THE ROLE OF TEMPERATE BACTERIOPHAGE IN THE PRODUCTION OF ERYTHROGENIC TOXIN BY GROUP A STREPTOCOCCI*

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PLATE 91

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In 1927 Frobisher and Brown (1) using doubly marked bacterial strains demonstrated that a filtrable agent isolated from scarlatinal strains of hemolytic streptococci could induce the formation of erythrogenic toxin by non-scarlatinal strains. This newly formed extracellular product was neutralized by standard antitoxins, and the capacity to produce erythrogenic toxin was carried through several subcultures. Bingel (2) essentially confirmed these findings, but the isolation of the filtrable agent responsible for the acquisition of this new property by non–toxin-producing strains was not reported in either of these studies. In view of the current knowledge of bacteriophages these earlier preliminary observations suggested that a streptococcal bacteriophage might play a role in the production of erythrogenic toxin.

This hypothesis was further strengthened by the series of classic papers published by Freeman (3, 4). He demonstrated that avirulent strains could be rendered both toxinogenic and lysogenic following exposure to bacteriophages obtained from virulent strains of *Corynebacterium diphtheriae*. The toxigenicity could be carried through many bacterial generations and did not depend on chance phage contamination of the organism. Freeman (4), therefore, concluded that an important relationship existed between the lysogenic state of the organism and its toxigenicity and suggested that the mechanism of the effect might be either mutant selection or an induced change in the bacterial metabolism as a result of lysogenization of the bacterial strain. These remarkable observations have been subsequently confirmed by Groman (5), Barksdale *et al.* (6), and Parsons (7), and the evidence now indicates that this property is indeed a cellular change under the genetic control of the phage.

It is known from the recent work of Krause (8) and Kjems (9) that lysogeny is quite common in Group A hemolytic streptococci, and this finding strengthened the possibility that the transmissible toxigenicity observed by Frobisher and Brown might depend on a relationship between streptococci and lysogenic bacteriophages similar to that described in the *C. diphtheria* lysogenic complex.

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The work reported here supports the validity of this concept: namely, that non-toxin-producing streptococci when lysogenized by temperate phage from scarlatinal toxin strains acquire the capacity to produce erythrogenic toxin.

Materials and Methods

Strains of Streptococci.—All of the strains were obtained from the Rockefeller Institute collection. They were grouped and typed by the Lancefield method (10). In certain instances, strains not typable by the acid extraction method for M protein were typed by Griffith's slide agglutination technique (11). Strains lysogenized in the laboratory bear a special designation indicating their composite nature. For example, in the designation $T25_3$ (T12g1), $T25_3$ refers to the phage recipient strain (T12g1) indicates the strain donating the bacteriophage used for lysogenization.

Todd-Hewitt Broth.—This beef heart infusion was prepared as described (12).

Dialysate Media.—Krause (8) had previously demonstrated that in order to obtain satisfactory plaque formation with hemolytic streptococci it was necessary to use a dialysate medium of beef heart infusion broth and peptone. Although this was excellent for the demonstration of virulent phages, the formation of temperate phage was more erratic and often not entirely reproducible. After many trials, the medium best fulfilling all the requirements was a dialysate medium described by Wannamaker (13) with the following modification. The medium was adjusted to pH 7.5–7.6 rather than 7.8 and filtered as described without the addition of sodium bicarbonate. The flasks could then be stored at 4°C for several weeks without change in pH. On the day of use, sodium bicarbonate was prepared in distilled water, filtered through a Coors No. 2 porcelain filter and added in a concentration of 200 mg per 100 ml of the dialysate medium used.

Preparation of Agar Plates.—Agar plates are prepared as follows: Difco agar is dissolved in distilled water at 2.4 per cent concentration, the pH adjusted to 7.5, and the agar then sterilized in the autoclave. For each liter of agar medium, 500 ml of 2.4 per cent melted agar at 50°C is added to 500 ml of dialysate broth warmed to the same temperature. Sodium bicarbonate was *not* added to the medium for the preparation of plates. Approximately 35 ml is poured into each Petri dish.

Agar medium for the soft agar layer is prepared in a similar fashion except that 1.2 per cent agar in distilled water is used instead of 2.4 per cent agar. Dialysate broth is added to the agar to make a final agar concentration of 0.6 per cent.

Plating and Counting Bacteriophage.—The soft agar layer method of plating phage and streptococci was used to demonstrate plaque-forming units (14). During preliminary experiments it was noted that the bacterial lawn growth was often irregular and spotty in the soft agar layer, making it difficult to detect plaques in the bacterial lawn. This difficulty was circumvented by the selection of bacterial variants capable of luxuriant growth in the surface layer of soft agar. These variants were picked, subcultured in dialysate broth, and used as stock cultures. The day before use, 0.1 ml of the stock culture was inoculated into 30 ml of dialysate broth with added 0.2 per cent sodium bicarbonate, incubated at 37°C for 18 hours, centrifuged and resuspended in 10 ml of fresh dialysate broth. One-tenth ml of this suspension was added to the soft agar layer tubes at the time of plating. All agar plates were incubated at 37°C under reduced O_2 tension provided by a candle jar.

Phage and Toxin Dilutions.—All dilutions were carried out in dialysate broth pH 7.5.

Optical Density.—Optical densities of bacterial suspensions were read at 560 μ in a Coleman junior spectrophotometer. Standard 19 x 105 mm tubes were used for all determinations.

Antitoxins.—The National Institutes of Health (NIH) standard antitoxin was supplied by

Biological Standards Division of the National Institutes of Health and contained 40 units of antitoxin per ml. Rabbit antitoxins containing 12 units per ml were obtained by injecting rabbits subcutaneously with 4 weekly injections of 30 mg of the concentrated lyophilized extracellular products of the C203 streptococcal strain in Freund's adjuvant. These lyophilized preparations were kindly supplied by Dr. S. Halbert (15) and were known to contain erythrogenic toxin. The animals were bled 7 to 10 days after the final injection.

Toxin.—A standard toxin containing 35,000 units toxin/ml was supplied by the Biological Standards Division of the National Institutes of Health.

