STUDIES ON THE VIRULENCE OF BACTERIOPHAGE-INFECTED STRAINS OF CORYNEBACTERIUM DIPHTHERIAE[1]

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The relationship of naturally occurring avirulent strains to virulent strains of *Corynebacterium diphtheriae* is an unanswered question in the epidemiology of diphtheria and in the evolution of the diphtheria bacillus. The detailed investigations reported here have revealed that avirulent strains of *C. diphtheriae* infected with bacteriophage have yielded virulent *C. diphtheriae strains.*

MATERIALS AND METHODS

Cultures. The avirulent cultures[2] of *Corynebacterium diphtheriae* used in the following experiments were part of a shipment of field cultures received from the Division of Laboratories of the California State Department of Public Health in 1949. The subcultures used in these experiments have been kept in the lyophilized state since receipt. Cultures no. 770 and no. 1180 were two different isolations from a single diphtheria contact. Cultures no. 1174, 411, and 444 represented three separate isolations from a single case of diphtheria. All five cultures were designated *mitis* and avirulent by the Division of Laboratories of the California State Department of Public Health. Epidemiologically, there was no known relationship between the two individuals from whom the five cultures were obtained.

To be certain that no change in virulence had occurred during transfer to and from the lyophilized state, all of these strains were tested both intradermally and subcutaneously in guinea pigs, and all were found to be avirulent. As a further check, the five avirulent strains were submitted to the routine virulence-testing procedures of two additional laboratories, and all results were reported as negative.[3] *In vitro* plate toxigenicity[4] tests conducted repeatedly on these cultures also were negative. Before use in final experiments, each avirulent strain was subjected to four successive single colony isolations.

Bacteriophages. The bacteriophages used in these experiments were designated

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[2] The criteria for "avirulent" strains are typical morphologic and cultural characteristics of *C. diphtheriae* without the property of toxigenicity.

[3] These tests were carried out through the courtesy of Miss Marie Mulhern, Director, Public Health Laboratory, Seattle-King County Health Department, and Miss Donna Kerr, Assistant Director, Division of Laboratories, British Columbia Department of Health and Welfare.

[4] The terms "virulence" and "toxigenicity" are used interchangeably throughout this paper.

arbitrarily as A and B, with no intention that such designations be regarded as nomenclature for phages of *C. diphtheriae*. Phage A was received from Dr. Allen Ferris of Melbourne, Australia. It is a weak phage that produces only partial clearing of susceptible cultures on agar media (figure 1). Phage B (figure 2) was received from Miss Sheila Toshach at the School of Hygiene, Toronto, Canada. The latter phage is more active than phage A, producing glass-clear lytic areas on agar media (figure 1). Lysis of the avirulent strains could be demonstrated in liquid media only with phage B. Both phages were received originally as filtrates of lysed virulent cultures of *C. diphtheriae*.

