RELATIONSHIPS BETWEEN ANTIGENIC VARIANTS OF BACTERIOPHAGE T3

by

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INTRODUCTION

The advance of genetics has been accelerated in recent years by the widespread use of microorganisms such as protozoa, fungi, bacteria and viruses. The techniques of genetic experiments have developed to a point where a highly detailed view of inheritance can be visualized. One of the key methods used for studying inherited characteristics is the analysis of the process of mutation. Mutation is the sudden transformation of a gene, which may be spontaneous or induced; the transformed gene is then inherited by subsequent generations. The character controlled by the gene may be one single biochemical capacity of the cell. The synthesis of a specific antigen is an excellent example of the final phenotypic result of gene action.

An antigen function depends on the presence in the protein of certain specific chemical groups or configurations, the "antigenic determinants", which induce the formation of antibody molecules. These antibodies are capable of distinguishing those details of organization on which functional specificity may depend. Serological reactions, therefore, can be considered as a refined tool for detecting very small changes in the amino acid sequence in the protein, which in turn may be caused by a small change in the gene structure.

The most sensitive analytical tool for the detection of an antigen is the neutralization of bacteriophage by specific antiserum. It is not surprising, therefore, that geneticists have kept a watchful eye on research dealing with antigenic mutations in bacteriophage. However, until the recent discovery of Eisenstark, et al (1961), there had been no case of a qualitative mutation of a neutralizable antigen in bacteriophage. Such a mutant is one which is completely resistant to antiserum prepared against the wild type.

The first task of this study was to determine whether the qualitative mu-

tation in phage T3 found by Eisenstark, et al (1961) was unique, or whether such a mutational situation might extend to other phage. For this purpose a series of 11 phages and their antisera were chosen. These antisera were the ones that were readily available. As may be seen from the results below, phage T3 turned out to be unique in respect to ability to mutate to a type with a new neutralizable antigen. Therefore, the second task of this study was to compare certain characteristics of this T3 antigenic mutant with T3 phage types obtained from other laboratories as well as from our own.

REVIEW OF LITERATURE

Geneticists have long recognized that antigens may be used as markers for the study of inheritance. Examples vary from the inheritance of blood type in humans to the inheritance of the specific antigenic surfaces in Salmonella; volumes have been written on each of these two subjects. To review the entire area of the inheritance of antigenic specificity would be far too great a task for this thesis. Therefore, the author has chosen to restrict the review of literature to the inheritance of antigenic structure in bacterial viruses. This is not to imply that these are the most important aspects of the genetics of antigenic structure; one need only be reminded of the research dealing with the transfer of antigen synthesis in transformation studies of Pneumococcus (Ephrussi-Taylor, 1958), as well as those involved in transduction (Hartman, 1957) and conversion (Hartman and Goodgal, 1959). Also, in any consideration of the inheritance of antigens in microorganisms, one cannot ignore three other fascinating areas of research.

One of these is the work of Beale (1954) on Paramecium aurelia, which showed that both genes and cytoplasmic factors play essential parts in the determination

of the antigens of P. aurelia. The general conclusion was that in each Paramecium stock, the cytoplasm could exist in one of a number of mutually exclusive states corresponding to each of the antigenic types which that stock had the potentiality of forming. Thus Beale demonstrated that the specificity of the antigens formed in conjunction with any particular cytoplasmic state was controlled by a particular gene.

In addition, Yanofsky (1960), and Yanofsky and St. Lawrence (1960) studied the antigenic specificity of enzymes synthesized by Neurospora crassa and Escherichia coli. He found that tryptophan synthetase mutants of these organisms represented mutations of the same gene and were antigenically distinguishable from one another. It was assumed that mutations in the genic region resulted in protein alterations which eliminated both the immunological identity and the tryptophan synthetase activity of the protein. Yanofsky's research presents an excellent illustration of the fact that a mutational event can result in an altered enzyme; the altered enzyme can be measured by immunological techniques.

Since T3 phage can multiply in the mating strains of E. coli, one should be aware of the investigations of Ørskov and Ørskov (1960), in which an antigen termed f+ and the genetic factor F, control sexual compatibility in E. coli. They stated that the relationship between the F particle and f+ antigen could be explained by one of three possibilities: (a) the genetic factor responsible for the f+ antigen was transferred along with the F particle; (b) the transfer of the F agent was followed by an uncovering of an already present f+ antigen; (c) only cells carrying an f+ antigen, i.e. cells where this antigen was not obscured by other antigens, could be converted to the F+ state.

While the above work has indirect application, it is only the work performed with bacterial viruses that is pertinent to this thesis and therefore it will be extensively reviewed.

There is no doubt that many pioneer bacteriologists saw and described phage action in bacterial cultures. However, no intensive investigation of this phenomenon was undertaken prior to the work by Twort (1915). His paper went unnoticed by scientists until d'Herelle (1926) published his independent discovery of "bacteriophage". These two men shared the credit for the discovery of phages and did much of the basic research concerning them. Bordet and Cuica (1921) demonstrated the antigenicity of bacteriophage by their ability to stimulate antibody production in experimental animals. The antigen was shown to be one of the most definite characteristics of the phages (Burnet, 1934; Burnet et al., 1937). It was also shown that the antigenic unit consisted of two portions, the larger part being responsible for the antigenic specificity and the smaller portion being non-specific (Pollard, 1953). Any variation or change in the activity of the small fraction did not change the antigenic specificity.

Kalmanson and Bronfenbrenner (1942) and Hershey et al. (1943 a,b) first reported the polyvalent neutralization in phage-antiserum reactions which they presumed was due to the heterogeneity of the antigenic structure of the phage particle. Small but well defined differences between the reactivity with coli phages of homologous antisera showed themselves as independent variations in the specific rate of neutralization, the neutralizing capacity, and the precipitating activity. With respect to these properties, individual antisera behaved like homogeneous solutions. This finding was of particular interest with respect to the rate of combination of antibody, which might be expected to reflect to a marked degree qualitative differences of any kind. The absolute rate of the phage-antiphage reaction at 0° C. was expressed as 2-10 molecules of antibody per second per phage in a "standard" antiserum 10^{-5} molar with respect to antibody. This corresponded to a typical bimolecular reaction, provided that one substance (the antiserum) was in large excess. Various factors influenced the

rate at which this bimolecular reaction could take place (Andrewes & Elford, 1933 a,b; Jerne and Skovsted, 1953).

Kalmanson and Bronfenbrenner (1943) established the concept that antiserum inactivated the virus by the deposition of antibody on the surface of the bacteriophage. This concept was confirmed by the reactivation of phage after digestion of the antibody coat with papain. Although the bacterial virus could be reactivated by special treatment, Hershey (1943a) showed that the reaction of the antigen with the antibody was irreversible by demonstrating no increase of infectivity of neutralized phage when the mixture stood for six weeks after diluting antiserum 1000 times beyond the concentration causing neutralization. The mode of inactivation had been thought to be the prevention of attachment of the bacteriophage to the surface of the bacterium. However, Hershey (1943b) demonstrated by direct and quantitative methods an instance in which the inactivated phage was as readily adsorbed to bacteria as an active particle, indicating that the inhibition of some step in the growth cycle subsequent to the attachment of the phage might also be responsible for the inability to multiply.

Several groups of investigators concentrated their attention on the same material, which consisted of one non-motile strain of E. coli, designated "B", and seven virus strains, all of which could multiply on "B". These viruses were obtained at various times by different investigators. Some of them were derived (by single plaque isolations) from old preparations. Bacteriophages, T1 and T2 had been used for many years in the laboratory of Prof. Bronfenbrenner. They were then named P28 and PC. Delbrück (1945) and Luria (1945) designated them alpha and gamma. A third virus was called delta. Demerec and Fano (1945) added four more viruses to the system and all seven viruses were given new number symbols, T1, T2, ..., T7, the T standing for "type". Possibly stimulated by the work of Burnet (1933), Delbrück (1946a,b) used serological cross-reactions

between the T viruses to determine relatedness. The serological specificity was so remarkably constant that the following grouping was obtained: (T1), (T5), (T3, T7) and (T2,T4,T6).

