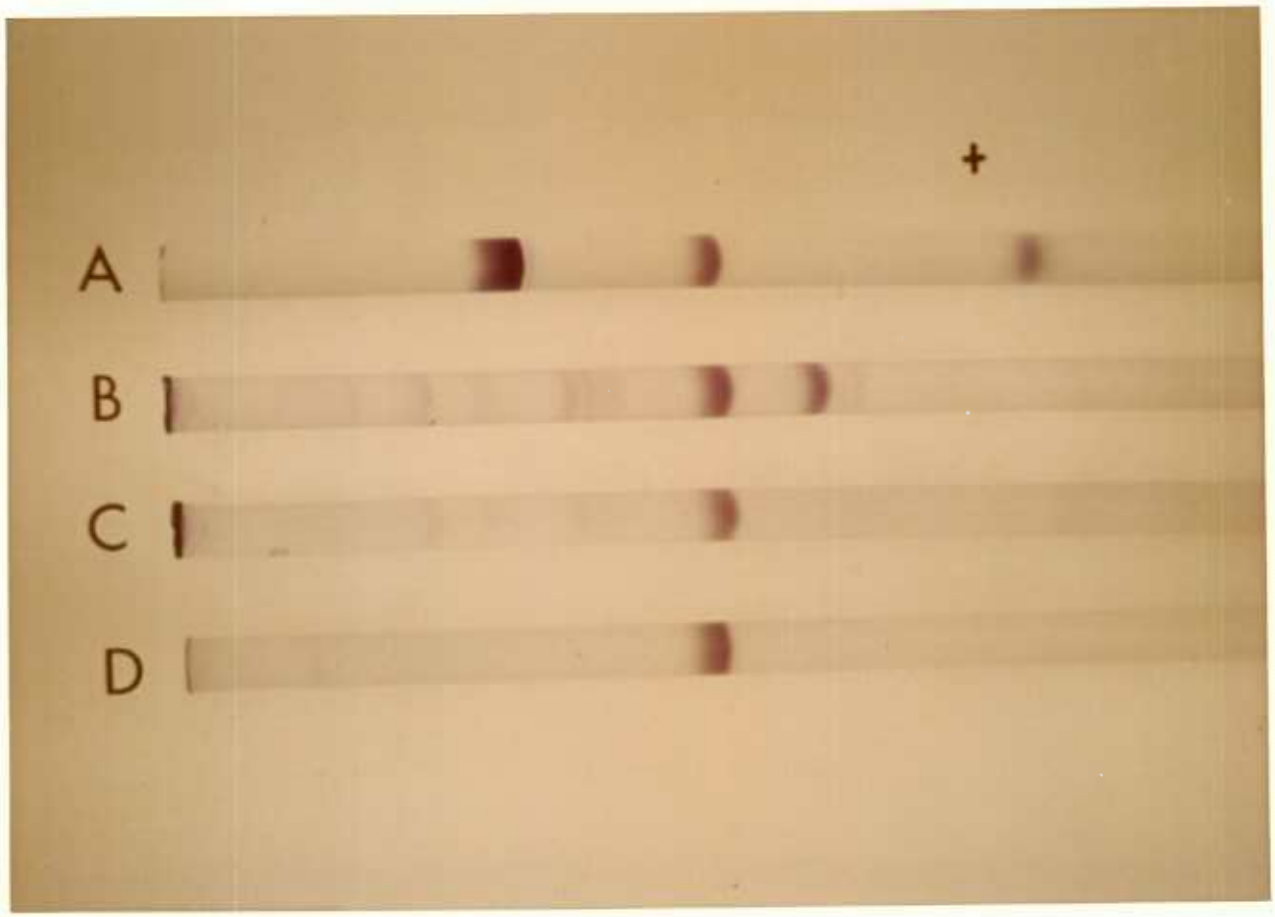
Purified βv tox$^+$ phage were extracted using a 6 M guanidine hydrochloride solution containing 5 mM DTT (see Methods) at 40 C for 2 hr. Phage remnants were centrifuged and the resultant supernatant (extracted phage protein) was dialyzed against PBS and concentrated twelvefold. Approximately 75 µg of the purified phage preparations and 50 µg of the extracted phage protein were dissolved in 30 µl of 0.1 M sodium phosphate containing 1% SDS and electrophoresed as described in Fig. 5.

Gel A: 100 µg of trypsinized diphtheria toxin.

Gel B: purified βv tox$^+$ phage preparation from which the extracted protein was derived.

Gel C: another βv tox$^+$ phage preparation.

Gel D: extracted βv tox$^+$ phage protein.


+
A
B
C
D

extracted protein preparation shown in gel D was obtained by a 2 hr extraction at 40 C which probably accounted for its relative homogeneity. However, a very faint band of protein of much larger molecular weight is discernible in this gel and will be discussed in the next section. Extracted protein preparations resulting from 6 hr extractions contained some smaller MW protein bands (Fig. 9; gel B) not seen in the 2 hr extract or in the intact, purified phage when analyzed in SDS-polyacrylamide gels. This may be evidence of contaminating peptidase in the phage preparations. If this hypothesis is correct, in the case of a 6 hr exposure to a protein denaturing agent like guanidine hydrochloride, the extracted phage protein would be more vulnerable, over a longer period of time, to proteolysis resulting in a higher level of low MW degradation products.

Six separate βv tox^+ phage preparations were extracted with 6 M guanidine hydrochloride. Gel D, Fig. 7 is an accurate representation of how all six extracted phage protein preparations appeared when electrophoresed in SDS-polyacrylamide gels.

4. Properties of the extracted phage protein:

An intriguing property of the extracted protein was its tendency to aggregate upon prolonged storage at 4 C or with repeated freezing and thawing of the preparation. Gel B in Fig. 8 is a case in point.

Figure 8

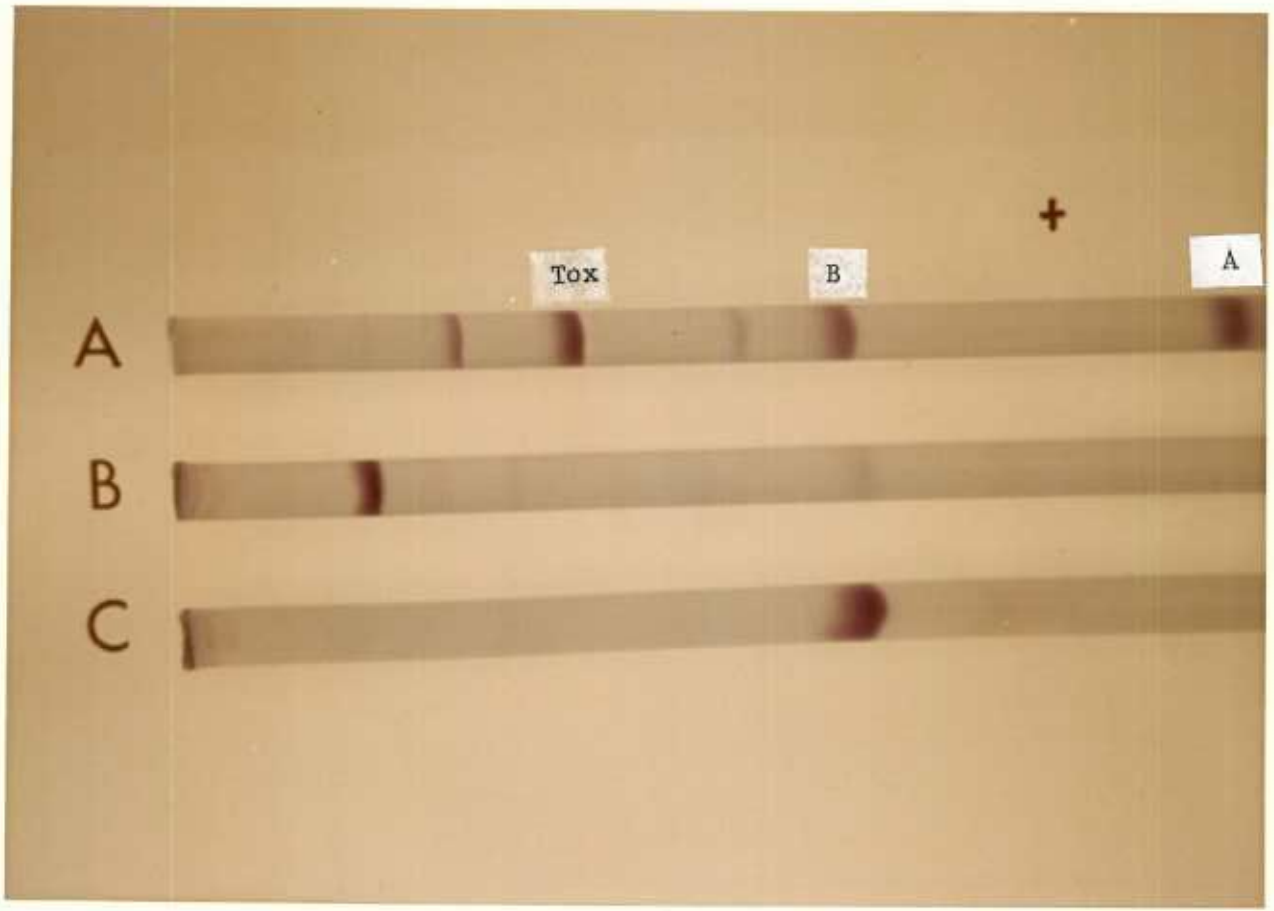
SDS-polyacrylamide gels of an "aggregated" extracted phage protein preparation with and without dithiothreitol (DTT).

Approximately 65 µg of an "aggregated" phage protein preparation was dissolved in 30 µl of 0.1 M sodium phosphate buffer containing 1% SDS, with and without 0.03 M (final concentration) DTT, and electrophoresed as described in Fig. 5.

Gel A: 100 µg trypsinized diphtheria toxin.

Gel B: extracted phage protein.

Gel C: extracted phage protein plus 0.03 M DTT.


+
Tox
B
A
A
B
C

This extracted phage protein preparation was 2 months old and had been repeatedly thawed and re-frozen. Whereas it had originally migrated in SDS-polyacrylamide gels as a single homogeneous band (MW of 38,000 daltons) after the conditions described above it demonstrated two bands on gel electrophoresis: the original band plus another much larger MW protein (approximately 77,000 daltons). To test whether this larger protein was an aggregate of the smaller one binding through disulfide linkages it was treated with 0.03 M dithiothreitol and re-electrophoresed. The result is seen in gel C. The reducing agent (DTT) dissociated all of the large MW protein species and the newly formed "monomers" now migrated as a single 38,000 dalton band, indicating that the original aggregation (probably a dimer) was indeed accomplished through disulfide bonds. Some phage protein preparations reacted differently to prolonged storage and/or thawing and re-freezing, namely in addition to aggregation a protein band appeared which was slightly smaller than the 38,000 MW extracted protein (Fig. 9; gel B). To support the hypothesis that this smaller MW protein was the result of some type of cleavage of the predominant protein, a relatively homogeneous extracted phage protein preparation was subjected to varying degrees of trypsinization. The resultant digests were analyzed in SDS-polyacrylamide gels. The results are shown in Fig. 9. Gel A contains trypsinized toxin. Gel B is a 1 month old extracted phage protein preparation. This phage protein was digested by the addition

Figure 9

SDS-polyacrylamide gels of trypsinized, guanidine hydrochloride extracted βv tox^+ phage protein.

