SOME EFFECTS OF IRON DEFICIENCY ON THE EXTRACELLULAR PRODUCTS RELEASED BY TOXIGENIC AND NONTOXIGENIC STRAINS OF *CORYNEBACTERIUM DIPHTHERIAE*[1]

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Received for publication February 27, 1957

Toxin production by *Corynebacterium diphtheriae* is closely associated with lysogenicity (Freeman, 1951; Groman, 1953; Barksdale and Pappenheimer, 1954). Indeed, only those strains of *C. diphtheriae* infected with the temperate bacteriophage β are capable of producing toxin. Nonlysogenic strains, or lysogenic strains infected with other temperate phages, even those closely related to β, are nontoxigenic (Groman, 1955; Barksdale, 1955). Toxigenicity appears to be associated with β-phage in much the same way that the E_2 serotype in Salmonella is associated with a particular prophage (Uetake *et al.*, 1955).

It is known that there is a relationship between toxin production and liberation of coproporphyrin III (Gray and Holt, 1948) by toxigenic strains of the diphtheria bacillus and that yields of both of these substances are in turn dependent upon a diminishing bacterial iron content (Pappenheimer, 1947, 1955). The present report is concerned with a comparison between the extracellular products released by certain nontoxigenic strains of *C. diphtheriae* and a toxigenic strain derived from one of them by infection with the temperate bacteriophage β. The strains were compared under conditions of decreasing bacterial iron content optimal for toxin production by the C7(β) strain. These studies were undertaken in the hope of gaining a clearer understanding of the relationship between prophage (i.e., lysogenicity) and the capacity to produce diphtheria toxin.

[1] Aided by a grant from the National Institutes of Health, U. S. Public Health Service. This work was conducted in part under the sponsorship of the Commission on Immunization, Armed Forces Epidemiological Board, and was supported in part by the Office of the Surgeon General, Department of the Army, Washington, D. C.

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MATERIALS AND METHODS

Cultures. Most of the experiments to be described used the toxigenic strain C7(β) of *C. diphtheriae* and the C7 indicator strain from which it had been derived by infection with β-phage. However, certain other nontoxigenic strains were also studied which included the lysogenic but nontoxigenic strain, C7(f), (Barksdale, 1955) and Halifax (Hf), a nonlysogenic variant of the *gravis* type.

Culture medium. The medium used was that of Mueller and Miller (1941) supplemented with 0.5 mg calcium pantothenate, 100 mg DL-tryptophan and 3 g sodium succinate/L. Deferration was carried out by the calcium phosphate gel method as described by Mueller and Miller. In the earlier experiments, 50 mg purified gelatin/L was added to prevent surface denaturation of toxin. Subsequently, this was shown to be an unnecessary precaution and was omitted. The final pH was adjusted to pH 6.8 to 6.9 and for most experiments the medium was dispensed in 200 ml amounts in 1 L Erlenmeyer flasks and sterilized at 10 lb for 10 min in the autoclave. Just before inoculation, 10 ml deferrated 50 per cent maltose (Pfanstiehl) solution was added aseptically.

Cultural methods. Mitsuhashi *et al.* (1949) studied the kinetics of toxin production in shake cultures and showed that toxin is only liberated from cells during the declining growth phase. Thus, if small inocula are used, a considerable length of time will elapse before measurable amounts of toxin will be released. The extent of this lag period will depend on the size of inoculum, the growth rate and the iron content of the medium. In order to obtain toxin with a minimum of delay, therefore, the following method was devised and found to be satisfactory.

The inoculum was prepared in medium containing a slight excess of iron. (One ml of 0.01 per cent $FeSO_4 \cdot 7H_2O$ in 0.01 per cent HCl was

added aseptically to each 200 ml of medium.) A small inoculum of a given strain was added so as to give a barely perceptible turbidity and the culture was incubated in the water bath at 32 to 33 C with constant mechanical shaking for about 15 hr. At the end of this time, the culture was harvested, centrifuged and washed twice with 50 ml changes of iron free medium. The washed bacteria were then suspended in iron free medium to give an optical density at 590 mμ which usually lay between 4 and 8 (250 to 500 μg bacterial nitrogen per ml). This heavy culture was then placed in the water bath at 32 to 33 C with shaking. Samples were taken at intervals for measurement of growth and for extracellular protein, toxin, porphyrin and nucleic acid analysis.