Luria et al. (1943) stated that T2, T4, and T6 were indistinguishable on electron micrographs, but T1 and T5 differed in structure both from each other and from the T2,T4,T6 group. The serological relationships in the latter group appeared to be close; heterologous neutralizing titers were generally not more than a factor of five lower than homologous ones. The relationship between T3 and T7, on the other had, though unmistakable, was evident only when high concentrations of antisera were used for the cross tests.

If several viruses were antigenically related, the antiserum from any one would inactivate the ability of the phage to produce homologous phage. Thus a method was needed that would measure the degree of relatedness. Adams (1950) mathematically expressed the reaction of the immune serum with virus particles by the formula $K=2.3D/t\ (\log p^o/p)$. K represented the empirical rate of virus inactivation, D the dilution, t the time in minutes the antigen and antibody were allowed to react, and p^o/p the survival ratio; p^o represented the number of active bacteriophage particles at the start of inactivation and p the number at the end. This equation held for 90-99 percent inactivation of the active phage present in the original mixture. The degree of relatedness was determined by the magnitude of the differences in K values; for example, the E. coli phages T2, T4 and T6 when neutralized by T2 antiserum had K values of 200, 50 and 90 respectively. Therefore, T6 was more closely related to T2 than was T4, and T4 more closely related to T6 than T2.

Lanni and Lanni (1953a,b) demonstrated that in the case of T2, there were multiple antigens. A non-neutralizing antibody agglutinated phage particles at concentrations of 10^8 per ml. and was directed against antigens located in the

phage head; the neutralizing antibody was directed against one or more antigens in the phage tail. These facts correlated many of the known serological properties of the phage. T2 cross-reacted in neutralizations and precipitated with the morphologically similar phages T4 and T6. The dual antigenic character of T2 required that these cross-reactions be restudied with reference to the separate antigens. Anti-T4 and anti-T6 rabbit sera were found to precipitate purified T2 "doughnuts" (empty head shells), a sign that T4 and T6 might also possess multiple antigens.

Tanami and Miyajima (1956) confirmed the results of Lanni and Lanni when they undertook to generalize the phenomenon of diphasic neutralization (two different rates of neutralization) in phage-antiserum reactions. This phenomenon was presumed due to the heterogeneity of the antigenic structure of the phage particle. A serological neutralization test was performed using anti-T2 rabbit serum. At various time intervals samples were taken and plated out on four hosts. They observed that the neutralization of T2 phage by its specific antiserum was polyphasic, e.i. many different rates of neutralization. The conclusion was that each phage particle had a complex structure in its site of attachment, which was neutralized in a polyphasic manner by the corresponding antibodies.

This conclusion may be compared with the results reported by Fodor and Adams (1955). In mixed infective experiments with related phages T5 and PB, they obtained several mutants which showed combined antigenic components of the two parental types in the neutralization reaction as well as in the complement fixation test. They concluded that the antigenic structure of these phage was quite complex and that a number of distinct genes must be involved in its control.

Lanni (1953, 1958) studied phage T5 from the standpoint of intracellular

formation of phage antigen and active phage. Broth cultures of E. coli B were infected with T5 and lysed prematurely at various time intervals. Infectivity was assayed by plaque count and phage antigen by complement fixation. During the first half of the latent period, the infectivity was that of unadsorbed phage (5% of the input), while the antigen titer corresponded to total phage input. Both quantities increased thereafter and reached levels characteristic of normal lysates. The rise curves were nearly parallel, with the midpoint for antigen preceding that for infectivity by about seven minutes. The results are in accord with the concept of synthesis of phage-specific material prior to phage maturation. This confirmed the findings with T2 (Hershey and Chase, 1952); namely, that there is dissociation of DNA from antigen during infection and external persistence of antigen, but in the case of T5 there is a slower injection of DNA.

Streisinger (1956a,b) found that the serological specificity characteristic of T2 and T4 could not be separated genetically from their host range characteristic. The host range and serological specificities which were controlled by one genetic locus were also linked phenotypically. It was suggested that the materials responsible for these specificities were produced in a pool and became randomly associated with the genetic material of phage during maturation. This could be explained by the concept that the phage tail, and specifically the sites of adsorption to bacteria, consisted of protein. Blocking the amino groups of phage T2 prevented their attachment to bacteria. Agents such as urea could activate the adsorptive capacities of certain strains of T4; the kinetics and conditions of activation resembled those found for protein denaturation.

These findings suggested that the host range properties of these phages might provide a good system for an investigation of the relationships between gene and product. Specifically, a very small genetic change (mutation) could

be measured precisely by scoring the change in host range of the phage.

Lanni and Lanni (1957) studied the relationship between serological mutations and structure of phage T5. They found that T5 stocks contained mutants B+F- and F+B-, the former lysed E. coli B and the latter, strain Fcb. The wild type (WT) infected both hosts with equal efficiency. Extensive cross-neutralization occurred in rabbit antisera against any one of the viruses; however, quantitative differences were apparent among the viruses and among the antisera. All sera neutralized F+B- approximately twice as fast as WT (assayed on Fcb). Hence, these two viruses probably differed in some organizational feature of the relevant antigen rather than in qualitative antigenic composition. WT antisera neutralized B+F- slightly more slowly than WT (assayed on B), while B+F- antisera neutralized this virus three to five times as fast as WT. Hence, these viruses probably possessed qualitatively different antigens and/or different proportions of multiple antigens. With WT the estimate of serum survivors in neutralizations by any of the antisera depended on the assay host. The ratio of plaques on B/plaques on Fcb became greater than units at the outset of neutralization and thenceforth increased progressively. Analysis showed that many or all of the virus particles passed through a stage in which they were effectively incapable of adsorbing to Fcb but still capable of adsorbing to and infecting B.

From these results, it was concluded that the antiserum-sensitive portion, probably the tail, of the T5 particle appeared to contain an ensemble of somewhat independently functioning, not necessarily heterogeneous antigens, whose collective structure could vary mutationally in at least two distinct ways.

The most recent work with the serology of the T phages has been done by Eisenstark et al. (1960) using variants of T3 phage. The initial T3 stock was found to contain at least three different phage types in high proportion which

were designated T3B, T3C, and T3Bhe. These variants differed with regard to antigenic, morphological, and host range properties. Experiments were performed to determine the relationship of these properties to each other, as well as to determine the origin of the mutant types. Antisera were prepared against the three variants by rabbit inoculation and were used for phage neutralization and serum blocking power experiments.

It was found that anti-T3B inactivated T3B, but not T3C; whereas anti-T3C inactivated both T3B and T3C. The explanation for this was that T3B contained B-type neutralizable antigen but not C-type; T3C, however, contained both B-type and C-type neutralizable antigens. This hypothesis was substantiated with tests for serum blocking power.

T3C not only differed from T3B antigenically but also had a different host range. Therefore studies were made to determine the relationship, if any, between neutralizable antigen and host range. The fact that these two phage differed in two properties that were believed to be associated with the protein at the tip of the tail raised the question as to the relationship between the host attachment site and the antigenic site. It was shown that possession of a C-type antigen was not a prerequisite for adsorption to HfrC cells. Phage T3Bhe did not possess C-type antigen but it did infect HfrC (the designation Bhe indicated that the phage possessed B-type antigen but its host range extended beyond that of T3B). The evidence that T3B and T3Bhe had the same neutralizable antigen was quite convincing because sera from each of these phages neutralized the other phage to the same extent as it did its homologous phage. Since T3Bhe did not possess a C-type antigen but did infect HfrC, it was assumed that C-type antigen was not a prerequisite for attachment to HfrC.

One of the most striking characteristics of T3C was its genetic stability. There was little or no detectable mutation to T3B antigenically or with respect

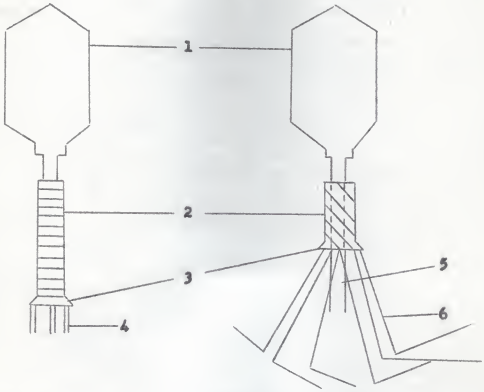
to host range. T3B, on the other hand, did not show such stability. There were numerous host range mutants in lysates of T3B and also phage which exhibited a C-type antigen. Recombination experiments were performed in an attempt to explain the stability of T3C and instability of T3B. No conclusions were drawn from these experiments but it was suggested that further investigation should be pursued on the assumption that T3C -- T3B represented a point mutation and that T3B -- T3C was a reversion.