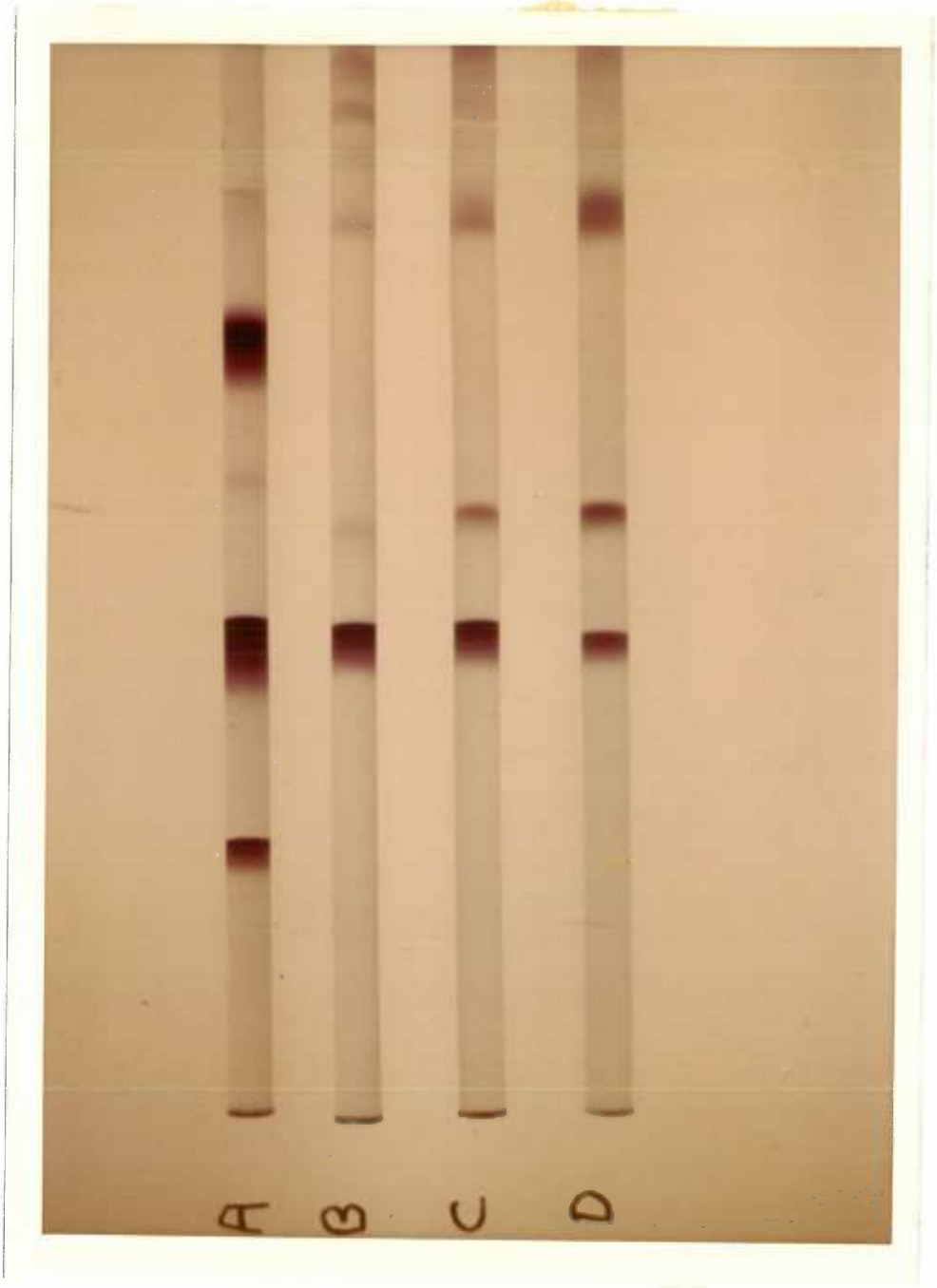
Freshly prepared trypsin was added to 65 µg of extracted phage protein dissolved in 0.05 M Tris-HCl, pH 8, 5% in glycerol and 0.05 M in DTT. After 20 min at 37 C the digestion was stopped by the addition of soybean trypsin inhibitor in mass double the mass of trypsin. The proteins were electrophoresed as described in Fig. 5.

Gel A: 160 µg of trypsinized diphtheria toxin.

Gel B: 65 µg of βv tox^+ extracted phage protein.

Gel C: 65 µg of βv tox^+ extracted phage protein with trypsin (0.01 mg per ml, final concentration).

Gel D: 65 µg of βv tox^+ extracted phage protein with trypsin (0.1 mg per ml, final concentration).


A
B
C
D

of trypsin to 0.01 mg per ml and after 20 min at 37 C the digestion was stopped by the addition of soybean trypsin inhibitor in mass double the mass of trypsin (gel C). Gel D is the phage protein digested with 10 times the trypsin concentration (0.1 mg per ml) as that used in gel C. The primary end-product (in addition to several small MW polypeptides) of the trypsinization of this extracted phage protein is a polypeptide of slightly smaller MW (approximately 31,000 daltons) which corresponds to the protein which spontaneously appeared (Fig. 9; gel B) in some phage protein preparations as a function of storage at 4 C coupled with repeated thawing and re-freezing. Furthermore, this polypeptide migrated in a position very similar to that of band VIII seen in some intact phage preparations (Fig. 6; gel D). The existence of this protein in some, but not all, phage preparations was disturbing in that it raised a serious question as to the purity of the phage preparations. This concern would be obviated if the protein in band VIII could be shown to be a cleavage product of the protein in band VII, the major phage protein. The results of the previous experiment supported this contention.

5. Toxin assay in cultured KB cells:

It has been established that the major protein seen in polyacrylamide gels of SDS-treated purified βv tox^+ phage co-migrates with fragment B of diphtheria toxin. Size identity could have been coincidental.

If fragment B is an integral part of, or closely associated with, βv tox^+ corynebacteriophage it would be expected that this phage would possess functional properties unique to fragment B. Cukor, Solotorovsky and Kuchler (1973) were able to delay the action of toxin on cultured KB cells by pre-incubating KB cells with fragment B, supporting the hypothesis that fragment B plays a role in the binding of toxin to susceptible cell membranes. βv tox^+ corynebacteriophage were therefore tested for the ability to protect KB cell cultures from diphtheria intoxication. Prior to doing those experiments however the parameters of the KB cell-assay system for toxin activity had to be established. Tenfold dilutions of toxin were added to tube cultures of KB cells. After an incubation period, C^{14} amino acids were added and protein synthesis was measured by the relative incorporation of the C^{14} amino acids into TCA-precipitable material. The specificity of this assay system was determined by neutralizing increasing concentrations of toxin with a constant amount of antitoxin and testing the ability of antigen/antibody mixtures to inhibit KB cell protein synthesis. The results can be seen in Fig. 10. The sensitivity of this assay system is quite apparent. As little as .06 µg of toxin inhibited protein synthesis of these cells by 95%. A constant amount of diphtheria antitoxin (0.1 ml of a 1:10 dilution) totally neutralized the ability of .06 µg of toxin to inhibit protein synthesis. At higher concentrations of toxin, antigen excess apparently occurred and

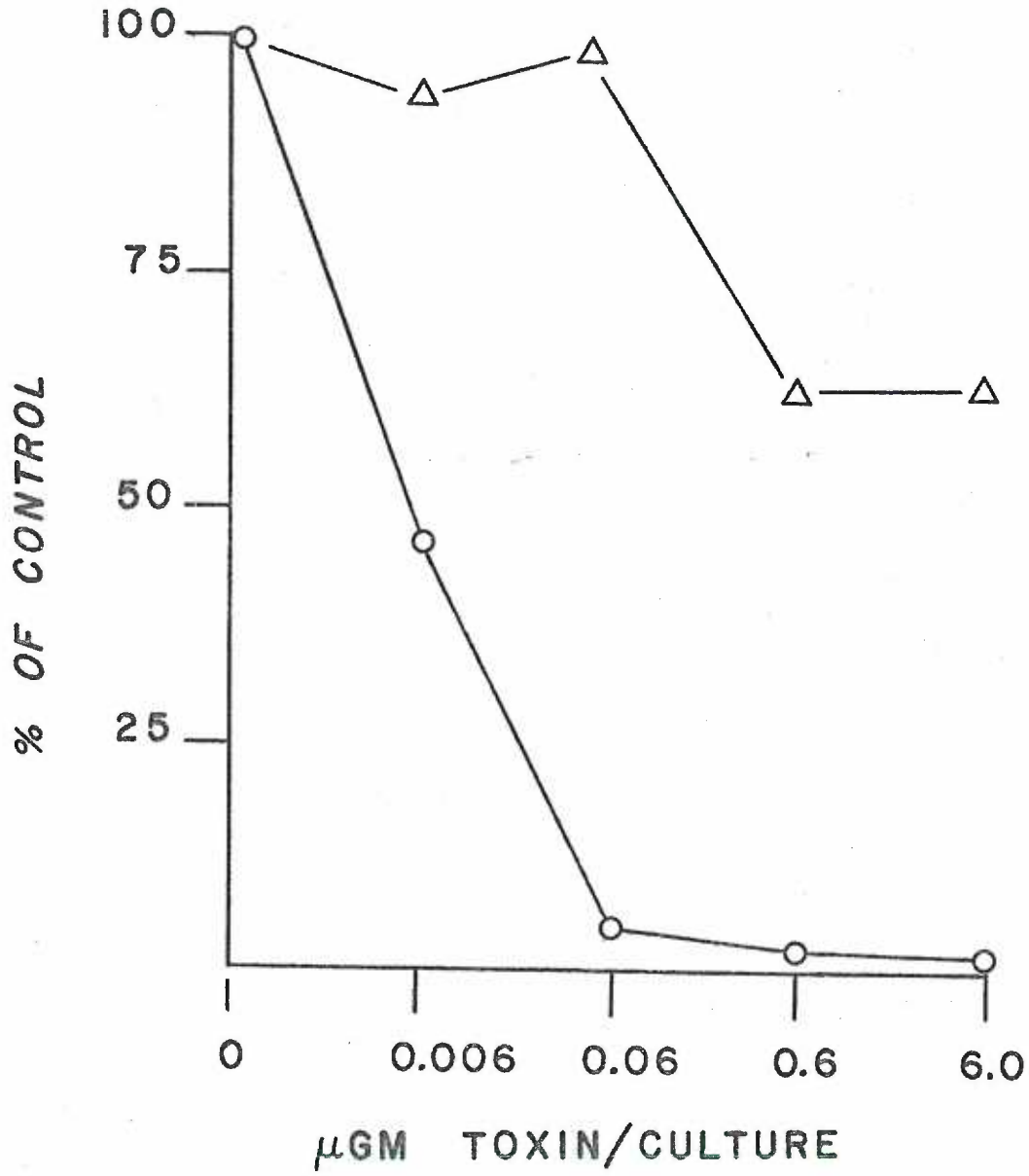
Figure 10

Diphtheria toxin assay in KB cell cultures.

Stationary tube cultures of KB cells were grown to confluency (1-3 x 10^5 cells per tube). Tenfold dilutions of diphtheria toxin were prepared and 0.1 ml of each dilution (in 0.9 ml Hanks BSS diluent) was added to the culture tubes in triplicate. Diluent was added in lieu of toxin to control cultures. After 3 hr incubation at 37 C, 0.1 ml of a C^{14} amino acid mixture was added to each tube (final specific activity: 0.1 µCi per tube). After 2 hr incubation cells were washed with PBS, trypsinized off the glass and TCA was added to a final concentration of 5%. The precipitates were collected on Millipore filters, washed with 5% TCA and 95% ethanol, dried and counted in a liquid scintillation counter. Each point represents the average TCA-precipitable radioactivity in triplicate samples.

o—o toxin dilutions.

Δ—Δ toxin dilutions neutralized by a constant amount (0.1 ml of a 1:10 dilution) of diphtheria antitoxin.


100
75
50
25
% OF CONTROL
0
0.006
0.06
0.6
6.0
μGM TOXIN/CULTURE

some cell intoxication was observed.

6. Ability of βhv64 tox$^+$ phage to inhibit KB cell intoxication:

The primary question could now be asked: can this βv tox$^+$ phage protect KB cells from diphtheria intoxication? Fig. 11 and 12 summarize the results obtained in two experiments in which increasing concentrations of purified phage were pre-incubated 1 hr with KB cells prior to the addition of toxin. Diphtheria toxin was added to cells at a concentration sufficient to inhibit protein synthesis by 95% (represented by the point touching the ordinate of the graphs, i.e., these cultures were not pre-incubated with either phage or BSA). In both experiments, there was a direct correlation between the concentration of βv tox$^+$ phage pre-incubated with KB cells and the degree of protection afforded these cell cultures from subsequent intoxication. In the first experiment, the addition of 270 µg βv tox$^+$ phage/KB culture resulted in an inhibition of intoxication of approximately 63%. In the experiment shown in Fig. 12, 360 µg of purified phage inhibited intoxication 98%. T4 coliphage and bovine serum albumin were pre-incubated with KB cells at concentrations of 270 µg and 360 µg per culture but neither showed any significant protective effect. This finding suggested that the protection was not merely due to non-specific binding of a protein or bacteriophage to KB cell receptor sites but was an inherent property attributable to βv tox$^+$ phage.

Figure 11

Ability of βv tox^+ phage to protect KB cells from diphtheria intoxication.

Increasing concentrations of purified βv tox^+ phage were pre-incubated 1 hr with tube cultures of KB cells prior to the addition of toxin. Diphtheria toxin was added to the cells at a concentration sufficient to inhibit protein synthesis by 95%. Diluent was added in lieu of toxin to control cells. All other operations were as described in Fig. 10. Each point represents the average TCA-precipitable radioactivity in duplicate samples.

