Diphtheria Toxin and Related Proteins

I. ISOLATION AND PROPERTIES OF MUTANT PROTEINS SEROLOGICALLY RELATED TO DIPHTHERIA TOXIN*

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TSUYOSHI UCHIDA,† ALWIN M. PAPPENHEIMER, JR., AND ROBIN GIEBAN

From the Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

SUMMARY

The kinetics of diphtheria toxin production by a mutant of the C7(β) strain of Corynebacterium diphtheriae has been studied. This strain, C7-262(β), produces unusually low yields of toxin, although its β-phage carries a normal tox gene.

The isolation of five strains of β-phage, each carrying a different mutation of its tox gene, is described. Lysogens carrying these mutants as prophages produce altered extracellular proteins (cross-reacting materials), serologically related to toxin, each of which has been isolated in purified form. Two of them, cross-reacting materials 30 and 45, are of lower molecular weight than toxin. Although Fragment A, which contains the enzymically active NH2-terminal region of the molecule, isolated from these mutant proteins could not be distinguished from Fragment A derived from toxin itself, cross-reacting materials 30 and 45 are nontoxic for susceptible animals. The other three mutant proteins are serologically indistinguishable from wild type toxin. Cross-reactive materials 197 and 228 are nontoxic because of amino acid substitutions in the Fragment A portion of the molecule causing loss of enzymic activity. Cross-reacting material 176 contains an altered Fragment A which retains about 8 to 10% of the enzymic activity of the wild type. In vivo, however, cross-reacting material 176 is only 0.2 to 0.4% as toxic as intact toxin.

Recent studies (1-3) have shown that suitably activated preparations of diphtheria toxin inhibit polypeptide chain elongation in eucaryotic cell extracts by catalyzing the splitting of NAD with transfer of its ADPR moiety1 to be linked covalently to EF-2 according to the following equation.

\[
\text{NAD}^+ + \text{EF-2} \rightarrow \text{ADPR-EF-2} \text{(inactive)} + \text{nicotinamide} + \text{H}^+
\]

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† Present address, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.
1 The abbreviations used are: ADPR, adenosine diphosphate ribose; EF-2, peptide chain elongation factor 2; CYE, casamino acid-yeast extract; TYE, tryptose-yeast extract; OD, optical density.

It has been shown that the cytoplasmic EF-2 content of cells and tissues of intoxicated animals decreases approximately in parallel with decreased protein synthesis (4) and in intoxicated HeLa cells ADPR-EF-2 accumulates (2).

Diphtheria toxin is synthesized and released extracellularly by Corynebacterium diphtheriae as a single polypeptide chain of 62,000 daltons (5, 6) containing two disulfide bridges (7). The intact molecule is enzymically inactive. However, it becomes activated following mild treatment with trypsin or other proteases in the presence of certain thiols (8, 9), a procedure which resolves the molecule into Fragments A and B, of 24,000 and 38,000 daltons, respectively. The fragments remain held together by weak interactions. All of the enzymic activity is associated with the A region which contains the NH2-terminal amino acid sequence of the toxin molecule (10).

Only strains of the diphtheria bacillus that are lysogenic for temperate corynephages that carry the tox gene (11-13), or that are infected by virulent mutant phages that carry the same gene (14), synthesize diphtheria toxin. Recent studies strongly suggest that the tox gene carries the structural information for synthesis of the toxin molecule (15, 16). Bacterial strains lysogenized with β-phage carrying a mutated tox gene, produce extracellular cross-reacting proteins that are either completely nontoxic or are of greatly reduced toxicity, but which are serologically related to or identical with toxin (17, 18). Tox gene products may be synthesized during the prophage stage (19-21), during the lytic cycle of phage replication (14, 22) or when the phage genome is present as a nonreplicating exogenote (23). Expression of the tox gene is thus under separate control from that which regulates genes concerned in phage replication. In fact it has long been known that toxin production is dependent on the physiological state of the host bacteria; in particular, toxin is only synthesized in significant amounts by bacteria of limited iron content (24, 25).