All of the glassware used was acid cleaned.

Analytical methods. (1) Estimation of growth:—Samples were diluted in distilled water and the optical density (OD) at 590 mμ was determined in a Bausch and Lomb spectrophotometer with 1 cm light path. The readings were converted to μg bacterial nitrogen per ml using the factor 56 μg N per unit OD (Barksdale and Pappenheimer, 1954). Some difficulty was encountered in obtaining homogeneous suspensions from packed centrifuged cells at the start of shaking. This difficulty could be largely overcome by using a Potter-Elvehjem type of homogenizer.

(2) Porphyrin analysis:—Samples were centrifuged and the culture supernatants adjusted to pH 5.0 with dilute acetic acid. One ml of supernatant was treated with an equal volume of alumina cream (Schmidt, 1931) and then diluted with 5 ml distilled water. The suspension was centrifuged and the precipitate, containing adsorbed porphyrin, was washed once with 5 ml distilled water. The washed precipitate was suspended in 5 ml 1 N HCl and the porphyrin eluted by placing in a boiling water bath for 2 min. After cooling, the precipitate was removed by centrifugation and the optical density of the supernatant at 403 mμ was determined. Porphyrin concentration was calculated using the value 5.3×10^5 for the molar extinction coefficient of coproporphyrin III (Jope and O'Brien, 1945).

(3) Toxin:—Determined by flocculation test and converted to μg per ml, assuming that 1 Lf is equivalent to 2.44 μg toxin protein (Pappenheimer and Yoneda, 1957).

(4) Total protein:—Precipitated from the culture supernatant by addition of an equal volume of 5 per cent trichloracetic acid. The precipitate was washed twice with 2.5 per cent TCA, dissolved in 1 N NaOH and the protein content was determined by the method of Lowry *et al.* (1951).

(5) Nucleic acid:—Schneider's (1945) method was used for extraction of nucleic acids from culture supernatants. The hot TCA soluble nucleic acid fraction was measured in the Beckman spectrophotometer at 257 mμ. Owing to the fact the TCA used showed considerable absorption at this wave length, the results obtained by this method must be regarded as approximate. Similar values were obtained in certain instances, however, by the colorimetric determination of desoxyribose according to Dische (1930).

Electrophoretic analyses. The electrophoretic behavior of fractionated extracellular proteins was examined on paper (method of Kunkel and Tiselius (1951) as modified by Kobayashi (1954)) and also in the Tiselius apparatus. Paper electrophoresis was carried out at 4 C for a period of 14 to 15 hr at 7 to 8 v and 0.3 or 0.5 milliamperes per cm width of paper. Each run used 2 or 3 parallel strips of Whatman no. 12 paper and highly purified toxin was always placed on one of the strips. Proteins were used in 1 to 2 per cent concentration and had been dialyzed against veronal buffer, ionic strength 0.1 and pH 8.5. When three solutions were examined simultaneously, the fraction from the nontoxigenic strain was compared with the corresponding fraction from the C7(β) strain and with purified toxin. The protein bands were stained with 0.05 per cent bromphenol blue in aqueous solution containing 1 per cent $HgCl_2$ and 2 per cent acetic acid as recommended by Kunkel and Tiselius.

RESULTS

Kinetics of growth and release of extracellular products by C7 and C7(β) strains. The following experiments, described in some detail, are representative of a large number of similar experiments.

In figure 1 are plotted results using the C7(β) strain. One liter of culture was grown with shaking on medium to which 1 mg $FeSO_4 \cdot 7H_2O$ had been added. When harvested after 14 hr at 32 to 33 C the OD 590 was 6.9. After centrifugation, the organisms were washed twice with iron free medium and resuspended in 400 ml of the iron free medium. The heavy suspension was then distributed equally between two 1 L Erlenmeyer

