

EVIDENCE FOR THE INDUCED NATURE OF THE CHANGE FROM NONTOXIGENICITY TO TOXIGENICITY IN *CORYNEBACTERIUM DIPHTHERIAE* AS A RESULT OF EXPOSURE TO SPECIFIC BACTERIOPHAGE¹

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It was reported by Freeman (1951) and Freeman and Morse (1952) that exposure of certain nontoxigenic strains of *Corynebacterium diphtheriae* to specific bacteriophage resulted in the appearance of toxigenic *C. diphtheriae* which were resistant to the bacteriophage. It was established further by Freeman (1951) that all toxigenic cells isolated from the resistant growth were lysogenic, carrying a phage which was capable of lysing the parent, nontoxin producing strain. Although the present work was undertaken to examine the kinetics of the population changes occurring in such a system, it has shed considerable light on the mechanism of the change from the nontoxigenic to the toxigenic state. Two possible explanations were advanced to account for the phenomenon (Freeman, 1951; Freeman and Morse, 1952). They were (1) the spontaneous development of toxigenic mutants and subsequent selection by phage, and (2) the alteration of bacterial metabolism as a result of the formation of the lysogenic complex. The possibility that the change was induced by an agent other than phage must be considered also. The evidence to be presented is best interpreted on the basis of an induced change, and the concurrence of lysogenicity and toxigenicity in the newly formed toxigenic cells suggests a significant role for phage.

MATERIALS AND METHODS

Bacterial cultures. Strain 444 of nontoxigenic *C. diphtheriae* (Freeman, 1951) was used throughout the study. This parent, nontoxigenic strain will be referred to as 444A and the lysogenic toxin producing strains derived from it as 444V. Strain 444A-1, to be described later, also was used in host range tests. Stock cultures were maintained on Loeffler's medium and were trans-

ferred monthly. All cultures were incubated at 34 C.

Bacteriophage. The phage used in the work to be described was designated 444V/A. It was produced originally by lysogenic strain 444V and propagated on strain 444A. Purification was accomplished by five successive single plaque isolations on 444A. In all probability phage 444V/A is identical with phage B described by Freeman (1951). It was derived from a lysogenic strain originating during an infection by phage B. All stock phage suspensions were checked for sterility just prior to their use.

Media. The *in vitro* agar medium (King *et al.*, 1950) was used for virulence testing of *C. diphtheriae* with the concentration of NaCl increased to 0.5 per cent as suggested by Hook and Parsons (1951). Pooled sheep serum stored frozen until use was added as the required supplement. Once thawed it was stored at 4 C. In all other experiments heart infusion broth (Difco) was used for both broth and solid media and as a diluent. The final pH of the media was 7.2 to 7.4. Difco agar was used where necessary.

Antitoxin. Commercial antitoxin (Lederle) was used throughout.

Methods employed with the phage-host system. High titer phage stocks were prepared in aerated broth cultures. Phage was assayed by the agar layer method and phage antiserum by an activity neutralization test. The techniques employed have been described by Adams (1950). Conditions for the quantitative determination of a closely related diphtheria phage were reported previously (Groman and Lockart, 1953) and were employed in the current work.

Bacterial counts. The pour plate method was employed for bacterial counts. For consistent results it was found advisable to add the aliquot of bacteria to the tube of media prior to pouring the plate. Since clumping is characteristic of the

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growth of *C. diphtheriae*, the plate counts represent clump counts.

Differentiation of nontoxin and toxin producing colonies. A means of differentiating nontoxin from toxin producing cells was required in order to follow population changes. Gross differences in nutrition between the nontoxic and toxic strains were not observed nor were differences in colonial or microscopic morphology (Groman, 1953a). Serological identity of the two strains (Freeman and Morse, 1952) precluded use of this characteristic for differentiation. Large scale screening for toxigenicity by the *in vitro* streak plate test was not feasible and would have necessitated a second and possibly a third transfer of the sampled colonies. In view of the nature of the phenomenon being studied this was not considered desirable.