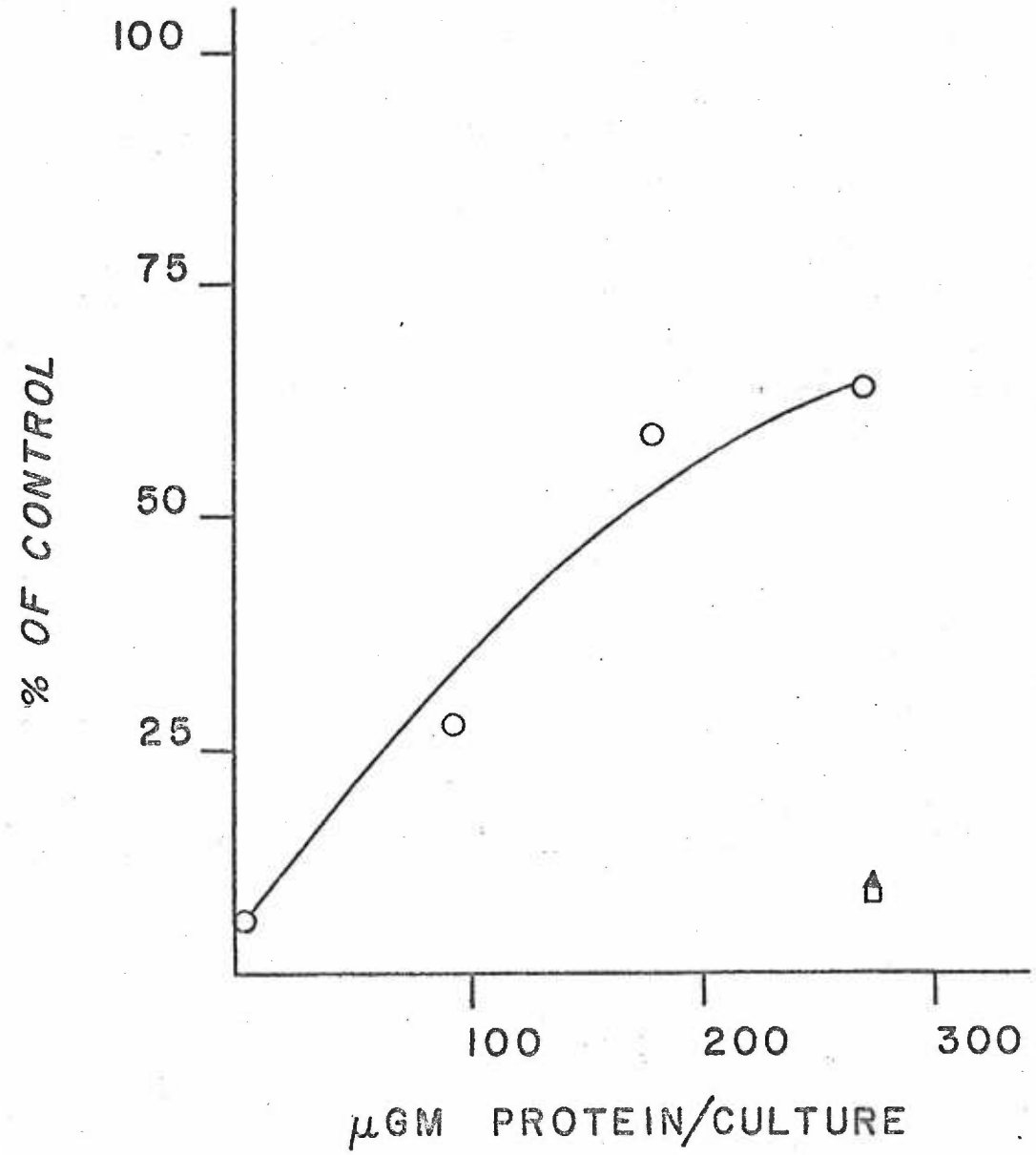
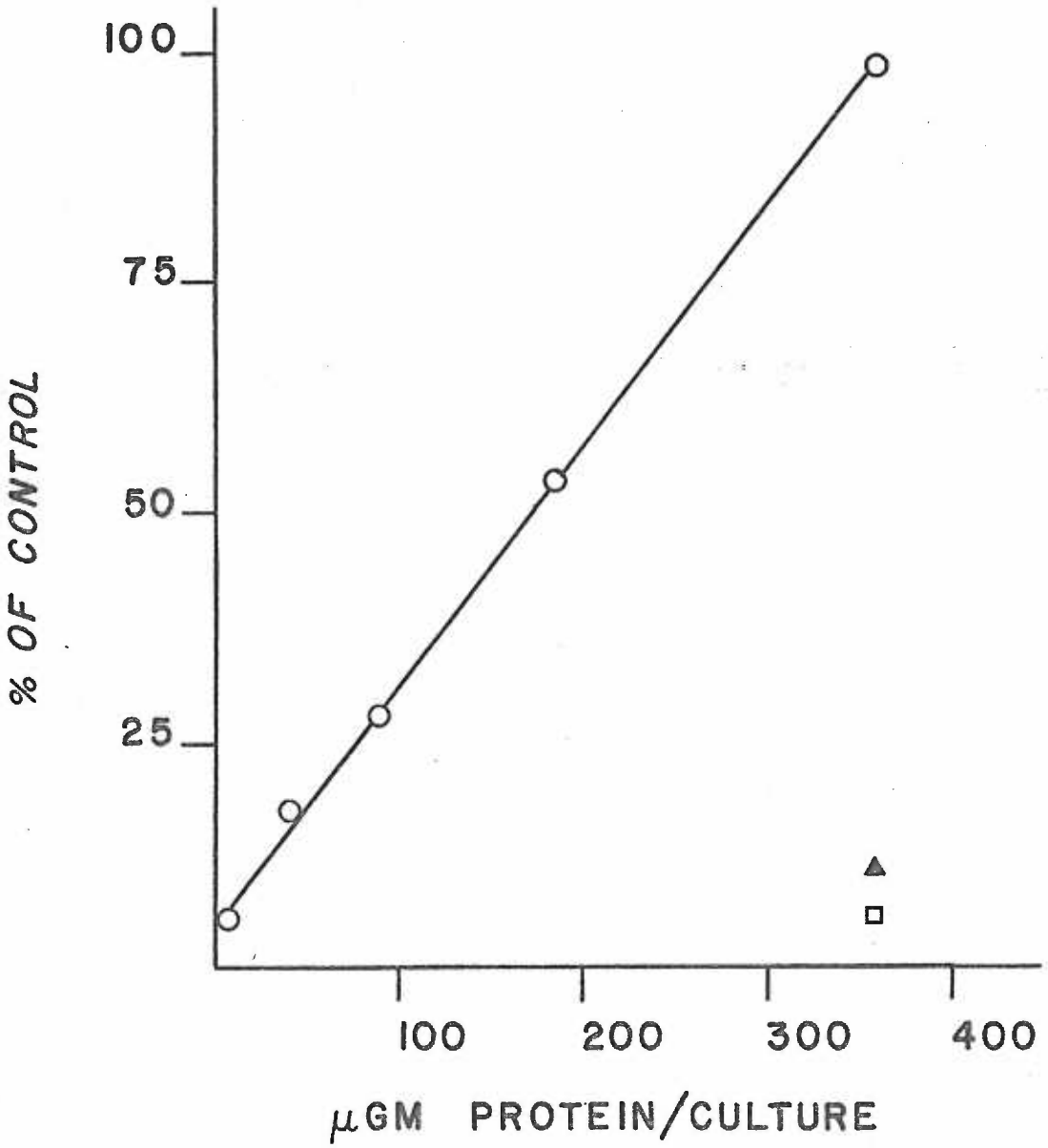

100
75
50
25
% OF CONTROL
100
200
300
μGM PROTEIN/CULTURE

Figure 12

Ability of βv tox^+ phage to protect KB cells from diphtheria intoxication.

The procedure is identical to that described in Fig. 11. Each point represents the average TCA-precipitable radioactivity in duplicate samples.


100
75
50
25
% OF CONTROL
100
200
300
400
μGM PROTEIN/CULTURE

7. Reconstitution of a toxic molecule using CRM45 protein and phage protein:

Two factors made the next set of experiments feasible:

(1) Uchida, Pappenheimer and Harper (1972) had succeeded in reconstituting a toxic molecule by adding trypsinized CRM197 (containing function 1 fragment B and non-functional fragment A) to trypsinized CRM45 protein (containing functional fragment A and non-functional fragment B). Toxic molecules were re-assembled from the functional fragment A and B derived from the two non-toxic CRM proteins.

(2) The major phage protein, the 38,000 dalton polypeptide that co-migrated with fragment B of toxin on SDS-polyacrylamide gels, could be isolated in relatively pure form from intact, purified βv tox^+ phage.

The rationale of the following experiments was as follows: if the 38,000 MW phage protein is functionally related to fragment B of diphtheria toxin then it should be possible to reconstitute a toxic molecule by mixing phage protein (fragment B?) with trypsinized CRM45 protein (containing functional A fragment and non-functional B fragment). Electrophoretically pure CRM45 protein was prepared and trypsinized in the presence of DTT. An extracted phage protein preparation, which migrated as a single 38,000 dalton protein on a SDS-polyacrylamide gel, was mixed with the trypsinized CRM45 protein under conditions (see p. 42 ; Methods) favoring the formation of hybrid

molecules (Pappenheimer, Uchida and Harper, 1972). The so-called "reconstituted product," CRM45 protein and the extracted phage protein were added to separate tube cultures of KB cells. A toxin control was included and the degree of protein synthesis-inhibition shown by any of the individually added test proteins was measured relative to the toxin control. Tables 3, 4 and 5 summarize the results of three experiments using three different preparations of "reconstituted hybrid" molecules. The numbers shown in the tables represent the average counts (CPM) of three tube cultures. The control cells were incubated throughout with MEM-tissue culture medium in lieu of toxin or other test proteins. In the first experiment the "reconstituted product" inhibited KB protein synthesis 16.6% whereas CRM45 protein and phage protein individually inhibited cell protein synthesis 3 and 8% respectively. The experiment summarized in Table 4 was more impressive in that the "hybrid" molecule was responsible for a 39% inhibition of protein synthesis. The KB cell controls which received equivalent amounts of CRM45 protein or extracted phage protein showed lower levels of protein synthesis inhibition. The experiment summarized in Table 5 included an additional control, an aliquant of "reconstituted product" which was pre-incubated overnight at 4 C with diphtheria antitoxin. Although the amino acid incorporation measured in radioactive counts per minute in this experiment was low compared to the two previous experiments, the trend was clear. The

Tables 3 and 4

The inhibition of KB cell protein synthesis by reconstituted hybrid molecules.

An extracted βv tox^+ phage protein preparation was mixed with trypsinized CRM45 protein under conditions favorable to the formation of hybrid molecules (see Methods). Tables 3 and 4 represent data derived from two separate hybrid molecule preparations. Equivalent amounts of the reconstituted hybrid protein, CRM45 protein and extracted phage protein were added to separate tube cultures of KB cells in triplicate. Control cells (MEM) were incubated throughout with tissue culture medium in lieu of toxin or other proteins. KB cell protein synthesis was assayed by the method described in Fig. 10. The numbers represent the average counts (CPM) of 3 KB tube cultures.

ADDED TO KB CELLS	CPM	% INHIBITION
MEM	32,080 ± 2,546*	-
TOXIN (10^{-4} DIL.)	4,360 ± 373	86.5
CRM45 PROTEIN	31,065 ± 1,586	3.2
PHAGE PROTEIN	29,400 ± 3,854	8.4
"RECONSTITUTED PROTEIN"	23,163 ± 2,127	16.6

TABLE 3

ADDED TO KB CELLS	CPM	% INHIBITION
MEM	8,325 ± 1,660*	-
TOXIN (10^{-4} DIL.)	212 ± 50.9	97.5
CRM45 PROTEIN	7,816 ± 158	6.7
PHAGE PROTEIN	7,939 ± 766	4.6
"RECONSTITUTED PROTEIN"	5,057 ± 287	39.3

Table 4

* 95% confidence level; $P > 0.05$ in all cases.

Table 5

The inhibition of KB cell protein synthesis by a reconstituted hybrid molecule in the presence and absence of diphtheria antitoxin.

A third reconstituted hybrid molecule was tested for its effect on KB cell protein synthesis as described in Tables 3 and 4. Before addint the protein to the tissue cultures, equivalent amounts of reconstituted protein were incubated, with either an equal volume of diphtheria antitoxin (1:10 dilution) or normal rabbit serum (1:10 dilution), at 37 C for 30 min. The numbers shown represent the average counts (CPM) of triplicate KB tube cultures.

ADDED TO KB CELLS	CRM	% INHIBITION
MEM	1,962 ± 124*	-
TOXIN (10^{-4} DIL.)	308 ± 28	84.3
CRM45 PROTEIN	2,064 ± 147	0
PHAGE PROTEIN	1,896 ± 165	3.4
"RECONSTITUTED PROTEIN" + NORMAL RABBIT SERUM	1,293 ± 203	34.1
"RECONSTITUTED PROTEIN" + ANTITOXIN	1,780$^{\xi}$	9.3

Table 5

* 95% confidence level; P > 0.05 in all cases.

$^{\xi}$ Done in duplicate; 95% confidence level not done.

"reconstituted product" inhibited KB protein synthesis by 34%, whereas this protein neutralized by antitoxin inhibited protein synthesis by 9%. CRM45 protein in this case was entirely non-toxic and extracted phage protein inhibited protein synthesis 3.4%.

The toxicity of these individual reconstituted "hybrid" molecules varied over a rather large range in the experiments reported here. This variance may be due to the molecular randomness of the protein-to-protein interactions involved in reconstitution experiments.