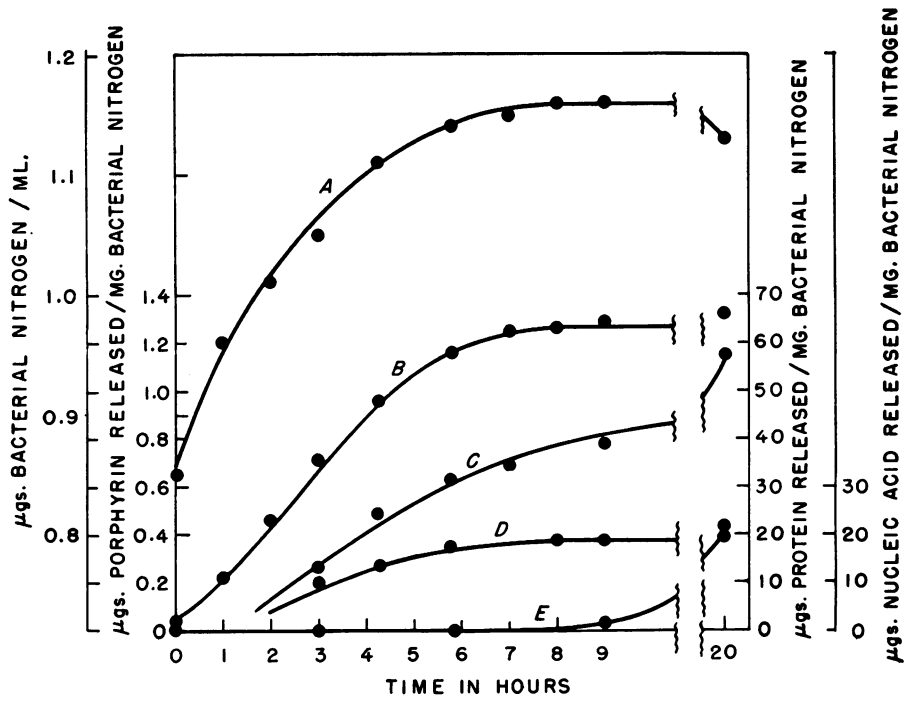

Figure 1. Kinetics of growth and release of total protein, toxin, porphyrin and nucleic acid by the C7(β) strain. Curve *A*: growth. *B*: coproporphyrin III. *C*: total extracellular protein. *D*: toxin. *E*: nucleic acid.

flasks and 10 ml deferrated 50 per cent maltose added to each flask. Both flasks were then placed in the water bath at 32 to 33 C with mechanical shaking. The initial OD 590 was 15.2, equivalent to 0.85 mg bacterial nitrogen per ml. Ten ml samples were taken at intervals from each flask and pooled. After measuring the optical density at 590 mμ, the pooled samples were centrifuged and the supernatants were analyzed for porphyrin, TCA precipitable protein, toxin and nucleic acid contents.

Figure 1 shows that the cultures entered the declining growth phase immediately after being placed in iron free medium. Iron is not the only rate limiting factor, however, when such a heavy bacterial suspension is used as inoculum, since in other comparable experiments, addition of iron failed to restore exponential growth. As seen from figure 1, measurable amounts of porphyrin were released within the first hr and of extracellular protein within 2 hr after inoculation. Almost 50 per cent of the total protein released before growth ceased could be accounted for as toxin. We may further note from the figure that, while liberation of toxin and of porphyrin were complete at the time of cessation of growth, the total extracellular protein continued to increase. It is probable that this continued increase in protein can be accounted for by autolysis, since it was only after growth terminated that significant amounts of nucleic acid appeared in the culture supernatant. Moreover, in this particular experiment nucleic acid release was associated with a significant decrease in turbidity of the culture.

The C7 strain was found to behave in a similar manner. In figure 2 is a comparison of results obtained with C7 and C7(β) cultures grown simultaneously under the same conditions as described for the previous experiment. The initial optical densities at 590 mμ after suspending in iron free medium were 4.1 and 3.6 for the C7 and C7(β) cultures respectively. In this experiment, both cultures continued to grow exponentially during the first hr and no measurable amounts of porphyrin or of protein were released. As soon as the cultures entered the declining growth phase, however, porphyrin appeared in the supernatants and within 2 hr, TCA precipitable protein could be detected. Figure 2 shows that, within the limits of experimental error, the growth rates and the

