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THE IMMUNITY FUNCTION OF BACTERIOPHAGE T4

by

James Bryce Cornett

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF MICROBIOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
WITH A MAJOR IN MOLECULAR BIOLOGY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1973
I hereby recommend that this dissertation prepared under my direction by James Bryce Cornett entitled The Immunity Function of Bacteriophage T4 be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy.

Dissertation Director Date

After inspection of the final copy of the dissertation, the following members of the Final Examination Committee concur in its approval and recommend its acceptance:

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ABSTRACT

The immunity (imm) gene of phage T4 was mapped between genes 42 (dCMP-hydroxymethylase) and 43 (phage DNA polymerase) on the standard T4 genetic map. The function of the imm gene was shown to be immediate early by the following criteria: (i) transcription of the imm gene initiated before 1.5 minutes under standard conditions (minimal glucose medium, 30° C); (ii) transcription of the imm gene occurred in the absence of phage protein synthesis.

The immunity function of phage T4 or phage T6 protected the host cell from the disruptive effects of superinfecting T4 phage ghosts, but did not protect against the lethal effects of the extracellularly acting, bactericidal agent, colicin K.

The imm gene protein of phage T4 was hypothesized to immunize the host cell against superinfecting phage ghosts by neutralizing the transmission of the normally disruptive effect on the cell membrane by phage ghosts. The imm gene protein acted stoichiometrically to immunize the host cell against superinfecting ghosts since partially developed levels of immunity did not increase with time after blocking further synthesis of the imm gene protein. For any particular time of immunity development in the phage infected cells, increasing the number of superinfecting ghosts increased the disruptive effects of the ghosts. For any number of superinfecting ghosts, increasing the time for immunity development up to 7 minutes, increased the protection against ghosts.
Full immunity (100% protection) in wild type T4 infected cells never developed against high numbers (>10 ghosts/cell) of superinfecting ghosts due to either shutoff of imm gene protein synthesis, or lysis of the phage infected cells by the ghosts.

The imm phage mutant could develop background levels of immunity to superinfecting ghosts at later times after phage infection. The background level of immunity could be increased by the exogenous addition of putrescine, a divalent cation normally present within the phage head. Putrescine, which also reduced the lethal effect of colicin K, protected the phage infected, or uninfected, cells from the lethal action of phage ghosts. Phage-injected putrescine could not be shown to account for the background level of immunity. The background level of immunity seen in imm infected cells was completely reduced when the phage carried a second mutation in the spackle (s) gene.

The spackle mutant phage exhibited levels of immunity less than developed in wild type infections, but greater than that developed in imm phage infections; but only when challenged with more than 7-8 ghost particles per cell. Spackle mutant phage infected cells also showed reduced ability to exclude superinfecting phage genomes, but at a level greater than seen with imm phage infections.

Cells infected with s phage were not lysis inhibited by superinfecting phage and as such were clearly distinguishable from imm infected cells which were lysis inhibited under these conditions.

Host cells infected with the s mutant phage were not resistant to lysis from without by superinfecting phage, and the extent of lysis
paralleled the loss in the ability to synthesize protein and to form infective centers. Cells infected with \textit{imm} phage were partially resistant to lysis from without but were severely "traumatized" by the superinfecting phage such that the loss in ability to synthesize protein and to form infective centers was much greater than the degree of cell lysis.

The hypothesized role of the spackle gene in developing immunity against superinfecting ghosts is to prevent lysis of the phage infected cells by the ghosts. In achieving this goal, the spackle gene product is viewed to possibly inhibit the contraction-injection process of the superinfecting phage ghosts.
INTRODUCTION

Infection of a susceptible host bacterium by wild type bacteriophage T4 leads very quickly to the inhibition of some of the cell's metabolic processes and redirection of others, resulting in the formation of several hundred progeny virions (cf reviews by Calendar, 1970; Mathews, 1971). Two or more genetically distinct T4 phage may initiate infection simultaneously by allowing adsorption of both to occur in the presence of a metabolic poison (Cohen, 1949) which is then diluted to non-toxic levels to allow simultaneous initiation of the infectious cycle. In these circumstances the different phage mutually participate in the infection from which both parental phage types as well as their recombinants emerge as progeny. However, once the infectious cycle has begun, addition of secondarily infecting (or superinfecting) T4 phage causes the infected cell to react in a characteristic manner. The reactions of the superinfected cell and the superinfecting phage are described below.