Rabbits.—New Zealand white rabbits were particularly satisfactory for skin tests of erythrogenic toxin and antitoxin, although New Zealand red and grey chinchillas were also adequate for preliminary titrations. Because of some variability in the skin response to the toxin in the three strains, it was found preferable to complete an experiment with a single rabbit breed.

Agar Diffusion Plates.—Double diffusion studies were carried out using a 1 per cent Noble agar (Difco Laboratories, Inc., Detroit) diluted in veronal buffer pH 8.35 0.1 mμ. The plates were kept at 4°C and observations made daily for 5 to 7 days.

Bacteriophage Antibody.—Phage antibody was prepared essentially as outlined by Adams (16). One to two ml amounts of phage lysates of $T25_3$ (T12g1) containing 1×10^8 plaque-forming units per ml were injected intravenously into rabbits daily for 5 to 7 days. The animals were bled 10 days following the last injection. Control bleedings were obtained prior to immunization.

EXPERIMENTAL

Demonstration of Lysogeny in Group A Streptococci.—A variety of methods have been employed, (Kjems, reference 17; Krause, reference 8), to demonstrate the presence of lysogenic streptococci. All of these procedures depend on the spontaneous synthesis of mature particles from the prophage in the propagating strain with subsequent release into the growth medium. The supernatants of these strains can thus be mixed with suitable "indicator" strains and plated by the soft agar layer technique. A modification employed by Kjems is the streaking of the growing culture over an agar plate followed by the use of chloroform vapor to kill selectively the bacteria leaving only the bacteriophages viable. Drops of cultures of indicator strains are then placed on the plates and, following incubation, plaques appear in the opaque lawns of those strains susceptible to the lytic action of the bacteriophage. The obvious advantage of this method over the soft agar layer technique is that many indicator strains can be tested on a single agar plate with a given lysogenic strain.

Our preliminary experiments utilized both these methods but the plaques produced were often indistinct and the results difficult to reproduce. In part, this was probably a reflection of the variation from strain to strain in the extent of spontaneous production of temperate phage; in part it may have been dependent on interference by components of the complex media utilized. Thus, the problem of demonstrating lysogeny was twofold: firstly, to find a satisfactory medium which would permit good streptococcal growth and possess factors favoring good plaque formation; and secondly, to find a method to induce large numbers of lysogenic organisms to produce temperate phage.

A dialysate medium similar to that utilized by Dr. Wannamaker (13) satisfied the first requirement. For the second, irradiation with ultraviolet light proved to be the most efficient method of enhancing the induction of a lysogenic strain. The following procedure was therefore adopted:

The strain to be tested is taken from the lyophilized state, placed in Todd-Hewitt broth containing 5 per cent rabbit blood, and grown for 18 hours in an incubator at 37°C. This is used as a stock culture. One-tenth ml of this culture is inoculated into 5 ml of dialysate medium to which sodium bicarbonate had been added at a concentration of 200 mg/100 ml.[1] The strain is grown for 4 hours at 37°C, usually reaching an OD of 0.10 to 0.15 on the spectrophotometer. One ml samples are then placed evenly over the surface of the agar plates, and the excess fluid removed by pipetting the fluid collected after tilting the plates slightly. The plates are then inverted and dried at 37°C for 15 to 20 minutes. The bacteria adhering to the surface of the plate are irradiated with ultraviolet light using a 15 watt General Electric germicidal lamp at a standard distance of 30 cm. To prevent fluctuations in the amount of irradiation given, the lamp is turned on 15 minutes prior to irradiation. The duration of irradiation varied but usually ranged from 20 to 40 seconds. Other strains, including the strain being tested for lysogeny, are grown for 18 hours in dialysate broth and serve as indicators. The irradiated plates are marked off in sections and 0.02 ml drops of each indicator strain are placed on the appropriate section and allowed to dry. Although the plates were originally incubated at 37°C in air, it was subsequently noted that the appearance of the plaques was more consistent when incubated in a candle jar.

After 19 hours a diffuse bacterial lawn appears in the inoculated areas, and the temperate phages released by the irradiated culture produce plaques in the opaque lawn (Fig. 1). As noted, plaques do not appear when non-susceptible strains are employed as indicators. Unirradiated controls were always included to be sure that temperate phage was not being spontaneously released by the indicator strain. This method permitted a rapid screening of many streptococcal strains for the presence or absence of temperate bacteriophage. Of a total of 88 strains tested in this manner approximately 85 per cent were positive for the presence of bacteriophage. Six different Lancefield types were included, but the majority of strains were Type 19 isolated from patients during an outbreak of scarlet fever at the Great Lakes Naval Station during World War II. Although this figure is considerably higher than that reported by others, (28 and 23 per cent in Kjems', reference 9, and Krause's, reference 8 series respectively) this is probably a reflection of the single lysogenic Type 19 strain. Kjems (9) also observed a striking change from non-lysogenic to lysogenic Type 12 organisms following an outbreak of streptococcal infection in a sanatorium, and the percentage of lysogenic strains during this period closely approaches the figures given here.

A majority of these phage-producing strains were detected using only a Type 25 and one or two Type 12 indicators. Subsequently certain Type 3 and Type 30 strains were also found to be suitable indicators. Furthermore, the phages did

[1] Unless otherwise stated all dialysate media for growth contain sodium bicarbonate added on the day of use at a concentration of 200 mg/100 ml of media used.

not appear to be type specific, as a number of Type 19 and Type 12 cultures produced plaques on a Type 25 indicator strain.

During these experiments it was noted that certain strains accepting bacteriophage from irradiated strains would upon irradiation themselves produce temperate bacteriophage acceptable to other indicator cultures. As a result, it was evident that a strain could carry more than one bacteriophage and for the studies on the relation of lysogeny to the production of erythrogenic toxin, it was important to attempt selection of a strain free of detectable bacteriophage.