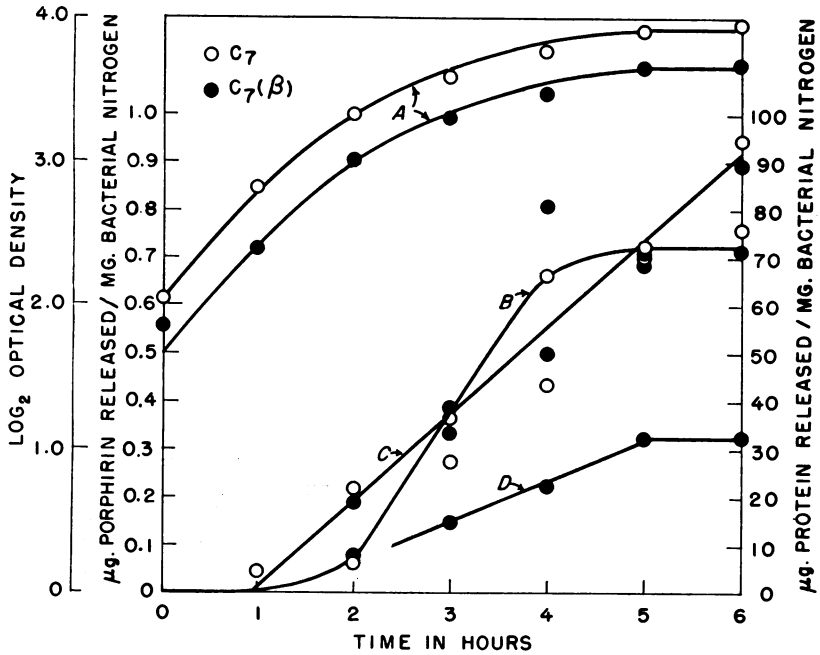

Figure 2. Kinetics of growth and release of total protein, toxin and porphyrin by C7 and C7(β) strains. Open circles: C7. Closed circles: C7(β). Curve *A*: growth. *B*: coproporphyrin III. *C*: total extracellular protein. *D*: toxin.

rates of liberation of coproporphyrin and of extracellular protein per unit bacterial growth were the same for both strains. Other experiments showed that slightly higher yields of total protein were sometimes released by the lysogenic strain.

Studies on the kinetics of porphyrin and extracellular protein release by 2 other nontoxigenic strains, C7(f) and Hf, gave results which were similar to those described for C7.

Liberation of porphyrin by C7 and C7(β) strains in relation to iron content of medium. The experiments just described demonstrate that C7 and C7(β) strains liberate almost identical amounts of coproporphyrin at identical rates when inoculated into iron free medium. It was of interest to determine if addition of iron to the culture medium would have an equal effect on porphyrin production by the 2 strains.

Suspensions of washed C7 and C7(β) strains were made in iron free medium as described in the preceding section. The suspensions (OD 590, 5.6 and 5.8 respectively) were distributed in 30 ml amounts into 300 ml Erlenmeyer flasks containing 0, 10, 30, 60 and 150 μg of $FeSO_4 \cdot 7H_2O$ and were incubated 14 hr at 32 to 33 C with continuous shaking. After measuring growth in each flask, the contents were centrifuged and the supernatants analyzed for their porphyrin content. As is shown in figure 3, with increasing concentrations of iron, porphyrin released into the culture supernatants decreased equally for both strains.

Fractionation of extracellular protein from nontoxigenic strains. The kinetic studies discussed above show that nontoxigenic diphtheria bacilli liberate extracellular protein in amounts and at rates comparable to the release of toxin by a toxigenic strain. It was therefore of interest to examine the chemical and immunological properties of extracellular protein prepared from nontoxigenic cultures and to compare these properties with those of diphtheria toxin.