The cross-reactive materials produced following lysogenic conversion of C. diphtheriae by phages carrying a mutated tox gene fall into two general classes. The first type contain one or more amino acid substitutions leading to loss or reduction in the NAD:EF-2-ADPR-transferase activity of toxin (17). These alterations are located in the thermostable 24,000-dalton Fragment A portion of the molecule that is unmasked by reduction of intact toxin by treatment with thiols and splitting of a single peptide bond in the loop formed by the disulfide bridge spanning the A and B regions (5, 6, 10). In the second class of cross-reacting materials, enzyme activity is normal and loss of toxicity
is due to alterations in the thermolabile COOH-terminal 38,000-dalton Fragment B portion of the toxin molecule, either by premature chain termination or by an amino acid substitution. Loss of toxicity in these cases is apparently due to inability of the mutant protein to cross the sensitive cell membrane.

In the present paper, we shall describe the isolation and properties of several strains of β-phage with mutations affecting their tox genes. The isolation, purification, and properties of cross-reacting materials produced by the C7 strain of C. diphteriae following lysogenic conversion with each mutant phage will also be described. Finally, evidence bearing on the expression of the tox gene will be discussed.

In the following paper (26) we will present studies on the kinetics of interaction of toxin and of related proteins with the sensitive HeLa cell membrane. In the final paper (27) we will discuss the formation and properties of hybrid molecules formed by interaction of normal and mutant A and B fragments.

**MATERIALS AND METHODS**

**Bacterial Strains**—C7(−), C7(β)tox+, C4(γ)tox−, and PWS (P) were stock cultures. Strains producing mutant proteins related to toxin were prepared by lysogenization of C7(−) with the appropriate mutant phage.

**Phage Stocks**—β- and γ-phage were prepared from ultraviolet-induced C7(β) and C4(γ) in the usual manner (13). Phage βc was a clear mutant of β. Stocks of coliphage λ and λb, provided by Dr. Nancy Hopkins. Phages carrying mutations in their structural gene were prepared as will be described below.

**Culture Media**—For plaque assay, we used a 1.5% agar base containing 10 g of tryptose, 5 g of yeast extract, and 5 g of NaCl per liter, with a soft agar overlay of PT medium (21) containing 30 g of casamino acids (Difco), 100 mg of N-tryptophan and 5 g of agar per liter. Deferred CYE medium and deferred TYE medium were prepared as previously described (18). The former was supplemented with 100 mg of L-tryptophan per liter.

Bacterial growth was followed by measuring the optical density at 590 nm in a Bausch and Lomb Spectronic-20. One OD unit was considered equivalent to about 109 bacteria per ml.

**Reagents**—N-Methyl-N'-nitro-N-nitrosoguanidine and phenylmethylsulfonylfluoride were obtained from Aldrich Chemical Co. Crystalline trypsin and soybean trypsin inhibitor were purchased from Sigma Chemical Co. Cesium chloride was “radio-tracer” grade from Harshaw Chemical Co. Na23HPO4 was obtained from New England Nuclear Corp. [U-14C]Adenine-labeled NAD (40 μCi per μ mole) was generously supplied by Dr. D. Michael Gill. Polyethylene glycol 20M (Carbowax) was purchased from Union Carbide Chemicals. The dry material was dialyzed against buffer before use.

**Bacterial Mutants of Low Toxigenicity**—Exponentially growing cultures of C7(β)tox− were centrifuged and washed once with sterile 0.05 M Tris-maleate buffer at pH 6 (28). The washed bacteria were resuspended in the same buffer to about 7 × 108 organisms per ml and treated for 15 min at 35° with 30 μg of N-methyl-N'-nitro-N-nitrosoguanidine per ml. The surviving bacteria (about 0.3%) were then plated on TYE agar. The following day, individual colonies were picked and inoculated into small tubes containing 0.6 ml of CYE medium supplemented with 2.5% deferrated maltose. After overnight growth at 35° on a rotary shaker (220 rpm), the cultures were diluted into fresh medium (one loopful per ml) and screened for toxicity by intradermal injection into rabbits (29). Out of more than 500 bacterial colonies tested in this way, a few appeared as nontoxin producers in the preliminary screening. However, on further cultivation under conditions optimal for toxin production, none proved to be completely nontoxinogenic, although in some cases the yield of toxin per unit of cell growth was very low.