Selection of Phage-Free Strains.—

Strain T25-41 was chosen for the following reasons. Previous work by Krause (8) indicated that this strain had accepted many heterologous group A bacteriophages, suggesting that it was particularly susceptible to the lytic action of virulent or temperate phages. While this strain gave evidence of being lysogenized, the possibility that it might be easily "cured" was strengthened by the fact that following surface irradiation the number of plaques produced were few. Since the Lederbergs (18) had noted a high incidence of non-lysogenic cells among the survivors following prolonged ultraviolet irradiation of a lysogenic strain, this was the method adopted for selection of a phage-free strain. Two-tenths ml of strain T25-41 stock culture was inoculated in 5 ml of dialysate broth and grown for 4 hours in a 37°C water bath. One ml samples were spread over agar plates, the excess fluid removed, and the plates dried as described in the previous section. The standard irradiation distance was employed, but the time of irradiation prolonged until 50 to 100 survivors remained on the surface. 200 to 300 seconds was usually sufficient. Twenty individual colonies were picked among the survivors, substreaked several times to remove contaminating phage particles and subsequently tested for the presence of bacteriophage by the method of surface irradiation.

Although the majority of the colonies picked still retained their lysogenic property, three colonies were found on repeated testing to be phage-free with the available indicators. It will be shown subsequently that one of the colonies, $T25_3$, lysogenized by phage from known scarlatinal toxin-producing strains now produced phage plaques upon irradiation.

Lysogenization of Phage-Free Strains.—The plaques noted in Fig. 1, produced by phage from a toxin-producing strain, were picked by repeated stabbings of plaque centers and transfer of the adhering virus particles to 0.5 ml dialysate broth with vigorous shaking. The phage suspensions were freed of contaminating bacteria by passage through a Coors No. 3 porcelain filter.

In order to avoid loss of phage particles in filtration it was necessary to "prime" the filters with 35 ml of Todd-Hewitt broth prior to filtration of the phage suspensions. Subsequently it was found that Millipore filters (MF type HA pore size 0.45 μ) could be used for the purpose without prior treatment. The sterile filtrates were then replated on the indicator strain. Occasionally, only a few plaques appeared on the original transfer, but the process could then be repeated on the indicator strain until phage stocks were obtained capable of lysing the bacterial lawn completely. These phage stocks, containing usually 1×10^6 particles per ml, were now used to lysogenize $T25_3$, the phage-free indicator strain. The method employed was as follows:

0.2 ml of a high titer phage stock obtained from strain T12 glossy,[2] containing 1×10^6 virus particles per ml was mixed with 0.1 ml of strain $T25_3$ containing 1×10^8 organisms per ml. These were plated by the soft agar layer method, and following incubation confluent lysis was noted except for areas where resistant colonies appeared within the confluent plaques. These colonies were picked and identified as $T25_3$ although differing from the original by virtue of lysogenization by phage from strain T12 glossy.

Although the chance of contamination by a temperate phage was minimized by the careful selection of single lysogenic colonies and repeated substreakings, the possibility existed that lysogeny was "apparent" rather than "true." The following experiment was designed to answer this question. A high titer rabbit antiserum was made by repeated daily intravenous inoculations of phage lysates of strain $T25_3$ (T12g1) and when diluted 1/100 this serum gave 99 per cent inactivation of (T12g1) phage plaques (see Text-fig. 7). Serial transfers of strain $T25_3$ (T12g1) were therefore carried out in broth containing phage antiserum at a final dilution of 1:10. Even after twelve transfers, $T25_3$ (T12g1) was still capable of producing bacteriophage when induced by ultraviolet irradiation.

This strongly suggested these strains were indeed lysogenic and not contaminated by temperate phage. Experiments were now designed to test the relationship between the state of lysogeny in Group A streptococci and the production of erythrogenic toxin.

Phage and Toxin Titers of Lysogenic Streptococcal Strains.—In the following experiments, a comparison of phage and toxin production was made between a phage-free strain $T25_3$ and the identical strain lysogenized by temperate phage from a known scarlatinal strain 3GL16.

0.5 ml of a stock culture of both strains was placed in 35 ml of dialysate medium and incubated simultaneously in a 37°C water bath for 4 to 6 hours. The cultures were centrifuged, washed once in saline, and resuspended in fresh saline until an OD of 0.50 to 0.55 was reached. The suspensions were then diluted with fresh dialysate broth until an OD of 0.15 was obtained; the cultures were then reincubated at 37°C. At hourly intervals, small aliquots were removed, centrifuged, and the supernatants tested for phage by the soft agar layer technique.

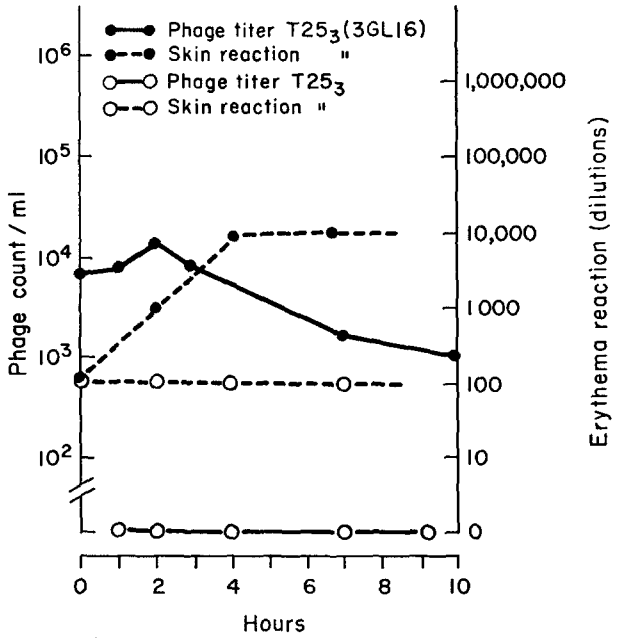
The initial test for toxin production consisted of serial tenfold dilutions of the broth supernatants in dialysate broth. One-tenth ml of the dilutions was then injected intradermally into the backs of shaved rabbits. The end point was read as the highest dilution giving a 10 x 10 mm area of erythemia. This has been defined as a "skin test dose" of toxin (19). A known toxin and broth control were always run simultaneously to establish the specific and non-specific reactivity of the individual rabbits. Positive reactions were considered valid only if neutralizable by standard scarlatinal antitoxin sera.