Several liters of culture supernatant were collected from strains C7, C7(f) and Hf grown in iron free medium as described above. The supernatants were collected after about 15 hr growth at 32 to 33 C and therefore had undergone some autolysis. A typical culture supernatant from C7, prepared in this way measured 1.4 L and contained 0.74 μg/ml coproporphyrin and 37.5 μg/ml TCA precipitable protein. The culture supernatant was dialyzed for 3 days in the cold against saturated ammonium sulfate adjusted to pH 7.1. The precipitate which formed was col-

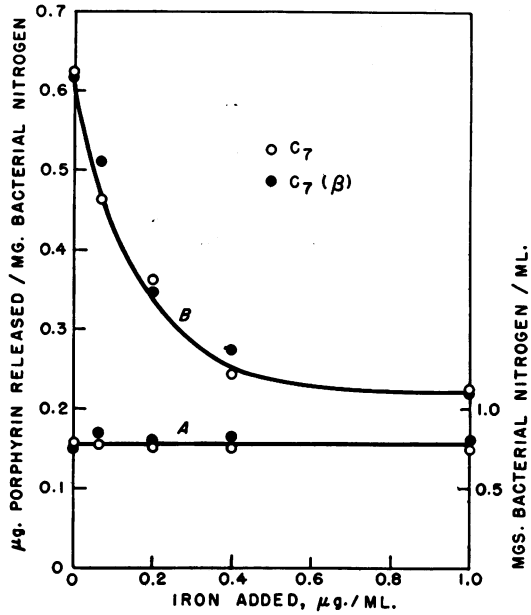

Figure 3. Effect of iron on porphyrin production by C7 and C7(β) strains. Open circles: C7. Closed circles: C7(β). Curve *A*: bacterial growth. *B*: coproporphyrin III.

lected by centrifugation and dissolved in M/10 phosphate buffer (pH 7). The brownish, turbid solution was centrifuged, a small amount of colored precipitate was discarded and the clear supernatant further fractionated with ammonium sulfate. The fraction soluble in ⅓ saturated but precipitated by ⅔ saturated ammonium sulfate was collected, dissolved in 2 ml of buffer and then dialyzed free of sulfate against changes of phosphate buffer. The over-all yield of protein was about 40 per cent. The final solution contained 7.6 mg protein/ml and was pinkish-brown in color. Similar fractions were prepared from the other two strains except that in the case of the Hf strain, a small amount of gelatin had been added to the original culture and was not completely removed by the fractionation procedure. The final yield and properties of protein fractionated in this way varied somewhat from lot to lot and from strain to strain. For example, it was observed that some lots contained little heat coagulable protein, whereas in others the protein was almost completely coagulated after a few minutes at 100 C.

Electrophoretic properties. Protein fractions from the C7 and C7(β) strains were compared with purified diphtheria toxin by simultaneous

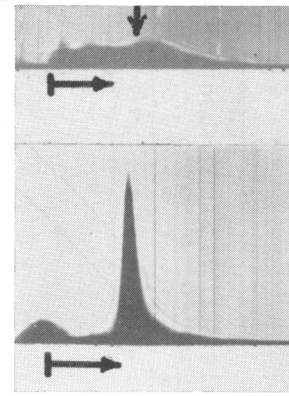

Figure 4. Descending electrophoretic patterns obtained in diethylbarbiturate buffer, pH 8.6, ionic strength 0.1. *Lower pattern:* Diptheria toxin after passage of 27.5 coulombs. Mobility of toxin, 4.1×10^5 cm^2 v^{-1} sec^{-1}. *Upper pattern:* C7 protein after passage of 31.1 coulombs. The arrow marks the position that a component with the mobility of toxin would occupy in this pattern.

electrophoresis on paper. The protein from the toxigenic C7(β) strain showed a sharp well-defined band with the same mobility as purified toxin. A second fast moving component was often present. Similarly prepared protein fractions from C7 and Hf strains invariably showed a component with approximately the same mobility as purified toxin. However, in all cases the band was broader and more diffuse than that of toxin.

The same type of inhomogeneity was apparent when C7 protein was examined in the standard Tiselius apparatus.[3] The upper electrophoretic pattern in figure 4 shows that even after salt fractionation, the C7 protein is extremely inhomogenous and contains many overlapping peaks. The arrow marks the position that toxin would occupy in this pattern. The lower pattern in figure 4 shows a similarly prepared fraction of toxin from the PW-8 strain showing the sharp, well-defined toxin peak together with a small amount of faster moving material.