**Isolation of Phage Mutants with Altered Toxin Gene**—Cells from cultures of C7(β) in exponential growth were collected and washed to remove free phage with 0.02 M Tris buffer, pH 7.4, containing 3 M MgCl2 and 0.1 M NaCl. The organisms were resuspended in the same buffer to approximately 5 × 109 cells per ml and induced by irradiation with an ultraviolet dose sufficient to kill about 90% of the cells. They were then diluted 10-fold in PT medium containing 2.5% maltose. After 15 min at 35°, 60 μg per ml of N-methyl-N'-nitro-N-nitrosoguanidine were added and the incubation was continued with shaking for a further 150 to 210 min. At this time the burst had occurred and the plaque titer was 0.3 to 0.0% of that expected for nonmutagenized cultures. The surviving phage from the bacteria-free supernatant were plated on C7(−) and, on the following day, resistant bacteria were picked with a sterile needle from the center of turbid plaques and spotted on fresh deferrated TYE agar plates. After overnight growth, the colonies were picked to 0.6 ml of CYE medium in small tubes. They were incubated overnight and tested for toxigeny by rabbit skin test.

Strains giving negative skin tests were restreaked and the isolated colonies were tested for toxigeny by rabbit intradermal test. Out of more than 500 plaques screened in this way, five lysogenized strains of C7 were isolated which consistently gave negative skin reactions. These strains were then grown under conditions optimal for toxin production (18) and 0.5-ml aliquots of bacteria-free culture filtrates were injected into guinea pigs. Four of the strains, C7(β30), C7(β45), C7(-228), C7(-228), showed no toxicity. The fifth strain, C7(-2176), produced a protein of greatly reduced toxicity per unit weight.

**Production and Purification of Toxin and Related Mutant Proteins**—Stock cultures of C7(β) and of the C7 strains lysogenized with the five mutant phages described in the preceding section, were grown at 34° with shaking in 3-liter lots of deferrated CYE medium with 2.5% iron-free maltose, as previously described (18). The optimal salt concentration for elution from DE 52 columns differed slightly for each mutant protein. Further purification of the various mutant proteins was accomplished by ion exchange chromatography and gel filtration as previously described (18). In order to minimize proteolytic degradation, cultures were harvested before growth had completely stopped, at a time (usually 15 hours or less) when the OD660 had reached 12 to 15. The bacteria were removed immediately by centrifugation and solid ammonium sulfate was added to 0.75 saturation (cross-reacting materials 30 and 45) or 0.65 saturation for cross-reacting materials 176, 197, 228, or for toxin itself.

Further purification of the various mutant proteins was accomplished by ion exchange chromatography and gel filtration as previously described (18). The optimal salt concentration for elution from DE 52 columns differed slightly for each mutant protein. Provided care was taken to work rapidly and at low temperatures, proteolysis was negligible and the final product in every case showed a single major component and only traces of minor components on sodium dodecyl sulfate gel electrophore-

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2 The CYE medium previously described contained no added tryptophan. We have since found that some lots of yeast extract are deficient in this amino acid which is essential for growth of C7 strains.

3 When the number of survivors fell below 0.3% we were unable to find any phage with an altered toxin gene.
Toxicity—Toxicity of the various preparations was calculated from the time of death following intraperitoneal injection into 250 ± 25 g guinea pigs, using the standard dose-response curve for intravenous injections of toxin of Baseman et al. (4). The kinetics of toxin production by low-toxin-producing strains was followed with a radioimmunoassay method recently described by Gill et al. (23).