The results of this experiment are depicted in Text-fig. 1. As noted, the phage titer of the "cured" strain $T25_3$ remained negative throughout the growth period. Minimal erythema reactions were noted in the skin of rabbits only at low dilutions, probably representing a skin reaction to streptococcal products other than erythrogenic toxin. Conversely, the lysogenic strain $T25_3$ (3GL16) showed the presence of phage throughout the growth period, usually reaching a peak titer at 2 to 3 hours and then falling to a constant level in 6 to 8 hours. Although at the time of the phage burst the toxin titer remained relatively

[2] Scarlet fever strain obtained from Dr. F. Griffith.

stable, it subsequently began to rise reaching its peak at 8 to 10 hours. Since the data strongly suggested a relationship between the production of bacteriophage and the appearance of erythrogenic toxin, ultraviolet irradiation of a liquid culture was carried out next to determine if enhanced phage production would result in increased toxin production.

Ultraviolet Enhancement of Bacteriophage Production.—Since studies in other phage bacterial systems (Barksdale *et al.*, reference 6) have indicated that there is an optimal dosage of ultraviolet irradiation for the enhancement of phage



TEXT-FIG. 1. A comparison of phage and skin reactions of the lysogenic strain $T25_3$ (3GL16) and the non-lysogenic $T25_3$ respectively. The skin reaction was defined as the highest dilution of toxin giving an area of erythema 10 x 10 mm in diameter.

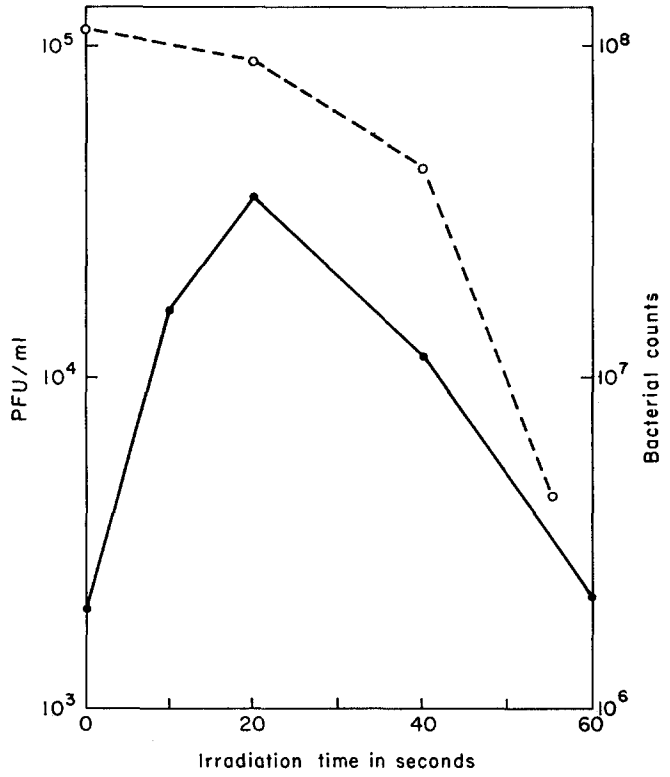
production, it was important to determine first the amount of ultraviolet irradiation necessary to insure maximal production of phage particles. Prolonged irradiation usually results in death of a large segment of the bacterial population and a concomitant decrease in phage production.

The lysogenic strain $T25_3$ (T12g1) was prepared for irradiation as follows: 1.0 ml of a stock suspension was inoculated into several hundred ml of dialysate medium and grown at 37°C for 4 to 6 hours. After centrifugation the sediments were pooled, washed once in a saline, and resuspended in saline to an OD of 0.20 to 0.25.[3] Ten-ml aliquots of this resuspension were next

[3] Saline suspensions were used for all irradiation experiments in liquid medium as broth suspensions proved to be highly erratic in the number of phage particles induced from experiment to experiment.

placed in Petri dishes and each dish subjected to irradiation for different time intervals. Five-ml samples from each dish were placed in 35 ml of fresh dialysate medium, and, following 3 hours incubation at 37°C, samples were removed from each culture and tested for phage production. Similarly aliquots were also appropriately diluted for bacterial survivor counts following irradiation.

Text-fig. 2 depicts the results obtained. As noted, the optimal dosage for

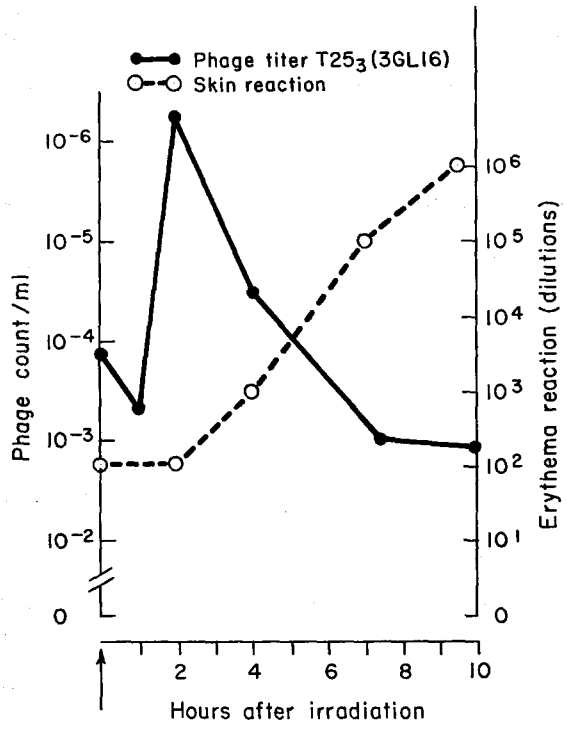


TEXT-FIG. 2. The effect of ultraviolet irradiation on viability of lysogenic streptococci and the production of phage particles. Bacterial counts are represented by the broken line, phage titers by the solid line.

maximal phage production was 20 seconds at a time when 10 per cent or less of the bacterial population had been killed. Higher doses result in a marked fall in bacterial counts and drop in phage titer. Selecting 20 seconds as an optimal irradiation dosage for the enhancement of bacteriophage production, experiments were now performed to test the enhancing effect of ultraviolet light on phage and toxin production.