8. Separation and purification of toxin fragments A and B:

Purified diphtheria toxin was reduced and trypsinized and fragments A and B were separated by sequential column chromatography employing Sephadex G-150 (Pappenheimer, Uchida and Harper, 1972) and diethylaminoethylcellulose DE-52 (Cukor, Solotorovsky and Kuchler, 1973). The purity of fragment B was especially important since it was to be used to immunize rabbits to obtain anti-fragment B antibody and also as an antigen in gel immunodiffusion slides. Fig. 13 is a photograph of the SDS-polyacrylamide gels of the toxin fragment preparations. Gel A contains trypsinized diphtheria toxin, gel B contains the fragment B preparation and gel C the fragment A preparation. The fragment B preparation appeared to be free of fragment A contamination but contained a minor, smaller MW protein band that migrated in a position similar to the minor protein band seen in several of the

Figure 13

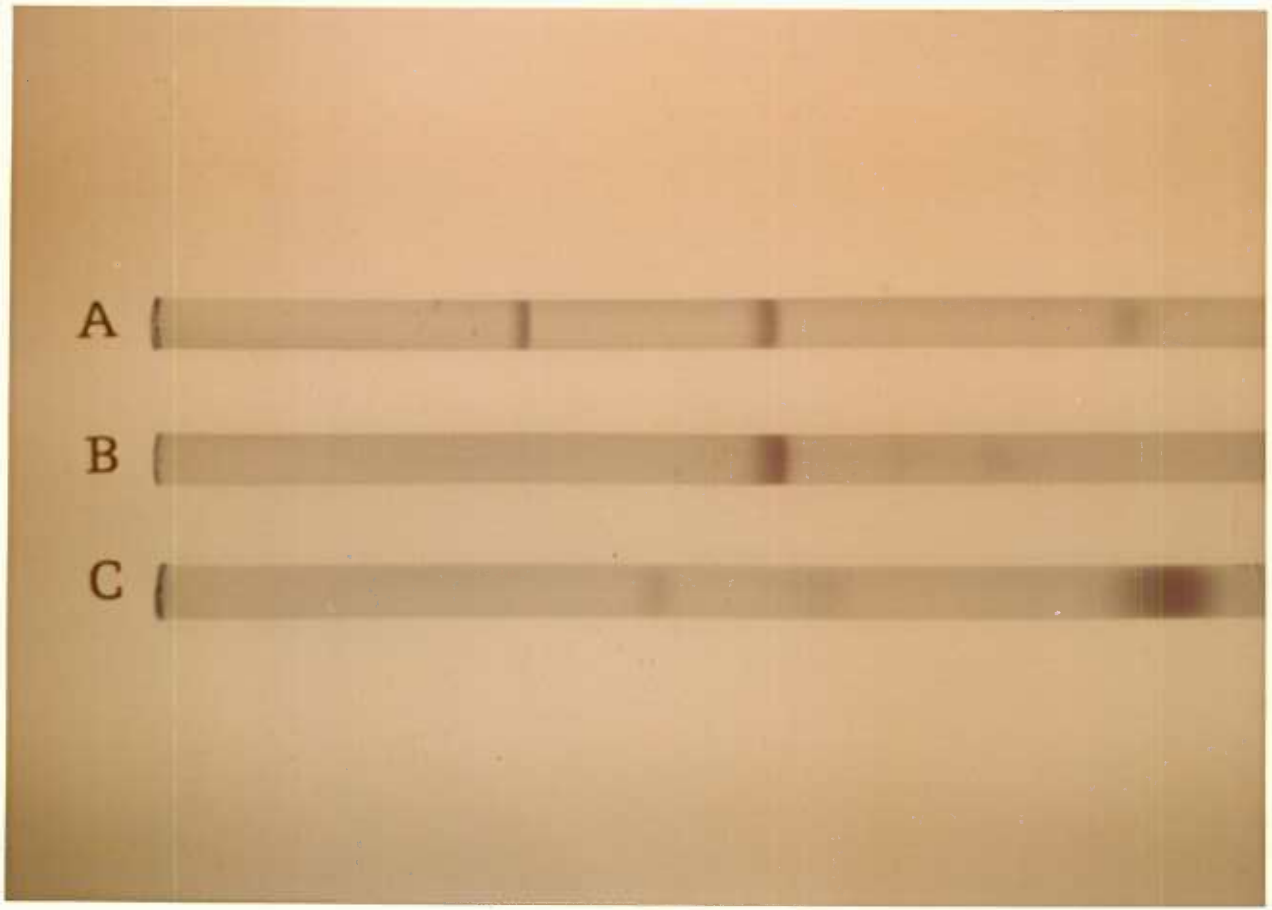
SDS-polyacrylamide gels of diphtheria toxin fragment A and fragment B.

Electrophoretically pure diphtheria toxin was trypsinized and toxin fragments A and B were separated according to the procedures outlined in Methods. The toxin fragments, dissolved in 0.02 M phosphate buffer, pH 6.8 containing 6 M urea and 1 mM DTT were electrophoresed as described in Fig. 5.

Gel A: 40 µg of trypsinized diphtheria toxin

Gel B: 50 µg of toxin fragment B

Gel C: 75 µg of toxin fragment A


A
B
C

extracted phage protein preparations (Fig. 9; gel B). The fragment A preparation (gel C) appeared to be equally free of contaminating B fragment. There was a second protein band of molecular weight larger than fragment B but smaller than intact toxin.

Fragment A of toxin has a unique enzymatic activity in that it covalently binds the adenosine-diphosphirobose moiety of NAD^+ to mammalian elongation factor 2. As little as .001 µg of fragment A can be detected in an assay system utilizing this enzymatic reaction. The fragment A and fragment B preparations shown in Fig. 13 were tested for their enzymatic activities. Purified, crystalline fragment A provided by Dr. R. J. Collier was included as a positive control. The results are shown in Table 6. The fragment A preparation had significantly more ADP-ribosylation activity than an equivalent amount of fragment B, although B fragment did have activity in excess of the background. When the background count was subtracted and the fractions compared, the fragment B preparation had 5.7% of the enzymatic activity of fragment A.

9. Immunological relationships of the extracted phage protein:

Antitoxin was prepared in rabbits by Dr. H. Lightfoot using formalin-treated, electrophoretically pure diphtheria toxin (kind gift of Dr. R. J. Collier). Four separate extracted phage protein preparations were tested for reactivity against antitoxin in double diffusion

Table 6

ADP-ribosylation of mammalian elongation factor 2 (EF2) by fragment A and fragment B.

Assay mixtures in total volumes of 250 µl contained in final concentrations: 50 mM tris-HCl pH 8.2, 0.1 mM EDTA, 40 mM DTT, 100 µl EF2 enzyme and 0.73 µM (C^{14}-adenine) NAD^+ (136 mCi/mM). The assay mixture also contained one of the following: 10 µl of fragment A or fragment B (see Fig. 13) or 10 µl of various dilutions of known fragment A (provided by Dr. R. J. Collier). A background control contained 10 µl distilled H_2O instead of fragment A. The incubation time was 30 min at 25 C.

Table 6

Additions to the ADP-Ribosylation Assay	µg Fragment A or Fragment B	CPM ADPR-EF2*
Collier Fragment A	.010	16,722
" " "	.005	14,926
" " "	.0025	11,415
" " "	.001	1,675
None	-	346
Fragment A (Fig. 13)	.0025	6,306
Fragment B "	.0025	638

* Adenosine-diphosphoribose elongation factor 2 precipitated by CCl_3COOH.

Ouchterlony slides employing ionagar in barbital buffer, pH 9.4. No lines were seen in any of these immunodiffusion slides, even though strong precipitin lines were seen when phage protein was reacted against diphtheria toxin in tube precipitin tests. Four extracted phage protein preparations were then tested for immunological reactivity against antitoxin employing the agarose/glycine-phosphate buffer method of Matsuda, Kanei and Yoneda (1972) with significant results. Fig. 14 is a photograph of an immunodiffusion slide in which extracted phage protein, toxin, toxoid, intact βv tox^+ phage, and guanidine hydrochloride-treated phage were allowed to diffuse against rabbit antitoxin. The precipitin lines between the extracted phage protein and antitoxin took 36 hr to appear, whereas the lines between antitoxin and both toxin and toxoid were strong within 18 hr. In the intervening 18 hr the latter two lines became diffuse. Fig. 14 shows:

(1) Two precipitin lines could be seen between the extracted phage protein preparation and diphtheria antitoxin. These lines demonstrated partial identity to both toxin and toxoid.

(2) Two precipitin lines occurred between the guanidine-HCl treated phage and antitoxin. Both lines showed complete identity with the extracted phage protein.

(3) Although less definitive, it appears that only one precipitin line was present between intact βv tox^+ phage and antitoxin. This line was common to the outer precipitin lines of both

Figure 14

Antigenic relationships between βv tox$^+$ phage, extracted phage protein and diphtheria toxin and toxoid.

Gel immunodiffusion slides were prepared employing 0.75% agarose in 0.01 M phosphate buffer pH 7.5, containing 0.5 M glycine and 0.14 M NaCl. Approximately 2.5 ml of the melted agarose solution was layered onto coated (1% ionagar) microscope slides, the agar allowed to harden and the appropriate wells cut out. The outer wells were filled with 40 µl of the various antigen preparations. The center well contained undiluted diphtheria antitoxin prepared from formalinized, electrophoretically pure diphtheria toxin (see Methods). The antigen concentrations were:

Toxin (electrophoretically pure): 400 µg/ml

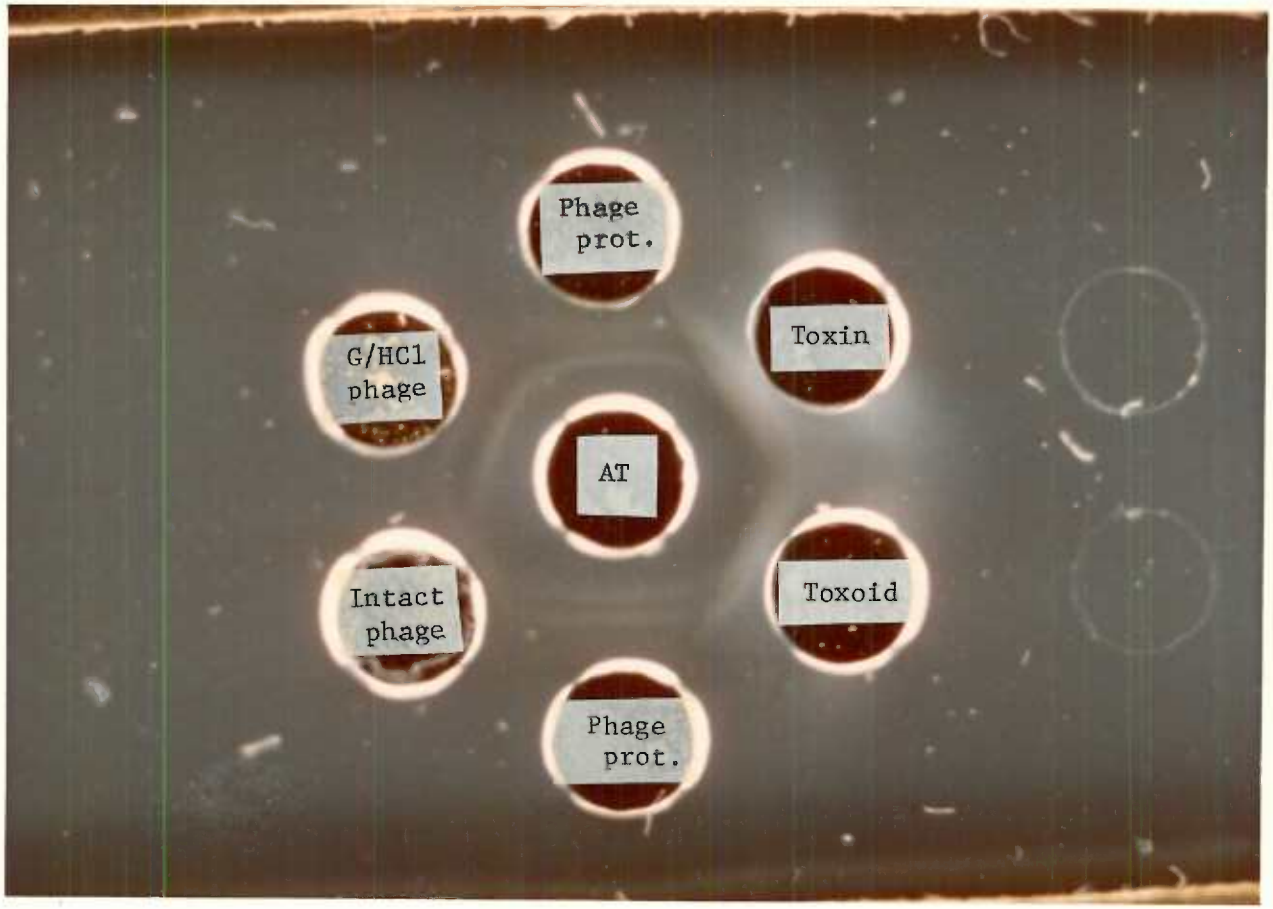
Toxoid (used to prepare antitoxin): 400 µg/ml

Extracted phage protein: 360 µg/ml

Intact βv tox$^+$ phage: 250 µg/ml

G/HCl βv tox$^+$ phage (an aliquant of purified phage was mixed with an equal volume of 6 M guanidine hydrochloride, incubated 10 min at 37 C and then used): 250 µg/ml

AT (antitoxin): undiluted rabbit anti-diphtheria toxoid antibody


Phage prot.
Toxin
G/HCl phage
AT
Intact phage
Toxoid
Phage prot.

the extracted phage protein and the guanidine-HCl treated phage.