Superinfection by Bacteriophage

Lysis Inhibition

Doermann (1948) observed that high cell density cultures of E. coli normally lysed 20-30 minutes after infection with T2r^- (r^- = rapid lysis phenotype), while infections employing T2r^+ did not lyse for several hours (were lysis inhibited). Doermann's study revealed
that the lysis inhibited state resulted from superinfection of cells primarily infected with an $rll^+$ phage. The role of the $rll$ gene in this phenomenon remains as elusive as the nature of the $rll$ gene product(s) and has not been identified. The high cell density infections which allowed Doermann's initial observation merely served to facilitate the process of superinfection. Lysis inhibition represents one phenomenon ascribed to superinfecting phage and it relies moreover on expression by the primary phage of the $rll^+$ function.

**Lysis From Without**

If very high (>40 phage/cell) multiplicities of infection of T-even phage are used in primary infections, the host cells are immediately killed without the usual intracellular multiplication of phage. The bacterial hosts under these conditions are literally destroyed by the overwhelming number of phage which adsorb and penetrate the host cell wall to cause *lysis from without* (LFW) (Delbrück, 1940). This phenomenon provides a second example where the process of superinfection differs from primary infection. These same high MOI's of T-even phage are unable to effect LFW in cultures preinfected with T2 phage (Visconti, 1953). As in the previous example of lysis inhibition, this inhibitory effect to LFW by the superinfecting phage relies upon expression of the primary infecting phage genome. T4 infected cells become resistant to LFW by an unknown mechanism through the expression of the spackle ($s$) gene (Emrich, 1968).
Superinfection Exclusion

A third distinction between the events resulting from infections by primary and superinfecting phage is that of genetic exclusion. Although the superinfecting phage is able to cause lysis inhibition, its genome is not expressed. The chromosome of the superinfecting phage is excluded from the infected cell and is thus unable to pass on either genetic markers or segments of its DNA to progeny. This characteristic was first noted by Delbrück and Luria (1942) as "interference" between different coliphages. With the advent of phage mutants and their selective hosts, exclusion between individuals of the same phage type was demonstrated by Delbrück and Bailey (1946) who showed that superinfecting phage were genetically excluded from the progeny bursts of superinfected cells.

Superinfection Breakdown

Another form of interference between two coliphages was described shortly thereafter by Lesley et al. (1951) who studied the breakdown of T2 phage DNA during infection of E. coli B. Following multiple infection with $^{32}$P-labeled T2 (whose DNA is radioactive), acid soluble radioactivity appeared in the medium. The extracellular appearance of the degraded phage DNA increased with greater MOI. Lesley et al. correctly reasoned that the breakdown products arose from secondarily adsorbing phage and went on to show an increase in breakdown of phage DNA if the labeled phage were added to cells that had been previously infected with T2. These results led to the hypothesis that the primary phage "stimulated" the cell to breakdown
superinfecting phage genomes. The property of superinfection breakdown, the fourth and last effect of superinfecting phage to be considered here, was subsequently observed to result following primary infections with coliphages T2, T4, T6 and T5, but not with T1, T3, or T7.

**Phage Induced Functions Related to Superinfection**

As mentioned above, certain functions expressed by the primarily infecting phage may govern the outcome of superinfection. Thus, the rII function allows lysis inhibition while the s gene specifies a protein to make the cell resistant to LFW. The s gene also has properties similar to the rII function since in the absence of s gene product lysis inhibition cannot occur (Emrich, 1968). The reciprocal case is not true, however, since the rII function has no effect on LFW (Visconti, 1953).