The protocol is similar to that described previously for the growth of the two

strains illustrated in Text-fig. 1. After appropriate dilution in saline, one aliquot of the suspension was irradiated for 20 seconds, and a second aliquot was held as the unirradiated control. The suspensions were transferred to fresh dialysate broth and reincubated at 37°C. Hourly samples were again tested for phage and toxin production. Text-fig. 3 depicts the results of these experiments. The un-



TEXT-FIG. 3. The effect of ultraviolet irradiation on augmentation of phage and toxin titers in strain $T25_3$ (3GL16). The arrow indicates the point at which ultraviolet irradiation was applied.

irradiated control gave results similar to that depicted in Text-fig. 1. In comparison the irradiated strain demonstrated a marked phage burst reaching a peak at 3 hours following irradiation. Concomitantly the erythema reaction had also increased considerably reaching a titer of 10^5 to 10^6 skin test doses per ml. The non-lysogenic strain $T25_3$ again failed to produce bacteriophage even after irradiation, and the minimal reaction noted in Text-fig. 1 was not augmented. Experiments similar to those illustrated in Text-fig. 3 were carried out on several strains lysogenized by phage from different scarlatinal cultures. The specific nature of the erythema produced by these culture supernatants

was now determined by utilizing a standard NIH scarlatinal antitoxin to neutralize the erythema reaction.

Toxin-Antitoxin Neutralization.—For the preliminary titration of the toxin reaction in each strain, culture supernatants selected at the time of maximal toxin production were serially diluted tenfold in dialysate broth.

0.1 ml of these dilutions was injected intradermally into the shaved backs of rabbits. In this manner two or three strains could be tested in the same rabbit. The highest dilution of each strain giving a reaction greater than 10 x 10 mm area of erythema was then serially titrated in twofold dilutions until a final end point was reached which just gave a 10 x 10 mm reaction. This was called a 1 skin test dose of toxin. To these twofold dilutions was added an equal amount of a standard NIH antitoxin appropriately diluted. The tubes were incubated for 1

TABLE I

Toxin-Antitoxin Neutralization Studies with an NIH Standard Antitoxin

Strain	Dilution required for 1 STD*	Amount of antitoxin neutralizing 1 STD
		ml
$T25_3$ (T12g1)	10,000	0.0075
$T25_3$ (T12g1)‡	2000	0.0075
$T25_3$ (B276)	40,000	0.0075
$T25_3$ (3GL16)	4000	0.0075
$T25_3$	20	0.01

* 1 STD is defined as the amount of toxin in 0.1 ml which will give an area of erythema 10 by 10 mm.

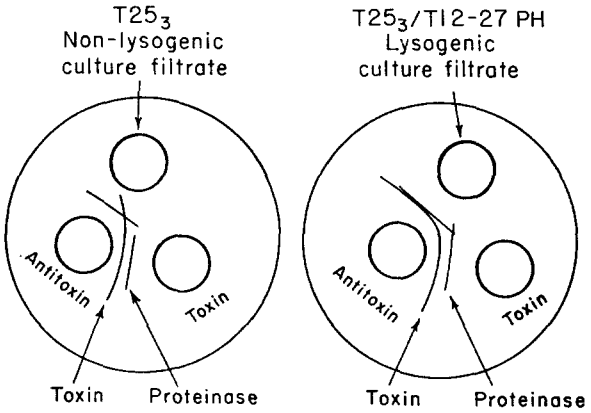
‡ Non-irradiated companion strain.

hour at 37°C at which time 0.1 ml of the toxin-antitoxin mixtures was injected into the skin of rabbits. A known toxin-antitoxin mixture was included in each series of tests as well as toxin and antitoxin alone. Knowing the highest dilution of toxin which was neutralized by a constant amount of antitoxin, the amount of toxin present in the original broth supernatant could then be calculated.

Table I demonstrates the number of toxin units found in the supernatants of several lysogenic strains. When compared to the non-lysogenic $T25_3$, the amount of toxin produced was 100 to 1000 times greater than that found in the phage-free strains. The fact that the supernatants of non-lysogenic $T25_3$ gave skin reactions only at low dilutions and required relatively more antitoxin for neutralization suggests that the reaction obtained represents a response to streptococcal products other than erythrogenic toxin.

Precipitin Studies of Toxin by Agar Diffusion.—Since it was highly suggestive that the lysogenic strains were producing a new extracellular product not found in non-toxin-producing cultures, agar gel experiments were performed in an attempt to demonstrate that this product was indeed related to erythrogenic toxin.

A partially purified toxin preparation of high potency (kindly supplied by Dr. Aaron Stock) served as the standard toxin and was stated to be primarily "A" toxin. When diluted 1/100 this preparation gave two distinct precipitin lines with a known rabbit antitoxin serum in agar double diffusion plates. One of the bands formed a line of identity with streptococcal proteinase (20) and the other presumably represented erythrogenic toxin. If the hypothesis that a new product had appeared in the lysogenic strains was correct, this should form a line of identity with the standard toxin, and no line should be present in the phage-free strain. The unconcentrated supernatants however gave only hazy and indistinct lines in agar gel, and concentrates were prepared in the following manner. The cultures were centrifuged at 2500 RPM to sediment the bacteria, the supernatant fluids decanted, and the extracellular products precipitated by the addition of ammonium sulfate (0.7 per cent saturation) to the broths. The mixtures were



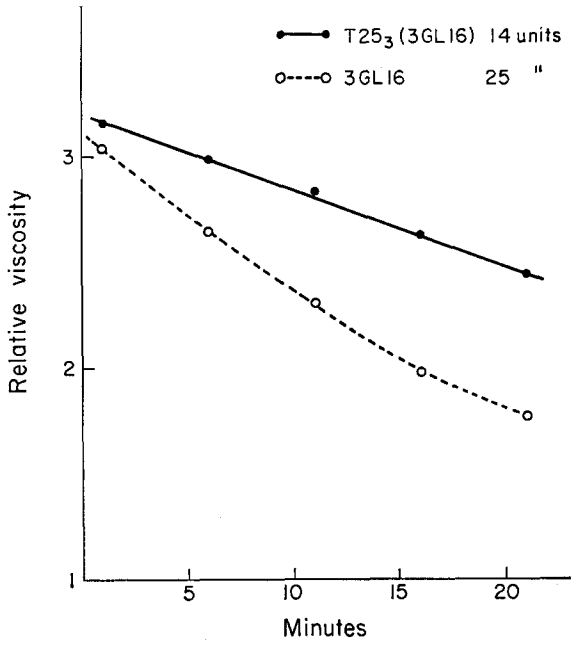
TEXT-FIG. 4. Tracing prepared from an Ouchterlony plate showing lines of identity between Stock's partially purified toxin diluted 1/100 and erythrogenic toxin from lysogenic strain T25$_3$ (3GL16). As noted a similar reaction does not occur in the non-lysogenic strain T25$_3$. The antitoxin was a known standard rabbit antitoxin.

