JOURNAL OF BACTERIOLOGY, Apr., 1967, p. 1220-1226
Vol. 93, No. 4

Inducible Lysis in *Clostridium tetani*

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Received for publication 15 December 1966

Lysis was induced in seven strains of *Clostridium tetani* by exposure to mitomycin C. The search for a suitable indicator strain to detect bacteriophage in lysates has, so far, been unsuccessful. Inhibition studies on macromolecular synthesis during induction have shown that deoxyribonucleic acid, ribonucleic acid, and protein syntheses are all involved in the lysis induced by mitomycin C. In experiments comparing toxin and protein content in induced and uninduced cells of *C. tetani*, the toxin-protein ratio proved to be the same in both systems up to the point of lysis. Several possible hypotheses deduced from these results are discussed.

Very little is known about the physiology or genetics of *Clostridium tetani*. A major portion of published material has been concerned with improved toxin or toxoid production (9–11, 13) or the structure and biochemical properties of the toxin (3, 8, 9), although the mechanisms of toxin formation and release remain obscure (16). The general purpose of this study was to detect lysogeny in *C. tetani*. A single paper (4) has appeared reporting bacteriophage active against this organism.

This work deals with induction of lysis with mitomycin C, the effects of inhibitors of macromolecular synthesis in the lytic system, and with some possible relationships of the lytic system to toxin formation and release. Finally, several techniques have been devised which should facilitate the handling and study of *C. tetani*.

MATERIALS AND METHODS

Bacterial strains. Six of the eight strains of *C. tetani* and one strain of *C. tetanomorphum* were received from the American Type Culture Collection, Rockville, Md. The numbers of *C. tetani* as given in Table 1 refer to their designations in that collection. *C. tetani* D9, Massachusetts strain, was kindly made available by Leo Levine of the Massachusetts Department of Public Health. *C. tetani* CCNIH was received from the Clinical Center, National Institutes of Health, Bethesda, Md., and *C. tetanomorphum* UMD was made available by F. Tyeryar of the University of Maryland.

The cultures were checked for purity, and their identities were established through growth characteristics, biochemical reactions, and toxin production (18; *Bergey's Manual of Determinative Bacteriology*.)

Media. The basic media used in this study were variations of Difco Fluid Thioglycollate Medium (FTM). Hard agar for double-layer plating contained 1% Difco agar in FTM.

The medium for long-term maintenance of stock cultures was composed of the following ingredients: N-Z Case (Sheffield Chemical Co., Norwich, N.Y.), 10 g; yeast extract (Difco), 3 g; NaCl, 5 g; agar (Difco), 3 g; and distilled water, 1 liter. The medium was heated to boiling and dispensed in 10-ml amounts in screw-capped tubes (16 by 125 mm). It was autoclaved for 15 min at 121 C, cooled quickly to 37 C, and inoculated with the bacterial cultures. The cultures were incubated at 37 C for 4 days and then were stored at 4 C.

Anaerobic incubation methods. A technique of anaerobiosis was adopted in which a pyrogallic acid-sodium hydroxide combination was used in 8-liter desiccators. Pyrogallic acid crystals (80 g) were placed in the bottom of the desiccator, and 200 ml of 40% sodium hydroxide was poured over the crystals. The desiccator was quickly sealed and incubated. Methylene blue was used as an indicator of the degree of anaerobiosis (5). All plates were incubated by this method. Broth cultures could be incubated statically in an air atmosphere.

Mitomycin C induction of lysis. The various strains used in this study were maintained through daily passage in FTM. Cultures of these strains in the logarithmic growth phase were prepared by inoculation of 8 ml of FTM with 1 ml of a 24-hr culture. These tubes were then incubated at 37 C until the optical density (Coleman Universal Spectrophotometer) at 650 mμ attained a value of 0.07 to 0.12, approximately 10^7 organisms. Mitomycin C was added to the desired concentration, the optical density was recorded, and the tubes were returned to the incubator. Optical density readings were taken at 1-hr intervals until the end of the experiment. All determinations were carried out in duplicate.