Late in the investigation, another mutant was found and was designated T3M (the designation M means that the plaques produced by this phage were minute). Its serological, morphological and host range characteristics were not presented in the report.

It is interesting to note that antigenic and host range variants of bacteriophage P22 active on Salmonella typhimurium have been discovered recently and compared with the T3 variants described on the preceding page (Yamamoto and Anderson, 1960). The relationship of T3C to T3B could be demonstrated because antisera against T3C would inactivate T3B. The relatedness of P221 (a long-tailed variant of P22) to P22 exhibited no detectable serological cross reactions. Relatedness could be inferred by the inheritance of P221 of parental P22 markers and it could be shown by demonstrating genetic recombination between them. A host range mutant of P22, designated P22h, was essentially identical morphologically and serologically to P22 particles, but differed from P221 in respect to these properties. Masked genomes were produced by mixed infection with P22h and P221. These were particles carrying P22h genomes in P221 capsids (protein coats) which could be inactivated by anti-P22, and particles carrying P221 genomes in P22h capsids, which could be inactivated by anti-P221. It was also found that P22h markers (those affecting lysogenization) could be transferred to P221 genomes and vice versa. The existence of these stable hybrids indicated that

P22 and P221 represented two morphologically and serologically distinct members of the same species of bacteriophage.

Finally, from the examination of the literature, it seems that the most intensively studied bacteriophage from every point of view is the T2 phage (Bradley and Kay, 1960; Brenner et al., 1959). In comparison with T2, little is known about other phages. Figure 1 is a diagrammatic description of T2 to enable a visualization throughout the remainder of this report of the structures believed to be concerned with attachment to the bacterial cell wall and thus the positions where antigen-antibody complexes might be formed that would inhibit phage attachment and subsequent infection of the host cell.



T2 type, active T2 type, inactive

Fig. 1. Description of the phage particle*.

1. Bipyramidal hexagonal prismatic head
2. Contractile tail sheath with cross-striations
3. Terminal base-plate
4. Prongs
5. Central hollow tubular core of the tail
6. Uncoiled fibers from sheath

*Diagram by D. E. Bradley and D. Kay (1960).

MATERIALS AND METHODS

Procedures for phage titration and preparation of phage stocks have now been standardized and descriptions are readily available (Adams, 1959).

Media

Difco nutrient broth with 0.1 percent added dextrose was used for all broth cultures and dilution blanks. Difco nutrient agar was used for all poured plates. Difco tryptose broth w/thiamine plus 8.5 grams Difco agar per liter was used for overlay merely for convenience rather than preference over nutrient agar overlay.

Phage

The following is a list of all the phage that were tested for antigenic mutants and the source of each:

Phage	Source
T1	K.S.U., stock culture collection
T2	R. Edgar, Calif. Inst. Tech., Pasadena, Calif.
T4	R. Edgar, Calif. Inst. Tech., Pasadena, Calif.
T7	Microbiology Inst., U. of Copenhagen, Denmark
PLT 22	P. Hartman, Johns Hopkins U.
X. pruni(strain XP4)	Isolated by A. Eisenstark from U. of Ill. phage stocks
T3(strain DF 18)	Dorothy Fraser, M.I.T., Cambridge, Mass.
T3(strain DF 32)	Dorothy Fraser, M.I.T., Cambridge, Mass.
T3(strain DF 3)	Dorothy Fraser, M.I.T., Cambridge, Mass.
T3B	Isolated by A. Eisenstark from T3 stock, U. of Copenhagen, Denmark
T3C	Isolated by A. Eisenstark from T3 stock, U. of Copenhagen, Denmark
T3Bhe	Isolated by A. Eisenstark from T3 stock, U. of Copenhagen, Denmark
T3M	Isolated by A. Eisenstark from T3 stock, U. of Copenhagen, Denmark
$T3B_{SL}$	Isolated by S. Lindell from T3M

Phage	Source
$T3B_L$	Isolated by S. Lindell from Templeton phage
$T3M$	Isolated by S. Lindell from Templeton phage
Templeton(N72)	M. Rakieten, Islip, Long Island, N. Y.

Bacterial Hosts

The bacterial hosts and their source used throughout this investigation were:

Bacteria	Code	Source
E. coli B	B	U. of Copenhagen, Denmark
E. coli B/1	B/1	U. of Copenhagen, Denmark
E. coli K12 strain HfrC	HfrC	U. of Copenhagen, Denmark
E. coli K12 strain C600	C600	U. of Copenhagen, Denmark
Templeton(*E. coli*)	Temp.	M. Rakieten, Islip, Long Island, N. Y.
Sal. typhimurium strain LT2	LT2	P. Hartman, Johns Hopkins U.
Xanthomonas pruni	*X. pruni*	U. of Ill.

Antisera

All the antisera used had already been prepared by this laboratory in the manner prescribed by Adams (1959). Table 1 indicates the approximate strength of the antisera used.

Isolation of Suspected Mutants

When plaques had been obtained on an agar plate, the ones that appeared to be mutants were stabbed with a sterile toothpick and then dipped into a bubbler tube containing 10 ml. of nutrient broth. Approximately 10^6 of the desired actively growing host cells were then added. The tube was aerated and incubated at 37°C for about 2 hrs. or until the tube was clear as compared to

a control tube of broth inoculated with 10^6 cells. A Servall angle-head super-centrifuge was used to remove bacteria and cell debris from the lysate. The supernatant was stored in a deep freeze for further testing.

Antiserum Tests

As each phage was plated it was tested for its antigen by a series of antiserum spots in the following manner. Ten minutes after adding the overlay containing phage and host cells, two drops of a 1:10 dilution of homologous antiserum in soft agar was placed on the surface of the assay plate (Figure 2). If plaques were visible within any of the serum spots, they were picked and tested further for their antigen. When it appeared that a phage might be an antigenic mutant, its neutralizable antigen was confirmed. To do this, the suspected antigenic mutant was grown up by lysis of broth cultures and tested by plating a small sample and adding antiserum directly to the overlay. The final confirmatory test was to run comparative neutralization curves between the mutant and the wild type phage against which the antiserum was originally prepared. This procedure was described by Adams (1959).

RESULTS

The Extent of Antigenic Mutants

The phage listed in Table 2 were tested for antigenic mutants by plating the phage with its homologous antiserum and collecting any survivors. The antigenic properties of the survivors were compared with those of the wild type by serum neutralization tests. If the degree of inactivation of suspected mu-

tant and wild type did not differ significantly, then the test was scored as negative insofar as the detection of an antigenic mutant was concerned. T3C, T3B and T7 were each incubated with their homologous antisera for 30 minutes and then plated out to detect any surviors.

As may be seen from Table 2, only T3B gave rise to serum-resistant mutants. In order to test whether the T3 survivors were true antigenic mutants, the offspring of thirty of these survivors were plated on HfrC and B/1, which are selective hosts for T3C and T3B respectively. As indicated by Table 3, 90 percent of the survivors of T3B treated with anti-T3B serum were T3C. Figures 3 and 4 illustrate the same point graphically. The serum merely acts as a selecting agent by eliminating the parental type (T3B) and permitting the mutant (T3C) to be observed. Thus, it may be concluded that T3B was unique among the phage tested insofar as mutation to a new neutralizable antigen is concerned.

A question then arose as to whether the number of survivors would differ if they were plated on different hosts. Other investigators (d'Herelle, 1926; Kalmanson and Bronfenbrenner, 1942; Friedman, 1954; Tanami and Miyajima, 1956) had already noted that different assay hosts might give different estimates of the fraction of unneutralized phage in a given phage-antiphage mixture. To determine if this were true in the case of T3B, an efficiency of plating experiment (Table 4) was performed with single plaque isolates of T3B on B, C600 and B/1 and was compared with the number of survivors from a quantitative serum neutralization test (Table 5). Figure 5 illustrates the same point when T3B survivors were plated on B/1 and C600 only. The results indicated that there was no significant difference regardless of which of the two hosts were used as indicators. This test merely indicated that the survivors had the host range of T3C; it does not specify whether these survivors would also have the T3C antigen.