left at 4°C for 24 to 48 hours and the light precipitates collected by centrifugation at 4000 RPM for 30 minutes. The supernatants were discarded and the precipitate resuspended in dialysate broth and concentrated *in vacuo* to 1/50 to 1/100 of the original volume. The concentrated supernatants were then dialyzed against veronal buffer pH 8.14, brought to equal volumes, and stored at 4°C until used. In the double diffusion test illustrated in Text-fig. 4 the upper wells contain the concentrates of the non-lysogenic culture filtrates respectively. The left lower wells contain the rabbit antitoxin and the right lower wells Stock's partially purified toxin preparation. The agar plates were developed at 4°C and observed daily.

Within 24 to 48 hours two distinct bands formed between the standard toxin and the rabbit antitoxin. As indicated, the band labeled proteinase was identified as streptococcal proteinase. The second line labeled toxin merged with a similar band emanating from the well containing the concentrate of the lysogenic strain. In contrast, the antigen-antibody complexes formed by the phage-free strain did not merge with the toxin-antitoxin band, and probably represented complexes of streptococcal products other than erythrogenic toxin.

Effect of Ultraviolet Irradiation on Other Extracellular Products.—During the experiments described above, the question arose as to whether other extracellular products might be similarly affected as a consequence of ultraviolet irradiation. To test this possibility, the production of deoxyribonuclease was measured quantitatively by a viscosimetric technique (21).

Samples of growing cultures were therefore withdrawn during the course of the experiments depicted in Text-fig. 3 at a time of maximal toxin production



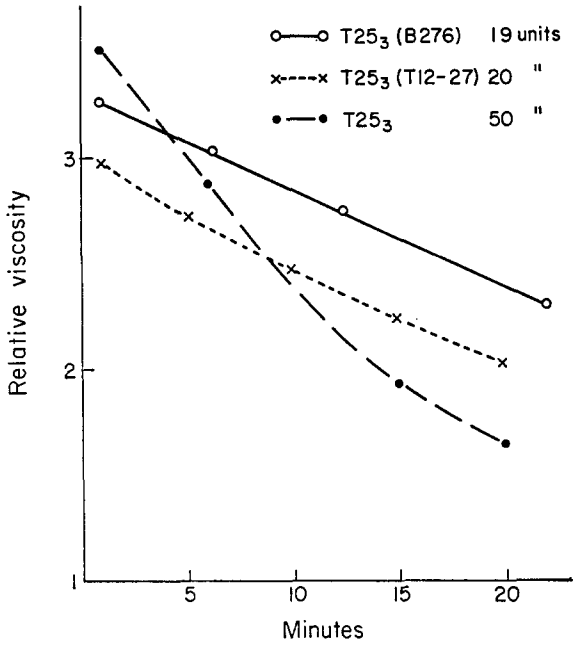
TEXT-FIG. 5. Deoxyribonuclease activity of broth culture supernatants of the phage-donating strain 3GL16 and the recipient strain $T25_3$ (3GL16). Samples obtained 7 hours following irradiation.

(usually 6 to 8 hours), and the supernatants after centrifuging were tested for deoxyribonuclease activity. Text-figs. 5 and 6 summarize the results obtained during these studies. Since the possibility existed that a lysogenic strain could be deficient in deoxyribonuclease production as a result of its phage infection, the amount of deoxyribonuclease present in a phage-donating strain was tested first. Text-fig. 5 demonstrates that the amount of deoxyribonuclease found in strain 3GL16 is actually greater than that observed in the phage recipient strain $T25_3$ (3GL16). Similar results were also obtained with strains B276 and T12-27.

Text-fig. 6 further demonstrates that the amount of the enzyme formed in the lysogenized strains is not in excess of the amount produced by the phage-free strain and in reality in considerably less than the non-lysogenic strain $T25_3$. It

would appear therefore that at least one other extracellular product is not being produced in excess in the irradiated lysogenic strains, and suggests that the phage-bacterium complex does not result in a general increase in production of extracellular products.

Serological Classification of Bacteriophages.—The toxin-antitoxin neutralization studies discussed in a previous section revealed that a single standard scarlatinal antitoxin neutralized the toxin produced by lysogenic strains infected with bacteriophages obtained from streptococci of widely differing origin. This



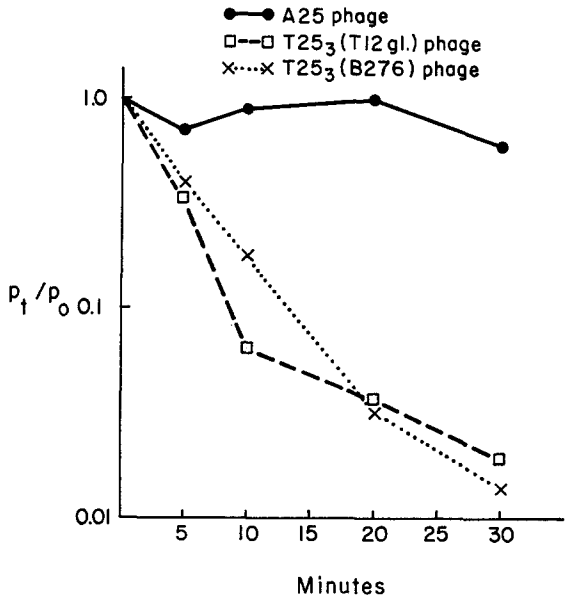
TEXT-FIG. 6. Deoxyribonuclease activity of two lysogenic strains and the phage-free strain T25$_3$.

suggested that these viruses might be related and would show serological similarities in viral neutralization studies.