Stock solutions of mitomycin C (0.1 mg/ml, Calbiochem, Los Angeles, Calif.), chloramphenicol (5 mg/ml, Parke, Davis & Co., Detroit, Mich.), actinomycin D (0.1 mg/ml, Merck Sharp & Dohme, Rahway, N.J.), and and 5-fluorouracil deoxyriboside

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(5-FUDR; 1 mg/ml) were prepared in 0.1 M Sorensen's phosphate buffer (*p*H 7.0) and were stored at 4 C until used (2).

Examination of lysate for presence of phage. At the conclusion of each induction study, those tubes which showed lysis were filtered through a sintered-glass filter, and the filtrate was examined for the presence of bacteriophage.

A 1-ml amount of a 24-hr indicator culture was added to 19 ml of melted FTM agar held at 45 C, and 0.5 ml of the lysate or one of its dilutions was added to the melted agar. The tubes were rolled to mix bacteria and lysate, and the agar was poured into a petri dish and allowed to harden. Another 19-ml layer of FTM agar was poured over the hardened agar layer and allowed to solidify. Anaerobic incubation of the plates at 37 C for 24 to 48 hr yielded satisfactory lawns for detection of bacteriophage plaques.

Agar-diffusion analysis of tetanus toxin production. The techniques used were similar to those of Thorne and Belton (17). Samples (12-ml) of purified Noble Special Agar (Difco) plus 0.1 g of Merthiolate per liter were dispensed in flat-bottomed petri dishes. Circular reservoirs were made in the solidified agar with a plastic template and cork borers. The agar circles were aspirated by use of a capillary pipette attached through a suction flask to a vacuum line. Plates prepared in this manner were stored at 4 C and used as required. The template consisted of three rows of holes in a linear pattern; the outer rows had 7-mm holes spaced 5 mm apart, and the inner rows had 5-mm holes spaced 7 mm apart. Outer rows were 6 mm from the inner row. The inner reservoirs were filled with tetanus antitoxin, USP (National Drug Co., Philadelphia, Pa.), by use of a stock solution of 1,500 units/ml. Actual amount in the reservoir was 30 units. The plates were incubated at 4 C for 24 hr. Test toxins were then added to the outer reservoirs in 0.01-ml serial increments. The center reservoir in each row contained 0.6 flocculation unit (Lf) of a highly purified tetanus toxoid prepared by the Massachusetts Department of Public Health Biologic Laboratories (stock solution, 600 Lf/ml). Only one line could be detected when this amount of purified toxoid was diffused against tetanus antitoxin. The plates were incubated at 20 to 25 C and read at 24 and 48 hr.

The smallest volume of test material giving a line with antitoxin contained, arbitrarily, one unit of toxin. The reciprocal of this value yielded the number of units of toxin per milliliter.

Preparation of sonic lysates for toxin-protein ratio studies. During induction of lysis with mitomycin C, 20-ml samples were taken at time-zero and every hour thereafter, and were centrifuged at 12,000 × *g* for 15 min. The supernatant fluid was discarded, and the cells were resuspended in 5 ml of 0.01 M Sorensen's phosphate buffer, *p*H 7.0. The cells were then disrupted by treatment for 60 sec with a model S-75 Branson sonifier at a setting of 6. The cellular debris was centrifuged at 12,000 × *g* for 15 min, and the supernatant fluid was analyzed for protein and toxin content. Protein determinations were carried out by the sulfosalicylic acid method.

For any preparation, the units of toxin per microgram of protein represent the toxin-protein ratio. It should be remembered that one unit of toxin is only a fraction of a microgram of protein.

RESULTS

Evaluation of growth curves. The rate and extent of growth of both *C. tetani* and *C. tetanomorphum* in FTM were determined both by optical-density changes and by most probable numbers count (7). The results in Fig. 1 reveal that *C. tetanomorphum* grows considerably faster than the strains of *C. tetani*. Mass doubling time for *C. tetanomorphum* was about 1.2 hr, whereas the minimal mass doubling time for *C. tetani* strains was in excess of 2 hr. Optical densities of *C. tetanomorphum* at stationary phase were approximately three times as great as those of *C. tetani* at stationary phase.