An observation made by Ouchterlony (1949) suggested a method of differentiation which overcame the basic handicaps of the streak plate method. He observed that when a mixed culture of virulent and avirulent cells was plated on an agar medium containing the proper concentration of antitoxin only the virulent cells produced colonies encircled by a halo of toxin-antitoxin precipitate. Guinea pig tests were confirmatory. Similar results with respect to halo formation and toxigenicity were obtained when mixtures of strains 444A and 444V, used in the present study, were plated on *in vitro* medium containing the proper concentration of antitoxin. The *in vitro* medium plus antitoxin was adopted as the differential medium for distinguishing between nontoxin and toxin producing colonies.² Final antitoxin concentrations were generally in the range of 1 to 4 flocculating (Lf) units per ml, and the amount of serum supplement varied from 0.5 to 2.0 ml per tube of medium. Excessive concentrations of serum inhibited colonial development. Best results were obtained if the plates were dried from 3 to 5 hours to overnight at 34 C prior to use.

To test the complete medium a 0.1 ml aliquot of strain 444V containing between 50 and 75 bacteria was spread over the surface of a plate and incubated. With a properly adjusted system it was possible to observe halos around all of

² The terms haloed and unhaloed will be used interchangeably with toxigenic and nontoxic when referring to colonies of *C. diphtheriae* on the differential medium.

the colonies within 36 hours. If the plates were held overnight at room temperature, the intensity of the halos was increased materially. Halos were observed most easily if the plate was held close to a shaded light bulb and viewed against a dark background.

It is assumed in using strain 444V as a standard for the test system that a majority of the toxic cells which appear will have the same level of toxin production as cells of strain 444V. That this assumption is reasonably true is indicated by analyses of the phage resistant population. Only a small percentage of the resistant cells did not produce halos. The data are discussed in a later section.

Testing for lysogenicity and host range. The spotting technique of Fisk (1942) was tried first. The results obtained with this method were inconsistent since surface lysis produced by low concentrations of phage 444V/A was faint at best and more often indeterminate.

The following technique, found much more reliable, was adopted for routine use. It was based on the observation that easily discernible plaques are formed in the overlay when plaque counts are made. An overnight broth culture of each strain to be tested for lysogenicity was prepared. The cells were removed by centrifugation, and the supernatant fluid was tested for phage as follows. Plates containing 20 ml of heart infusion agar (1.5 per cent) were prepared. A dozen wells were punched out of the agar with the open end of a sterile culture tube, and the cut out discs were removed. A drop of each supernate was placed in a separate well, and after all the wells had been seeded, 5 or 6 drops of a bacterial indicator mixture were added to each well. Indicator mixture for one plate was prepared by adding 0.5 ml of bacteria (strain 444A) at a concentration of approximately 1×10^8 per ml to 4 ml of melted heart infusion agar (0.7 per cent). As a control the supernate of a known lysogenic strain was used. After hardening, the plates were incubated overnight and plaques could be discerned easily with the lysogenic strains. Occasionally complete clearing occurred and in order to avoid confusing this with a lack of growth, a control well with indicator strain alone was included.

The "well" method was used also for host range tests. In this case 2 to 3 drops of phage and 5 to 6 drops of each of the various host strains sus-

pended in 0.7 per cent heart infusion agar were added to separate wells. Lysis could be observed after overnight incubation, and controls of the indicator strains were included for comparative purposes.

Phage sensitivity. One-tenth ml of high titer phage (10^8 to 10^9 per ml) was spread over the surface of a heart infusion agar (1.5 per cent) plate. The plates were dried in the incubator for 15 to 30 minutes with lids tipped. A single loopful of the bacterial strain to be tested was spotted on the surface and the liquid allowed to adsorb. After overnight incubation lysis was observed. Approximately 20 cultures could be tested per plate in this manner. Control strains of *C. diphtheriae* were included on each plate in order to verify the activity of the phage suspension and to indicate the degree of lysis to be expected.