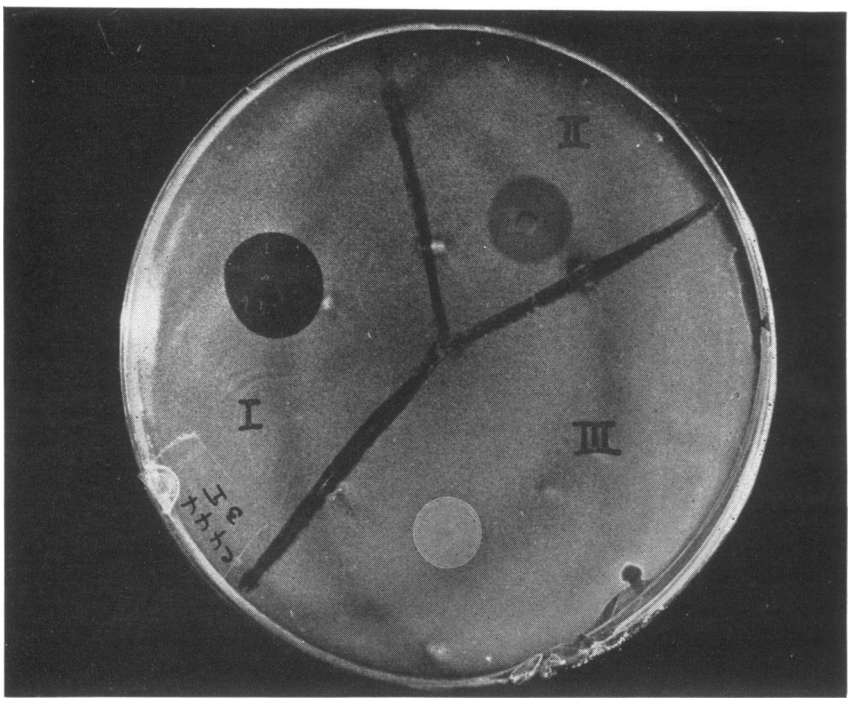

Figure 1. Bacteriophage lysis of an avirulent strain of *C. diphtheriae* (no. 444) growing on infusion agar. I, lysis by phage B. II, lysis by phage A. III, lysogenesis demonstrated by a strain newly rendered virulent.

The phages were prepared for use by making three successive single plaque isolations from agar plate cultures. The first plate was inoculated with a mixture of strain no. 444 and one of the bacteria-free phage filtrates. The resulting "adapted" phages were then produced in quantity on solid media, after which they were passed through Seitz EK filter pads. These filtered lysates were used undiluted in the subsequent experiments.

Media. Difco heart infusion broth was used for both liquid and solid media. The final pH of both broth and agar was 7.2 to 7.4. The agar medium for the *in vitro* virulence tests was prepared according to the recent modification of King *et al.* (1950). Agar plates made up from Mueller's serum tellurite medium (Difco)

were used for the isolation of cultures. Incubation of all media was carried out at 37 C.

Preparation of "lysates." The phage "lysates" used for testing toxigenicity contained whole bacterial cells as well as bacteriophage, extracellular products, and the products of cell lysis. These lysates were prepared in two different ways. In the first method, a solid medium was employed. Several agar plates were inoculated and spread with approximately 0.5 ml of an 18-hour broth suspension of the appropriate avirulent culture. The excess fluid was removed with a pipette. The inoculated plates were then dried at 37 C for approximately half an hour and were afterwards inoculated with 0.5 ml of the appropriate filtered bacteriophage

Figure 2. Electron micrograph of bacteriophage B. Preparation by replication method, followed by chromium shadowing. Magnification × 21,200. (This photomicrograph was prepared with the technical assistance of Mr. Oliver Rowe, electron microscopist, Engineering Experimental Station, University of Washington.)

lysate. Following removal of the excess fluid, the plates were incubated at 37 C overnight. The next morning the surface growth on the agar plates was washed off with 3 to 5 ml of 0.85 per cent saline. Suspensions prepared in this manner were tested by the injection of 0.1 ml intradermally and 1.0 ml subcutaneously into guinea pigs. Control suspensions were prepared in the same way except for the use of saline in place of the bacteriophage lysate.

In the second method, tubes containing 3 ml of broth were inoculated with 3 drops (approximately 0.15 ml) of an 18-hour culture and incubated until growth was just visible (usually about 1 hour). The tubes were then inoculated with 3 drops of the appropriate filtered bacteriophage lysate and returned to the incubator until the next morning. Control broth cultures were left in the incubator after the initial inoculation. Broth from the incubated culture tubes was tested

by the injection of 0.1 ml intradermally and 1.0 ml subcutaneously into guinea pigs. Lysis in broth occurred fairly consistently when the susceptible avirulent strains were mixed with phage B in the manner described. The lysis was incomplete, but a reduction in turbidity was readily evident on comparison with control cultures. Phage A failed to show any evidence of lysis of these strains growing in broth.

Virulence tests. Intradermal testing was done on 450- to 500-g guinea pigs, according to the method of Fraser (1937). Usually 8 to 12 tests were made on each animal. In all instances a naturally virulent strain of *C. diphtheriae* was included as a positive control. Subcutaneous tests were conducted in guinea pigs weighing 250 to 300 g by inoculating 1.0 ml of test fluid beneath the skin of the animal's flank. Care was taken to avoid the introduction of any fluid into the peritoneum. Since preliminary experiments did not reveal any difference in results when different brands of commercial antitoxin were used, the particular brand has not been specified in the account of results given below. Test animals in both intradermal and subcutaneous methods received 1,000 units of diphtheria antitoxin, the controls in the subcutaneous group receiving theirs 4 hours before, and all of the intradermal group 4 hours after, inoculation of the initial test lysates.