Effect of mitomycin C concentration on induction of lysis. Seven strains of *C. tetani* and two strains of *C. tetanomorphum* were examined for

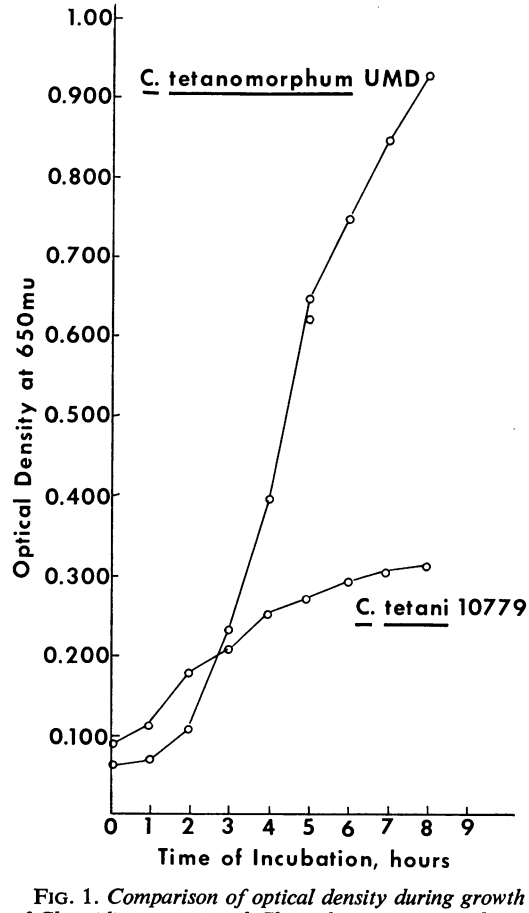

FIG. 1. *Comparison of optical density during growth of Clostridium tetani and Clostridium tetanomorphum.*

TABLE 1. *Concentration of mitomycin C effective in inducing maximal lysis in various strains of Clostridium tetani*

C. tetani strain	Concn of mitomycin C
	μg/ml
ATCC 9441	1.0
CCNIH	3.0
ATCC 454	3.0
ATCC 10779	3.0
ATCC 453	5.0
ATCC 8033	5.0
Mass. DPH D9	5.0

lysis caused by mitomycin C. Neither strain of *C. tetanomorphum* was lysed with concentrations of mitomycin C up to 10 μg/ml. Lysis was, however, induced in all strains of *C. tetani* tested (Table 1). Maximal lysis was caused by 1 to 5 μg of mitomycin C per ml, and both growth and lysis were prevented by 10 μg of mitomycin C per ml (Fig. 2).

The cell concentration at which mitomycin C was an effective inducer of lysis was rather critical. At concentrations equivalent to an optical density at 650 mμ of 0.07 to 0.12, lysis was achieved. Above these values, however, little lysis was obtained.

Addition to the basic FTM of large concentrations of supplemental amino acids, such as cystine (0.5 mg/ml), alanine (0.05 mg/ml), or tryptophan (0.5 mg/ml), singly or in combination, enhanced growth, but also inhibited induction of lysis.

Induction of lysis in *C. tetani* can also be effected by exposure to ultraviolet radiation by a previously described procedure (1). After irradiation, the latent period and the rate of lysis are identical to those obtained after induction with mitomycin C (*unpublished data*). Lysis could not be induced in *C. tetanomorphum* by irradiation with ultraviolet light.

Effect of macromolecular synthesis inhibitors on mitomycin C-induced lysis. The effect of various inhibitors on lysis caused by mitomycin C was determined. The induction process was sensitive to chloramphenicol, an inhibitor of protein synthesis. Bacterial cultures were induced with mitomycin C, and chloramphenicol at a final concentration of 50 μg/ml was added at zero-time and at various time intervals thereafter. Chloramphenicol was effective in preventing lysis up to the actual point of lysis (Fig. 3). When added just prior to lysis, no blocking effect was seen.