The Relationship of T3M to T3

Eisenstark et al. (1961) demonstrated that T3B and T3C were serologically related but antigenically different phage. They also found that when T3C was plated on C600, minute plaques were produced in a very low frequency on a number of occasions.

This investigator continued the study by attempting to relate T3M to the other T3 phages. As an initial step, T3M was titered on C600, B, HfrC and B/1 and was found to plate with equal efficiency on each. All of the plaques were typical minutes with regard to morphology. In an attempt to determine whether T3M could mutate back to T3C or T3B, T3M was plated on C600, B and HfrC and overlayed with anti-T3M. In every case, large plaques developed. This led to the temporary hypothesis that T3B and T3C could be isolated from T3M. Another attempt to prove this was done by picking 25 plaques from the control plate above (T3M on C600 w/o anti-T3M) and each was plated out on a mixed indicator containing equal amounts of HfrC and C600. One drop of anti-T3M serum in soft agar was placed on each plate. This method would not only permit the detection of any antigenic variants, but also would be a control on the antigenicity of the parent type (T3M). It was found that 24 plates contained large cloudy plaques, a portion of which appeared within the serum spot, and clear minute plaques, none of which appeared within the serum spot. One plate contained only typical minute plaques, both morphologically and serologically.

In order to determine the antigenicity of the phage that formed the large cloudy plaques, one or more cloudy plaques from each plate were picked and plated out on HfrC and C600, which are selective hosts for T3C and T3B respectively. As a control, a clear mintue plaque (the parent type) was also picked from each plate and plated out on HfrC and C600. The results of this experiment

(Table 6) led this investigator to believe that T3M was truly related to T3B and T3C, since one T3B and numerous T3Cs were recovered. The T3B that was recovered will hereafter be designated $T3B_{SL}$. Broth lysis stocks were made of each stock listed in Table 6 for further investigation.

The above results were encouraging enough to warrant an additional critical test with the original stock of T3M. It was plated on B, B/1, C600, HfrC and Templeton to obtain well isolated plaques. Somewhat larger plaques were formed on B and B/1 than on C600 and HfrC but T3M did not plate at all on Templeton. From the B/1 plate, 100 plaques were picked and each was placed in 1 ml. of nutrient broth containing approximately 10^6 B cells. The tubes were incubated at 37°C for 2 hrs. and then put in a 60°C water bath for 30 minutes to kill the host cells. Since T3B and T3C will plate on Templeton and T3M will not, 3 ml. of overlay were added to each tube together with one drop of Templeton cells and then each tube was poured over nutrient agar plates and incubated at 37°C. It was reasoned that if T3M were a mutant of T3B and/or T3C, large plaques might develop on one or more of the plates. However, no large plaques developed on any of the 100 plates. Therefore, if T3M mutates to T3B or T3C, it does so at a very low frequency.

A final critical test was performed on T3M in an attempt to relate it to T3B or T3C. In the case of the latter, it was established (Eisenstark et al., 1961) that T3B contained B-type neutralizable antigen but not C-type; whereas, T3C contained both B-type and C-type neutralizable antigens. Such an hypothesis was substantiated with tests for serum blocking power. This method involves the exhaustion of an antibody (or antibodies) by a particular phage. After a proper period of adsorption, the serum is tested for remaining antibodies; i.e., antibodies which were not adsorbed because of the absence of homologous antigen on the blocking phage.

If T3M possessed an antigen in common with either T3B or T3C, then a serum blocking power test should be able to detect it. The results indicated, however, that there was no apparent neutralizable antigen in common, as T3M did not block any of the antibodies in either the anti-T3B or anti-T3C serum.

All of the T3C stocks (Table 6) isolated from T3M were scored as T3C on the basis of host range only (plating on HfrC). These could also be T3Bhe, since T3Bhe resembles T3C as to host range. Therefore, all of the T3C stocks were plated on HfrC and C600 and spotted with anti-T3B in order to establish the stocks as T3C and not T3Bhe. With the exception of sample 10c, all of the stocks were T3C. This sample (10c) was further tested and found to be T3M.

Recombination Studies

Since on one occasion T3Cs arose from single plaque isolates of T3M and on numerous other occasions they arose from T3B stocks, recombination experiments were performed to determine the possible type of mutation that brought about the occurrence of T3C. A mutation from T3B to T3C or T3M to T3C might involve a deletion of genetic material that cannot be readily reinserted upon subsequent growth of T3C on *E. coli* B. On the other hand, it might involve a point mutation of normal frequency. If so, each T3C might differ from other T3Cs with regard to the exact site at which the mutation occurred. Five of the T3 stocks obtained from T3M were recombined to see if they might yield wild types (T3B or T3M) from the mutant (T3C). Although this was not a large number to test, there should have been a fair chance that recombination of two of these should yield some wild type phage if a point mutation were involved. The results (Table 7) indicated that no recombination occurred and therefore, T3C might represent a deletion instead of a point mutation.

Attention was then turned to $T3B_{SL}$. It was compared to the T3B stock on hand in this laboratory as to rate of inactivation with T3B antiserum (Table 8). Both $T3B_{SL}$ and T3B were inactivated at approximately the same rate. Therefore, it was concluded that T3B and $T3B_{SL}$ were antigenically the same. It was also noted that T3B and $T3B_{SL}$ both gave rise to T3C when each was plated with anti-T3B in the overlay. This suggested that the $T3B_{SL}$ survivors of the neutralization test were probably T3C, which was a property of T3B described earlier. With this information and the fact that T3B and $T3B_{SL}$ exhibited the same host range, it was assumed that although the sources of each were different, they were identical phage as far as neutralizable antigen was concerned.

Comparative Study of Other T3 Stocks

T3 phage stocks were obtained from other laboratories (see MATERIALS AND METHODS for source) in order to compare the characteristics of these with those described above.

Three T3 stocks obtained from Dorothy Fraser were tested for the presence of T3C. Each sample (DF 18, DF 3 and DF 32) was plated out on C600 to obtain single plaque isolates. At the same time, each plate was spotted with anti-T3B, anti-T3C and anti-T3M to determine the antigenic type. It was then discovered that DF 3 would not plate on C600. Further testing indicated that it was a T3C. However, single plaques of DF 18 and DF 32 (both were T3B) were isolated and grown up by broth lysis on B cells. After titering each stock on C600, 0.1 ml. of each (approximately 10^9 phage per ml.) was plated on HfrC to select for any T3Cs present. No plaques arose on either plate, thus suggesting that the T3Bs obtained in this laboratory were unique in their ability to mutate from T3B to T3C.

The next logical step was to screen the original phage material from which T3 phage was isolated (designated W72), a sample of which was received from M. Rakieten. This material was tested for different antigenic types in order to compare them with those found in this laboratory. A sample of W72 was grown up on hosts B, C600, HfrC, B/1 and Templeton; the phage stocks were designated W72 (C600), etc.. Since W72 would neither broth-lyse nor plate on B/1, that host was no longer used. This difference in host range was the first thing that was notably unlike our own T3 stocks. These samples were then titered on each of the hosts mentioned above and tested for antigen by serum spots (Table 9). From these plates, plaques were picked from within the serum spots and further tested for their antigen (Table 10). By this method of screening, 4 different antigenic types were detected. One phage type, designated $T3B_{NA}$, appeared to be a phage with a new antigen, which was not common to T3B, T3C or T3M, against which the antisera had been prepared. It was also tested against anti-T1, anti-T2, anti-T4 and anti-T7, none of which had any effect on it. Only its host range properties seemed to relate it to the other T3 phages. No further attempt was made to find its relationship to T3. Only one phage in this group, designated $T3B_L$, differed antigenically from all the other T3s found thus far. Serum neutralization tests comparing $T3B_L$ and T3B, using the sera previously prepared against T3B and T3C, indicated that $T3B_L$ was antigenically different than T3B in that anti-T3C had very little effect on $T3B_L$. Another difference between the two B-types was the fact that neutralization survivors of T3B are normally T3C. This was not true of $T3B_L$. Samples from a plate containing $T3B_L$ and anti-T3B, after incubation at 37°C for 30 min., were tested and found to be B-type. The last interesting difference was the host range of $T3B_L$. It would only plate on Templeton, whereas ordinarily T3B would plate on Templeton with only 1 percent efficiency. Antiserum is presently being prepared against $T3B_L$ and will

be tested by this laboratory at a later date.