The preparation of the phage lysates for injection and viral neutralization studies was essentially as outlined by Adams (16) with a few minor modifications as follows. One of the lysogenic strains T25$_3$ (T12g1) was prepared in dialysate medium as described previously for ultraviolet enhancement of bacteriophage production. Following irradiation for 20 seconds, the strain was diluted in fresh dialysate medium until an OD of 0.10 to 0.15 was obtained. This culture was, however, incubated in a *28°C* water bath instead of the customary 37°C as preliminary experiments had indicated less reabsorption of phage particles at this temperature. Phage titers of 10^7 were obtained as a routine in this manner. Following centrifugation at 2500 RPM for 20 minutes to sediment the bacteria, the lysates were filtered through Millipore filters and the filtrates placed in Visking casing No. 24 sacs and concentrated by dialysis

against carbowax M-20[4] until suspensions containing 10^8 plaque-forming units per ml were obtained. The concentrated lysates were spun at 4000 RPM to remove cellular debris. Following a small preliminary test dose of 0.5 ml of the lysate, 1 to 2 ml doses were injected intravenously in New Zealand white or hare brown rabbits daily for 5 days. The rabbits were bled 10 to 14 days following the last injection.

For the phage neutralization studies, the lysates prepared as described above were diluted to contain 1 x 10^6 phage particles per ml in the final phage-antibody mixture. The phage antiserum was diluted to achieve a final concentration of 1/100 in the reaction mixture. The



TEXT-FIG. 7. Phage inactivation studies utilizing phage antiserum 1/100 dilution prepared against $T25_3$ (T12gl) phage particles.

mixture was incubated in a 37°C water bath, and 5 minute samples were withdrawn and tested for phage particles by the plaque count method. A virulent A25 phage (supplied by Dr. Krause) was also included for comparison.

The results are depicted in Text-fig. 7. The plots are the ratio of the plaque counts at time t over plaque counts at time zero charted logarithmically. The rate of inactivation of two temperate phages was remarkably similar and indicated a definite serological relationship between these viruses. In contrast, the virulent A25 phage was not inactivated by the phage antiserum. Although virulent A25 phage did not appear to be related antigenically to the temperate phages, the possibility that the scarlatinal toxin was preformed in the cell and released by the lytic action of any bacteriophage was still undetermined.

[4] Union Carbide and Carbon Corp., New York.

Cellular Disruption Studies.—

The disruption of the streptococcal cells was achieved in two ways. First, the phage-free $T25_3$ and its toxigenic counterpart $T25_3$ (T12g1) were grown for 4 to 5 hours in dialysate medium, centrifuged, and the sediment washed in saline. The washed streptococci were then disrupted by a Mickle disintegrator for 15 minutes followed by centrifugation at 12,000 RPM for 40 minutes. The supernatants were diluted in dialysate broth, and tested for the presence ot toxin in the rabbit skin. Supernatants of the saline suspensions of streptococci prior to Mickle disentegration served as controls.

Secondly, a virulent A25 phage was used to infect the strains noted above during log phase

TABLE II

Mickle Disintegration

Strains	Before disruption	After disruption
$T25_3$	100*	100
$T25_3$ (T12g1)	1000*	1000

* Skin test dose units.

TABLE III

Virulent Phage Infection

Strain	A25 phage*	OD‡	Colonies per ml§	Toxin units per ml
$T25_3$	−	0.26	3×10^8	100
$T25_3$	+	0.04	1×10^5	100
$T25_3$ (T12g1)	−	0.25	4×10^8	10,000
$T25_3$ (T12g1)	+	0.02	6×10^5	5000

* A25 virulent phage containing 10^8 plaque-forming units per ml was added at a 3:1 ratio of bacteria to virus.

‡ Optical densities were obtained with a Coleman junior spectrophotometer.

§ Bacterial counts were made by the pour plate method.

growth, and after overnight incubation almost complete lysis had occurred in the experimental tubes as evidenced by a marked drop in optical density when compared to the controls as well as a 2 to 3 log drop in bacterial counts. The supernatants of the control as well as the virulent phage-infected tubes were then diluted in dialysate broth and also tested for toxin production by the rabbit skin erythema method.

The results are summarized in Tables II and III. The saline suspension of washed streptococci showed as expected little evidence of toxin production in the rabbit skin tests prior to Mickle disintegration. Following disruption of the cells, there was no further increase in the minimal skin reactions observed in the phage-free and toxigenic strains. Essentially identical results were obtained following infection with a virulent A25 phage not related to the temperate

phages (see Text-fig. 7). Although lysis of both the scarlatinal strain, $T25_3$(T12g1), and the phage-free $T25_3$ was quite successful, the toxin titer did not increase after lysis by virulent bacteriophage. In fact, the titer of the lysogenic strain was somewhat less than its companion non-infected strain. It would thus appear that disruption of the cells either by mechanical means or unrelated phage lysis is not important in the production of scarlatinal toxin.

DISCUSSION

The experiments described in this report confirm the observations made nearly 40 years ago by Frobisher and Brown. The nature of the "filtrable agent" described by these authors can now be more clearly defined as a streptococcal bacteriophage capable of lysogenizing strains of Group A hemolytic streptococci. Following infection with temperate bacteriophages isolated from toxin-producing streptococci, non-toxinogenic strains are altered so that they now produce scarlatinal toxin. The toxin formed by these newly induced strains can be measured by the skin erythema reaction in rabbits and can be neutralized by standard scarlatinal antitoxins. In contrast, culture filtrates of non-lysogenic strains cause skin erythema only in low dilutions and the reactions are not readily neutralizable by antitoxin, suggesting a reaction to other streptococcal products present in the growth medium. Furthermore, agar diffusion studies indicate that a new extracellular product is formed only in the lysogenic strains, and that it appears to be identical with that found in a standard toxin preparation.