The effects of 5-FUDR, an inhibitor of deoxyribonucleic acid (DNA) synthesis, and actinomycin

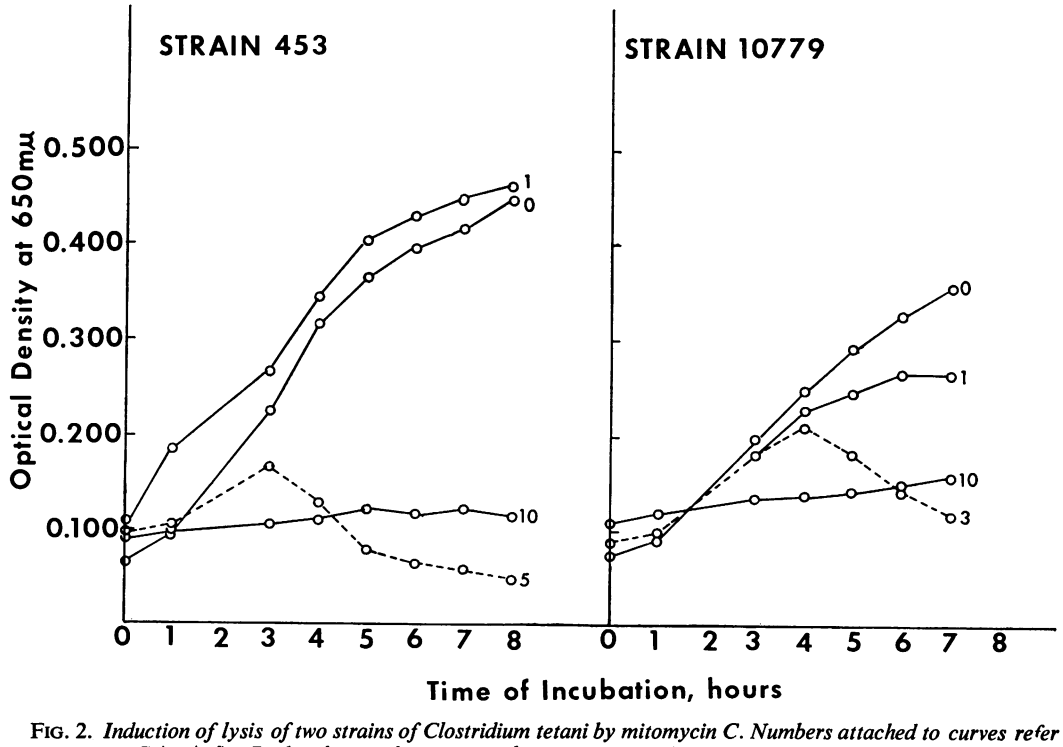

FIG. 2. *Induction of lysis of two strains of Clostridium tetani by mitomycin C. Numbers attached to curves refer to mitomycin C (μg/ml). Broken lines indicate optimal concentration of mitomycin C.*