Enzyme Activity in Culture Filtrates—Before estimation of NAD:EF-2-ADPR-transferase activity, any NAD present was removed by preliminary dialysis of the bacteria-free culture filtrate against 0.01 M sodium phosphate, pH 7.2, for 20 hours at 0°. Bovine serum albumin, 2 mg per ml, was added as stabilizing agent. Before assay the NAD-free dialyzed toxin or mutant proteins were diluted with an equal volume of phosphate buffer and were activated by treatment of 0.5-ml aliquots with 5 μl of trypsin solution containing either 2, 5, or 10 μg per ml of enzyme in the presence of 10 mM dithiothreitol at 37° for 10 min, at which time the reaction was stopped by addition of 2 μl of soybean trypsin inhibitor (25 mg per ml). Assay for NAD:EF-2-ADPR-transferase activity was then carried out on 2- and 5-μl samples. The method of Gill and Pappenheimer (8) was used for preparation of EF-2 and for the assay itself. Three different amounts of trypsin were tested because the optimal amount of trypsin required varied somewhat for each mutant protein tested.

Extracts from sodium dodecyl sulfate polyacrylamide gel slices (8) were assayed for enzymic activity directly without preliminary activation with trypsin.

RESULTS

Mutations in C7 Affecting Toxin Yield

When this work was first undertaken, no lysogenic diphteriaal strain producing an altered toxin molecule was known. It was still not known whether the toxin structural gene was carried by the bacterial host or by the phage genome. In attempting to isolate a strain producing an altered toxin molecule we first screened more than 500 N-methyl-N′-nitro-N-nitrosoguanidine-induced mutants of C7(β). Not one of these mutant strains failed to show some toxin production when tested under suitable conditions, although as others had already observed, mutations affecting the yield of toxin were not infrequent. As an example, we may consider the mutant strain C7-262(β). This strain forms colonies that are smaller than wild type on TYE agar and bacterial suspensions tested for toxigeny in rabbits caused minimal skin lesions. Fig. 1 shows that strain 262 produced only 10 to 12% as much toxin as did the C7(β) strain from which it was derived. The figure also suggests that the tox gene carried by the bacterial strain 262 was unaltered. At any rate, when C7(−) was lysogenized with the phage liberated following ultraviolet irradiation of strain 262, the kinetic data of toxin production by the resulting lysogenic C7(262) were indistinguishable from that of C7(β) itself. It is clear from this experiment that a mutation in the host genome may profoundly affect expression of the β-phage tox gene.

Mutations in β-Genome Affecting Toxin Structural Gene

Several hundred strains of C7, lysogenized with mutagenized β-phages, were screened for toxigeny by rabbit skin test. Of the strains tested, four, C7(β30), C7(β45), C7(β197), C7(β228), proved to be completely nontoxic and the fifth, C7(β176), was one of greatly reduced toxigenicity. Each of these lysogenic strains carries a modified toxin structural gene in its prophage, as evidenced by the fact that each directs the synthesis of a different and distinctive extracellular protein that is serologically related to toxin. The five mutant proteins, cross-reacting materials 30, 45, 176, 197, and 228, have each been isolated and purified.

Table I

<table>
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<tr>
<th>Protein</th>
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<th>Toxicity</th>
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<th>Blocking activity</th>
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<td>0</td>
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<td>0</td>
<td>100</td>
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</tr>
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<td>10</td>
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<tr>
<td>CRM197</td>
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<td>0</td>
<td>100</td>
<td></td>
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<tr>
<td>CRM228</td>
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<td>0</td>
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<td>Fragment A</td>
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<td>0</td>
<td>100</td>
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</table>

a Guinea pig minimum lethal dose per μg. The minimum lethal dose is defined as that amount of toxin required to kill a 250-g guinea pig on the 4th or 5th day after subcutaneous injection.

b NAD:EF-2-ADPR-transferase activity per mole after activation by trypsin and reduction (8) relative to Fragment A taken as 100.

c Ability to inhibit competitively the action of toxin on HeLa cells. The blocking activity of cross-reacting material 197 is taken as 100 (27).

d CRM, cross-reacting material.