Design of the experiment for determining population changes in phage and bacteria. The following experiment was designed to provide a description of the changes occurring from the time that strain 444A of *C. diphtheriae* and the phage 444V/A were mixed until the time resistant growth reached substantial concentrations. Heart infusion broth was inoculated with an overnight culture of 444A and phage 444V/A to a final concentration of from $1-5 \times 10^8$ bacterial per ml and 5×10^6 to 5×10^7 phage per ml. Duplicate 50 ml samples of the mixture were pipetted into 200 ml bottles and placed on a reciprocating shaker kept in the incubator. The bottles were slanted to ensure adequate aeration. A control bottle without bacteriophage was incubated concurrently in order to provide visual evidence of the relative stage of development of the experimental flasks. From seven to nine 0.5 ml samples were removed from each flask during the course of the experiment.

The individual sample was handled in the following manner. An aliquot was diluted immediately with broth containing phage antiserum at a concentration sufficient to inactivate 90 per cent of the phage in ten minutes. During the inactivation period, tests for host range activity of the phage were performed with strains 444A, 444A-1, and 444V. A total phage count was performed on every other sample using strain 444A as the indicator strain. After ten minutes the phage inactivated suspension was diluted further and plated for total bacterial count. The dilutions were refrigerated for 24 to 36 hours until the

plate count was determined. An aliquot of the proper dilution then was plated on the differential medium. It was convenient to wait for the plate count since during much of the experiment there was no measurable turbidity by which to judge bacterial concentrations. In control experiments no detectable change in total bacterial count or in the relative numbers of toxigenic and nontoxigenic colonies occurred over a 2 to 3 day period of refrigeration. Prior to plating on the differential medium slight adjustments in bacterial concentration were always necessary. In order to decrease interference further by phage on the plates, this dilution was made as above with media containing phage antiserum.

After approximately 36 to 48 hours of incubation colonial growth and halo production on the differential medium were advanced sufficiently for the plates to be examined. The total colony count of each plate was recorded. A significant proportion of the colonies showed evidence of "nibbling", an effect interpreted as being due to phage contamination. As a result a distribution count of nontoxigenic intact, nontoxigenic contaminated, toxigenic intact, and toxigenic contaminated colonies was made. Concern about the numbers of contaminated colonies was centered about the interpretation of their origin, in particular the origin of contaminated haloed colonies. This problem will be discussed later.

Following these distribution counts a number of representative colonies from each sample were restreaked on heart infusion agar in order to obtain clones free from external phage. Colonial isolates were picked to heart infusion broth and incubated overnight. The following day these cultures were checked for their sensitivity to the original phage preparation and for lysogenicity. Thus, the information collected for each colony included toxin producing ability, phage sensitivity, and lysogenicity.

RESULTS

Bacterial population. The results of a typical experiment are presented in figure 1. They represent changes that occurred over a period of approximately 24 hours. Data from the duplicate flasks are plotted continuously. During the first 5 to 7 hours of incubation, over 90 per cent of the cells of strain 444A were lysed by phage 444V/A. Within only $1\frac{1}{2}$ hours after the start of the experiment the presence of toxigenic, lyso-

genic cells (strain 444V) was revealed. Protected by lysogenicity from further phage attack the toxigenic cells reproduced unhampered and became established as the major population component. In this and other experiments the original avirulent phage susceptible population was never destroyed completely and following the initial period of lysis continued to comprise from 2 to 15 per cent of the total population. This observation concurs with that made by Freeman (1951) in which he reported that after overnight incubation of unshaken cultures of avirulent bacteria and phage some 20 per cent of the cells present were still of the original phage susceptible type.