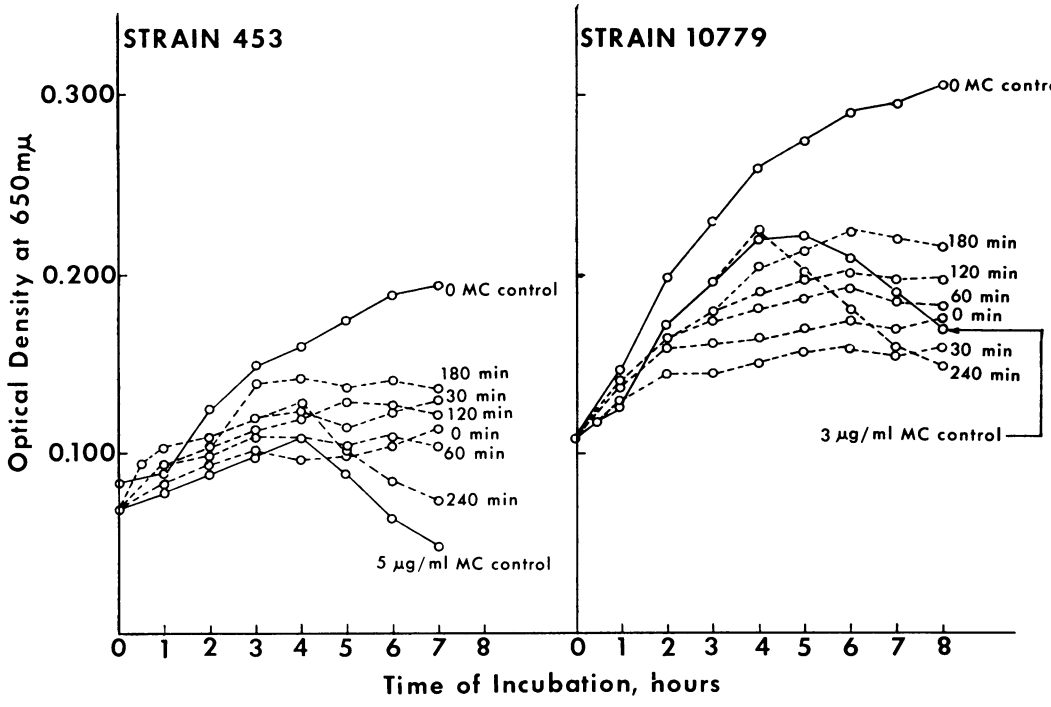

FIG. 3. *Effect of addition of chloramphenicol on the lysis induced by mitomycin. All inductions have chloramphenicol added to 50 μg/ml final concentration at the times indicated. Solid lines are control cultures.*

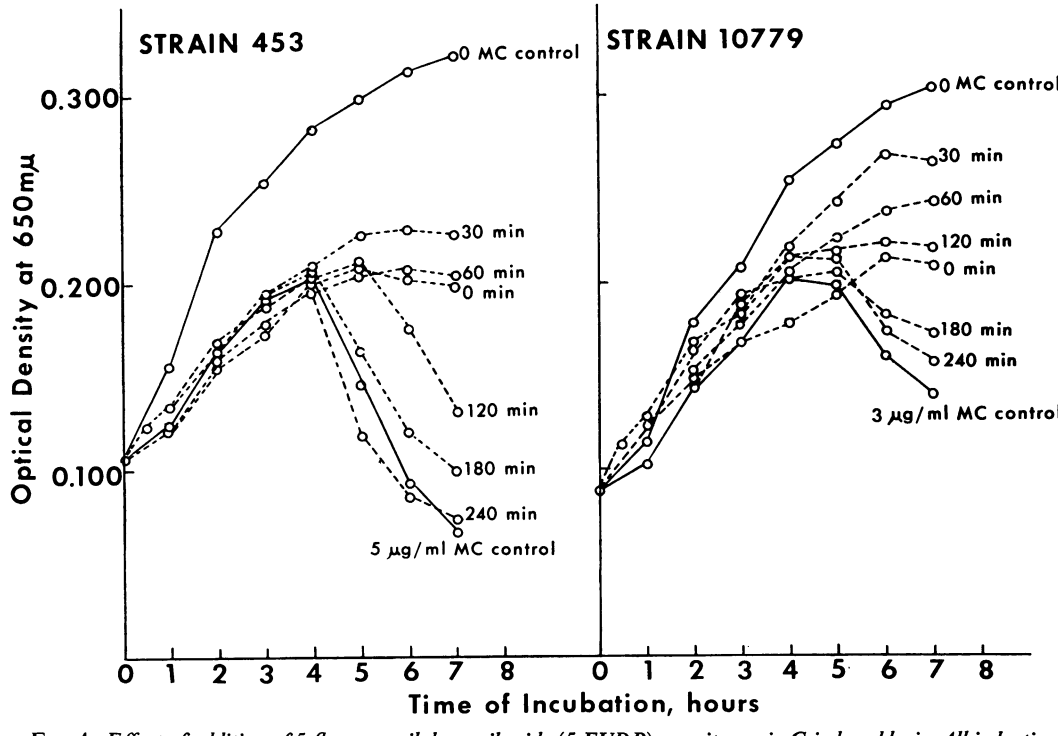

FIG. 4. *Effect of addition of 5-fluorouracil deoxyriboside (5-FUDR) on mitomycin C-induced lysis. All inductions have 5-FUDR to 20 μg/ml final concentration at the times indicated. Solid lines are control cultures.*

