isotope effect, reported by Baker (*6, 7*), for B-NADD of 0.50 ± 0.10 based on kinetic measurements, it is in complete agreement with our most recent kinetic results (*9*). Our observations also indicate that the B-NADD preparation is more sensitive to deterioration on storage than the usual NADH sample. This is probably the cause of the discrepancy between Baker's results and those reported here.

The isotope effect for equilibrium dissociation constants with A-NADD was determined by both the Sephadex and the fluorimetric methods, and the two results are identical within experimental error:

$$\overline{K}_d \equiv K_d^{E\text{-}NADH}/K_d^{E\text{-}(A\text{-}NADD)}$$
$$= 0.65 \pm 0.14 \text{ (Sephadex method)}$$
$$= 0.52 \pm 0.07 \text{ (fluorimetric method)}$$

This kind of ("inverse") isotope effect indicates that A-NADD is bound less tightly to the enzyme than is NADH:

$$\text{LADH} + \text{NADH} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{LADH} - \text{NADH}$$

and that the zero point energies for the isotopically sensitive vibrations are lower in the complex than in the free NADD.

Since $\overline{K}_d$ (observed) $\equiv K^H/K^D = \overline{k}_2/\overline{k}_1$; $\overline{k}_1 \equiv k_1^H/k_1^D$, $\overline{k}_2 \equiv k_2^H/k_2^D$, then if $\overline{K}_d < 1$ and $\overline{k}_2 > 1$ it follows that $\overline{k}_1 > \overline{k}_2 > 1$ (conversely for $\overline{k}_2 < 1$, $\overline{k}_1 < \overline{k}_2$). Therefore if the isotope effect on the dissociation reaction (rate constant k_2) is normal ($\overline{k}_2 > 1$), a possibility suggested from kinetic data (*6, 7, 9*), then the isotope effect for the binding step (rate constant k_1) must also be normal and even larger, thus indicating an ability of the enzyme to discriminate kinetically against the coenzyme deuterated at the transferable hydrogen.

Previously Mahler *et al.* (*7*) reported $\overline{K}_d$ for the LADH-(A-NADD) dissociation constant to be 1.31 ± 0.64; however, this value was based on an extrapolation from kinetic constants derived from initial rate measurement and as indicated, exhibited a large experimental uncertainty. Isotope effects for the corresponding equilibrium constants for yeast alcohol dehydrogenase (*1*) and malate dehydrogenase (*3*) were reported to be of the same magnitude as that for LADH. However, "inverse" deuterium isotope effects similar to the one reported here, but based on inverse rate effects for k_1, were reported from Thomson's laboratory (*4*) for the binding of A-NADD to heart lactate dehydrogenase ($\overline{K}_d = 0.69$) and muscle lactate dehydrogenase ($\overline{K}_d = 0.43$).

These various results in conjunction with the absence of any real difference in the binding constant between NADH and B-NADD, which eliminates the possibility of secondary interaction between the B proton and the enzyme, indicate that dehydrogenases are capable of recognizing and discriminating against coenzymes isotopically substituted in the appropriate position ("A" for A enzymes) even in the initial binding reaction. The properly deuterated coenzyme is bound less tightly than is its protonated counterpart, so that during the binding reaction the equilibrium (and perhaps also the rate) constant already reflects the specificity of the subsequent hydrogen transfer step.

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Bacteriophage and the Toxigenicity of Clostridium botulinum Type C

Abstract. *Nontoxigenic and bacteriophage-sensitive bacterial cultures have been isolated from toxigenic* Clostridium botulinum *type C, strain 468C, after treatment with either ultraviolet light or acridine orange. Two bacteriophages, designated CEβ and CEγ, were isolated from toxigenic strain 468C. Both of these bacteriophages were capable of infecting the nontoxigenic type C cultures, but only bacteriophage CEβ was involved in the change from nontoxigenicity to toxigenicity.*

Nontoxigenic clostridia resembling toxigenic *Clostridium botulinum* have frequently been isolated from marine and terrestrial environments (*1*); occasionally, pure cultures of toxigenic *C. botulinum* have also become nontoxigenic (*2*). It has been suggested that the production of toxin by *C. botulinum* might be governed by the presence of a specific converting bacteriophage in a system analogous to the production of diphtheria toxin by *Corynebacterium diphtheriae* (*3, 4*). In a recent report, Inoue and Iida (*5*) presented data which strongly suggested that bacteriophage may be involved in the toxigenicity of *C. botulinum* type C. They were able to recover toxigenic isolates from nontoxigenic cultures incubated in broth with filtrates of a toxigenic type C (strain Stockholm). Marked lysis occurred in the cultures; however, plaques were not demonstrated on solid medium.