Fig. 1. Growth and toxin production by C7-262(β), C7(262), and C7(β). Growing cultures (25 ml) of C7(β)(O), C7-262(β)( ), and C7(262)(X) were centrifuged and the cells resuspended in approximately 12 ml of CYE medium containing 2.5% deferrated albumin, 1 OD at 490 nm = 2.5, and placed in 125-ml Erlenmeyer flasks. The flasks were incubated at 36° with rotary shaking. At intervals samples were taken for determination of OD and for flocculation titer by radioimmunoassay. ——, growth; ——, toxin.
cross-reacting material 45 (mol wt = 45,000) with trypsin in the presence of a thiol, yielded enzymically active 24,000-dalton Fragments A30 or A45 which appeared to be identical with Fragment A released by similar treatment of toxin itself. On sodium dodecyl sulfate gels in the presence of a thiol, nicked cross-reacting material 45 showed an inactive 21,000-dalton B component (B45) in addition to Fragment A. Under the same conditions the 9,000-dalton B component presumably liberated from nicked cross-reacting material 30 either moved too fast to be detected or had been further degraded. In contrast to intact toxin which has little or no enzymic activity (8, 9), both cross-reacting materials 30 and 45 show considerable enzymic activity even without nicking. The fact that both Fragment A30 and A45 show full enzymic activity suggests that Fragment A, which contains the NH2 terminus of the toxin molecule. Strong supporting evidence that this is indeed the case comes from the recent work of Michel et al. (10) who have demonstrated an NH2-terminal alanine in both Fragment A and in intact toxin. Cross-reacting materials 30 and 45 contain only a single disulfide bridge; the second disulfide present in toxin itself must therefore be located in the COOH-terminal 17,000-dalton amino acid sequence (15). The fact that cross-reacting materials 30 and 45 are released into the extracellular medium in amounts comparable to toxin, shows that an intact COOH-terminal B fragment is not needed for their secretion.

While cross-reacting material 45 could be purified without difficulty, cross-reacting material 30 appears to be more sensitive to degradation by proteolysis during purification. Sodium dodecyl sulfate gels of purified cross-reacting material 30, even in the absence of a thiol, invariably show considerable amounts of free Fragment A (24,000 daltons) and of products of molecular weight intermediate between Fragment A and 30,000 daltons.

Proteins of Reduced Enzymic Activity—Purified cross-reacting materials 176, 197, and 228 form lines of identity with toxin when tested against antitoxin by immunodiffusion (8). Moreover, the three mutant proteins are indistinguishable from native toxin on sodium dodecyl sulfate gels. These three cross-reacting materials all have the same molecular weight as toxin, therefore, and their low toxicity is presumably the result of one or more missense mutations. The amino acid substitutions causing loss of enzymic activity. We conclude that the production of mutant proteins is under the same iron-dependent control as is toxin.

It can also be seen from Fig. 2 that compared to the corresponding A and B bands of the other three proteins, B228 is weak relative to A228 and at least two new bands appear that move faster than Fragment A. The possibility that B228 might be more sensitive to trypsin than is normal Fragment B was supported by the following experiment. Toxin, cross-reacting materials 176, 197, and 228 were each treated with trypsin and dithiothreitol as described in Fig. 2; soybean inhibitor was then added and the dithiothreitol removed by dialysis. The flocculation titers with antitoxin of treated toxin, cross-reacting materials 176 and 197 remained almost constant at 80 to 100% of their initial values, whereas that of cross-reacting material 228 dropped to about 50% its initial titer. In the two following papers (26, 27), it will be shown that Fragments B176 and B197 do not differ functionally from wild type B since they can interact with Fragment A from cross-reacting material 45 to yield a fully toxic hybrid molecule.