The abilities of T2 to (i) exclude secondary phage markers from appearing in the progeny (genetic exclusion) and (ii) to effect the degradation of the superinfecting phage DNA were correlated by Dulbecco (1952), who suggested that both effects resulted from the same phage function. This short-lived hypothesis was convincingly disproven by Lesley's group who indirectly measured genetic exclusion by determining the amount of $^{32}\text{P}$ in DNA of superinfecting phage which was transferred to progeny virions. In this study, French et al. (1952) were able to demonstrate that exclusion of superinfecting DNA occurred under conditions where breakdown was not observed and they concluded that
degradation of the superinfecting phage genome was not the only mechanism for exclusion. This conclusion was later confirmed in an independent manner by Hershey et al. (1954) and most recently by Fielding and Lunt (1970), who actually identified the enzyme responsible for superinfection breakdown of T4 as *E. coli* endonuclease I. Host strains lacking endonuclease I are capable of genetic exclusion but are incapable of degrading the superinfecting T4 phage DNA\(^1\) (Anderson and Eigner, 1971).

Thus T4 infected cells are able to resist superinfecting T4 phage by two means; each of which requires the expression of a viral gene(s) as demonstrated by the requirement for protein synthesis to effect superinfection exclusion (Mufti, 1972; Vallée, Cornett and Bernstein, 1972) and superinfection breakdown (Anderson and Eigner, 1971). This does not, however, imply the need for two phage functions since in the case of T4, the breakdown of superinfecting phage DNA results from a host enzyme. The superinfecting phage T4 DNA is probably first excluded (by a virus-induced function) and then becomes susceptible to degradation by a host enzyme that is present prior to infection. It should be noted that this method of exclusion at the cell's periphery is distinct from that effective against RNA coliphages which occurs at an intracellular level (Hattman and Hofschneider, 1967; Goldman and Lodish, 1971). The phage induced barrier not only serves

---

1. T5 presents an unusual case in that this phage appears to code for a second enzyme which can degrade superinfecting phage genomes to large acid insoluble fragments (Fielding and Lunt, 1969, FEBS Letters 5; 214).
to exclude the DNA of superinfecting phage but also prevents the normally disruptive effects of superinfecting phage "ghosts" i.e., phage particles lacking DNA (Duckworth, 1971a; Vallée, Cornett and Bernstein, 1972).

**Bacteriophage Ghosts**

T4 phage ghosts are produced from intact phage by rapid dilution into a medium of lower osmotic strength (Anderson, 1950). This procedure ruptures the phage head membrane, releasing the DNA but leaving the adsorbing apparatus of the virus intact (Hershey and Chase, 1952; Lanni and Lanni, 1953; French and Siminovitch, 1955; Herriott and Barlow, 1957a). The study of phage ghosts began with Herriott's observation that while these DNA-less phage could not produce progeny, they retained the capacity to lyse their host cells (Herriott, 1951). Although it was previously known that UV or X-ray inactivated phage could kill their host cells (Watson, 1950), Herriott's observation provided a basis for studying the biological function of a viral protein coat without interference from the viral genome. Subsequent studies with ghosts have shown that they are capable of generally disrupting the metabolism of host cells by inhibiting: respiration; the synthesis of protein, RNA and DNA; the uptake of several amino acids and carbohydrates; and inducing the leakage of intracellular material (French and Siminovitch, 1955; Herriott and Barlow, 1957b; Lehman and Herriott, 1958; Duckworth, 1970a, 1970b, 1971a, 1971b; Winkler and Duckworth, 1971). The mechanism by which ghosts kill their hosts is similar to, but distinct from, that of the colicins K and E1 (Winkler
and Duckworth, 1971). Although both colicin K and ghosts act extra-cellularly to exert their lethal effect, it is not known how the effect is transmitted.