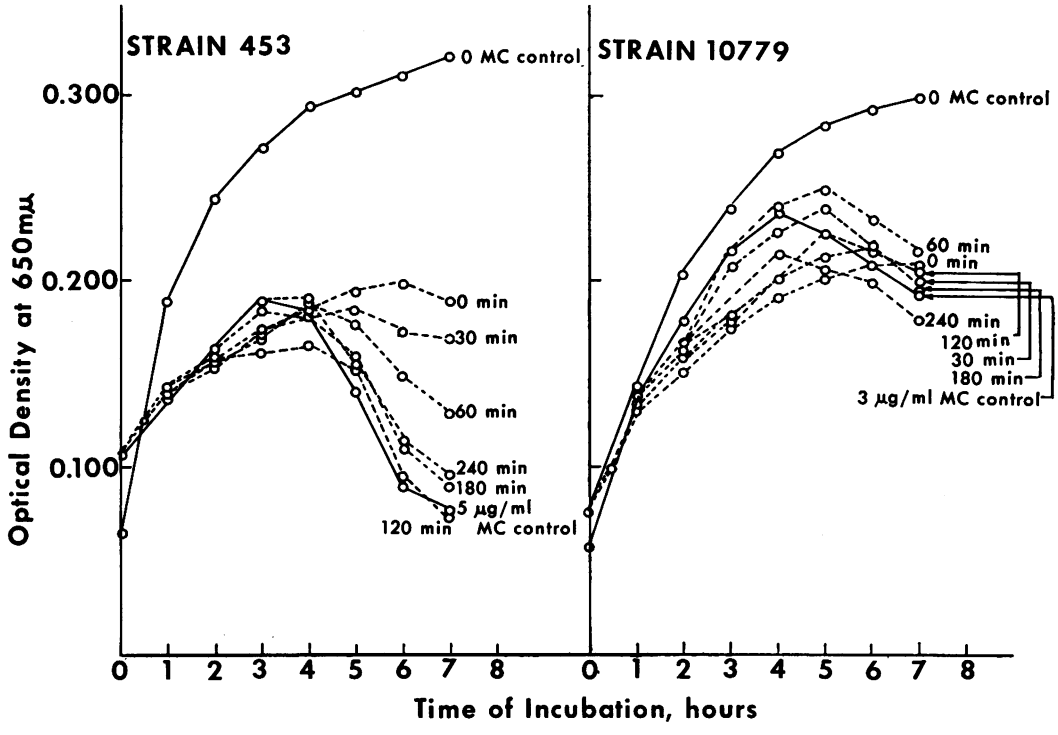

FIG. 5. *Effect of addition of actinomycin D on mitomycin C-induced lysis. All inductions have actinomycin D added to 5 μg/ml final concentrations at the timed indicated. Solid lines are control cultures.*

D, an inhibitor of ribonucleic acid (RNA) synthesis, were also investigated. Mitomycin C-induced lysis was inhibited by 5-FUDR at a final concentration of 20 μg/ml during the first 60 to 120 min of induction (Fig. 4). If 5-FUDR was added after this time period, lysis continued at the same rate as in the untreated control. Actinomycin D also prevented mitomycin C-induced lysis when added during the first 30 min after induction (Fig. 5). In cultures treated with actinomycin D 60 to 240 min after induction, initiation of lysis was delayed for about 1 hr.

Examination of toxin production versus protein formation during early growth and mitomycin C induction. Repeated attempts to isolate bacteriophage from filtrates of mitomycin C-lysed cultures were unsuccessful. All strains of both organisms listed in Table 1 were tested as indicators.

If the toxin gene resides in a temperate phage, inducible by mitomycin C, the intracellular toxin-protein ratio should increase rapidly during induction and should be very high prior to lysis, whereas this ratio in an uninduced organism should remain constant. Experiments comparing toxin and protein produced in induced and uninduced cells have shown the toxin-protein ratio to be the same in both systems up to the point of lysis (Fig. 6). No toxin could be demonstrated in cells of *C. tetanomorphum*, either exposed or unexposed to mitomycin C.

In control experiments, the Branson Sonifier treatment did not measurably reduce the titer of the purified toxoid. Agar diffusion analyses were clear-cut, and the toxin line was easily distinguishable.