In our studies, two bacteriophages, designated CEβ and CEγ, were isolated from toxigenic *C. botulinum* type C,

strain 468C (received from W. P. Segner, Continental Can Co., Chicago). This report presents conclusive evidence for the involvement of CEβ in the toxigenicity of *C. botulinum* type C. Bacteriophage-producing cultures were obtained from nontoxigenic type C cultures that had been reinfected with either bacteriophage. However, only bacteriophage CEβ caused the change from nontoxigenicity to toxigenicity, and these toxigenic isolates invariably carried bacteriophage CEβ. Cultivation of the CEβ-infected isolates in medium containing antiserum against bacteriophage CEβ resulted in the loss of bacteriophage CEβ and loss of toxigenicity. These isolates were then reinfected with bacteriophage CEβ and they again became toxigenic.

Nontoxigenic cultures were isolated after treatment of toxigenic strain 468C with either ultraviolet irradiation or acridine orange (AO). Irradiation with ultraviolet light is a standard method for inducing lysis of bacteria that harbor prophages (the form in which bacteriophages are perpetuated in lysogenic bacteria), whereas AO has been used to remove extra chromosomal elements from bacteria. Both procedures can result in bacterial isolates that are "cured" of either prophages in the former or extra chromosomal elements in the latter.

For irradiation, about 10^6 cells of strain 468C in the logarithmic phase of growth were spread onto the surfaces of trypticase, yeast-extract, glucose (TYG) blood agar plates and irradiated for 60 seconds at a distance of 20 cm (General Electric germicidal lamp, 15 watts). The agar plates were incubated anaerobically in Brewer jars for 48 hours at 37°C. Isolated colonies surviving the treatment (approximately 10^2 cells) were tested for toxigenicity by the mouse assay (*6*) and for sensitivity to the bacteriophages of the toxigenic parent culture by the agar-layer procedure (*7*). The base agar used in the agar-layer procedure was TYG agar, and the overlay was soft agar (0.7 percent) prepared from the filtered broth of Segner's fortified egg meat (SFEM) medium (*8*). The addition of blood (0.2 ml) to the overlays was essential for confluent growth. Cysteine hydrochloride (0.1 percent) was used as the reducing agent in all media.

For curing with AO, about 10^5 cells of strain 468C in the logarithmic phase of growth were added to TYG broth (*p*H 7.4) containing 20 μg of AO per milliliter and incubated at 37°C for 18 hours. The AO culture was streaked onto TYG blood agar plates, and isolated colonies were tested for toxigenicity and sensitivity to bacteriophages by the same procedures that were described for the colonies treated with ultraviolet light.

Table 1. Reinfecting cured nontoxigenic bacterial cultures with bacteriophage CEβ and the effect on toxigenicity. UV, ultraviolet.

Nontoxigenic cured culture	Method of obtaining toxigenic cultures	No. of cultures	
		Toxigenic	Tested
AO28	Plaques*	40	40
AO28	30-minute contact†	4	40
AO28	240-minute contact†	40	40
UV171	Plaques	20	20
UV171	30-minute contact	2	40
UV171	240-minute contact	40	40

* Material from plaques produced by bacteriophage CEβ on culture AO28 or culture UV171 was checked for toxigenicity and bacteriophage production. † Cultures AO28 and UV171 were exposed to bacteriophage and isolated colonies were checked for toxigenicity and bacteriophage production.

Ultraviolet treatment was more efficient than AO was in curing strain 468C of its prophage. After a 60-second treatment with ultraviolet light, 15 of the 106 surviving colonies were cured of their prophages and concomitantly became nontoxigenic. In comparison, only 2 of the 68 colonies surviving the AO treatment were cured of their prophages and toxigenic character. The cured cultures were subcultured ten times over a period of 2 months, and all of them remained nontoxigenic and sensitive to the bacteriophages of the toxigenic parent strain 468C.