**Immunity Induced by Phage to Superinfecting Ghosts**

If cells first challenged with ghosts are superinfected with infectious phage, few cells will go on to form infective centers (French and Siminovitch, 1955; Herriott and Barlow, 1957b). This example of "exclusion" by ghosts is merely due to the lethal action of the ghost infection and not due to exclusion as described above which requires phage gene(s) expression. If the phage infection precedes that of the ghosts by as little as one minute, then an appreciable number of infective centers are formed; the number being at least 90% if there is a 2-3 minute lag between the initial phage infection and the super-infecting ghosts (Duckworth, 1971a; Vallée, Cornett and Bernstein, 1972). Similar to superinfection exclusion and breakdown, the ability of phage T4 to make its host "immune" to the disruptive effects of ghosts also requires protein synthesis (Duckworth, 1971a; Vallée, Cornett and Bernstein, 1972), indicating the requirement for phage gene(s) expression. As mentioned earlier, the two properties of superinfection exclusion and superinfection breakdown were demonstrated to be distinct but it was not known whether superinfection exclusion and immunity to superinfecting ghosts were separate phenomena. This separation could be best made through the use of a mutant phage unable to exclude or to confer immunity upon the host cell. Such a mutant, however, had never been reported among the many T4 phage mutants isolated.
since the initial discovery of the exclusion phenomenon in 1942. In retrospect, this is not surprising since most mutants of T4 were isolated as conditionally lethal mutations and there is no compelling reason that an exclusion-negative or immunity-negative mutation would result in lethality for the phage. Logically, a mutation of this type would inactivate an early gene function of the phage since both exclusion and immunity develop within a few minutes after phage infection.

An Immunity Negative Mutant of T4

The first successful detection of such a T4 mutant type was reported by Mufti (1972) who laboriously screened a large number of mutations in early genes whose functions were yet unknown. Among 22 amber mutations in the DNA-delay gene 39 (function unknown), Mufti (1972) detected a single amber mutant which failed to exclude superinfecting phage. An independent search, employing a different procedure, detected the same phage strain unable to confer immunity upon cells to superinfecting ghosts (Vallée, Cornett and Bernstein, 1972). This unique amber strain was subsequently shown to contain, in addition to the amber lesion in gene 39, a second mutation (imm\(^-\)) responsible for both the loss of phage-induced immunity to superinfecting ghosts and the inability to exclude superinfecting phage (Vallée and Cornett, 1972). There was no other demonstrable phenotype of the imm\(^-\) strain, indicating that the imm gene was not essential for phage development.
In addition to this fortuitous isolation of an immunity-negative phage mutant, John Childs (1973) independently isolated an immunity-negative T4 mutant by an elegant exercise in classical genetic technique employing "petit" mutants of T4 which contain incomplete chromosomes. The mutant isolated by Childs is allelic (see Results) to the imm mutant described by Vallée and Cornett (Cornett and Vallée, 1973).

This dissertation describes studies on the immunity function of phage T4 with the goal of determining how the immune state is induced by the phage and the role of the imm gene in this process. Some of the experiments to be described here were conceived by Dr. Myriam Vallée and are so indicated in the text. Whenever the immunity-negative mutant (imm) is used, it refers to the imm2 mutant isolated by Vallée and Cornett (1972) unless otherwise indicated.
MATERIALS AND METHODS

Media

The bacteria were grown in either Hershey's (H) broth (Steinberg and Edgar, 1962) or minimal glucose medium (M9G) prepared with M9 salts (per liter of distilled deionized water: 5.8 g Na₂HPO₄, 3.0 g KH₂PO₄, 0.5 g NaCl, 1.0 g NH₄Cl) supplemented with 0.25 g MgSO₄·7H₂O, 2.7 mg FeCl₃·H₂O and 0.4 g glucose.

Bacteria

The Enterobacterium *Escherichia coli* was used throughout these studies. Most of the experiments used strain B/5 (Su⁻) for the experimental host and a derivative of strain B, *E. coli* S/6 (Su⁻), as the plating indicator strain except where amber (am) mutants were employed, in which case the K strain CR63 (Su⁺UAG) or the B strain, BB (Su⁺UAG), were used as indicator strains. The above strains are from the laboratory of Dr. Harris Bernstein with the exception of the BB (Su⁺UAG) strain which was obtained from S. Beckendorf, California Institute of Technology. Later experiments employed strain B (Su⁻) obtained from the Pasteur Institute, France. This strain proved superior to B/5 when plating on broth agar plates after growth in M9G medium. Strain B207 (Su⁻), a K strain unable to retain intracellular potassium (Lubin and Kessel, 1960), was provided by Dr. Paul S. Cohen, University of Rhode Island. Restrictive hosts for rII⁻ mutants were strain K112λh8 (a Su⁻ strain from this laboratory) or CR63(λ), a Su⁺UAG λh8 lysogenic
strain derived in this laboratory from strain CR63. The colicin K producing strain, K-235, was provided by Dr. S. E. Luria, Massachusetts Institute of Technology.