Cross-reacting material 228 appears to be a double mutant. Not only does A228 fail to show enzymic activity, but cross-reacting material 228 is only 15 to 20% as effective as cross-reacting material 197 in competing with toxin for entry sites on the HeLa cell membrane and the hybrid molecule formed with A45 and B228 is only about 15% as toxic as would be expected if it contained a normal Fragment B (27).

Effect of Iron on Cross-Reacting Material Production

In order to determine whether the biosynthesis of prematurely terminated toxin molecules is subject to the same control by iron as is toxin, we have followed over a 2.5-hour period extra cellular release of NAD:EF-2-ADPR-transferase activity in cultures of C7(2), C7(230), and C7(245) under conditions optimal for toxin production. Fig. 3 shows that the rate of growth and of enzyme production was the same for all three strains within limits of experimental error. Enzymic activity was measured on dialyzed aliquots that had been activated with trypsin in the presence of a thiol. In each case, addition of 2 μg of Fe(II) per ml to the cultures completely arrested enzyme synthesis within a matter of minutes. Although not shown in Fig. 3, the production of toxin by C7(2) was also followed by radioimmunossay. The amount of toxin produced at each time interval measured by this method closely paralleled the enzymic activity. We conclude that the production of mutant proteins is under the same iron-dependent control as is toxin.
FIG. 3. Cross-reacting material production by lysogenic C7 strains. Fifty milliliters of growing cultures of C7(β), C7(β30), and C7(β45), OD590 nm = 3.6, were removed from the incubator and centrifuged at 8000 rpm for 7 min. The cells from each culture were resuspended in 25 ml of CYE medium containing 2.5% deferrated maltose and placed in 250-ml Erlenmeyer flasks. After 60 min at 36° in the shaking water bath, 1.5-ml aliquots were removed from each culture and placed in test tubes, each containing 15 µg of FeSO₄·7H₂O. The tubes were incubated a further 90 min with rotary shaking and then tested for growth (OD₆₀₀), and for enzyme activity after dialysis of the bacteria-free supernatant to remove NAD. Samples were also removed from the flasks, at intervals, for determination of growth and enzyme activity.

We also conclude from this experiment that COOH-terminal amino acid sequences of toxin are not essential for its extracellular release.

Buoyant Density of Phages Carrying Altered Toxin Gene

Fig. 4 shows the distribution of phages β, β45, and γ in a CsCl gradient. β (buoyant density = 1.4466) could not be distinguished from either β45 or β30 by density gradient centrifugation. Thus, if either β30 or β45 contains a deletion, it is probably less than 2% of the β-genome. The serologically related nontoxigenic phage γ, which may possibly contain a deletion in the lox region (30) is slightly heavier than β with buoyant density = 1.470. In the experiment shown in Fig. 4, the peak titer for the coliphage marker λ2 (density = 1.491) which carries an 18% deletion, was collected nine tubes after the X+ peak (density = 1.508).

Kinetics of Phage Multiplication

Cultures of C7(β), C7(β30), C7(β45), and C7(β197) were irradiated with ultraviolet and the kinetic data of phage release were followed at 35° under identical conditions. Fig. 5 shows that in every case the titer of free phage began to increase sharply 120 min after irradiation. The burst size was 70 to 80 and the one-step growth curves obtained were superposable. C7(β176) and C7(β228), although not shown in the figure, behaved similarly. We conclude that the toxin gene plays no essential role in phage multiplication under ordinary laboratory conditions.

Superinfection Experiments with Mutant Strains

We reported previously that when C7(β45) was superinfected with βvir, both cross-reacting material 45 and toxin were synthe-
The present studies confirm our earlier report (15) that there is a single structural gene carried on the \( \beta \) phage genome that directs the synthesis of the diphtheria toxin polypeptide chain. That a single viral gene is involved was confirmed by superinfection experiments showing that cross-reacting materials 45 and 197 do not reassemble to yield toxin in vivo even when both are produced within the same cell.