Thus, it may be seen that of the four phage types found in N72, two were antigenically different from any other T3 phages found thus far. A comparison of all the T3 isolates as to their properties may be found in Table 11. From this table it may be seen that, with the exception of $T3B_L$ and $T3B_{MA}$, all of the variants differed only in host range and, in one case, in plaque size from the phage type to which it was compared. $T3B_L$, however, differed not only antigenically but also in plaque size from the T3B previously found in this laboratory, as may be seen in Table 11. It also exhibited an extremely different host range.

DISCUSSION

It is necessary to assume similarities to the better known T phages when considering properties of T3 associated with their structure. As suggested by a number of investigators (Lanni, 1953; Fodor and Adams, 1955; Tanami and Miyajima, 1956; Streisinger, 1956a,b), the antigenic structure of such phage as T5, T2 and T4 is quite complex. The serum-sensitive portion, probably the tail, contained an ensemble of somewhat independently functioning, but not necessarily heterogeneous antigens, whose collective structure could vary mutationally in several distinct ways. This appears to be the situation that exists in the T3 phage group but the present study indicates that the complexity of this phage warrants further investigation.

The T3 group appeared to be the only one which produced antigenic mutants. This is in contrast to other phages. For example, Hershey (1946) could not detect an antigenic difference between host range mutants and their ancestors, confirming the work of Luria (1945). However, Hershey was able to detect

significant immunological differences in various races of T2 which had been subcultured in different laboratories for some time, indicating that antigenic differences in phage do develop, probably by mutation, during repeated subculture. Whether this has any bearing on the results found in this laboratory is not known at the present time. It was noted, however, that in some instances stocks of T3B and T3M, which were presumably free of contaminants when stored, turned out to be T3C when subcultured at a later date. This could have been due to the existence of a mutant in the population; with unequal inactivation rates during storage, the proportion of mutants could arise. On the other hand, the possibility of air contamination upon subculturing is ever present, since so much work is done with all of the T3 phage types in this laboratory.

The relationship of T3M to the other T3 phages proved to be an interesting though puzzling problem. In one instance, single plaques from a T3M stock, which had been tested and found to be pure by plating on all hosts, gave rise to one T3B and numerous T3Cs when plated on a mixed indicator; nevertheless, when one T3M plaque (the parent type) was picked and grown up from each plate that contained mutants (T3B and T3C), no mutation to T3B or T3C was observed when again plated on a mixed indicator. There are at least two possible explanations for this. One is, again, the possibility of contamination. The other possibility is that only an occasional T3M clone will produce a mutant; if this were the case, it would explain why the chance of picking one T3M plaque, from a plate containing several hundred T3M plaques that would produce mutants would be highly improbable. However, even when 100 plaques were tested, no mutant plaques arose; so mutations, if they occur at all, must do so at an extremely low frequency.

Even though there is indirect evidence for the relationship of T3M to the other phages, conclusive direct evidence is still lacking.

The fact that the original T3 phage stock prepared many years ago contained variants that were antigenically identical to the ones found in this laboratory led this investigator to believe that the findings were valid. The most interesting observation, however, was the appearance of a new antigenic type ($T3B_{NA}$) and an antigenically similar, though strikingly different type ($T3B_L$). The former type cannot be discussed as it was not thoroughly tested. $T3B_L$, however, was investigated and found to be completely inhibited by anti-T3B serum. This was not significant in itself, but what was significant was the striking difference that anti-T3C had very little inhibitory power on it. Also, $T3B_L$ serum survivors (using anti-T3B) were not T3C, but $T3B_L$. Both of these properties were different from our own T3B. Another interesting difference was that of host range; $T3B_L$ would plate only on Templeton. Could the B antigen of this variant have been so minutely changed that it could not be detected until it was exposed to anti-T3C serum? Would this difference in antigen have any correlation with the extreme difference in host range properties of this phage? Were the results of the serum neutralization tests distorted by a peculiar batch of T3C antiserum? All of these questions are as yet unanswered. More information is needed before an adequate explanation and discussion can be made. An interesting phenomenon did occur occasionally, however, which might have a bearing on the last question posed. It was observed that when T3C was injected into rabbits, the serum produced from the first bleeding (after 10 injections) contained antibodies which would neutralize only T3C. After several booster injections, however, the B-type antibodies built up to the same level as the C-type. An explanation for this could be that the T3B antigens on the T3B phage are all exposed and readily soluble, whereas the T3C phage protein of the tail is coiled in such a way that the C-type antigens are exposed but the B-type antigens are relatively hidden. Therefore, until the C-type antigen is dissolved away, the

B-type antigen cannot stimulate antibody formation. This concept, of course, is purely hypothetical; an extensive study of this phenomenon alone would be necessary before any conclusions could be drawn for this difference in the rate of antibody production.

CONCLUSION

In order to determine whether qualitative mutations in neutralizable antigen were very extensive, eleven different phage species were tested. It was found that only T3 phage exhibited antigenic variants.

Four variants of T3 phage had been discovered previously in this laboratory, namely, T3B, T3C, T3Bhe and T3M. The relationships between T3B, T3C and T3Bhe had also been established previously; however, the relationship of T3M to the other T3 phages was not yet established. This task was undertaken by this investigator. The evidence obtained was only indirect: occasionally a T3C stock, when plated on C600, would give rise to minute plaques, which were antigenically different than either T3B or T3C. Attempts were made to determine the type of mutation (T3C to T3M) that took place. Large quantities (10^9 phage particles per ml.) of T3M phage were plated on selective hosts for T3B and T3C. This method would detect any reversion to the wild type that might occur. Recombination studies and serum blocking power experiments were also performed with single plaque isolates of T3M. The results indicated that T3M would not revert, recombine, or block the antibodies in the serum of T3B or T3C. Therefore, it was concluded that the type of mutation that took place might have been a deletion.

The T3B phage found in this laboratory seemed to be unique in that 90 percent of the serum survivors obtained were T3C. T3B phage obtained from other

laboratories did not appear to possess this property of mutation from T3B to T3C in the tests performed by this investigator.

An attempt was then made to find out if these four and possibly other variants could be found in T3 stock that had been prepared 16 years ago by Rakieten upon examination of this material. Indeed, three types were found which were neutralized by the sera prepared against the ones found earlier. This meant that Rakieten's phage and those variants in the KSU laboratory were antigenically similar but exhibited a different host range. In addition, two new antigenic types were found in Rakieten's material. One type ($T3B_L$) was neutralized by anti-T3B but not anti-T3C, indicating that the B-type antibody within the T3C antiserum was produced by a different B-type antigen than the one found. The other type ($T3B_{NA}$) did not react with any of the sera on hand but it did infect the same hosts as the other phage. No further attempt was made to find its relationship to the T3 phages.

From this study, it was concluded that the T3B and T3M phage had only one demonstrable neutralizable antigen; whereas, the T3C phage exhibited two neutralizable antigens, one of which was similar to the B-type. It was also concluded that the antigenic site had no strict relationship to the attachment site, both of which are presumably located at the tip of the phage tail, since different host ranges were exhibited by the same antigenic type.

APPENDIX

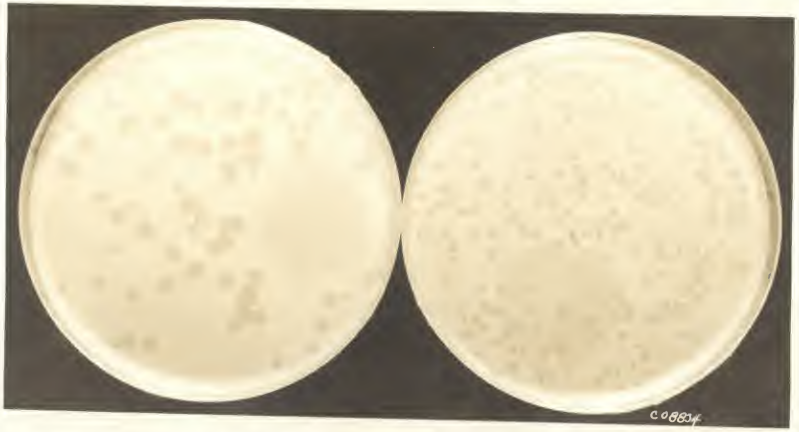


Fig. 2. Antiserum spot test. Left, assay plate of T3B. Plaques are absent in area which contains anti-T3B serum. Right, assay plate of T3C. Plaques are present in area which contains anti-T3B serum.