In vitro toxigenicity tests were carried out as previously described (Freeman, 1950), except for the modification in medium mentioned above. The *in vitro* plates were inoculated with the same test fluids as were used in the animal virulence tests, both series of tests being started on the same day. As with the *in vivo* tests, the brand of commerical antitoxin used has not been specified. Differences similar to those previously described for the production of secondary lines (Freeman, 1950) were observed in these experiments when the appropriate antitoxin was employed. A naturally toxigenic strain was included on all plates as a positive control.

Final experiments. In order to ensure that all possibility of contamination of the avirulent strains with virulent cultures of *C. diphtheriae* might be excluded, an entirely separate and previously unused laboratory was set up for the final experiments. In this final series, the same test sample harvested from solid medium was inoculated intradermally and subcutaneously in guinea pigs and on *in vitro* plates all within the same day. To further ensure comparability of results, the mixtures of avirulent culture with virulent culture filtrate were prepared in exactly the same manner as that already outlined for preparation of the culture-bacteriophage lysates.

RESULTS

Phage susceptibility of avirulent strains employed. Table 1 shows the susceptibility of the five avirulent strains of *Corynebacterium diphtheriae* to the phages A and B. The readings given are for visible lysis only. It is conceivable that some lysis might have occurred in the broth cultures to which phage A was added, but only quantitative analysis would reveal this. Control materials spotted on agar plates included the sterile broth medium and homologous culture grown in

broth. All such controls were negative for lysis. Culture 411 obviously is differentiated from the other four avirulent strains by reason of its phage resistance. This was the only characteristic observed that provided such differentiation. In all other respects, both cultural and morphologic, all five avirulent strains were indistinguishable.

TABLE 1

Bacteriophage susceptibility of avirulent strains of C. diphtheriae

STRAIN NO.	ON AGAR MEDIUM		IN BROTH	
	Phage A*	Phage B*	Phage A	Phage B
444	2+†	4+	0	1-2+
1174	2+	4+	0	1-2+
1180	2+	4+	0	1-2+
770	2+	4+	0	1-2+
411	0	0	0	—0

* Material spotted on plate previously inoculated with culture indicated in first column.

† Degree of clearing: 0 = none; 1+ = slight; 2+ = moderate; 3+ = marked; 4+ = complete.

TABLE 2

*Intradermal tests of bacteriophage lysates in guinea pigs**

STRAIN NO.	AVIRULENT CULTURE GROWN ON AGAR						AVIRULENT CULTURE GROWN IN BROTH					
	Control†		Phage A filtrate†		Phage B filtrate†		Control		Phage B added		Seitz filtrate of incubated culture phage B mixture	
	T	C	T	C	T	C	T	C	T	C	T	C
444	0	0	0	0	4+‡	0	0	0	4+	0	2+	0
1174	0	0	0	0	4+	0	0	0	4+	0	2+	0
1180	0	0	0	0	4+	0	0	0	4+	0	2+	0
770	0	0	0	0	4+	0	0	0	4+	0	2+	0
411	0	0	0	0	0	0	0	0	0	0	—	—

* The intradermal method used involves a second inoculation of the test substances 4 hours after the initial injections and immediately following an intraperitoneal injection of 1,000 units of diphtheria antitoxin. The test results are recorded under "T" and the control antitoxin results under "C."

† Material used for the second inoculation of the plates (details described under "Preparation of Lysates"); saline was used in the control cultures.

‡ The symbol 4+ indicates the presence of both erythema and necrosis involving an area greater than 1 cm^2; 2+ indicates a similar reaction, except for the absence of necrosis.

Demonstration of Toxigenicity of Lysates in Vivo

Intradermal tests. Table 2 summarizes the effects of the different culture lysates inoculated intradermally in guinea pigs. All positive reactions were similar and were indistinguishable from typically positive reactions caused by characteristically virulent strains of *C. diphtheriae*. From these results it can be seen that

a dermal necrotic factor was present in the phage B lysates of the avirulent strains. Even though phage A did show partial lysis on four of the avirulent strains, the phage A lysates failed to show any evidence of dermal toxicity. That the necrotic factor in the phage B lysates may have been true diphtheria toxin was suggested by the fact that diphtheria antitoxin invariably prevented the necrosis from occurring.