Fig. 15 is a photograph of an immunodiffusion slide in which toxin, electrophoretically pure fragment B and extracted phage protein (a different preparation than that used in the immunodiffusion slide shown in Fig. 14) were reacted against rabbit antitoxin. This slide shows:

(1) Two precipitin lines, very close to each other, formed between fragment B and diphtheria antitoxin.

(2) Two lines were visible between the extracted phage protein and antitoxin. The outer line showed identity with one of the fragment B precipitin lines and appeared to join the toxin precipitin line.

(3) The juncture between the fragment B and toxin precipitin lines was too diffuse to determine whether a spur of partial identity was present.

Since immunological cross-reactivity was demonstrated between intact βv tox^+ corynebacteriophage, extracted phage protein (four separate preparations) and antitoxin it followed that a similar relationship ought to exist between anti-βv tox^+ antibody and toxin. The immunodiffusion slide shown in Fig. 16 consists of the following proteins reacted against anti-βv tox^+ antibody: extracted phage protein, intact phage and toxin. It can be seen that:

Figure 15

Antigenic relationship between diphtheria toxin, toxin fragment B and extracted βv tox^+ phage protein.

A gel immunodiffusion slide was prepared as described in Fig. 14. The outer wells were filled with 40 µl of the various antigen preparations. The center well contained diphtheria antitoxin. The antigen concentrations were:

Toxin (electrophoretically pure): 350 µg/ml

Fragment B: 280 µg/ml

Extracted phage protein: 310 µg/ml

AT (antitoxin): undiluted rabbit anti-diphtheria toxoid and antibody

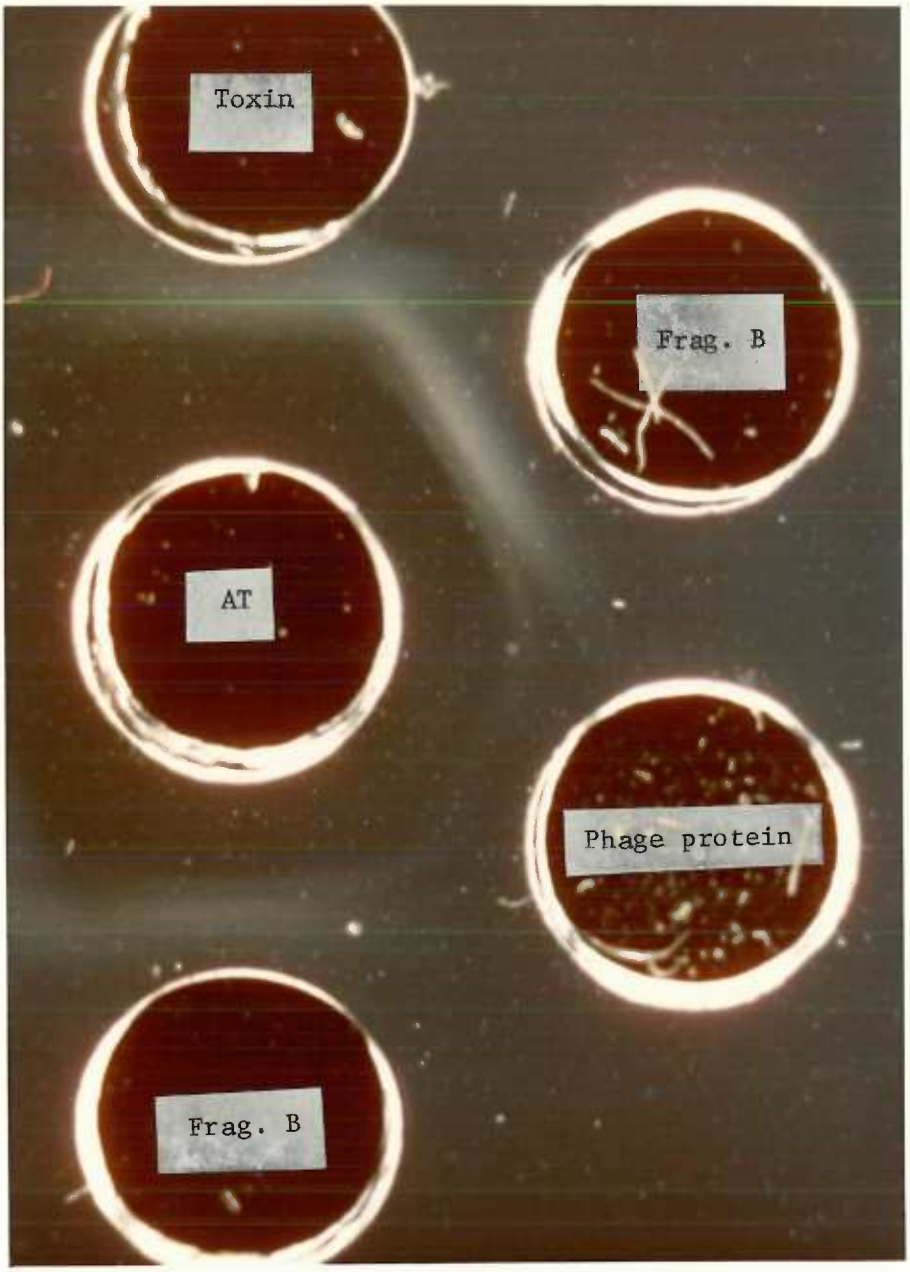

Toxin
Frag. B
AT
Phage protein
Frag. B

Figure 16

Antigenic relationships between βv tox^+ phage, extracted phage protein and toxin.

A gel immunodiffusion slide was prepared as described in Fig. 14. The outer wells were filled with 40 µl of the various antigen preparations. The center well contained undiluted anti-βv tox^+ phage antibody prepared in rabbits as outlined in Methods. The antigen concentrations were:

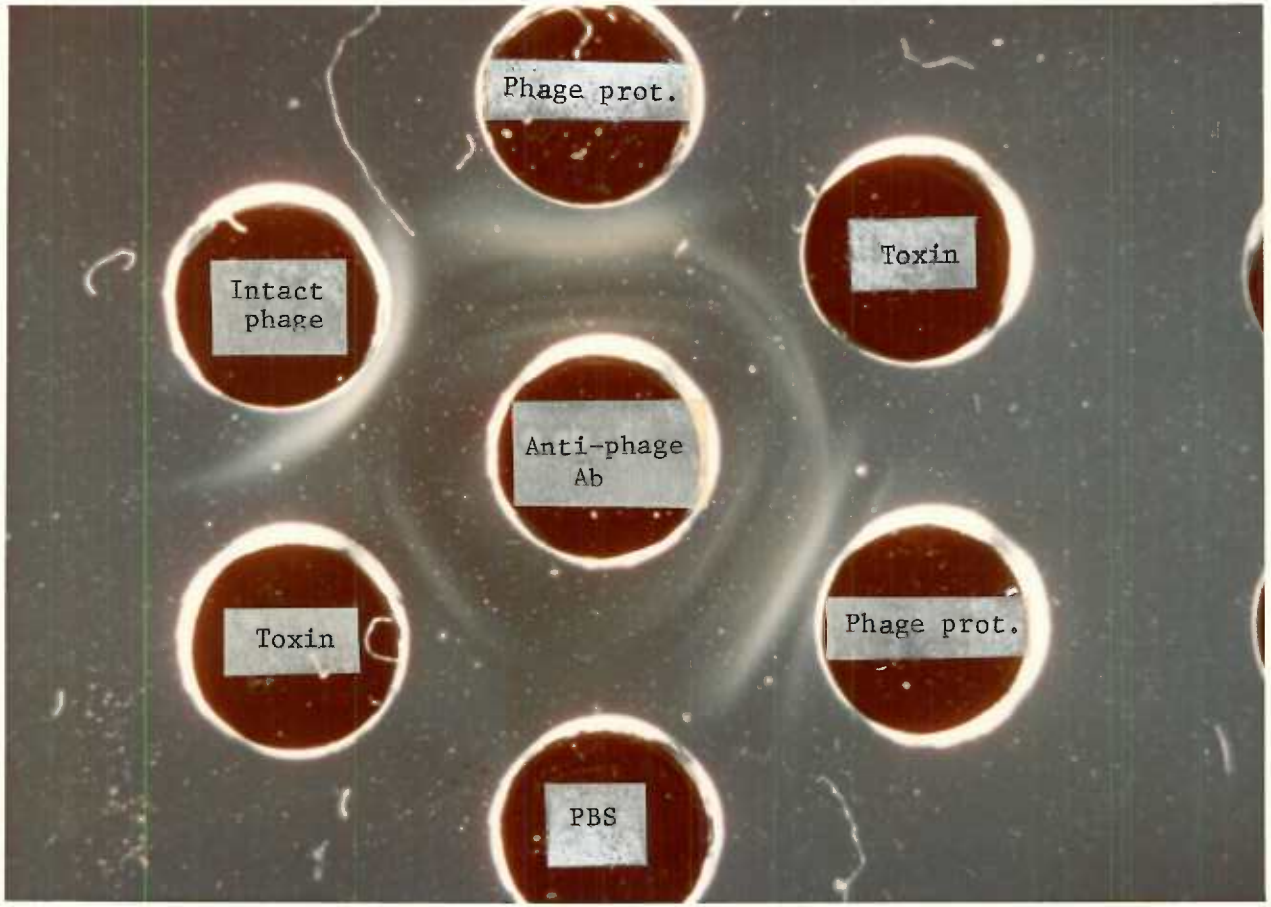
Extracted phage protein: 310 µg/ml

Toxin (electrophoretically pure): 400 µg/ml

Intact βv tox^+ phage: 360 µg/ml

PBS: phosphate buffered saline

AT (antitoxin): undiluted rabbit anti-βv tox^+ phage antibody


Phage prot.
Intact phage
Toxin
Anti-phage Ab
Toxin
Phage prot.
PBS

(1) There are at least two lines of precipitation, which appear to merge, between anti-phage antibody and diphtheria toxin.

(2) Significantly, the toxin precipitin lines clearly showed a line of partial identity with the major precipitin line of the extracted phage protein with the spur indicating additional antigenic determinants in the phage protein not shared with toxin.

(3) There were several precipitin lines between the extracted phage protein and anti-phage antibody.

(4) There was one strong and unique line between the intact βv tox^+ phage and anti-phage antibody. This line was very close to the antigen well.

10. Peptide fingerprint analysis of fragment B and phage protein:

Zanetta, Vincendon, Mandel and Gombos (1970) have reported a rapid method for partially analyzing the primary structure of proteins. Their method consisted of digesting the protein thoroughly with trypsin, dansylating the resultant peptides with 1-dimethylamino-naphthalene-5-sulphonylchloride and then chromatographing the dansylated-peptides in two directions on a silica gel thin-layer plate. The final peptide locations were determined by virtue of their fluorescence on the chromatogram under UV light. Approximately 310 µg of the "pure" extracted phage protein preparation and 280 µg of

electrophoretically pure fragment B (see Fig. 17) were analyzed employing the method of Zanetta et al. Fig. 18 and 19 are the resultant peptide maps of digested fragment B and extracted phage protein. The maps are remarkably similar. Sixteen peptides are common to both proteins. In addition, the extracted phage protein contains four peptides which have no counterpart in the map of fragment B and there are two peptides in the map of fragment B which are not seen in the extracted phage protein chromatogram.

Figure 17

SDS-polyacrylamide gels of the toxin fragment B and the extracted βv tox^+ phage protein preparations used in the peptide fingerprint analysis.

Fragment B and the guanidine hydrochloride extracted phage protein, in 0.02 M phosphate buffer, pH 6.8 containing 6 M urea and 1 mM DTT, were electrophoresed as described in Fig. 5.