Overnight cultures were grown in H broth from slant innocula once a week. Fresh cultures for daily use were obtained by diluting the weekly overnight 1000-fold into H broth or M9G medium for growth overnight at 30° C. Experimental hosts were prepared by diluting the fresh overnight cultures 100-fold into the appropriate medium. Often, experimental cells were initially grown in M9G medium at 37° C and then shifted to 30° C for at least two generations prior to performing experiments at this temperature. The cell density of the experimental cultures was routinely determined by turbidity as measured in a Klett-Summerson colorimetric photometer at 520-580 nm wavelength and confirmed by plating for viable colonies. Plating indicators were prepared from fresh overnight cultures diluted 100-fold into H broth, grown for two hours at 37° C (cell density equal to 2-4 x 10^8 cells/ml) and concentrated 25-fold by centrifugation and resuspension in fresh H broth. All plating was carried out with enriched Hershey agar (EHA) plates and overlays (Steinberg and Edgar, 1962) for both bacteria and bacteriophage by the method of Adams (1959). Strain B/5 grown in M9G medium was titered on M9G medium plates and overlays containing 1.5% and 0.7% agar respectively.

**Bacteriophage**

Bacteriophage T4D was used throughout these studies with the exception of the T4B strains rIIA105 (deletion of the rII A and B
cistrons; Benzer, 1961) and T4BO₁, a phage whose head membrane is more permeable to salts than wild type T4, obtained from Dr. Carol Bernstein, University of Arizona. The amber (am) and temperature sensitive (ts) mutants used in this study are from the California Institute of Technology collection. The immunity-negative (imm⁻) strain, imm2, was isolated in this laboratory (Vallée and Cornett, 1972) while the imm⁻ mutant, imm1, was isolated and kindly provided by Dr. John Childs, Atomic Energy of Canada Ltd. The T4D spackle (s⁻) mutant (Emrich, 1968) was provided by Dr. Joyce Emrich Owen, University of Oregon.

Phage stocks were prepared from plate lysates obtained by adding $1 \times 10^5$ phage and $2 \times 10^9$ bacteria to 2.5 ml of EHA overlay agar and poured over EHA plates. After incubation overnight at 30° C, several drops of chloroform (CHCl₃) were added to each plate followed by 10-15 ml of M9 salts. The plate was held overnight at room temperature before the phage-containing fluid was decanted and the cellular debris removed by centrifugation at 3,800 x g for 10 minutes. The phage were then sedimented by centrifugation at 23,000 x g for two hours and resuspended by overnight dissolution of the phage pellet in 1/10 volume of M9 salts containing 100 μg/ml of gelatin. Particulate material was removed by low speed centrifugation at 3,800 x g for 10 minutes. The phage stocks contained between $5 \times 10^{11}$ and $2 \times 10^{12}$ plaque forming units (PFU) per ml.

**Bacteriophage Ghosts**

Ghost suspensions, purified by rate zonal centrifugation in sucrose gradients, were prepared according to the procedure of
Duckworth (1970b) and titered by their ability to kill cultures of E. coli B/5 grown in H broth. When a population of susceptible cells is challenged with phage, the number of infected cells is statistically related to the number of phage particles added, according to the Poisson distribution (Adams, 1959). The titer of a phage ghost preparation was determined from the number of cells surviving the ghost challenge by employing the zero term of the Poisson equation,

\[ p(0) = e^{-x} \quad \text{or} \quad x = (\log N_0/N)(2.3) \]

where \( x \) = the multiplicity of killing (MOK), \( N_0 \) = the initial titer of cells and \( N \) = the titer of surviving cells. The titer of the ghosts in such an assay is numerically equal to the MOK multiplied by the initial titer of cells. The ghost preparations stored in M9 salts at 5° C were stable for over a year.