Immunological properties of protein released by nontoxigenic strains. Numerous unsuccessful attempts were made to demonstrate interaction between diphtheria antitoxin and nontoxic protein liberated by the C7, C7(f) and Hf strains

[3] The authors are grateful to Dr. Robert Warner for carrying out the experiments in the Tiselius electrophoresis apparatus.

under conditions of decreasing bacterial iron content. Ammonium sulfate fractionated atoxic proteins from these strains, even when added in considerable excess, failed either to precipitate or to neutralize horse, rabbit or human antitoxin.

The following experiment shows that protein from a nontoxigenic strain is not co-precipitated with the toxin-antitoxin complex. In each of 10 centrifuge tubes 22.5 units of a human antitoxin were placed and increasing amounts of protein prepared from strain Hf were added (0 to 260 μg Hf protein nitrogen per tube). After incubating for 1 hr at 37 C, 10 Lf of diphtheria toxin was added to each tube. The total volume per tube was 1 ml. Visible precipitation first occurred in the control tube 20 min after addition of toxin, but required 70 min in those tubes containing 13 or more μg Hf protein-N.[4] The tubes were placed in the cold overnight and then centrifuged. The precipitates were washed twice with chilled saline, dissolved to a volume of 5 ml in 0.25 N acetic acid and protein determined by absorption at 277 mμ (Gitlin, 1949). Within limits of experimental error, all tubes contained close to 240 μg specifically precipitated protein. That the same amount of antitoxin was precipitated in all tubes was confirmed by analysis of the supernatants for antitoxin by the rabbit intracutaneous test (Fraser, 1931). Each of four supernatants so analyzed contained 5 to 7.5 units per ml of excess antitoxin.

Six rabbits were immunized with protein preparations from the C7, C7(f) and Hf strains incorporated in Freund adjuvant (Cohn, 1952). As shown in table 1, the antisera obtained gave weak precipitation when tested by ring test against protein fractions from all three nontoxigenic strains. Weak precipitin reactions were also obtained with both crude and purified toxoid prepared from the PW-8 strain and with proteins from a nontoxigenic strain grown in a medium containing excess iron. A rough quantitative determination was carried out using what appeared to be the strongest antiserum (anti-C7 protein). More than 100 μg TCA-precipitable C7 protein were required per ml of antiserum for maximal precipitation. Less than 150 μg total antigen-antibody complex were precipitated per ml serum at this point and the supernatant contained an excess of both antigen and antibody indicating the presence of more than one antigen-antibody system. None of the antisera contained demonstrable antitoxin. From our study of these antisera, we conclude that they contain several antibodies in low concentration directed against proteins common to both toxigenic and nontoxigenic strains. Since relatively large amounts of protein were required for formation of a relatively small amount of specific precipitate, we are forced to conclude that the major protein components released by the nontoxigenic strains were nonantigenic in rabbits under the conditions used for immunization.

TABLE 1

Precipitin reactions (ring tests) of diphtherial proteins with antisera from rabbits immunized with protein fractions from nontoxigenic strains of Corynebacterium diphtheriae

Antiserum*	Antigen†				
	C7-protein	C7(f)-protein	Hf-protein	Purified‡ toxin	Crude§ toxin
	(113) μg/ml	*(126) μg/ml*	*(129) μg/ml*	*50 Lf/ml*	*50 Lf/ml*
Anti-C7	++¶	++	+	+	+
Anti-C7(f)	++	++	+	+	+
Anti-Hf	tr	+	+	tr	tr

* Antisera heated at 56 C for 30 min.

† Figures in parenthesis denote protein concentration at which tests were carried out.

‡ 95 per cent specifically precipitable by antitoxin.

§ 25 per cent specifically precipitable by antitoxin.

¶ + = slight zone of precipitate; ++ = moderate zone of precipitate; tr = trace.

Relation of spontaneous phage release to toxin production. Barksdale and Pappenheimer (1954) showed that when the C7(β) strain is irradiated during exponential growth, phage development is induced and lysis occurs without release of toxin. The possibility remained that toxin might only be released as a result of phage lysis of iron deficient cells. The following experiment shows that this is not the case.[5] Shake cultures of the C7(β) strain in exponential growth were centrifuged and washed with iron free medium, and then re-

[4] Small amounts of protein from nontoxigenic strains delay precipitation of toxin by rabbit and human antitoxins. This effect on precipitation time is not specific, however, since the nontoxic protein causes a similar delay in precipitation of ovalbumin by rabbit anti-ovalbumin serum.