Gel A: 40 µg of trypsinized diphtheria toxin

Gel B: 36 µg of toxin fragment B

Gel C: 30 µg of extracted phage protein

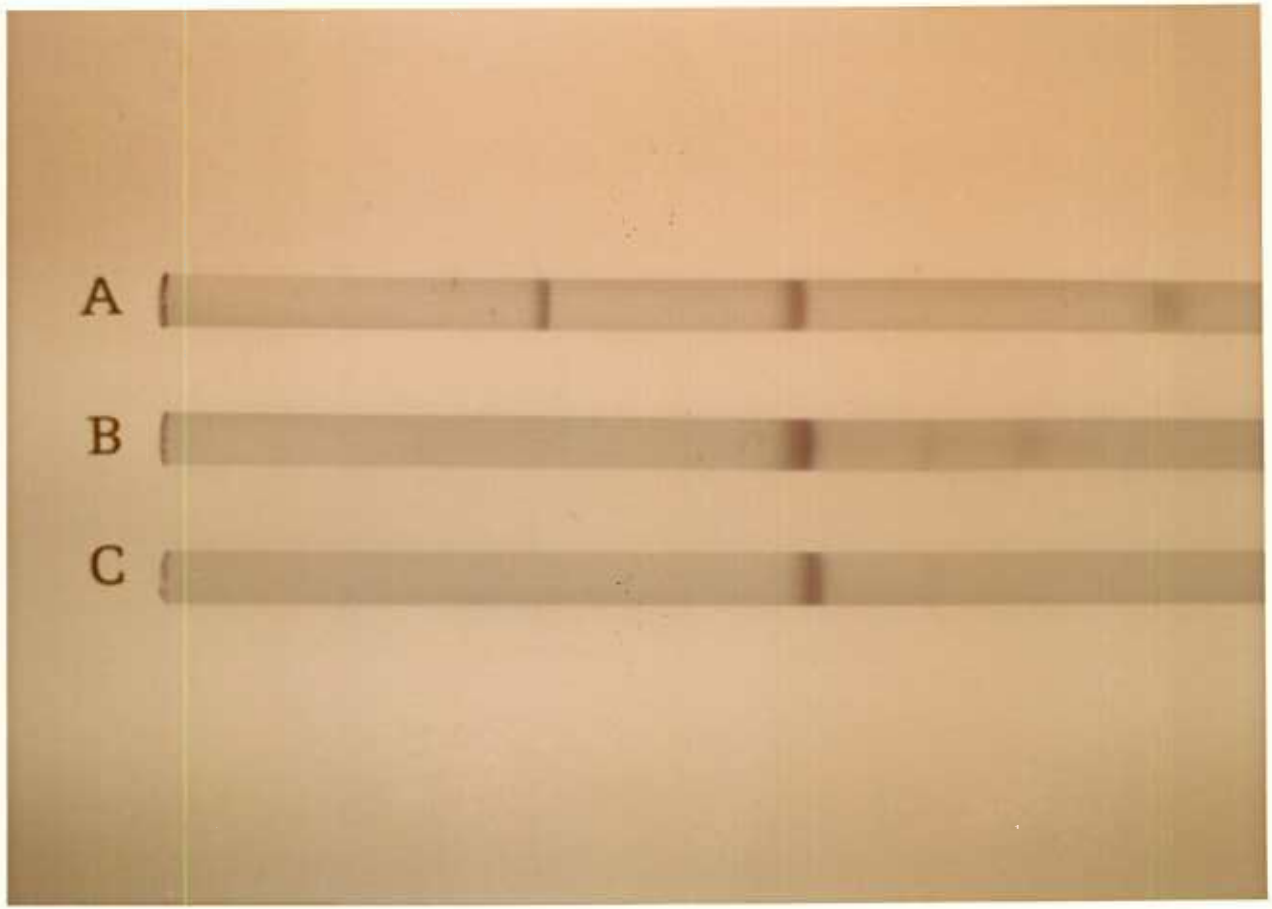
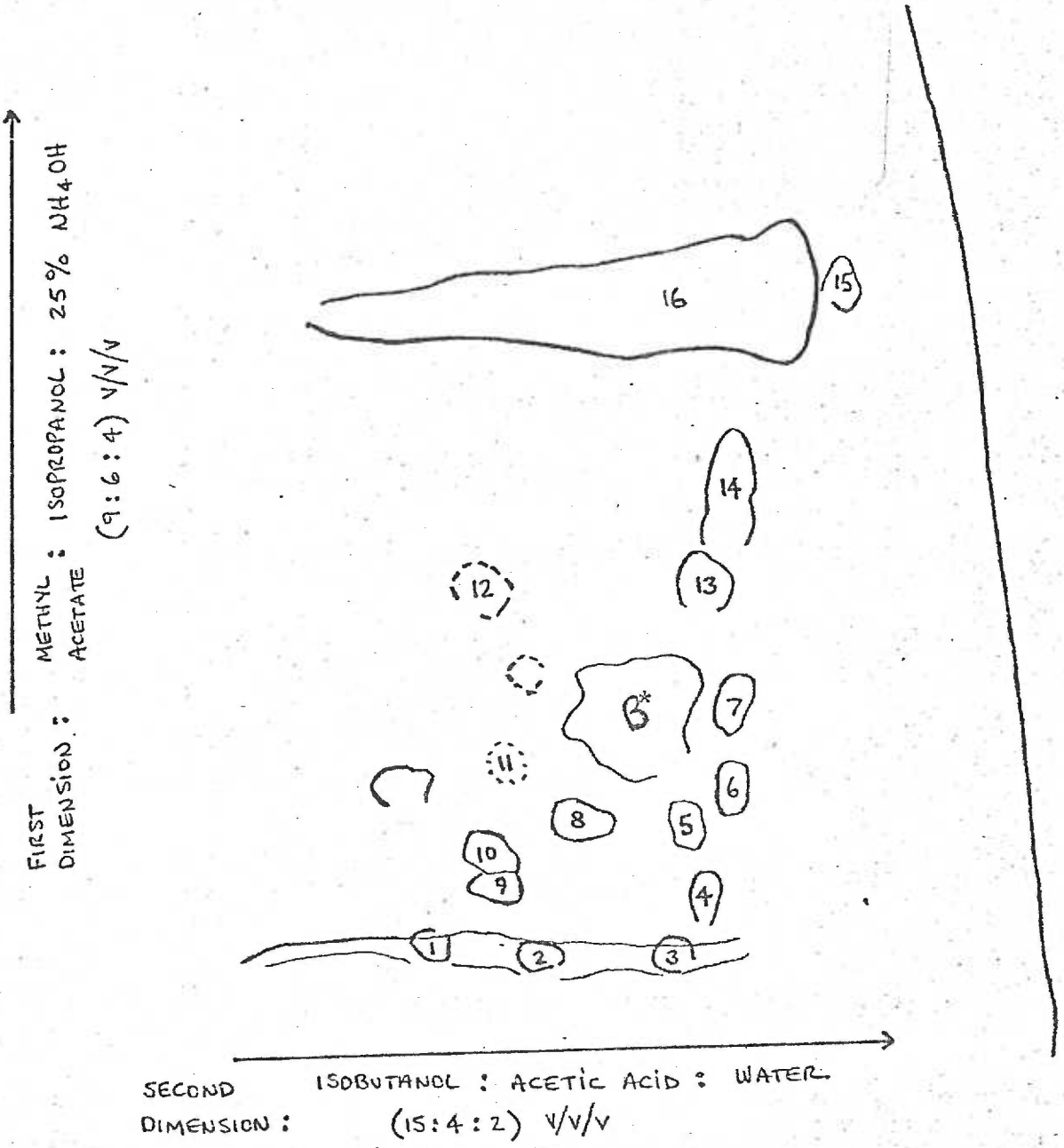

A
B
C

Figure 18

Fingerprint of a dansylated tryptic digest of toxin fragment B on a silica gel thin-layer.

Approximately 280 µg of fragment B, dissolved in 6 M urea, was hydrolyzed and dansylated according to the procedure outlined in Methods. A 100 µl sample was placed on a silica gel plate. The chromatogram was developed in the first dimension with methylacetate-isopropanol-ammonia (9:6:4). The plate was dried and developed in the second dimension using isobutanol-acetic acid-water (15:4:2). After drying overnight the chromatogram was examined under UV light and the dans-peptide spots were outlined in pencil. The peptides considered common to both digests are numbered.



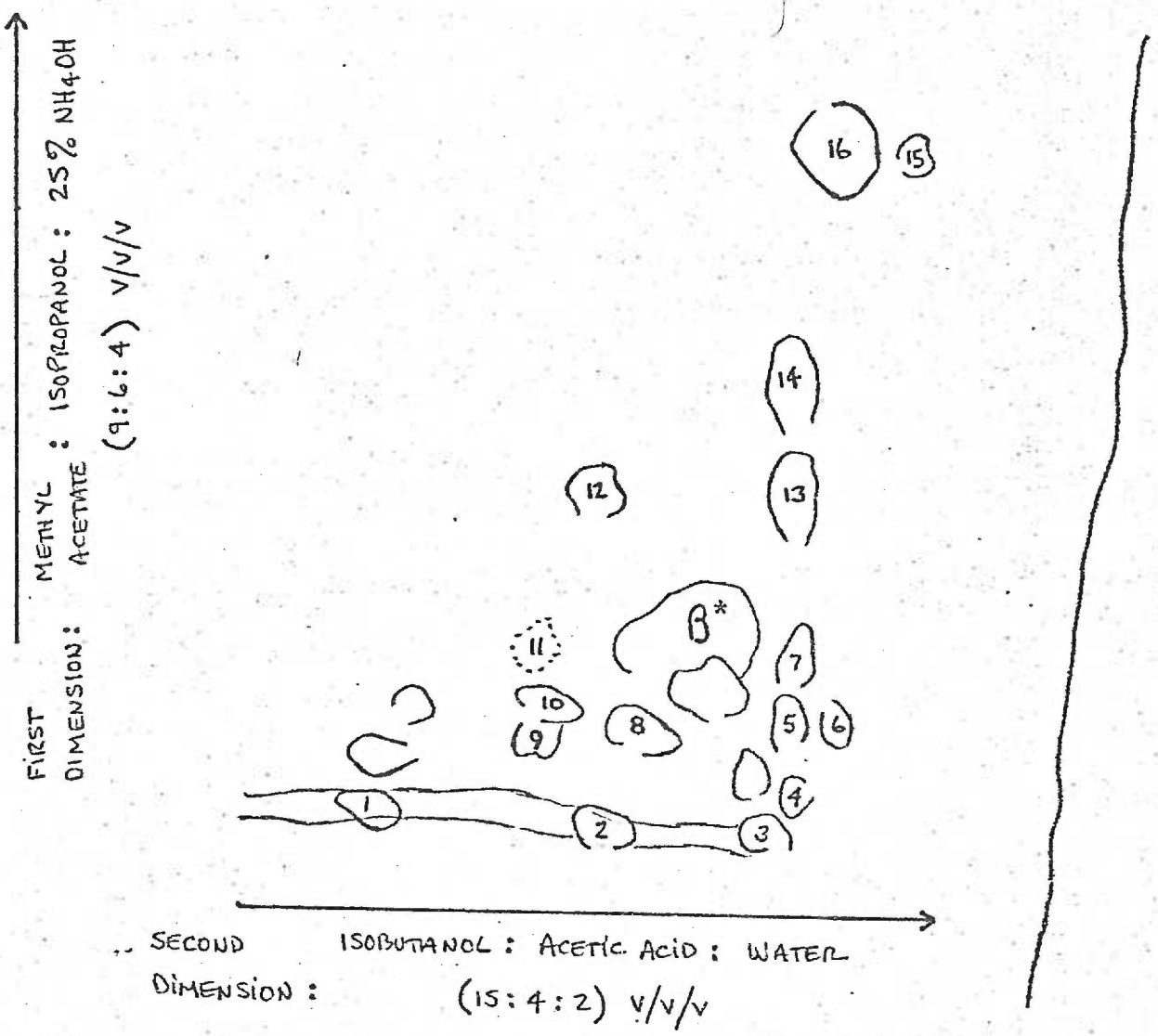
B* : dans-OH

Figure 18

Figure 19

Fingerprint of a danslyated tryptic digest of the extracted βv tox^+ protein on a silica gel thin-layer.

Approximately 280 µg of extracted phage protein, dissolved in 6 M urea, was hydrolyzed, danslyated and chromatographed on a silica gel plate as described in Fig. 18.



B* : dans-OH

Figure 19

DISCUSSION

To determine whether any polypeptide(s) resembling diphtheria toxin or toxin fragments were associated with βv tox^+ corynebacteriophage, purified phage preparations were treated with SDS and the dissociated proteins were analyzed on SDS-polyacrylamide gels. The protein composition of corynebacteriophage had never before been characterized on acrylamide gels. The first βv tox^+ phage preparations so analyzed were apparently not pure and approximately 20 proteins were visible in SDS-acrylamide gels which is high for a phage the size of β (Buchwald, Murialdo and Siminovitch, 1970). The major phage protein in these impure preparations co-migrated with fragment B of diphtheria toxin, both in phage propagated in a medium containing a low iron concentration (allowing maximum toxin synthesis) and in high iron medium (sufficiently high to inhibit toxin synthesis 60-75%). As can be seen in Fig. 5, there was no apparent decrease in the amount of the major phage protein relative to the iron concentration of the propagation medium. It appeared, therefore, that although extracellular toxin synthesis was under inorganic iron control (Righelato and Van Hemert, 1969) the 38,000 MW protein found in the phage preparations was independent of this control.

In order to analyze the protein profiles of more highly purified phage βv tox^+ corynebacteriophage were purified using a preliminary

two-phase, polyethylene glycol-dextran sulfate separation step followed by two CsCl equilibrium centrifugations. These phage preparations were found consistently to contain 7 or 8 protein bands when SDS-treated and electrophoresed on SDS-acrylamide gels. The major protein in all of the latter preparations analyzed also co-migrated with toxin fragment B. In subsequent experiments βv tox^+ phage were considered sufficiently pure for further experimentation only if the following criteria were met:

(1) the preparations had a DNA to protein ratio no greater than 1:1.5 that remained unchanged following any further purification step (Iglewski, unpublished data).

(2) the SDS-treated phage had a maximum of 8 protein bands when electrophoresed on SDS-polyacrylamide gels (Fig. 6).