The broth lysates that caused dermal toxicity were retested after being passed through Seitz EK filter pads to determine whether the factor under study was extracellular. The reactions produced by the filtered lysates were reduced in intensity but were comparable to a control of filtered diphtheria toxin equally diluted.[5] The filtered lysates were actively lytic for the original susceptible strains, in addition to being toxic under the conditions described.

TABLE 3

Subcutaneous tests of bacteriophage lysates in guinea pigs*

STRAIN NO.	CULTURE PLUS SALINE	CULTURE PLUS PHAGE A	CULTURE PLUS PHAGE B	CULTURE PLUS PHAGE B AND ANTITOXIN
444	0/3†	0/1	4/4	0/2
1174	0/1	0/1	2/2	0/1
1180	0/1	0/1	2/2	0/1
770	0/1	0/1	2/2	0/1
411	0/1	0/1	0/1	0/1
Total.....	0/7	0/5	10/11	0/6

* All cultures and culture lysates were washed off agar media with 0.85 per cent saline and inoculated in 1.0-ml doses.

† The numerator represents the number of guinea pigs that died; the denominator, the total number tested.

The absence of toxicity manifested by strain 411 was recorded repeatedly. This negative result was regarded as a twofold control. First, it provided a limited control on the phage per se, for one could conclude that, at least in the quantity of the original inoculum, the phage was not toxic. Secondly, this result controlled the possibility that any "carry-over" toxin might have been responsible for the reactions. In other words, even though the phage B filtrates were known to be toxic (see last column, table 2), the degree of dilution involved in the preparation of the lysates was adequate to prevent necrosis occurring from any added toxin. It would seem extremely doubtful that the amount of toxin carried over from the original virulent culture filtrate could have maintained measurable potency through three successive single plaque isolations on avirulent cultures.

Subcutaneous tests. The subcutaneous tests recorded in table 3 confirm the results of the intradermal tests and in addition demonstrate that the toxin concerned had lethal as well as necrotic properties. All animals in each of the con-

[5] The diphtheria toxin was provided through the courtesy of Dr. W. E. Ward of Cutter Laboratories.

trol groups and in the group receiving phage A lysate survived and showed no evidence of ill effect from the inoculations other than slight induration, which disappeared within 48 hours. The only survivor of the unprotected phage B lysate group received a suspension of the one avirulent strain in this series (411) that was resistant to phage lysis. This animal also showed no significant reaction to the inoculation.

All 10 animals that succumbed to infection with the phage B lysate died within 48 hours. Autopsy findings in all instances revealed a moderate to marked gelatinous hemorrhagic exudate at the site of inoculation, markedly hemorrhagic adrenal glands, and distension of some or all of the following abdominal organs: stomach, gall bladder, small bowel, large bowel, and urinary bladder. Gelatinous exudate and hemorrhagic adrenal glands have been reported consistently as characteristic of diphtheria intoxication (Topley *et al.*, 1946). Isolation of cultures from these animals is described below.

An additional 300-g guinea pig was inoculated with 1 ml of undiluted phage B filtrate. Although local necrosis resulted, the animal survived without any permanent residual effects. As pointed out above, the phage B filtrates were, of necessity, toxic. Consequently, local necrosis was to be expected. But the fact that death did not result was regarded as further evidence that the phage itself was not the virulent factor.

The probability that the toxic factor (or factors) demonstrated in the above experiments was true diphtheria toxin was considered great. Both dermal necrotic and lethal reactions produced by the phage B lysates were indistinguishable from such reactions produced by naturally toxigenic strains. Also, in all instances where diphtheria antitoxin was employed, necrotic and lethal reactions were entirely prevented. In addition, the fact that filtered phage lysates were still toxic showed that, as with true diphtheria toxin, the lysate toxin was extracellular. Preliminary heat stability tests have shown that the lysate toxin, like diphtheria toxin (Smith and Martin, 1948), was thermolabile, being destroyed by heating to 56 C for 15 minutes.