[5] This experiment was carried on in collaboration with Dr. W. L. Barksdale.

TABLE 2

Effect of iron on growth, toxin and spontaneous phage release by C7(β)

$FeSO_4.7H_2O$	Bacterial-N	Viable Count	Free β-phage	Per Cent of Bacteria Lysed*	Toxin
μg/30 ml	*mg/ml*	*× 10¹⁰*	*× 10⁷*		*μg/ml*
0	0.62	0.92	5.8	0.023	12
2	0.66	1.0	3.1	0.01	6
6	0.67	1.1	4.6	0.014	3
10	0.67	1.1	3.1	0.009	0
30	0.66	1.2	1.6	0.005	0

* Assuming burst size = 30 phage particles per bacterium.

TABLE 3

Toxin production by C7(β) when grown in mixed culture with C7 strain

Per Cent C7 in Inoculum	Optical Density of Mixed Culture C7 + C7 (β)		Toxin	Porphyrin Release
	Initial	Final		
			Lf/ml	*μg/ml*
0	8.9	16.0	5	0.28
0.12	8.9	15.0	5	0.28
1.2	9.0	16.0	5	0.26
11	10.0	16.5	5	0.23
100	9.5	18.0	0	0.23

suspended in iron free medium and distributed into 5 flasks containing increasing amounts of $FeSO_4 \cdot 7H_2O$. The cultures were incubated with shaking at 34 C for 7½ hr. At this time the optical density was measured and viable counts were carried out on each flask. Samples were centrifuged and the supernatants assayed for free β phage and for toxin. The results are shown in table 2. From this table it can be calculated that when 12 μg of toxic protein are released per ml, the number of bacteria spontaneously lysed by phage development is about 2×10^6 per ml (assuming burst size of 30). This would correspond to less than 1 μg of total protein per ml. It is clear that phage lysis can not account for the toxin released. Recently Hatano (1956) has carried out experiments along somewhat similar lines and arrived at the same conclusion.

Does the C7 strain produce a factor which inhibits or destroys toxin? Because of the inhomogeneity of the extracellular protein liberated by the C7 strain and because of our failure to demonstrate an antigenic protein analogous to toxin but characteristic of nontoxigenic strains, it seemed possible that C7 cultures might liberate a factor, perhaps a protease, capable of causing breakdown of toxin.

Cultures of C7 and C7(β) in exponential growth were centrifuged and washed with iron free medium. They were then mixed in varying proportions in iron free medium and incubated with shaking at 34 C for 16 hr. As shown in table 3, toxin production by the C7(β) strain is unaffected by the simultaneous presence of the C7 strain. Since only one division occurred (i. e., doubling of bacterial mass) it was impossible for any appreciable proportion of the C7 population to be converted to lysogenicity; hence, it seems unlikely that the C7 strain liberates an extracellular enzyme capable of breaking down toxin. If such a factor is formed by nontoxigenic strains, it must be intracellular.

DISCUSSION

Earlier studies (Pappenheimer, 1955) have suggested that diphtheria toxin may be related in some way to the protein moiety of diphtherial cytochrome *b*. Comparative studies of the C7 strain before and after its conversion to toxigenicity by infection with β-phage have provided no evidence either for or against this hypothesis. Both C7 and C7(β) have identical cytochrome *b* contents as determined by difference spectra of cell extracts. The specific activities of the succinoxidase and DPNH oxidase systems are identical and these systems are equally reduced in both strains grown under conditions in which iron becomes limiting.

The introduction of β-prophage into a strain of *C. diphtheriae* causes no known alteration in the biochemical properties of cells maintained in the exponential growth phase. Under normal conditions of exponential growth of C7(β), for example, the presence of prophage can only be detected by demonstration of free phage β in the culture filtrate owing to spontaneous phage lysis of occasional cells within the population. Once a toxigenic culture enters the declining growth phase, due to limitation of its iron supply, free coproporphyrin appears in the supernatant accompanied by extracellular protein. The major protein component released from cells of decreasing iron content is diphtheria toxin. Nontoxigenic strains, whether lysogenic or nonlysogenic, also

liberate porphyrin when the iron supply becomes limiting. The present studies clearly show that introduction of prophage β has no effect on the amount or rate of free porphyrin release by the C7 strain. Moreover, the addition of increasing amounts of iron to the culture medium reduces porphyrin production equally by the two strains.