The absence of nontoxic, nonlysogenic, phage resistant mutants from the final population deserves comment since one might anticipate growth competition between such mutants, if present, and the lysogenic strain. Such a strain (444A-1) was isolated following lysis of strain 444A with a phage closely related to phage 444V/A. It is interesting to note that phage 444V/A as originally released from the lysogenic strain has a limited lytic activity against strain 444A-1. If a mutant similar to 444A-1 does arise concurrently with the lysogenic strain, then the lysogenic strain has an obvious selective advantage.

Phage population. During the experimental period the total phage population increased, and as expected the first significant rise in titer corresponded to mass lysis of the nontoxic cells. The factors underlying the behavior of the phage population during the remainder of the experimental period are not known. The drop in phage titer following initial lysis may be due to inactivation or adsorption of phage by the cellular debris. The subsequent increase can be attributed partially to continued lysis of some of the remaining susceptible cells and may be aided in part by lysis of some of the lysogenic cells. Insofar as could be determined by host range activity the type of phage produced throughout the experiment remained the same.

Correlation of characteristics. The distribution of colony types (parts B and C figure 1) is based on observation of 1,840 colonies on the differential medium. An average of 153 colonies was observed per sample, and no less than 95 colonies per sample was checked for toxigenicity. A total of 76 colonies representing the various types present at each sample interval was checked further for

lysogenicity and susceptibility to the original phage. In a second experiment with results of an identical nature some 2,082 colonies were observed on the differential medium and 71 representative colonies were checked for lysogenicity and phage susceptibility. Combined results of

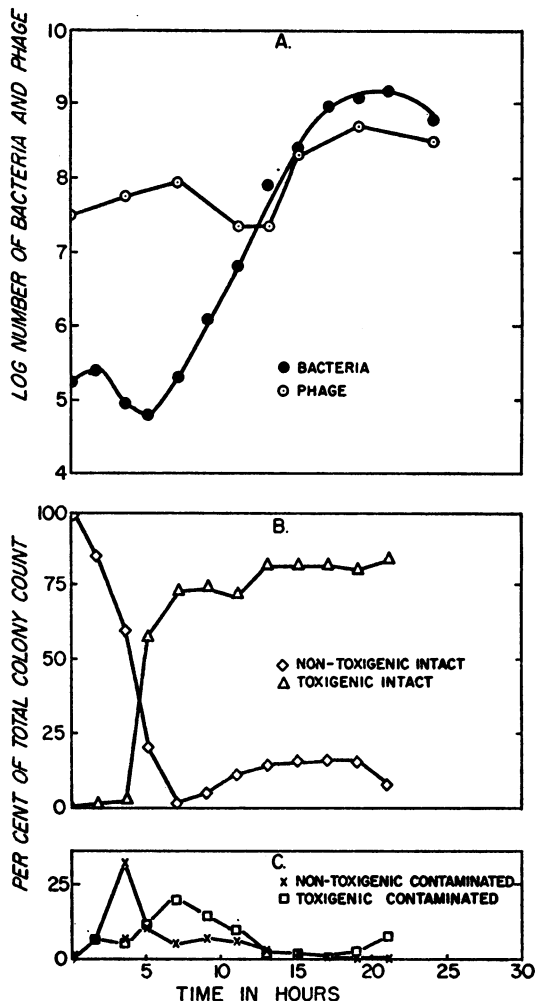


Figure 1. Changes which occurred during the incubation of *Corynebacterium diphtheriae*, strain 444A, with bacteriophage 444V/A.

these two experiments show that halo formation was correlated with lysogenicity and phage resistance in over 94 per cent of the colonies whether the colony was intact or contaminated. In 93 per cent of the intact and the contaminated colonies having no halo, lysogenicity was not demonstrated and the cells were susceptible to

the original phage. In the few instances of combinations other than those mentioned it is possible that a halo was not formed even though the colony was toxigenic or that nonspecific halo formation had occurred.