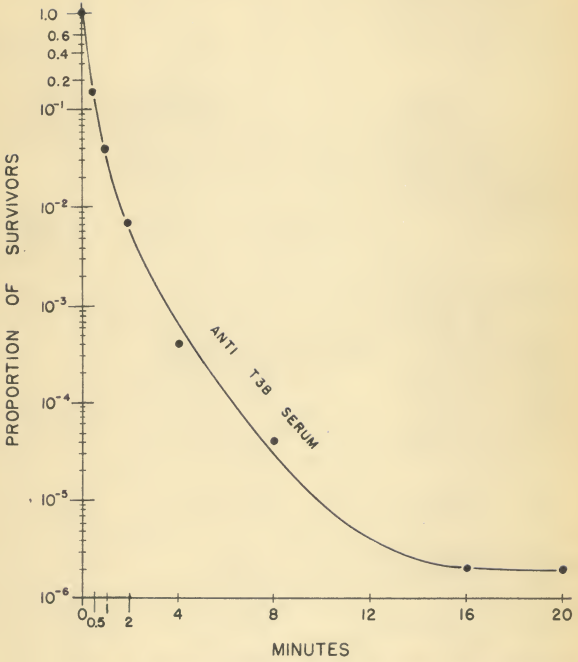


Fig. 3. Antiserum inactivation of T3B phage. Surviving phage at "tail" of curve possess a neutralizable antigen different from the inactivated phage, as may be seen in figure 4.

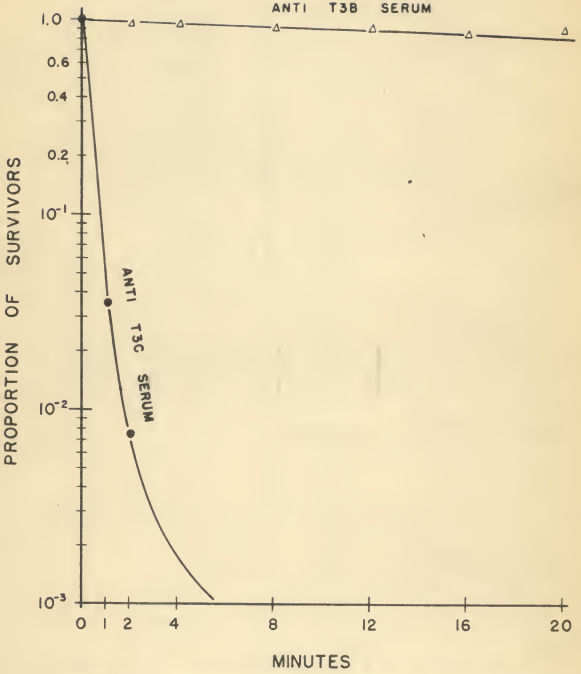


Fig. 4. Antiserum inactivation of phage recovered from "tail" portion of curve in figure 3. These phage, designated as T3C in the text, possess an additional neutralizable antigen.

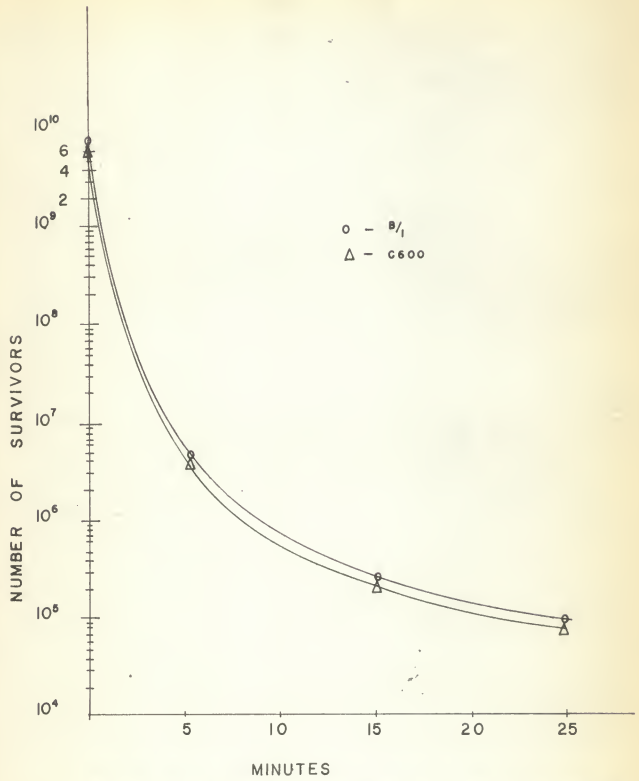


Fig. 5. Neutralization of T3B phage with anti-T3B serum plated on hosts B/1 and C600. The results indicate that there is no significant difference in neutralization rate regardless of which of the two hosts are used as indicators.

Table 1. Minimum K values (serum inactivation constants) after neutralization of phage with homologous antiserum (1:100).

Phage	P^o count	P count	Time in Min.	K(minimum)
T1	4.5×10^9	3×10^3	30	46
T2	9.25×10^9	1.6×10^5	30	31
T3B	1.0×10^9	1.2×10^5	30	31
T3C	2.24×10^9	1×10^3	30	46
T3M	1.76×10^7	$< 10^3$	30	31
T4	5.87×10^7	$< 10^3$	30	31
T7	2.35×10^9	4×10^3	30	46
PLT22	7.43×10^9	8.5×10^7	30	15
X. pruni	5.7×10^9	5.6×10^3	30	46

$$K = \frac{2.3(d)}{T} \log \frac{p^o}{p}$$

K = serum inactivation constant
d = dilution of antiserum
T = length of exposure of phage to antiserum
p^o = phage assay at zero time
p = phage assay at time (T)

Note: The K values presented in this table are minima. 30 min. inactivation at 1/100 dilution of antiserum was chosen arbitrarily in order to obtain rough comparative values. Had precise determinations been made at several shorter time intervals and higher dilutions, more precise and higher K values could have been obtained.

Table 2. Phage tested for the presence of antigenic mutants.

			Original stock		"Survivor" stock	
Phage	Serum	Host	count w/o serum	count w/serum	count w/o serum	count w/serum
T1	anti-T1	B	4.5×10^9	0	-	-
T2	anti-T2	B	7.2×10^9	2.5×10^5	1.5×10^9	1.2×10^9
T3B	anti-T3B	B	1.0×10^9	1.2×10^5	1.9×10^5	1.8×10^5
T3C	anti-T3C	B	3.0×10^9	2.3×10^7	1.6×10^9	1×10^7
T3M	anti-T3M	B	5.1×10^7	1.5×10^4	7×10^9	7×10^9
T4	anti-T4	B	3.0×10^5	0	-	-
T7	anti-T7	B	2.7×10^9	6.4×10^4	2.8×10^9	7.7×10^4
PLT22	anti-PLT22	LT2	7.4×10^9	8.5×10^7	10×10^9	10×10^7
XP-4	anti-XP	X. pruni	6.0×10^9	5.6×10^3	7×10^9	4.6×10^9
$T3B_{SL}$	anti-T3B	C600	7.0×10^9	3×10^6	4×10^7	4×10^7
$T3B_L$	anti-T3B	Temp.	2.6×10^{10}	1.5×10^6	3.5×10^7	1×10^3

Each phage listed above was tested for the presence of antigenic mutants by plating each phage with its homologous antiserum. If any plaques appeared on this plate, they were considered as the "survivor" stock. This stock was then compared with the original stock by adding the same antiserum to both stocks and comparing the number of plaques on the plates w/o serum (control) and the plates w/serum.

Table 3. Identification of T3B serum survivors.