**Colicin K**

*E. coli* K-K235 was grown in M9G medium supplemented with casamino acids (1%) plus thiamine (0.002%) at 37° C to a cell density of \( 2 \times 10^8 \) cells/ml. Ten ml of the cells in a 90 mm glass Petri dish were exposed to ultraviolet (UV) light irradiation from a GE germicidal lamp (GE G8T5) from a distance of 23 inches (5 ergs/mm²/second) for 90 seconds. The irradiated cells were incubated an additional three hours at 37° C, centrifuged to remove the cells (12,000 x g, 15 minutes) and the resulting supernatant, containing approximately \( 2 \times 10^{12} \) killing units per ml, was retained as the colicin preparation. The colicin preparation was titered by the same procedure used to titer phage ghosts. The colicin proved effective in killing all *E. coli* B strains except strain S/6.
Phage Crosses

Crosses between phage strains were performed according to the procedure of Fisher and Bernstein (1970) except that the KCN concentration was reduced by one-half to 1 mM.

Replica Plating of Phage

Determination of phage genotypes by replica plating onto the appropriately seeded bacterial plates by transfer with sterile toothpicks has been described by Fisher and Bernstein (1970).

Complementation Spot Testing

Determination of phage genotypes by spotting a portion of phage onto appropriate phage infected bacterial lawns has been described by Vallée and Cornett (1972).

Measurement of Immunity Developed in T4 Infected Cells to Superinfecting Ghosts

The level of immunity in T4 infected cells to the disruptive effects of superinfecting T4 ghosts was routinely determined by the amount of protein synthesis following superinfection by ghosts (Vallée and Cornett, 1972). Host cells were grown in M9G medium at 30° C to a cell density of 4 x 10^8 cells/ml from which duplicate 1.0 ml samples were infected with T4 phage (MOI = 5). After ten minutes at 30° C, a time interval sufficient to develop greater than 90% immunity in imm^+ infected cells, one sample received superinfecting ghosts (MOK = 7) followed by the addition of ^3H-leucine (final concentration 5 x 10^{-5} M, 0.5 uC/ml) to both samples five minutes later. The reaction was
stopped after five minutes by adding one-half volume of ice cold tri-chloroacetic acid (TCA) (20% w/v) and the suspensions were chilled on ice for at least twenty minutes before filtering. The filters (type HA, 0.45 μ pore diameter, Millipore Corp., Bedford, Massachusetts) carrying the TCA-precipitated bacteria were washed three times with room temperature 5% TCA and dried. The radioactivity associated with each filter was determined by liquid scintillation spectrometry in a toluene based cocktail containing Spectrafluor (Amersham/Searle Corp., Arlington Heights, Illinois). All values were corrected for non-specific adsorption of ³H-leucine by subtraction of the amount of radioactivity adsorbed by an equal volume of TCA-killed bacteria under the same conditions.

The values for immunity presented in the Tables and Figures represent the amount of ³H-leucine incorporated by the phage infected cells which were superinfected with ghosts expressed as percentage of the amount incorporated by the duplicate control which did not receive ghosts. It should be noted that the value, percent immunity, reflects differences in the rates of protein synthesis under these conditions. This may be seen in Fig 1 where continued protein synthesis was monitored in T4 infected cells in the presence and absence of superinfecting ghosts. The percent immunity, determined as described above, in this experiment was 78% and 11% for the wild type (imm¹) and imm⁻ infections respectively.
Fig 1. Continued Protein Synthesis in T4 Infected *E. coli* B/5 After Superinfection by Ghosts.