Demonstration of Toxigenicity of Lysates in Vitro

Additional confirmation of the production of toxigenicity from avirulent strains of *C. diphtheriae* was provided by the *in vitro* plate tests. Such tests also provided a control against host factors that otherwise might have been difficult to exclude. As illustrated in figure 3, the toxin-antitoxin precipitate formed by the avirulent culture phage B lysate was more distinct than that formed by the control virulent culture. This has been a consistent observation with all the *in vitro* tests. The precipitate produced by the phage B lysate appeared earlier than that produced by the control virulent culture, sometimes being evident as early as 18 hours. It was produced with all brands of commercial antitoxin tested, a result that was found true only for virulent diphtheria cultures (Freeman, 1950). Secondary lines were produced by both control and test cultures when the appropriate antitoxin was employed, but here again these lines appeared earlier and were definitely more pronounced with the phage lysate than with either the avirulent or virulent culture controls.

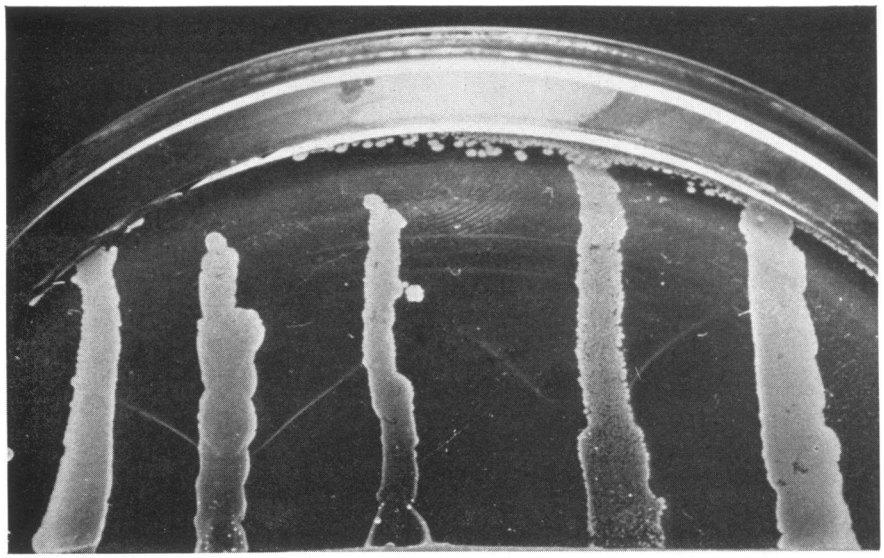

Figure 3. In vitro plate virulence test. Culture *A* shows a negative reaction. It is strain 444 mixed only with saline. Culture *B* is strain 444 mixed with phage B. The characteristic positive arrowhead precipitate is readily evident. *C* is strain 444 mixed with phage A, giving a negative reaction. *D* is a naturally virulent control strain showing a positive reaction. *E* is strain 444 mixed with a virulent culture filtrate, showing a negative reaction.