Survivor No.	No. of plaques on hosts HfrC	B/1
1	12	0
2	13	0
3	100	0
4	53	0
5	98	0
6	70	0
7	50	0
8	75	0
9	38	0
10	50	0
11	250	0
12	200	0
13	63	0
14	0	0
15	0	144
16	150	0
17	80	0
18	65	0
19	150	0
20	0	0
21	150	0
22	250	0
23	150	0
24	50	0
25	150	0
26	250	0
27	250	0
28	175	0
29	200	00
30	150	0

T3B was neutralized with anti-T3B serum. There were a number of survivors which appeared as indicated by Fig. 3. Each survivor was stabbed with a sterile toothpick and dipped into 1.0 ml. of broth. Two samples (0.1 ml. each) were plated with HfrC and B/1 cells, which are indicator hosts for T3C and T3B respectively. As may be seen from this table, only one of the survivors was T3B.

Table 4. Efficiency of plating of T3B from single plaques on three different indicator hosts.

	Hosts		
Plaque No.	B	C600	B/1
1	100	107	92
2	97	110	116
3	89	101	107
4	90	126	123
5	98	108	108
6	109	106	111
7	115	101	117
8	96	103	80
9	99	130	100
10	92	104	97
Average	98	109.6	103.1

Numbers are plaque counts x 10^2 of samples taken from initial plaques. As may be seen from this table, there is no apparent difference in efficiency of plating, irrespective of indicator host.

Table 5. Neutralization test of T3B with anti-T3B serum (1:1000) when plated on three different hosts. Time indicates number of minutes of phage-antiserum combination.

	Hosts		
Time in min.	B	C600	B/1
0	9.5×10^9	1×10^9	8.1×10^8
5	1.18×10^7	1.9×10^7	8.5×10^6
10	1.11×10^7	1.10×10^7	9.4×10^6
15	1.1×10^7	8.1×10^6	8.6×10^6
20	7.3×10^6	1.3×10^7	1.2×10^7
25	1.2×10^7	8.9×10^6	1.2×10^7
30	2.6×10^6	4.5×10^6	2.5×10^6

Table 6. Identification of types of T3 phages obtained from single plaque isolations of T3M. These determinations were made to see if T3B and T3C phage types could be isolated from T3M plaques.

Plate and sample No.[1]	No. small plaques on HfrC	No. small plaques on C600	No. large plaques on HfrC	No. large plaques on C600	Probable T3 type
1	TNTC[2]	TNTC	0	0	T3M
1-A	0	5	CL[3]	0	T3C
1-B	0	200	CL	7	T3B
2	TNTC	TNTC	-	-	T3M
2-A(s)	0	0	CL	0	T3C
2-B	0	4	CL	0	T3C
2-C	0	0	CL	0	T3C
2-D	0	0	CL	0	T3C
3	630	645	0	0	T3M
3-A	0	35	CL	0	T3C
3-B	0	1	CL	0	T3C
4	TNTC	TNTC	0	0	T3M
4-A(s)	0	0	CL	0	T3C
4-B(s)	0	0	CL	0	T3C
4-C(s)	0	0	CL	0	T3C
4-D	0	3	CL	0	T3C

Twenty-five single plaque isolates of T3M (plated on C600) were plated out on a mixed indicator containing equal amounts of HfrC and C600. One drop of anti-T3M serum in soft agar was placed on each plate. After incubation, clear minute and large cloudy plaques appeared on all but one plate. In order to determine the antigenicity of the phage that formed the large cloudy plaques, one or more of these plaques from each plate were picked and plated out on HfrC and C600, which are selective hosts for T3C and T3B respectively. As a control, a clear minute plaque was also picked and plated on the same hosts.

[1]The numbers without letters represent the minute parent from each plate; the letters represent large cloudy plaques obtained from the same plate as the minute. (s) represents large cloudy plaques which appeared within the anti-T3M serum spot.

[2]TNTC = too numerous to count

[3]CL = confluent lysis

Table 6. (cont.)

Plate and sample No.[1]	No. small plaques on HfrC	No. small plaques on C600	No. large plaques on HfrC	No. large plaques on C600	Probable T3 type
5	TNTC	TNTC	0	0	T3M
5-A(s)	0	0	CL	0	T3C
5-B(s)	0	0	CL	0	T3C
5-C(s)	0	TNTC	CL	0	T3C
6	TNTC	TNTC	0	0	T3M
6-A(s)	0	0	CL	0	T3C
6-B	0	150	CL	0	T3C
6-C	0	200	CL	0	T3C
7	TNTC	TNTC	0	0	T3M
7-A	0	TNTC	CL	0	T3C
7-B	0	400	CL	0	T3C
8	TNTC	TNTC	0	0	T3M
8-A	0	10	CL	0	T3C
8-B	0	3	CL	0	T3C
9	TNTC	TNTC	0	0	T3M
9-A	0	600	CL	0	T3C
9-B	0	10	CL	0	T3C
9-C	0	75	CL	0	T3C
10	TNTC	TNTC	0	0	T3M
10-A	0	350	CL	0	T3C
10-B	0	600	CL	0	T3C
10-C	0	800	CL	0	T3C
10-D	0	750	CL	0	T3C
11	TNTC	TNTC	TNTC	0	
11-A	0	800	CL	0	T3C
12	TNTC	TNTC	0	0	T3M
12-A	4	50	TNTC	0	T3C
12-B	0	850	TNTC	0	T3C
12-C	4	75	TNTC	0	T3C
12-D	0	2	CL	0	T3C
13	TNTC	TNTC	0	0	T3M
13-A(s)	0	0	TNTC	0	T3C
13-B(s)	0	0	CL	0	T3C
13-C	0	TNTC	CL	0	T3C

Table 6. (cont'd)

Plate and sample No.[1]	No. small plaques on HfrC	No. small plaques on C600	No. large plaques on HfrC	No. large plaques on C600	Probable T3 type
14	TNTC	TNTC	0	0	T3M
14-A(s)	0	1	TNTC	0	T3C
14-B	50	800	600	0	T3C
15	TNTC	TNTC	0	0	T3M
15-A	0	45	CL	0	T3C
15-B	0	0	TNTC	0	T3C
15-C	0	75	CL	0	T3C
16	TNTC	TNTC	0	0	T3M
16-A	0	50	CL	0	T3C
16-B	25	800	TNTC	0	T3C
16-C	10	125	600	0	T3C
16-D	0	900	TNTC	0	T3C
17	TNTC	TNTC	0	0	T3M
17-A(s)	0	0	TNTC	0	T3C
18	TNTC	TNTC	0	0	T3M
19	TNTC	TNTC	0	0	T3M
19-A	0	100	TNTC	0	T3C
19-B	0	85	TNTC	0	T3C
19-C	100	TNTC	600	0	T3C
20	TNTC	TNTC	0	0	T3M
20-A	15	300	TNTC	0	T3C
20-B	0	500	CL	0	T3C
20-C	0	0	0	0	-
21	TNTC	TNTC	0	0	T3M
21-A	0	250	CL	0	T3C
22	TNTC	TNTC	0	0	T3M
22-A(s)	0	300	CL	0	T3C
23	TNTC	TNTC	1	0	T3M
23-A	0	150	CL	0	T3C
24	TNTC	TNTC	0	0	T3M
24-A	0	5	TNTC	0	T3C
25	TNTC	TNTC	0	0	T3M
25-A(s)	3	0	300	0	T3C

Table 7. Recombination experiment crossing independent isolates of T3C and plated on C600.

Isolate No.	Isolate No. C1	C2	C3	C4	C5
C1	0	0	0	0	0
C2		0	0	0	0
C3			0	0	0
C4				0	0
C5					0

Each of five independent isolates of T3C were plated with each of the other four isolates. C600 host cells were used to detect any T3B or T3M phage that might arise from a recombination of the T3C phages. It is obvious from the results that no recombination occurred upon crossing T3C isolates.

Table 8. Comparative rates of inactivation of T3B and $T3B_{SL}$ using anti-T3B (1:1000) serum. The results indicate identical neutralizable antigens for the two phage.

	T3B		$T3B_{SL}$	
	B/1	C600	B/1	C600
No serum	8.85×10^9	7.02×10^9	4.63×10^9	7.2×10^9
5 min.	5.5×10^6	5.8×10^6	4.4×10^6	1.19×10^7
15min.	5.5×10^6	3×10^5	3.2×10^5	3.1×10^6
30 min.	1.04×10^5	1×10^5	3.2×10^5	4.2×10^5

Table 9. First screening of Templeton phage (M72) from Rakieten from which original T3 was isolated.