Phage contaminated colonies. Examination of part C of figure 1 shows that during the 2 to 12 hour period a considerable number of phage contaminated colonies, both haloed and unhaloed, were present. The contaminated colonies are of significance in determining the toxin producing population present in the broth cultures at each sample interval. If the contaminated

developing colonies have indicated that colonies attacked by phage while still very minute (0.5 to 0.25 mm) can still be recognized as having been attacked when they reach maturity (1.5 to 2 mm). It is true that lysis might have occurred so early as to leave no visible "scar", but in this case the possibility of selecting for a toxigenic mutant would not be very great.

Short exposure of nontoxigenic cells to phage preparations. In order to test the hypothesis that toxigenicity is induced rather than the result of mutant-selection, the following experiment was performed. Cells of strain 444A suspended in

TABLE 1
The production of toxigenic Corynebacterium diphtheriae after a short exposure of nontoxigenic strain 444A to bacteriophage 444V/A

EXPERIMENT NO.	PHAGE PER ML	BACTERIA PER ML	PHAGE BACTERIAL RATIO	CONDITIONS DURING ADSORPTION	NUMBER OF PHAGE INFECTED CELLS PLATED	HALOED (TOXIGENIC) COLONIES DEVELOPING			
						Intact		Intact and contaminated*	
						No.	Per cent	No.	Per cent
1	1.7×10^9	4.0×10^7	42/1	20 min, room temp, cells clumped	52	4	7.7	8	15.4
2	1.7×10^9	1.0×10^8	16/1	30 min, 37 C, cells clumped	108	3	2.8	14	12.9
3	1.4×10^9	8.4×10^7	17/1	30 min, 37 C, cells clumped	142	7	4.9	31	21.8
4a	1.1×10^9	1.6×10^8	7/1	30 min, 37 C, cells clumped	99	1	1.0	16	16.0
4b	1.1×10^9	4.2×10^8	2.5/1	30 min, 37 C, cells dispersed	268	4	1.5	21	7.8
5	8.0×10^8	1.7×10^8	4.7/1	30 min, 37 C, cells dispersed	701	1	0.1	34	4.8

* This includes colonies listed as haloed intact plus colonies that showed zones of partial lysis. No haloed colonies were observed on control plates of strain 444A in any of the experiments.

haloed colonies arise due to selection on the plate of a toxigenic mutant, then these colonies should not be included in calculating the toxin producing population present in the broth culture. However, if contamination resulted from phage production by a lysogenic cell which was deposited on the plate, then these colonies could be counted legitimately as having reflected the presence of another toxigenic cell in the broth culture. Logical arguments can be devised for either possibility, but there is insufficient evidence to support or exclude one or the other. It may be asked whether an intact colony might not actually be a "healed" phage contaminated colony. Observations on

heart infusion broth were exposed to various concentrations of phage 444V/A for periods of from 20 to 30 minutes. The mixture was diluted and aliquots plated directly to the differential medium. Suitable controls with strain 444A alone were included. In all cases the dilution of the mixture was sufficient to bring the phage population on the plate low enough so that the probability of contact between a developing colony and a bacteriophage particle was extremely small. The experimental period was so short as to preclude the possibility of any selection occurring. The number of phage infected cells deposited on the plate was determined as the difference be-

tween the total plate count of an uninfected aliquot and the number of unhaloed phage susceptible colonies on the experimental plate.

The data presented in table 1 represent a series of experiments of the type described. Various phage preparations were employed. In experiments 1, 2, and 3 suspensions of normally clumped cells were used. Contaminated haloed colonies continued to develop in spite of negligible phage on the plate. The interpretation to be placed on them was unknown. It was felt that clumping of the cells might be involved and that dispersing the clumps might remedy the situation. Cell clumps were broken up by shaking the cells by hand for 5 to 10 minutes, a procedure which reduced over 90 per cent of the cells to pairs or singles. No noticeable reclumping was observed during the experimental period. In experiments 4a and 4b the results are given for a clumped cellular suspension and for the same suspension after shaking. It will be noted that the per cent of toxigenic colonies which developed, based on the intact, haloed colonies, is similar for both preparations. However, there was a 50 per cent drop in the number of contaminated toxigenic colonies after shaking, an observation which supports a concept of endogenous phage contamination.