Each nontoxigenic culture cured of its prophage was tested for its sensitivity to lysates of the other cured nontoxigenic cultures by the agar-layer procedure. Only one culture, AO28, was sensitive to the lysates of the other nontoxigenic cultures. Culture AO28, therefore, had been cured of two of its prophages, whereas the other cultures continued to carry a second prophage.

Bacteriophages were isolated from an 18-hour broth culture of the toxigenic parent strain 468C. The broth culture was centrifuged at 6000*g* for 10 minutes and sterilized by filtration. Colony-centered plaques and turbid plaques produced by bacteriophages designated as CEβ and CEγ, respectively, were observed when the filtrate was plated with

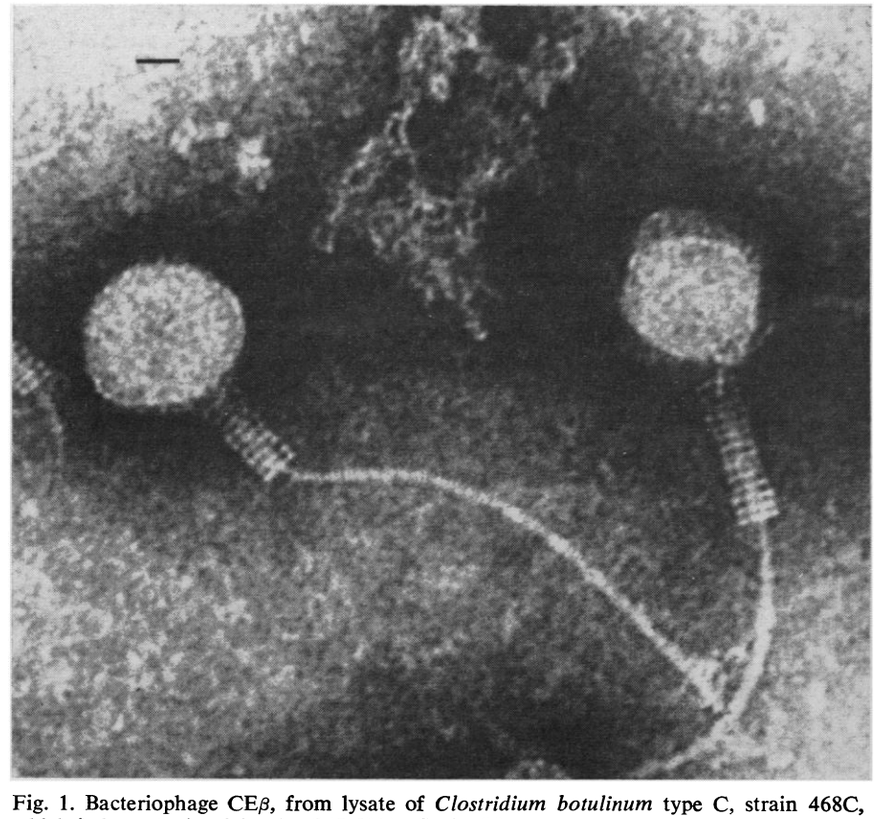

Fig. 1. Bacteriophage CEβ, from lysate of *Clostridium botulinum* type C, strain 468C, which induces toxigenicity (× 247,500). Scale, 20 nm.

culture AO28. These two different bacteriophages were purified by five successive single-plaque isolations on culture AO28.

Electron micrographs were prepared of the two bacteriophages according to the procedure of Eklund *et al.* (*9*). Bacteriophage CEβ exhibited a hexagonal head 100 nm in diameter and a tail 400 nm long and 11 nm in diameter surrounded by a contracted sheath 30 nm in diameter (Fig. 1). Bacteriophages comparable to CEβ in size and morphology have been observed in lysates of other strains of type C (*5, 10*). Bacteriophage CEγ exhibited a hexagonal head 60 nm in diameter and a tail 185 nm long and 6 nm in diameter surrounded by a contracted sheath 20 nm in diameter (Fig. 2).

Both bacteriophages were studied for their relation to the toxigenicity of type C strain 468C. Bacteriophage stocks used in these studies were produced by propagating the purified bacteriophages with culture AO28 in TYG broth at 30°C. Bacteriophage stocks were treated with 25 μg of crystalline deoxyribonuclease II (Sigma) per milliliter for 3 hours at 37°C and then sterilized by filtration. This step was used to rule out the possibility of a transformation principle of the deoxyribonucleic acid type. Sterility checks were made by inoculating several milliliters of filtrate into SFEM broth and then incubating the medium for several weeks at 37°C.