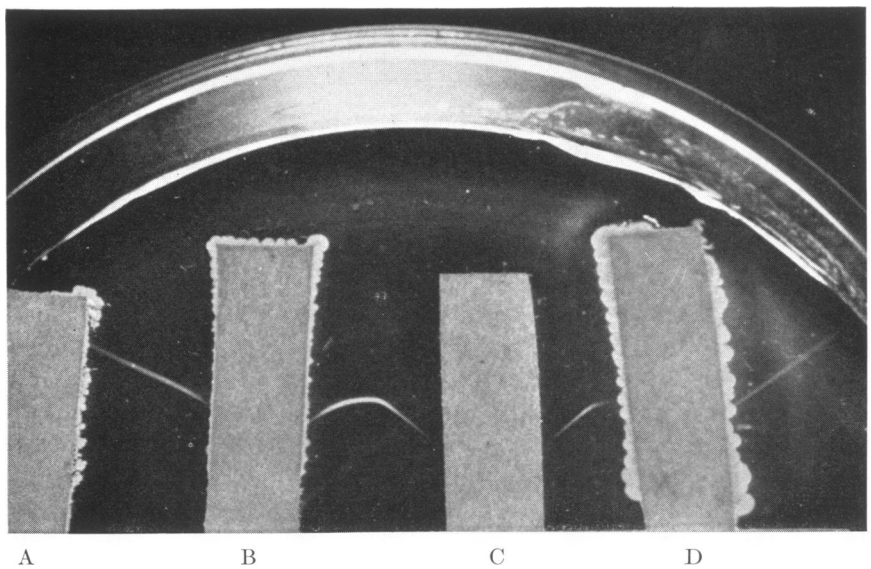

Figure 4. Double diffusion gradient analysis. Filter strip *A* was soaked in a broth culture of strain 444; *B* was soaked in a culture of strain 444 mixed with phage B; *C* was soaked in known diphtheria toxin; *D* was soaked in a culture of a control virulent strain. Note the fusion of the known diphtheria antitoxin precipitates with the toxins from the virulent culture and the avirulent culture-bacteriophage mixture.

Further evidence suggesting that the filtrable toxic factor produced by the avirulent culture phage B lysate was true diphtheria toxin was obtained by applying Elek's principle of double diffusion gradients (1949*a*) to the antigen-antibody system involved in the production of the precipitates just described. Figure 4 illustrates the results. The linear precipitates produced by the avirulent culture lysate, the diphtheria toxin, and the virulent diphtheria culture have fused with one another. Such fusion is indicative of the homologous nature of the toxic antigens diffusing out, since heterologous antigen-antibody precipitates cross one another instead of fusing (Elek, 1949*b*; Ouchterlony, 1949).

Properties of Cultures from Lysates and Autopsies

In vitro toxigenicity tests. These were carried out to assess the relative proportions of virulent and avirulent organisms in the cultures resulting from the mix-

TABLE 4

In vitro toxigenicity tests on single colonies from avirulent cultures and culture lysates

		NO. COLONIES TESTED	POSITIVE	NEGATIVE	PER CENT NEGATIVE
I	Avirulent cultures without phage B	107	0	107	100.0
II	Avirulent culture phage B lysates—isolations from animal autopsies	156	129	27	20.9
III	Avirulent culture phage B lysates—direct isolations without animal passage	61	53	8	13.1
IV	Subculture from one of the virulent colonies in group II	72	72	0	0.0

ture of avirulent strains with phage B, both before and after animal passage. Also tested were unlysed avirulent control cultures and the progeny of a known toxigenic single colony culture isolated from a guinea pig autopsy strain. *In vivo* checks on some of the *in vitro* tests always yielded confirming results. Cultures isolated from guinea pigs that died following inoculation with the phage B lysates were obtained from the gelatinous exudate and the peritoneal cavity. The majority of *C. diphtheriae* cultures were isolated from the gelatinous exudates, although an appreciable number of cultures isolated from the peritoneum also were positive for *C. diphtheriae* organisms.

Table 4 summarizes the results of the toxigenicity tests. It can be seen that in the culture lysates the majority of organisms isolated both before and after animal passage were toxigenic. No toxigenic strain has yet been isolated from the avirulent control cultures. Tests on 72 single colony subcultures, the progeny of a toxigenic single colony isolated from a guinea pig autopsy, were 100 per cent positive.

Phage susceptibility and lysogenicity of culture isolations. All virulent cultures isolated from lysates or autopsies were found to be resistant to lysis by phage B, but, like the avirulent cultures from which they originated, they showed partial lysis by phage A. When tested by the method of Fisk (1942) for lysogenicity, these same phage-B-resistant virulent strains all were found to carry a phage that, in its gross characteristics, could not be distinguished from phage B. Apparently, exposure of the phage-susceptible avirulent cultures to phage B resulted in the production of virulent lysogenic strains of *C. diphtheriae.*

A representative sampling of the 35 avirulent cultures isolated from the single colony subcultures of the phage B lysates invariably yielded strains that were still phage-B-susceptible and capable of producing toxigenic cultures on exposure to phage B. These avirulent strains were not lysogenic.

Stability of newly isolated virulent strains. Evidence of the stability of these newly isolated virulent strains was indicated by the fact that repeated stock culture passage did not reduce their virulence to any appreciable degree. Also, second and third animal passages with these "new" virulent strains in even smaller doses and under more rigid conditions (applied to a scratched skin surface after the method of Orskov, 1948) still caused rapid death with the characteristic post-mortem picture.