The mechanism of the toxin formation however remains obscure. Chance contamination by bacteriophage, *i.e.* "apparent" as opposed to "true" lysogeny, does not appear to play a part, since the lysogenized strain can still form toxin even after several transfers in antiphage serum. Rather it seems to be intimately associated with the synthesis of mature phage particles in the infected strain in a manner remarkably similar to the production of diphtheria toxin (6). The evidence for this relationship between phage synthesis and the production of erythogenic toxin is twofold. Firstly, the formation of toxin follows the appearance of phage particles, and methods of enhancing phage production will concomitantly increase toxin production. Secondly, the cellular disruption results indicate that this toxin is not preformed in the cell requiring only physical disruption for its release. These factors strongly suggest that the metabolic processes involved in the production of mature phage secondarily result in the production of scarlatinal toxin.

The question as to whether the expression of toxin + character is possible only in certain streptococcal strains is at present unanswered. The selection of $T25_3$, a phage-free strain, was accomplished quite easily, but subsequent attempts to obtain other "cured" strains capable of accepting bacteriophages from scarlatinal strains have been completely unsuccessful. A variety of other

methods such as replica plating techniques of the Lederbergs (22) and the use of temperate phage spraying to indicate sensitive (phage-free) colonies (23) have also been unrewarding. The importance of finding a convenient method of curing streptococcal strains must not be minimized as it would provide an important tool for the understanding of the genetic mechanisms governing toxin production.

Although these experiments were concerned only with the lysogenic complex involved in the production of erythrogenic toxin the role that bacteriophages might play in the formation of other streptococcal products remains relatively unexplored. That not all extracellular products are affected by lysogenization (resulting in toxicogenicity) is demonstrated by the finding that deoxyribonuclease production is not enhanced in the strains examined in the present study. On the other hand Kjems (24) has recently reported that another extracellular product, the enzyme hyaluronidase, appears in lysates following phage propagation in non–hyaluronidase-producing strains of Group A streptococci. Its presence is not related to lysogenization of the strains *per se* but rather is associated with virus multiplication in the host organism. On the basis of his serological data Kjems (25) has identified several antigenically distinct hyaluronidases, the specificity being dependent on the phage type used for infection. Also these phage-induced hyaluronidases appear to be distinct from the previously described streptococcal hyaluronidase since a human serum with a high titer of antibody to the streptococcal enzyme failed to inactivate the phage-induced hyaluronidases.

Although Kjems' distinction between phage-induced *versus* bacterial hyaluronidase is not fully established since only one patient's serum was used, nevertheless his work further demonstrates the important role bacteriophages may play in the release of streptococcal products into the surrounding medium. In contrast to the production of serologically distinct hyaluronidases by different bacteriophages noted by Kjems, the temperate phages responsible for scarlatinal toxin production in the present study were antigenically similar and the toxin produced in each case was neutralized by a single antiscarlatinal antitoxin. However, these studies deal with only one of the known toxins, erythrogenic toxin "A."

The exact nature of the toxin and its mechanism of action continues to be an enigma. That it causes an erythematous flush in both man and animals is well documented (19), but the mechanisms by which it produces the characteristic erythema and the basis for its *in vivo* reversibility by antitoxin are as yet unknown. It is apparent that the measurement of toxin on the basis of production of skin erythema is a non-specific procedure, since other streptococcal products may conceiveably cause some skin reaction and even the neutralizing antitoxin is prepared by the injection of multiple streptococcal products into an animal. However, the development of a more specific assay procedure will probably de-

pend on a better understanding of the mode of action of the toxin. The preparation of toxin in a highly purified state represents an important step for study of both the mode of action of the toxin and its relationship to the lysogenic complex.

SUMMARY

Non-lysogenic, non-toxinogenic Group A streptococci when infected by temperate bacteriophages isolated from known scarlatinal toxin–producing strains acquire the capacity to form erythrogenic toxin. This toxin causes a characteristic erythematous reaction in the skin of rabbits and is readily neutralizable by standard scarlatinal antitoxins.

The production of toxin appears to be related to the synthesis of mature phage particles since ultraviolet enhancement of phage production results in a concomitant increase in toxin titer. In contrast, there is no increase in the production of another extracellular product, deoxyribonuclease, by these lysogenized streptococci. Furthermore, cellular disruption studies indicate that the toxin probably does not exist in a preformed state within the cell.

Double diffusion reactions in agar indicate that a newly formed protein appears in the lysogenic culture filtrate and is absent in the non-lysogenic filtrates.

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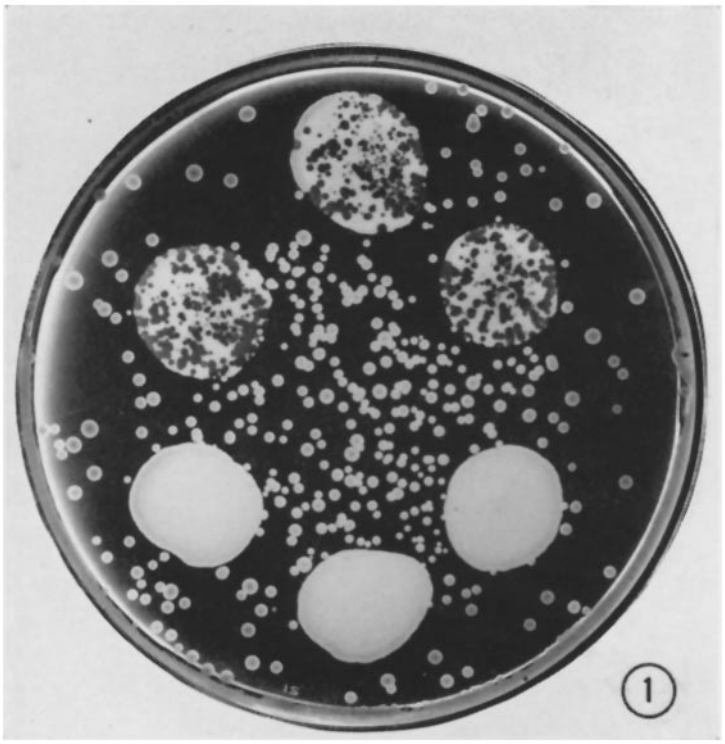
EXPLANATION OF PLATE 91

FIG. 1. Strain $T25_3$(T12g1) was irradiated for 60 seconds. Plaques can be readily seen in the "indicator" lawns on the upper half of the plate. As noted other indicator lawns were resistant to phage lysis.



(Zabriskie: Lysogeny and erythrogenic toxin)