From the per cent conversion to toxigenicity noted in these experiments and the conditions of the experiments, there would seem little doubt that the appearance of the toxigenic cells is the result of an induced change rather than the selection of a toxigenic mutant. The correlation of toxigenicity and lysogenicity was verified for the toxigenic colonies produced in this series, and toxin production was checked by guinea pig intradermal tests. The results given in the table tend to indicate a higher conversion ratio with higher phage-bacterial ratios. The data are insufficient at present to allow any conclusions to be drawn regarding the conditions most suited to this change.

Other arguments in support of an induced change can be derived from the long term experiments described previously. From a plot of the numbers of toxigenic cells present during the experimental period it is possible to determine by extrapolation the numbers of toxigenic cells which should have been present in the initial inoculum. Such a plot for the experiment described in figure 1 is presented in figure 2.

In this experiment approximately 0.23 per cent of the original population should have been toxigenic in order to account for the subsequent development of the toxin producing population. This value is derived from a plot of the toxigenic population based on the percentage of intact haloed colonies (figure 2, section A). If both intact and phage contaminated haloed colonies are included in the toxigenic population, then approximately 0.47 per cent of the original popula-

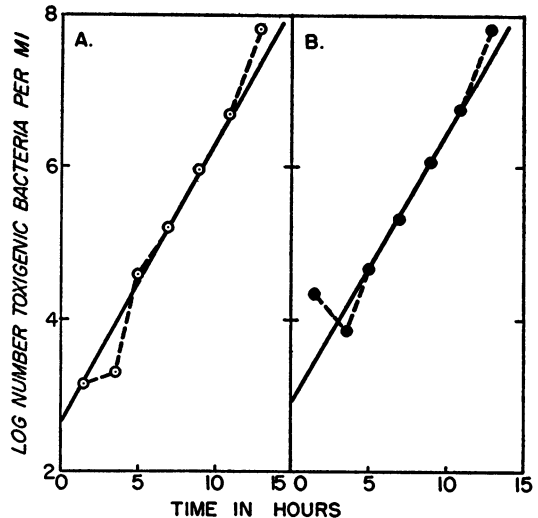


Figure 2. Development of toxigenic *Corynebacterium diphtheriae*, strain 444V, during the incubation of strain 444A and bacteriophage 444V/A. The unbroken line represents a theoretical semilogarithmic plot based on the experimental points; the open circle, experimental points based on intact, haloed colonies only; the solid circle, experimental points based on the total haloed colony count, i.e., intact plus phage contaminated.

tion should have been toxigenic (figure 2, section B). Similar plotting and calculation for the data of the second experiment cited earlier give values for toxigenic cells in the original inoculum of 0.07 per cent and 0.33 per cent, respectively.

The values calculated for the initial toxigenic population would represent a very high population status for a mutant. In observations extending over the period of a year not a single haloed colony has been observed on control plates of the nontoxigenic strain. It is estimated that at least several thousand of these colonies have been examined. Even if the value of 0.07 per cent is accepted as representative, it is anticipated that

some haloed colonies would have been observed in the controls. If the values of 0.23 per cent, 0.33 per cent, and 0.47 per cent are more nearly valid, then the absence of haloed colonies in the controls is even more significant. This argument and others (Groman, 1953b) are suggestive of the probability that the toxigenic population did not develop from mutants present in the initial inoculum but as a result of an induced change.