A comparison between βv tox^+ corynebacteriophage and coliphage λ is of interest in the context of this structural analysis. These phage share many characteristics. Lambda phage has an icosahedral head, 65 nm in diameter and a long non-contractile tail, 150 nm in length (Kemp, Howatson and Siminovitch, 1968). The molecular weight of the λ DNA genome is 32×10^6 daltons (Mathews, 1971; Echols and Joyner, 1968). βv tox^+ corynebacteriophage has an icosahedral head, 58 nm in diameter, a long non-contractile tail 260 nm in length and contains a DNA genome of approximately 22×10^6 daltons (Uchida, Pappenheimer and Greany, 1973; Nagington and Carne, 1971). Buchwald, Murialdo and

Siminovitch (1970) found that purified, SDS-treated λ phage contained eight protein bands when electrophoresed in 10% SDS-acrylamide gels. Seven or eight protein bands were evident in four separate preparations of purified βv tox^+ phage analyzed here. In addition, the major constituent of the λ phage head was a 37,500 dalton protein that constituted 57% of the total phage protein. The major βv tox^+ protein has a molecular weight of 38,000 and constituted 60.6 ± 10% of the four separate purified preparations analyzed by densitometry. However, it remains to be determined if the 38,000 dalton βv tox^+ phage protein is associated with the phage head.

From the foregoing evidence, it can be concluded that the major protein associated with βv tox^+ phage and fragment B of diphtheria toxin were of equal molecular weight as determined by SDS-acrylamide gel electrophoresis. The migration of proteins in SDS gels is inversely related to the molecular weight of the proteins (Shapiro, 1967) and such migration is relatively insensitive to the charge and shape of the molecule (Dunker and Rueckert, 1969). This method has been reported by Weber and Osborn (1969) to yield molecular weights with an accuracy of ± 10% for polypeptide chains with molecular weights between 15,000 and 100,000. It could well be argued however, that this size identity was merely coincidental and by itself does not prove that this phage protein is related to fragment B of diphtheria toxin. Therefore, evidence was required to show that the βv tox^+

protein shared functional properties with toxin fragment B. When KB cells were pre-incubated with toxin fragment B and then exposed to toxin, protein synthesis was inhibited at a much slower rate compared to control cells in which the pre-incubation was carried out with buffer (Cukor et al., 1973). Fragment B was thought to compete with toxin for binding sites on susceptible cell membranes, thereby inhibiting cell intoxication. To determine if the 38,000 MW polypeptide associated with βv tox^+ phage shares this property of fragment B, purified phage were pre-incubated for 1 hr with KB cells and a concentration of toxin sufficiently high to inhibit protein synthesis 95% was added to the cultures. In two such experiments summarized in Figs. 11 and 12, increasing concentrations of phage resulted in increasing protection of KB cells from intoxication. T4 coliphage and bovine serum albumin gave no protection indicating that the protection afforded by βv tox^+ phage was specific. In one experiment the phage dilutions were decanted and the KB cells were washed several times with phosphate buffered saline prior to the addition of toxin and the βv tox^+ still afforded some protection (63%) against subsequent intoxication. These results suggest that the protection observed involved a relatively stable phage-cell interaction.

The ability of purified βv tox^+ phage preparations to protect KB cells varied over a wide range. The phage preparations shown in Figs. 11 and 12 protected KB cells against subsequent intoxication to

63% and 98% respectively. Four other phage preparations (not shown in Results) protected KB cells to a maximum level of 26%, 27%, 21% and 30% relative to toxin controls. As a rule, freshly purified phage showed higher levels of protection than those phage preparations that had been stored for long periods at 4 C. Two of the purified phage preparations that had been stored at 4 C for approximately 30 days were SDS-treated and analyzed on SDS-acrylamide gels. They were found to contain, in addition to the major 38,000 dalton protein, a relatively large amount of a smaller (31,000 MW) protein (Fig. 6; gel D). This protein (band VIII of the densitometry profiles) was seen in phage preparations which had been carefully purified using the two-phase separation method followed by CsCl equilibrium centrifugation and then stored at 4 C. One plausible explanation for the presence of this 31,000 MW protein is that it is a degradation product of the major protein (band VII). Evidence supporting this hypothesis resulted from the discovery that the major βv tox^+ protein could be extracted in a homogenous state from phage virions using 6 M guanidine hydrochloride and dithiothreitol. The 38,000 dalton phage protein thus isolated from the virion was hydrolyzed with varying amounts of trypsin and the resultant digests were analyzed on SDS-polyacrylamide gels. The primary end-product of this controlled proteolysis was a polypeptide migrating in a position corresponding to a molecular weight of 31,000. This protein was very similar, if not identical, to the band VIII

protein observed in the SDS-gels of some purified βv tox$^+$ phage preparations. It is possible that the diminished capacity of the stored phage preparations to protect KB cells against intoxication is a function of the degradation of the 38,000 dalton, fragment B-like protein associated with the virion. The polypeptide(s) lost as a result of such proteolysis might contain the critical hydrophobic residues thought to be necessary for the binding of fragment B to susceptible cell membranes. Pappenheimer and Gill (1973) have shown that all toxin preparations undergo limited proteolysis from the action of a serine protease present in the crude filtrate which co-purified with the toxin. It is conceivable that this or a related protease co-purifies with the βv tox$^+$ phage and causes partial degradation of the virion e.g., cleaves the 38,000 dalton major phage protein. This hypothesis might be tested by experiments in which the extent of cleavage of the 38,000 MW protein is related to the loss of phage infectivity and/or the ability of βv tox$^+$ phage to protect KB cells against intoxication.

The second set of experiments which attempted to show that the 38,000 dalton phage protein possessed functional properties of fragment B was made possible by the experiments of Uchida, Pappenheimer and Harper (1972). They reconstituted a toxic molecule by mixing trypsinized CRM197 protein (containing functional fragment B and non-functional fragment A) with trypsinized CRM45 protein (possessing functional fragment A and non-functional B fragment). These

experiments were performed to determine if a toxic molecule could be reconstituted by mixing functional fragment A molecules (derived from CRM45 protein) with extracted phage protein ("fragment B"). CRM45 protein was prepared, purified, mildly trypsinized in the presence of DTT and mixed with the extracted phage protein. The resultant hybrid molecules were assayed for toxicity by measuring their ability to inhibit protein synthesis in KB cell cultures. Three separate reconstitution experiments were performed resulting in the formation of molecules that inhibited KB cell protein synthesis 17, 39 and 34% respectively (Tables 3, 4 and 5). The individual control proteins i.e., CRM45 protein and extracted phage protein inhibited protein synthesis from 0 to 9.4%. In one experiment (Table 5), the addition of diphtheria antitoxin to the "reconstituted protein" resulted in the neutralization of 2/3 of its ability to inhibit KB cell protein synthesis. Uchida, Pappenheimer and Harper (1972), using a CRM45-CRM197 mixture estimated the yield of their reconstituted toxin to be between 40 and 80% of the maximum yield possible in terms of the amount of CRM197 protein originally added, which is a high order of reconstitution compared to the CRM45 protein/phage protein system reported here. According to Gill and Pappenheimer (1971), all attempts to reconstitute a toxic molecule using a mixture composed of isolated fragments A and B have been unsuccessful. The most successful reconstitution experiments have involved mixtures of larger

polypeptides i.e., CRM197 (MW 62,000) and CRM45 (MW 45,000) which have been mixed prior to being nicked, then reduced and finally reconstituted. Perhaps efficient reconstitution of toxic molecules is in large part a function of the extent of physical contact between the reacting polypeptide species. Therefore, the greater the degree of association and non-covalent binding between the non-functional segments of the polypeptides, the more likely a toxic molecule will result. Interactions favoring efficient hybridization would be less likely to occur between CRM45 protein (45,000 daltons) and the phage protein (38,000 MW) than between the CRM197 (62,000 MW) and CRM45 (45,000 MW) proteins. The least likely mixture to hybridize, if this hypothesis is valid, is fragment A (24,000 daltons) and fragment B (38,000 MW). This hypothesis is more compatible with the limited experimental data available on reconstituted toxic proteins. An alternative hypothesis to explain the inability to reconstitute a toxic molecule from isolated A and B fragments is that the isolated fragments undergo subtle configurational changes in solution during purification so that they no longer "fit" together in a toxic conformation. Such changes might have occurred during the isolation of the phage protein.

The major 38,000 MW phage protein associated with βv tox^+ phage possessed a functional property of fragment B of toxin in that it appeared to bind to the membrane receptor sites on KB cells and

interacted with fragment A (from CRM45) to form a toxic molecule. Another way of testing whether two proteins are identical or closely related is to determine whether or not they cross-react immunologically. If the 38,000 dalton phage protein is related to the B fragment of toxin, it should react with diphtheria antitoxin, since most of the antibodies in antitoxin are directed against immunodeterminant groups on fragment B (Gill, Pappenheimer and Uchida, 1973). Double immunodiffusion slides were prepared using 0.75% agarose in a phosphate-glycine buffer (Matsuda, Kanei and Yoneda, 1972). The extracted phage protein, dialyzed against the immunodiffusion agar buffer to eliminate salt rings, formed two lines of immune precipitation when reacted against diphtheria antitoxin. The antitoxin was prepared in rabbits injected with formalin-treated, electrophoretically pure diphtheria toxin (courtesy of Dr. R. J. Collier). The phage protein precipitin lines showed partial identity with the precipitin lines of toxin and toxoid in contiguous wells. In addition, guanidine hydrochloride treated, purified βv tox^+ phage also produced two lines against the antitoxin and these lines shared complete identity with the phage protein precipitin lines. Several possible explanations for these double precipitin lines are suggested. The extracted phage protein tends to polymerize as shown in Fig. 8; gel B. This aggregate was shown to have a molecular weight of 77,000 daltons by its mobility in SDS-acrylamide gels (Weber and Osborn, 1969) and could be completely

dissociated by DTT as seen in Fig. 8; gel C. Perhaps the dimeric and monomeric forms of the phage protein precipitate at different positions in immunodiffusion slides. This hypothesis could be tested by carboxymethylating the SH groups on the phage protein, thus preventing disulfide formation. An alternative explanation is based upon the assumption that the fragment B-like phage protein is actually a mixture of two closely related antigenic species. If one assumes that the toxoid antigen contains three immunodeterminant groups on fragment B (a, b and c) and all the sites are fully exposed to the rabbit's immune system, the antiserum will contain antibody molecules specific to each of the three antigenic sites. Perhaps the phage protein contains a mixture of the two protein species; one set of polypeptides contains three immunodeterminant groups, the second set has only two. This latter polypeptide may be shorter; hence the b determinant is missing altogether or the b site may be hidden, thus immunologically "silent" within the molecule. The possibility of a shorter molecular species fits well with the observed tendency of the 38,000 MW phage protein to be degraded to a 31,000 MW polypeptide (Fig. 9; gels B, C and D). It is suggested that the phage protein species containing only a and c immunodeterminant groups diffuses more rapidly than the other polypeptide and the anti-a and anti-c antibodies react with it and a precipitin line is formed. All of the anti-b antibody and some of the anti-a and c antibody molecules diffuse through the zone of precipitation and

react with the slower migrating phage protein containing determinants a, b and c, resulting in a second line of precipitation slightly closer to the antigen well.

Irrespective of the reason for two precipitin lines, it is clear that an immunological relationship exists between βv tox^+ corynebacteriophage and diphtheria toxin because both the intact phage and the extracted phage protein form precipitin lines against antitoxin and both lines show partial identity with toxin.

To determine if fragment B and the extracted phage protein are immunologically related, they were allowed to diffuse against diphtheria antitoxin in a gel immunodiffusion slide. A precipitin line of identity was observed between the phage protein and fragment B. The purified fragment B formed two precipitin lines against antitoxin similar to the double lines observed between phage protein and antitoxin. In the case of fragment B it is likely that double precipitin lines is a function of antigenic polymerization since it contains three SH-groups and is known to aggregate in all but denaturing solvents (Gill and Dinius, 1971).

If fragment B of toxin is intimately associated with βv tox^+ phage, it follows that anti-phage antibody should immunologically cross-react with diphtheria toxin. More specifically, the extracted phage protein would be expected to form a precipitin line of partial identity with toxin when both are allowed to diffuse against anti-βv tox^+ antibody. These predictions were verified by the data shown in

Fig. 16.

Biochemical evidence for a close structural relationship between toxin fragment B and the extracted phage protein was derived from peptide fingerprint analyses of the proteins. Purified fragment B and phage protein preparations were hydrolyzed by trypsin, the resultant peptides dansylated, the dans-peptides were chromatographed in two dimensions on silica gel thin-layer plates and the peptide maps were compared. There were 20 peptide spots on the phage protein chromatogram and 18 spots on the fragment B chromatogram. Michel et al. (1972) reported that fragment B contains 25 lysine and 9 arginine residues. From this, it can be predicted that if fragment B were completely digested and all of the peptides recovered, one should find 34 peptide spots. Matsuda et al. (1972) recovered 31 peptides in their fingerprint map of fragment B. The low peptide yield reported here can be explained by the fact that some dansylated peptides in both preparations remained at the origin of the cation exchange column (see Methods). Presumably then, the missing peptides were not eluted from the resin. Nevertheless, both proteins were subjected to identical treatments and conditions making a comparison between the two meaningful. The peptide fingerprint map of purified toxin fragment B was very similar to that of the extracted phage protein. Sixteen peptides were found to be common to both proteins. The phage protein contained four additional and unique peptides. At least two

explanations are suggested for the additional peptides of the phage protein. In the first place, polypeptides smaller than 10,000 daltons are excluded with the tracking dye in 10% acrylamide gels. Therefore, even though the isolated phage protein preparation migrated as a single band on SDS-gels it may have been contaminated with low amounts of a short polypeptide(s) which would have gone undetected in gels. The second explanation assumes that toxin is a phage structural precursor. In this case the fragment B-like phage component might be cleaved at a position beyond the arginine-rich hinge region of the toxin molecule to include a part of the fragment A portion of the toxin. This would result in a slightly larger polypeptide than fragment B isolated from trypsinized toxin. This alternative is supported by the immunodiffusion slide represented in Fig. 16. The spur at the intersection of the phage protein and toxin lines indicates that there are additional antigenic determinants in the phage protein that are not present in toxin. If one assumes several lysines and/or arginines in this region, several more dansylated peptides would appear on the fingerprint chromatogram.