	B			C600		
Phage	Titer	Morph.	Effective Antiserum	Titer	Morph.	Effective Antiserum
M72(B)	8.6×10^9	Med	0	no growth		
M72(C600)	1.0×10^1	Med	BS & CS	1.3×10^4	M	MS
					1L	
M72(HfrC)	4.0×10^9	Med	0	2.0×10^1	M	MS
M72(T)	6.0×10^5	Med	0	no growth		

	HfrC			Temp.		
Phage	Titer	Morph.	Effective Antiserum	Titer	Morph.	Effective Antiserum
M72(B)	9.1×10^9	Med	CS	9.3×10^9	Med	0
M72(C600)	6.0×10^1	Med	CS	8.0×10^1	Med	CS
M72(HfrC)	3.0×10^9	Med	CS	3.0×10^9	Med	CS
M72(T)	7.0×10^5	Med	0	4.0×10^9	L	BS & CS
				3.0×10^9	M	BS

Samples of the Templeton phage (M72) were grown up by lysis of broth cultures of B, C600, HfrC and Templeton. Each of these samples were then titered on each of the hosts mentioned above and tested for antigen by serum spots of anti-T3B, anti-T3C and anti-T3M.

Code: Identification of the phage.
M72 = phage
M72(B) = phage grown on B as first host
M72(B)B = phage grown on B as first host and B as the 2nd host

Morph. = Morphology
L = Large plaque
M = Minute plaque
Med = Medium plaque

Antiserum
BS = anti-T3B
CS = anti-T3C
MS = anti-T3M

Table 10. Identification of phage plaques picked from plates listed in Table 9.

Phage	Host	Morph.	Effective Antiserum
W72(B)B	B	Med	0
W72(C600)B	B	Med	0
W72(C600)C600	C600	M	MS
W72(C600)HfrC	HfrC	Med	CS
W72(C600)Temp	Temp	L	BS & CS
Large plaque		M	BS
W72(C600)Temp	Temp	L	BS & CS
Small plaque		M	BS
W72(HfrC)C600	C600	M	MS
W72(HfrC)C600	C600	L	BS & CS
Large plaque		Med	CS
W72(Temp)B	B	Med	0
W72(Temp)HfrC	HfrC	Med	0
W72(Temp)Temp	Temp	L	BS & CS
Large plaque		M	BS
W72(Temp)Temp	Temp	L	BS & CS
M plaque(CS)		M	BS

Plaques which appeared within the serum spots on the plates listed in Table 9 were picked and further tested for their antigen by a series of serum spots of anti-T3B, anti-T3C and anti-T3M. What host cells were used depended upon what host cells were used on the plate from which the plaques were picked.

Code: Same as for Table 9.

Table 11. Characterization of all T3 variants that were isolated and tested.

Phage	Serum			Host range					Plaque Morphology
	BS	CS	MS	B	C600	HfrC	B/1	Temp	
T3B	+	+	-	+	+	-	+	+	medium
T3Bhe	+	+	-	+	+	+	+	+	medium
$T3B_{SL}$	+	+	-	+	+	-	+	+	medium
T3B*	+	+	-	+	+	-	-	-	large
$T3B_L$*	+	-	-	-	-	-	-	+	large
T3C*	-	+	-	+	-	+	-	+	medium
T3C	-	+	-	+	-	+	-	+	large
T3M	-	-	+	+	+	+	+	-	minute
T3M*	-	-	+	-	+	+	-	+	minute
$T3B_{NA}$*	-	-	-	+	-	+	-	+	medium

*These phage types were isolated from N72.

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RELATIONSHIPS BETWEEN ANTIGENIC VARIANTS OF BACTERIOPHAGE T3

by

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The inherent characteristics of bacteriophage make them excellent tools for the investigation of biological phenomena. One of the most specific characteristics of a phage species is its serological reactions. This specificity enables the determination of antigenic relationships, perhaps even the detection of small differences in the amino acid sequence of phage protein, which may in turn be the result of a small change in the gene structure. Therefore, the synthesis of a specific phage antigen is an excellent example of the final phenotypic result of gene action.

Geneticists have long recognized that antigens may be used as markers. Examples vary from the study of inheritance of blood type in humans to the inheritance of the specific antigenic surfaces in Salmonella. A great deal of research on the inheritance of antigens has also been performed on other microorganisms, particularly Diplococcus pneumonia, Neurospora crassa and Escherichia coli. While those studies have given valuable information, the review of literature in this thesis was restricted to the inheritance of antigenic specificity in bacteriophages since this was the area of experimentation.

The first task of this study was to determine whether qualitative mutations in neutralizable antigen were extensive in phage of different species. For this purpose, a series of 11 phages and their antisera were chosen. These antisera were selected because they were the ones that were readily available. Since the results showed that phage T3 was unique among the 11 phages in respect to ability to mutate to a type with a new neutralizable antigen, the second task of this study was to compare additional properties of these mutants with other T3 phage types including those from other laboratories.

The method used to detect antigenic variants was to overlay a nutrient agar plate with a mixture of phage, bacterial host cells and soft agar. The antigenicity of the phage was tested by means of a series of antiserum spots

(1:10 dilution of antiserum in soft agar) placed on the surface of the overlay. Failure to develop plaques within the serum spot demonstrated that phage was neutralized and that the antiserum was specific. If plaques were visible within any of the serum spots, it was assumed that the antiserum was negative. Plaques were picked from within the serum spot, grown up by lysis of broth cultures and tested further for their antigen in a manner similar to the one described above. The final confirmatory test was to run comparative neutralization curves between the mutant and the wild type phage against which the antiserum was originally prepared.

As mentioned previously, of the 11 phages tested in the manner described above, only the T3 phage seemed to mutate to types which possessed different neutralizable antigens than the wild type. Therefore, attention was directed toward the comparison of the properties of these variants with other T3 phage types.

Four antigenic variants of T3 phage had been discovered previously, namely, T3B, T3C, T3Bhe and T3M. The relationships between T3B, T3C and T3Bhe had been established previously; however, an investigation was needed to establish the relationship of T3M to the other T3 phages. It was found that when T3C was plated on E. coli C600, minute plaques (T3M) were produced in a very low frequency on a number of occasions. Attempts were made to discover the type of mutation (T3C to T3M) that took place. Large quantities (10^9 phage particles per ml.) of T3M phage were plated on selective hosts for T3B and T3C. This method would detect any reversion that might occur. Recombination studies and serum blocking power experiments were also performed using single plaque isolates of T3M. The results indicated that T3M would not revert, recombine or block the antibodies in the serum of T3B or T3C. Therefore, it was concluded that the type of mutation that took place might have been a deletion. Actually, there

was no direct evidence for the relationship of T3M to the other T3 phage.

T3 phage stocks were obtained from other laboratories in order to compare the characteristics with those found in our laboratory. It was discovered that our T3B was unique in its ability to mutate from T3B to T3C.

The next step was to examine a sample of phage obtained from Dr. M. Rakieten; this stock was the progenitor of the original T3. This material was tested for different antigenic types in order to compare them with those found in this laboratory. Two new antigenic variants were in this material. One ($T3B_{NA}$) appeared to have an entirely new type of antigen, as none of our antisera would neutralize it. It was tested for host range properties but no further attempt was made to find its relationship to the other T3 phages. The other new type ($T3B_L$) was antigenically different than T3B in that anti-T3C serum had very little effect on it. Another difference between T3B and $T3B_L$ was the fact that neutralization survivors of T3B are normally T3C. This was not true of $T3B_L$. The last interesting difference was the host range of $T3B_L$. It would only plate on the host Templeton, whereas ordinarily T3B would plate on Templeton with only one percent efficiency.

A comparison was made of all the T3 isolates as to their antigenic, host range and plaque size properties. With the exception of $T3B_L$ and $T3B_{NA}$, all of the variants differed only in host range and, in one case, in plaque size from the phage type to which it was compared. $T3B_L$ and $T3B_{NA}$, however, differed not only antigenically but also in plaque size and host range from the T3B previously found in this laboratory.