Preliminary immunity tests. Frobisher *et al.* (1947) have shown that animals inoculated with avirulent diphtheria cultures demonstrate some resistance to subsequent challenge with virulent strains. It was decided to test the 12 survivors listed in table 3 that had had avirulent culture mixed with saline or phage A. A week following the initial inoculations, the animals were given second inoculations of the same mixtures. Two weeks after the second inoculations, the animals were challenged with 0.5-ml doses of broth suspensions of newly isolated virulent strains inoculated subcutaneously. All animals died within 48 hours and showed on post-mortem examination the characteristic picture of diphtheria intoxication referred to above.

Six additional guinea pigs, one that had received two doses of 1 ml each of diphtheria fluid toxoid (Cutter), one that received heart infusion broth in the same doses, two that received 1,000 units each of diphtheria antitoxin, and two uninoculated, were challenged with one of the newly isolated virulent cultures applied according to the Orskov scratch method. The animal that had received the diphtheria toxoid and those that received the antitoxin survived without ill effect. The two uninoculated animals and the guinea pig inoculated with the broth died within 48 hours, showing the characteristic picture of diphtheria intoxication.

Effect of Virulent Culture Filtrate on Avirulent Strains

In order to determine whether all naturally virulent strains of *C. diphtheriae* possibly were associated with a filtrable factor capable of producing virulence in previously avirulent strains, the following experiment was conducted. A naturally virulent culture of *C. diphtheriae* growing in broth was passed through a Seitz EK filter pad, and the filtrate was handled in the same manner as described

above under "Preparation of Lysates," except that the virulent culture filtrate was substituted for the phage filtrate. Mixtures were prepared for the five avirulent strains and tested for toxigenicity both *in vitro* and *in vivo* at the same time as the lysate tests recorded above, and on the same plates and animals. All such tests were negative for toxigenicity. These results would tend to rule against any extracellular product of virulent organisms as the agent involved in rendering avirulent strains virulent and suggest that the phage per se or phage by-products were implicated.

It would have been desirable to test the filtrate of a virulent strain that had been lysed by phage B and rendered free of phage as a control on phage by-products. But the difficulty of obtaining such a phage-free filtrate that has not at the same time been divested of possibly significant phage extracellular factors is obviously great.

DISCUSSION

In 1917 Rosenau and Bailey reported on their investigations into the problem of avirulent diphtheria bacilli and their relation to diphtheria immunity. They concluded that the avirulent bacilli constituted a group so different from the typical Klebs-Loeffler organism as to throw doubt on the appropriateness of the name "avirulent diphtheria bacilli." In 1929 Okell noted that practically all observers agreed that avirulent diphtheria bacilli could never become virulent either *in vivo* or *in vitro*. On the basis of serologic investigations, Okell concluded that there seemed no reason to modify the prevailing view that the avirulent diphtheria bacillus was not even potentially a cause of disease. On the other hand, Frobisher and his group have emphasized the probable importance of nontoxigenic diphtheria strains in naturally acquired diphtheria immunity. They observed, not infrequently, the isolation of totally avirulent strains from undoubted cases of clinical diphtheria, and their experiments have been designed to investigate what they describe as the "subtle, unexplained, pathogenic power" of these nontoxigenic strains (Frobisher, Parsons, and Updyke, 1947). The material reported herein would appear to support the Frobisher group.

The question of the mechanism by which the transition of avirulent to virulent diphtheria organisms occurs is one that will require careful and extensive investigation. The possibility that a mixture of avirulent organisms with a very few virulent cells might occur either as a natural mixture from the nasopharynx or as the result of accidental contamination has been considered. An extremely small proportion of virulent organisms could be mixed with the avirulent cells, and still it might be conceivable that the whole culture would be regarded as avirulent. Since phage B lyses the avirulent but not the virulent form, a mixture of such a culture with the phage would allow the virulent strain to predominate. However, the fact that the avirulent cultures used in all of the reported experiments had been subjected to four successive single colony isolations, and that subsequent subcultures from these "purified" strains in every one of 107 single colony isolations failed to yield a virulent culture, would argue against the possibility of initial mixture with virulent cells. It would be desirable to re-

peat some of the foregoing experiments with avirulent cultures that had been prepared from single cell isolations.