A trivial explanation could account for all of the preceding results. That is, toxin and/or toxin fragments co-purify with the phage and are adventitiously sticking to the virion. Hence intact phage and phage components would form immunoprecipitin lines with antitoxin and anti-βv tox^+ antibody would react with diphtheria toxin.

The possibility that intact toxin was adhering to the purified phage was ruled out by injecting 1 mg of purified phage intradermally into the skin of a rabbit without resulting in toxin-like necrosis. Matsuda and Barksdale (1967) also found no toxicity associated with their purified βv tox^+ phage using the rabbit skin reaction assay. Fragment A is not non-specifically sticking to the virion because no line of immune precipitation was seen when anti-βv tox^+ antibody was allowed to diffuse against purified fragment A (immunodiffusion slide not shown). Furthermore, 75 μg of purified phage possessed no *in vitro* ADP-ribosylation activity when tested in a system sensitive enough to detect .001 μg of fragment A. The other alternative is that fragment B is sticking to the phage and this possibility will be discussed in more detail later.

On the basis of three independent kinds of evidence, functional, immunological and biochemical, fragment B of diphtheria toxin was shown to be intimately associated with βv tox^+ corynebacteriophage.

Is this protein a *structural component* of βv tox^+ phage? The experimental evidence pertinent to this important question is indirect. It is clear that this polypeptide comprises a very large percentage of the total βv tox^+ phage protein. In three separate purified phage preparations this 38,000 MW protein represented an average of 60 ± 10% of the total phage protein. If one accepts the evidence that the protein in band VIII results from a degradation of the band VII

protein (Fig. 6; gel D), the proportion represented by this protein is raised to 72 ± 7%, a value too high to be accounted for by a protein adventitiously sticking to a phage of this size having a DNA to protein ratio of 1:1.5. Furthermore, the SDS-polyacrylamide gel analyses showed that impure phage (Fig. 5) contained at least 20 protein bands and the major protein co-migrated with fragment B. Upon further purification of the phage, 11 or 12 of the proteins were eliminated from the phage preparations. The amount of the 38,000 dalton protein did not decrease (Fig. 6; gels B and C), suggesting that it does not represent a contaminating protein.

Arguments that favor the proposition that the tox gene is not involved in phage replication are well summarized by Uchida, Pappenheimer and Greany (1973). Prominent among the arguments is the isolation of several phage mutants, each carrying a different mutation in the tox gene (Uchida et al., 1973). One lysogenic mutant in particular, β30, produces an <u>extracellular</u> protein with a molecular weight of 30,000 which possesses enzymatic activity and cross-reacts immunologically with toxin. CRM30 protein therefore appears to contain a normal A fragment and a much smaller fragment B (6,000 daltons). The burst size of β30, when induced by ultraviolet irradiation, was found to be identical to that of wild type β tox^+ phage. Uchida et al. reasoned that since mutations in essential genes are usually lethal, the tox gene must not be involved in any critical way in phage replication.

This would be true if the CRM30 protein was the consequence of a mutation resulting in premature chain termination, however, there is no evidence to support this. As mentioned before, there are as yet no suppressor strains of C. diphtheriae. Uchida, Gill and Pappenheimer (1971) were not able to induce phenotypic suppression by growing lysogens in the presence of 5-fluorouracil or streptomycin. An equally likely interpretation is that the mutagen (nitrosoguanidine) induced one or more point mutations in the fragment B portion of the toxin molecule, rendering it more vulnerable to proteolytic attack. Fragment B is inherently more susceptible to protease activity to begin with (Drazin, Kandel and Collier, 1971). Since the CRM proteins are routinely isolated extracellularly, generally after extended incubation times, indigenous proteases would have ample time to reduce these proteins to polypeptides of constant length. The resultant size of the CRM proteins therefore would be determined by the site or sites of the point mutation(s). In light of this, it may be proposed that the toxin molecule is a structural precursor molecule of βv tox^+ phage. The phage genome would therefore code for a 62,000 MW protein which is ultimately cleaved into a 24,000 MW fragment and a 38,000 MW polypeptide, the latter fragment being incorporated into the phage structure. A corrolary assumption must be made: that is, phage precursor molecules are overproduced and many are not cleaved for phage processing but are released extracellularly by the bacteria (especially when

the cells are iron-depleted) into the medium where they are eventually cleaved to a final MW of 30,000 in the case of $C_7(\beta 30)$ or 45,000 MW in the case of $C_7(\beta 45)$. This hypothesis can be tested. The $\beta 45^{crm+}$ phage can be analyzed on SDS-acrylamide gels to determine whether its major structural protein is related to fragment B of toxin. Preliminary evidence (Elwell and Iglewski, unpublished data) suggests that one of the major protein constituents of $\beta 45^{crm+}$ phage does co-migrate, in SDS-gels, with toxin fragment B. However, the yields of this lysogenic phage are low, making it difficult to isolate and characterize the major phage protein. Dr. J. Murphy (Murphy and Pappenheimer, 1973) has isolated a virulent mutant of this phage which makes analyses of its constituent protein components more feasible.

An alternative possibility is suggested to explain why mutations in the tox gene do not affect βv tox^+ phage maturation and burst size. Perhaps the 38,000 MW phage protein is coded by its own gene, a fragment B-like gene. Therefore mutations of the tox gene, resulting in CRM proteins, leave the phage unaffected. The phage structural B-gene may have evolved as a copy of that part of the toxin gene that codes for toxin fragment B. The fact that fragment B and the phage protein do not have identical peptide fingerprint maps suggests that both the toxin gene and the phage fragment B-like structural gene have undergone separate and apparently "neutral" mutations.

The virulent, toxigenic corynebacteriophage system described in this thesis might also be employed to study potential precursor-product relationships between toxin and phage structural protein. Hosada and Cone (1970) were able to follow precursor protein processing in T4 phage by co-electrophoresing ^{14}C proteins from phage capsids and ^{3}H proteins from phage infected cells over a carefully controlled time sequence. Such an approach would be particularly valuable in the βv $tox^{+}/C_{7}(-)$ system in which toxin and phage are synthesized simultaneously within the host bacterium.

The fact that a bacteriophage contains the genetic information for a 62,000 dalton protein whose only known biological function is to inactivate an enzyme involved in eukaryotic protein synthesis poses an evolutionary dilemma. Pappenheimer and Gill (1973) are of the opinion that the toxin gene was incorporated into the phage genome during a chance association with a eukaryotic cell. Several bi-functional T4 bacteriophage-coded enzymes have recently been reported by Mathews, Collinsworth and Capco (1973) and Mathews, Crosby and Kozloff (1973). Two specific enzymes involved in DNA precursor synthesis, dihydrofolate reductase and thymidylate synthetase, were found to occur as structural elements of the mature T4 virion. These enzymes fulfilled a dual role, in that they were synthesized as early proteins to build phage DNA and then were inserted into the virion as structural components. An ancestral β tox^{+} bacteriophage may have coded for such a bi-functional

enzyme: one portion of the molecule inhibited bacterial protein synthesis (ancestral fragment A) and the other portion (fragment B) eventually being incorporated into the phage. Over the eons the enzymatic half of the molecule could have been slightly modified to the extent that it no longer affects prokaryotic protein synthesis. Fragment B has been retained as a structural component.

The location of the fragment B-like protein on the βv tox^+ virion has not been established. It is not located at the phage attachment site because diphtheria antitoxin does not neutralize corynebacteriophage (Barksdale and Pappenheimer, 1953). Binding of antibody to either the head or tail components does not lead to T4 inactivation, whereas antibody binding to tail fibers (Brenner, Champe, Streisinger and Bernet, 1962) or to the tail plate inactivates phage infectivity (Kozloff, Verses, Lute and Crosby, 1970). Since in a typical diphtheria antitoxin preparation two thirds of the antibody is directed against immunodeterminant groups on fragment B (Gill, Pappenheimer and Uchida, 1973), it follows that antitoxin would neutralize βv tox^+ phage only if the fragment B-like protein was located on or very near the site of phage attachment. A hyperimmune, commercial antitoxin (Behringwerke) was tested for its ability to neutralize βv tox^+ phage infectivity. This antiserum had a K value of .480 min^{-1} (Adams, 1959). Anti-βv tox^+ antibody prepared in our laboratory had a K value of 7.62 min^{-1}. Since the fragment B-like protein does react immunologically with antitoxin the inability of antitoxin to neutralize phage

suggests that the fragment B-like protein is located in a position other than the phage attachment site.

Two different approaches might be used to determine whether this protein is located in the head or the tail of βv tox^+ phage. Cummings et al. (1968) succeeded in fractionating T4 coliphage with DMSO and thereby isolated large amounts of pure heads and pure tails. If the same procedure is applicable to corynebacteriophage, the protein components of phage heads and tails could be separately analyzed on SDS-polyacrylamide gels. An alternate approach to this problem is genetic. Buchwald, Steed-Glaister and Siminovitch (1970) have obtained pure tail and head preparations using headless and tailless mutants of bacteriophage lambda. Singer (1972) has isolated several temperature-sensitive mutants of toxinogenic corynebacteriophage. Some mutants produce only tails at the non-permissive temperature whereas other mutants produce only phage heads. If the tail and head yields from these mutants are sufficiently high, this method might provide a system whereby the location of the fragment B-like protein could be established.

In summary, the 38,000 dalton βv tox^+ phage protein constitutes a major proportion of the protein content of purified phage, some 60-70%. It is functionally related to fragment B of diphtheria toxin, in that a moderately toxic molecule can be reconstituted by the addition of the extracted phage protein (fragment B?) to trypsinized CRM45

protein (a source of functional A fragments). This protein is immunologically related to diphtheria toxin, in that it forms a line of immune precipitation, demonstrating partial identity with toxin, when diffused against antitoxin in gel immunodiffusion slides. Furthermore, purified diphtheria toxin forms a precipitin line, showing partial identity with the major phage protein, when diffused against anti-βv tox^+ antibody. Finally, the 38,000 MW phage protein is biochemically related to fragment B of toxin. The peptide fingerprint maps of the two purified proteins show that 16 (of 20) peptides are common to both polypeptides.

Many questions remain unanswered. Is this phage fragment B-like protein truly a structural component of the virion? If so, where is it located? Is it coded for by the tox gene or a separate, closely related gene?

SUMMARY AND CONCLUSION

A hypervirulent, toxigenic corynebacteriophage was propagated in large volumes and successfully purified using a two-phase, polyethylene glycol-dextran sulfate preliminary separation followed by equilibrium centrifugation in cesium chloride. The βv tox^+ phage thus purified was SDS-treated and analyzed on 10% SDS-polyacrylamide gels. Seven or eight protein bands were evident in the gels of six purified phage preparations so analyzed. The major protein constituent of this phage was a 38,000 dalton polypeptide that co-migrated with fragment B of mildly trypsinized diphtheria toxin. Intact, purified βv tox^+ phage were capable of protecting monolayers of KB cells against subsequent diphtheria intoxication. Presumably, the fragment B-like protein associated with the phage competed with toxin for the binding sites on the KB cell membrane. The major phage protein was extractable from the virion in a relatively pure and homogeneous form by treating purified phage with 6 M guanidine hydrochloride. A moderately toxic "hybrid" molecule could then be reconstituted by the addition of the extracted phage protein (fragment B?) and trypsinized CRM45 protein (source of functional fragment A).

The phage protein and diphtheria toxin were found to be immunologically related. The fragment B-like phage protein formed a line of precipitation, showing partial identity with toxin, when reacted against antitoxin in gel immunodiffusion slides. The phage

protein also formed a precipitin line of complete identity with purified fragment B when both were allowed to diffuse against diphtheria antitoxin. Furthermore, purified diphtheria toxin was found to form a precipitin line, showing partial identity with the major phage protein, when allowed to diffuse against anti-βv tox^+ antibody.

The major phage protein and toxin fragment B are also related biochemically. The peptide fingerprint map of the fragment B-like phage protein was similar to the map of known toxin fragment B. Sixteen out of 20 peptides were common to both polypeptides. The phage protein contained four peptides with no counterparts in the fragment B map.

In summary, a polypeptide that constitutes 60-70% of the total protein of a purified βv tox^+ corynebacteriophage was found to be functionally, immunologically and biochemically related to the B fragment of diphtheria toxin. Whether this phage-associated protein originated in the toxin molecule, being subsequently cleaved and then inserted into the virion or whether it is coded for by a separate but closely related fragment B-like phage structural gene remains to be elucidated. The data that have been presented here raise questions as to the validity of the theory that the tox gene plays no role in the development of βv tox^+ corynebacteriophage.

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