DISCUSSION

Mitomycin C-induced lysis of bacteria has been well documented. Otsugi and co-workers (16) were able to induce phage formation in a lysogenic strain of *Escherichia coli*. Reich et al. (15) demonstrated the lytic effect of mitomycin C in *E. coli*, blue-green algae, and *Euglena*, and Altenbern and Stull (2) reported inducible lytic systems in the genus *Bacillus*. Mitomycin C was capable of eliciting a pattern of behavior similar to those reported above in all strains of *C. tetani* tested (Table 1).

Two major differences can be discerned between *C. tetani* and previous organisms studied. First, larger concentrations of mitomycin C are needed to induce lysis, and, second, a much longer latent period is obtained. This second factor is probably due, at least in part, to the long generation time of *C. tetani*.

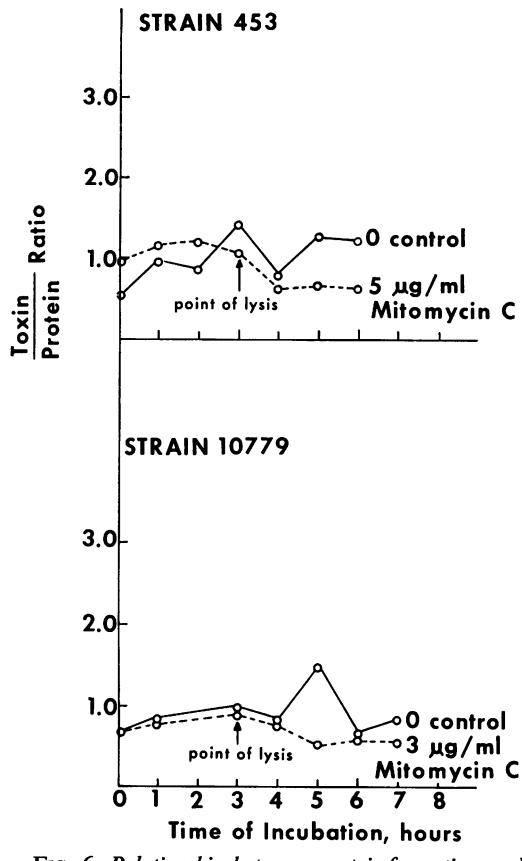

FIG. 6. *Relationship between protein formation and toxin production during induction of lysis with mitomycin C.*

C. tetanomorphum was used in this study because of its close resemblance to a nontoxinogenic *C. tetani*. Mitomycin C treatment failed to lyse *C. tetanomorphum*; however, it is not an indicator of any phage released by *C. tetani*. So far, a suitable indicator strain that could detect phage particles has not been found.

Induction of lysis by a radiomimetic agent such as mitomycin C may offer presumptive evidence of lysogeny even in the absence of a suitable indicator strain to detect free phage (2). Efforts to detect bacteriocin-like killer particles in mitomycin C-induced lysates of *C. tetani* have so far been unsuccessful.

The inhibition studies on macromolecular synthesis during induction have presented some clues to the mechanism of lysis. Chloramphenicol effectively blocked postinduction lysis, presumably by preventing synthesis of the lytic enzyme(s). Inhibitors of RNA and DNA synthesis were effective early in induction but were ineffective when added later in the induction. Lysis inhibition by chloramphenicol very late after induction resembles the late-function genes of bacteriophage, including the phage lysozyme necessary to lyse the infected cell. DNA, RNA, and protein syntheses all appear to be definitely involved in the lysis induced by mitomycin C.

The data have shown that the amount of intracellular toxin in cells of *C. tetani* is not affected by mitomycin induction, indicating that there is no marked increase in transcription or translation of the toxin gene(s). In contrast, a recent paper by Matsuda and Barksdale (12) showed that, after phage infection, the amount of diphtherial toxin per unit of protein increased enormously, since this toxin is programmed in the bacteriophage genome. Although a correlation between lysogeny and toxinogeny has been suggested in several bacterial genera (1, 6, 19), it can be tentatively concluded that in *C. tetani*, the mechanism of lysis following mitomycin induction is unrelated to the synthesis of intracellular toxin.

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