Two techniques were used to test the relation of the two bacteriophages to the toxigenicity of cultures AO28 and UV171 (which carries bacteriophage CEγ). In the first, dilutions of bacteriophage CEβ were plated with the recipient nontoxigenic culture according to the agar-layer procedure. Material from the plaques was transferred into TYG broth and incubated at 30°C for 5 days. Cultures arising from the plaques produced by CEβ on cultures AO28 and UV171 produced bacteriophage and were toxigenic (Table 1). Cultures arising from the plaques produced by CEγ on culture AO28 also produced bacteriophage but were nontoxigenic (data not shown).

In the second procedure, bacteriophage CEβ was added to actively growing nontoxigenic cultures of AO28 and UV171 at a multiplicity of infection (ratio of adsorbed bacteriophage to bacteria) of 1.8 and 2.6, respectively. After 30 and 240 minutes of exposure to the bacteriophage, the cultures were

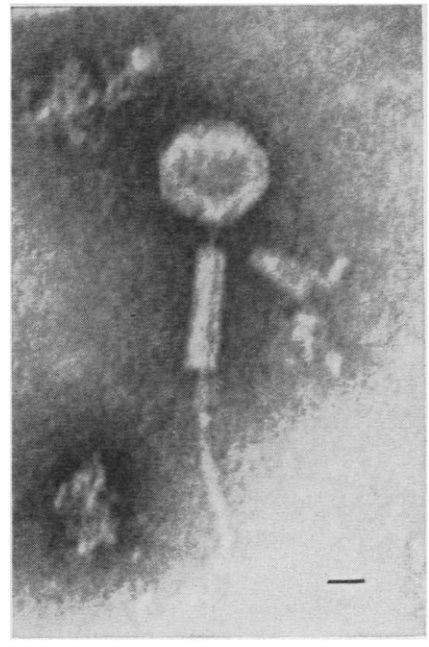

Fig. 2. Bacteriophage CEγ, from lysate of *Clostridium botulinum* type C, strain 468C, which does not induce toxigenicity (× 247,500). Scale, 20 nm.

streaked onto TYG blood agar plates, and isolated colonies were transferred into TYG broth, incubated, assayed for toxin, and tested for bacteriophage production, with culture AO28 as the indicator strain.

After a 30-minute contact with bacteriophage CEβ, 10 percent of the colonies of culture AO28 and 5 percent of the colonies of culture UV171 were toxigenic (Table 1). The toxigenic cultures were resistant to bacteriophage CEβ. The nontoxigenic cultures continued to be sensitive to bacteriophage CEβ, but when they were reinfected with this bacteriophage they also became toxigenic and produced bacteriophage. After a 240-minute contact between bacteriophage and bacteria, all of the isolates were toxigenic and produced bacteriophage (Table 1).

Several of the CEβ-infected cultures were subcultured in TYG broth containing 50 percent antiserum against bacteriophage CEβ (produced by immunizing rabbits with CEβ bacteriophage). Four successive transfers in antiserum resulted in the simultaneous loss of bacteriophage CEβ and toxigenicity. When these nontoxigenic cultures were reinfected with bacteriophage CEβ, they again became toxigenic.

The toxins of the different isolates were neutralized with type C antitoxin. These cultures were subcultured in SFEM six times over a period of 2 months, and all of them maintained their toxigenic and bacteriophage-producing characteristics.

Experiments reported in Table 1 were repeated with bacteriophage CEγ and with bacterial culture AO28 (data not shown). Colonies from a 240-minute contact between bacteriophage CEγ and culture AO28 were all bacteriophage-producers but nontoxigenic.

There is little doubt that in our experiments toxigenicity of *C. botulinum* type C (strain 468C) is in some manner dependent on the presence of bacteriophage CEβ. Groman (*4*) also reported that conversion of toxigenicity of *Corynebacterium diphtheriae* is bacteriophage specific. The curing of CEβ-infected cultures by antiserum against the bacteriophage indicates that this relation between bacteriophage CEβ and host is probably pseudolysogeny, similar to that which occurs with *Bacillus subtilis* (*11*), instead of true lysogeny.

Some of the other types of *C. botulinum* that we have studied have been cured of their prophage but still remain toxigenic. It is possible either that they carry other bacteriophages or that not all *C. botulinum* toxins are induced by bacteriophage.

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