DISCUSSION

The data which have been presented show that the change from nontoxigenicity to toxigenicity occurs in a relatively high proportion of nontoxigenic *C. diphtheriae* exposed to phage. The conversion of 0.1 to 7.7 per cent of nontoxigenic cells to toxigenicity after a 30 minute exposure to phage virtually excludes the possibility that this change is the result of selection of toxigenic cells from a mixed population. The most reasonable interpretation is that the change is induced. Other quantitative data presented in this paper also support this hypothesis.

Although the role of phage is not established unequivocally in the conversion to toxigenicity nor its exact function defined, there seems to be little doubt that it is concerned with this change. The possibility that there is another filterable agent present in the phage preparation which is capable of inducing the change to toxigenicity is minimized by the following considerations. If such an agent were present, then one would expect to find toxigenic colonies which were nonlysogenic when nontoxigenic cells were exposed to phage for a short period of time and during which time less than 100 per cent of the cells adsorbed phage. Under the conditions cited no selection against nonlysogenic cells would be anticipated. Nevertheless, all toxigenic colonies which did develop under these conditions (experiments 1 to 5, table 1) were lysogenic even though 20 per cent to 35 per cent of the cells exposed to phage were not infected. In order to account for the observed facts it would be necessary to postulate that infection of a bacterial cell by phage and by an agent inducing toxigenicity coincides in 100 per cent of the cases. It seems highly improbable that such complete concurrence would result if conferral of toxigenicity is independent of phage infection.

It is possible that the present work is related to that reported by Zinder and Lederberg (1952)

and Berry, McCarthy, and Plough (1952). Both groups of investigators have presented evidence which demonstrates the transfer of various nutritional characteristics from one strain of salmonella to another by an agent designated FA (filterable agent). This new mode of character transfer has been called transduction.

Zinder and Lederberg (1952) have presented evidence which implicates bacteriophage as a passive carrier of the transducing principle. It is not possible at this time to decide with certainty whether the salmonella and *C. diphtheriae* systems are analogous. The relative numbers of *C. diphtheriae* on which toxigenicity was conferred as indicated in the experiments thus far performed are of a much higher order of magnitude than any single transduced character reported by Zinder and Lederberg. Nevertheless, this difference could be quantitative rather than qualitative in nature.

The exact origin of the principle inducing toxigenicity still remains unsettled. The phage B suspension used by Freeman (1951) originated from the lysis of a virulent strain of *C. diphtheriae*. The loss of the ability to induce toxigenicity after a series of single burst infections of phage B on strain 444A has been observed and would tend to support the concept that the inducing agent cannot be regenerated on the nontoxigenic strain. However, selection of a virulent phage mutant incapable of establishing lysogenicity also could explain this finding. On the other hand, inducing activity was maintained through five successive single plaque isolations on strain 444A, a fact which would indicate maintenance of the inducing agent on nontoxigenic cells. Restudy of these isolated observations is necessary before a clear picture will be available.

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SUMMARY

The origin of toxigenic *Corynebacterium diphtheriae* resulting from the action of phage 444V/A, on nontoxigenic *C. diphtheriae*, strain 444A, has been studied by following population changes which occurred during a 24 hour incubation period.

The development of toxigenic, lysogenic cells

was observed within 1½ hours after mixing the nontoxigenic, phage susceptible strain with bacteriophage. The numbers of toxigenic cells which developed could not be explained reasonably by a selection hypothesis. In order to account for the development of the toxin producing population from an original inoculum of 1×10^8 cells per ml, some 7×10^4 to 4.7×10^5 cells per ml should have been toxigenic. This high concentration of toxigenic mutants was not revealed in suitable control experiments.

Short exposures (20 to 30 min) of nontoxigenic *C. diphtheriae* to bacteriophage also resulted in the development of toxigenic colonies when aliquots were plated immediately on a differential medium. In this manner 0.1 per cent to 7.7 per cent of the nontoxin producing cells infected by bacteriophage were found converted to toxigenicity.

On the basis of the evidence and arguments presented it is proposed that the change to toxigenicity is induced and that bacteriophage is concerned with this change.

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