Of some 500 \( C7 \) strains lysogenized with \( N \)-methyl-\( N' \)-nitro-\( N \)-nitrosoguanidine-mutagenized \( \beta \)-phages, five were isolated that produced cross-reacting materials due to alterations in their \( t\)ox gene. The high frequency of mutated \( t\)ox genes of altered function among the surviving phage is easily explained. (a) The \( \beta \)-phage chromosome is relatively small and contains about 2.2 \( \times \) 10^6 dalton double stranded DNA, or sufficient to code for only 25 to 30 proteins. (b) The \( t\)ox gene thus represents about 5% of the entire phage genome.

The experiments on the rate of cross-reacting materials 30 and 49 production and extracellular release show that the biosynthesis of these mutant proteins is under the same regulatory control by iron as is toxin. Just as in the case of toxin synthesis by \( C7(\beta +) \), cross-reacting materials 30 and 49 are only produced by \( C7(\beta 330) \) or \( C7(\beta 45) \) bacteria of low iron content and cross-reacting material synthesis stops within minutes after addition of only 2 \( \mu \)g per ml of Fe(II). On the other hand, excess iron does not appear to affect the expression of other phage genes and maximal yields of \( \beta \)-phage are obtained from induced cultures growing at iron concentrations sufficient to suppress all but traces of toxin synthesis. It is clear that host metabolism is involved in regulating expression of the \( t\)ox gene. It is equally clear that the \( t\)ox gene can be expressed in the absence of phage multiplication, and is therefore under separate control from the systems regulating expression of other phage genes. This is true when the phage is present as a superinfecting, nonreplicating exogenote (23) or when in the prophage state and integrated with the bacterial host genome (19, 20).

The fact that toxin is only produced by bacteria in which iron becomes limiting and the fact that yield of \( t\)ox gene product is determined by the bacterial host could be explained if \( t\)ox gene expression were regulated by a bacterial iron-containing protein. Were such a host protein to lose its iron, it might be expected to undergo a conformational change permitting the \( t\)ox gene to be expressed. Certain recent observations are consistent with such an hypothesis. Thus bacterial mutants such as \( C7-201 \) which produce low yields of toxin when lysogenized with \( \beta^* \) are encountered frequently. It may be significant that most bacterial mutants lysogenic for wild type \( \beta \) but producing low yields of \( t\)ox gene product, grow poorly relative to normal strains on dehydrated agar (31). On the other hand, despite a good deal of effort, phage mutations leading to insensitivity of \( t\)ox gene expression to inhibition by iron have not yet been found and would thus appear to be rare. Finally, studies now in progress by Murphy (32) have shown that \( \beta \)-phage proteins, including \( t\)ox gene products, may be synthesized in good yield in vivo by \( E. \) coli extracts prepared according to Zubay et al. (33) and supplemented with purified \( \beta \)-phage DNA. Iron has no inhibitory

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**Fig. 6.** Sodium dodecyl sulfate gel patterns of proteins from crude culture filtrates of \( C7(\beta 197) \) and of \( C7(\beta 197) \) superinfected with \( \beta 45 \). To 25 ml of culture of \( C7(\beta 197) \) in exponential growth (OD = 2.6) was added 1.5 ml of chloramphenicol (2 mg per ml) and 0.01 M sodium phosphate, pH 7. The final solution contained all of the genetic information needed for synthesis of tox gene product, cross-reacting material 45, is a chain-terminating protein containing a normal Fragment A. 

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\( J. S. \) Wolfson and D. S. Dressler, personal communication.
effect whatever in this system contrary to what might be expected
if iron acted directly on the phage genome or on a phage gene
product.

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    bor, New York
Diphtheria Toxin and Related Proteins: I. ISOLATION AND PROPERTIES OF MUTANT PROTEINS SEROLOGICALLY RELATED TO DIPHTHERIA TOXIN

Tsuyoshi Uchida, Alwin M. Pappenheimer, Jr. and Robin Greany


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