If one assumes that the avirulent cultures were absolutely pure, and if one recognizes the results recorded above concerning the failure of a virulent culture filtrate to elicit toxigenicity in avirulent strains, then the most likely hypothesis to explain the phenomenon of virulence conversion is the spontaneous development of toxigenic mutants, with selection by phage lysis. The finding that all the newly isolated virulent strains tested for phage B susceptibility were resistant to lysis by phage B is consistent with such a hypothesis. The inability of phage A lysates to demonstrate toxigenicity might have been due to quantitative limitations, since lysis of the avirulent cultures was only partial, and therefore they may still have overgrown the virulent mutants.

The theory of mutation in the study of *Corynebacterium diphtheriae* is not a new concept. Crowell (1926) obtained an avirulent culture by a single cell isolation from a virulent parent strain that had been cultured from a single cell. He attempted in a great variety of ways to cause the avirulent strain to revert to its toxigenic form, but without success. As a consequence, Crowell concluded that the mutation was irreversible. There are many other instances cited in the literature of the isolation of avirulent strains from virulent parent cultures, but the reverse situation apparently has not been described. Variation from the virulent to the "atoxic" form of *C. diphtheriae* in the presence of bacteriophage has been reported by Blair (1924) and Stone and Hobby (1934).

The release by phage lysis of preformed toxin or of endotoxin that conceivably might be oxidized to toxin (Frobisher *et al.*, 1947) is a hypothesis that cannot be excluded at present, but on the basis of preliminary work it is considered less likely. If the inability of phage A lysates to demonstrate toxigenicity is qualitative rather than quantitative, when compared with the action of phage B, then the possibility that the complex phage-bacterium relationship per se is involved must be considered. Investigations concerning these various hypotheses are in progress.

Regardless of the fact that the underlying mechanism is not understood, the knowledge that avirulent cultures of *C. diphtheriae* can become virulent in the presence of specific bacteriophage is of importance to any consideration of the many perplexing problems that have confronted bacteriologists and epidemiologists interested in the study of diphtheria. If the virulence of the diphtheria bacillus should prove dependent not only on its toxigenic ability and its invasive power but also on the degree of its association with a specific bacteriophage, then some of the difficulties involved in understanding the complex problems of bacterial metabolism and immunity as they occur in the diphtheria case or carrier might be partially solved.

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SUMMARY

Virulent strains of *Corynebacterium diphtheriae* were isolated from four of five avirulent cultures of *C. diphtheriae* when the four cultures were incubated with a specific bacteriophage B filtrate by which they were readily lysed. No virulent strains could be isolated when these same cultures were incubated with a phage A filtrate by which they were only partially lysed. The fifth avirulent culture was not susceptible to either phage and yielded no virulent strains when incubated with either phage filtrate.

Virulence (toxigenicity) tests were carried out *in vivo* by both intradermal and subcutaneous methods and *in vitro* by the plate technique, complete correlation being obtained by all methods. Controls of cultures incubated with saline were negative in all instances when tested for virulence.

The extracellular nature of the toxic factor produced by the newly isolated virulent strains was demonstrated by filtration through a Seitz EK filter pad. Occurrence of the toxic reactions in guinea pigs inoculated with these virulent strains was shown to be preventable by the administration of diphtheria antitoxin.

Tests on single colonies isolated from avirulent cultures and culture lysates revealed that 100 per cent of colonies isolated from the avirulent cultures without phage were avirulent, whereas over 80 per cent of colonies from the avirulent culture phage B lysates were virulent.

The virulent strains isolated from the avirulent culture lysates were found to be stable. Also they were phage-B-resistant and lysogenic. The phage they were found to carry could not be distinguished from phage B.

A filtrate of a naturally virulent strain of *C. diphtheriae* was tested on the avirulent cultures in a manner identical with that of the phage filtrates. No virulent strains were isolated by this method.

It is suggested that an initial mixture of virulent with avirulent cells is unlikely, and therefore that the most likely hypothesis is the spontaneous development of toxigenic mutants with selection by phage lysis.

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