Diphtheria bacilli liberate only traces of extracellular protein during exponential growth. In the present experiments, organisms were grown to very high population densities under conditions where exponential growth could no longer be maintained. Nevertheless, no appreciable amount of protein was released into the culture supernatants by either lysogenic or nonlysogenic strains until iron became limiting. Once iron became a limiting factor, protein appeared in the culture supernatant, but significant amounts of nucleic acid were not detected until the complete cessation of growth. This observation suggests that protein release from bacteria of decreasing iron content occurs without lysis of any important proportion of the cells. Our experiments also confirm those of Hatano (1956), in showing that proteins released during the declining growth phase do not originate from phage-lysed cells. In the case of the C7-C7(β) pair, the amount and rate of protein release is the same or nearly so for both strains. While the amount of protein released is approximately the same, the presence of prophage β causes profound changes in its properties, i. e., toxicity, antigenicity, electrophoretic behavior, etc.

If it could be demonstrated that a distinct antigenic protein component were liberated by a nontoxigenic strain during the declining growth phase, this would provide evidence that prophage β is directly concerned in synthesis of a specific toxic protein by toxigenic strains. This would be true regardless of whether or not the antigenic protein produced by the nontoxigenic strain, cross-reacted with diphtheria antitoxin. However, all attempts to demonstrate an immunologically active protein that is characteristic of nontoxigenic strains, whether lysogenic or nonlysogenic, have been unsuccessful. Further evidence against the existence of such a protein was provided by Uhr *et al.* (1957). Their experiments showed that while intradermal infection of guinea pigs with toxigenic strains of *C. diphtheriae* induced marked delayed hypersensitivity to toxin, infection with nontoxigenic strains merely lead to slight sensitivity to proteins common to both toxigenic and nontoxigenic strains.

The possibility exists that prophage β is in some way concerned with the manner in which protein is released from the cell when iron becomes limiting. Diphtheria toxin is a heat coagulable protein of well-defined chemical and immunological properties. On the other hand, the proteins liberated by nontoxigenic strains of *C. diphtheriae* of decreasing bacterial iron content, are ill-defined and inconstant in their properties. Even working with a given nontoxigenic strain, we have found great variation in heat coagulability, and electrophoretic patterns show a number of poorly defined and spreading peaks. This variability would be consistent with the notion that nontoxigenic strains are more proteolytic than are toxigenic ones. However, evidence in favor of this hypothesis has not been forthcoming as yet.

The present study has provided us with certain definite information with respect to the comparative behavior of toxigenic and nontoxigenic strains of *C. diphtheriae* grown under conditions of iron limitation. Whatever the mechanism by which infection with β-phage confers the property to toxigenicity upon a sensitive strain may be, introduction of prophage β causes no change in the amount or rate at which either coproporphyrin III or proteins are released from the cells. The presence of prophage β does result in a profound change in the nature of the protein liberated, however. Only when this prophage is present do the organisms release a toxic antigenic protein. The mechanism by which prophage β brings about this alteration has not been elucidated and the solution of this problem awaits further study.

SUMMARY

A kinetic study has been made of the release of total extracellular protein, toxin, coproporphyrin III and nucleic acid by certain strains of *Corynebacterium diphtheriae* under conditions in which iron had become limiting as a growth factor. Under these conditions, the yields and rates of total protein and of porphyrin liberated by the toxigenic C7(β) strain and by the nontoxigenic C7 strain from which it had been derived by infection with β-phage, were found to be the same. Other nontoxigenic strains, whether lysogenic or not, behaved in a similar manner. Nucleic acid did not appear in the culture supernatants until growth had come to a complete standstill.

In contrast to diphtheria toxin, the protein released by nontoxigenic strains was polydisperse on electrophoresis and variable in its properties. It was nonantigenic when injected into rabbits and failed to interact with diphtheria antitoxin.

The significance of these findings and their bearing on the problem of relating lysogenicity to toxigenicity are discussed.

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