*E. coli* B/5, grown in M9G medium (30°C) to a density of $4 \times 10^8$ cells per ml, was divided into two portions and infected with wild type (*imm*<sup>+</sup>) or *imm*<sup>2</sup> (*imm*<sup>-</sup>) phage (MOI = 6). One minute after infection, $^3$H-leucine was added (final concentration, $1 \times 10^{-4}$ M, 1 µC/ml) and at the times indicated 100 µl samples were pipetted into 2 ml ice cold 5% TCA (open symbols). Ten minutes after infection, superinfecting ghosts (MOK = 7) were added to a portion of each culture (closed symbols). The samples were filtered (Millipore, 0.45 µ) and washed with 5% TCA, dried, and their radioactivity determined by liquid scintillation counting in a toluene based fluor. The filtrates from the samples taken at 2, 45, and 90 minutes were neutralized with NaOH and 50 µl added to 5 ml scintillation fluid for counting (toluene 666 ml, Triton X 333 ml, PPO 5.5 g, POPOP 0.1 g). There was no decline in the level of $^3$H-leucine in the filtrates over the 90 minute course of the experiment.
Since ghosts also inhibit the uptake of leucine (Duckworth, 1970b; Vallée, Cornett and Bernstein, 1972) as well as incorporation of leucine into protein, uptake of $^3$H-leucine was used to measure immunity in experiments using inhibitors of RNA and protein synthesis as described in the legends accompanying the Tables and Figures.

**Chemicals**

Crystalline chloramphenicol (CAM) and puromycin dihydrochloride (PM) (Grade II) were purchased from Sigma Chemical Co., St. Louis, Missouri. Rifampicin (RIF) (B grade) was purchased from Calbiochem, Los Angeles, California. L-leucine-4,5-$^3$H ($^3$H-leu or $^3$H-leucine) of specific activity 62.5 curies per millimole (C/m mole) was purchased from New England Nuclear, Boston, Massachusetts. Phage T4 antiserum, prepared in sheep, was a gift from Dr. Harris Bernstein.
RESULTS

Map Position of the imm Gene

The genetic determinant for superinfection exclusion in T4 infected cells had been localized to a region between genes 41 to 44 on the standard T4 genetic map by Childs (1970). This region was identified as being absent in those incomplete phage genomes which were most efficiently rescued by superinfecting phage (i.e., the more efficient rescue required the inability to exclude by the primary infecting incomplete phage genome). By implication from these results, it was considered likely that the imm phage mutant of Vallée and Cornett (1972) might reside in the gene 41 to 44 region. The imm phage mutant now provided an opportunity to map more precisely the gene responsible for exclusion.

Since the imm2 mutation is not conditionally lethal to the phage, the conventional method of mapping by determining the recombinational distance from known markers could not be used, since this method ordinarily depends upon the ability to selectively plate for wild type recombinants. The method employed for mapping allowed determination of the immunity phenotype of temperature sensitive (ts) and amber (am) segregants from crosses between am-ts double mutants in the gene 41 to 43 region, and the imm2 mutant. The double mutants used in these crosses were constructed to contain an am and a ts mutation which would easily be distinguished from each other by selective plating. In each
case a \textit{ts} mutation was chosen which was clearly restricted at a temperature (40° C) which allowed unimpaired growth of the \textit{am} mutant in a \textit{Su}^+UAG host (E. coli BB). The respective positions of the different mutations used in this study are shown in Fig 2.

To determine the position of the \textit{imm} gene with respect to gene 43, the double mutant \textit{am}E4314(43)-\textit{ts}P39(43) was crossed to \textit{imm}2 (Fig 3). All \textit{am} segregants\(^1\) (10/10) were \textit{imm}~\textsuperscript{−}, while the \textit{ts} segregants (10/10) were \textit{imm}~\textsuperscript{+} (Table 1, A) indicating that the \textit{imm} gene was to the left of gene 43.

To locate the \textit{imm} gene with respect to gene 41, the double mutant \textit{ts}A14(41)-\textit{am}NG205(42) was crossed to \textit{imm}2 and the resulting \textit{am} and \textit{ts} segregants scored for their \textit{imm} phenotype (Table 1, B). The results indicate that the \textit{imm} gene resides to the right of gene 41, although they do not determine if the \textit{imm} gene is located in the interval between genes 41 and 42, or between genes 42 and 43. The following crosses were performed to provide more information on this point.

The \textit{am}-\textit{ts} combination in gene 42, \textit{am}NG352(42)-\textit{ts}L66(42) gave unexpected results. In the initial experiment although 9 of the 10 \textit{ts} segregants were \textit{imm}~\textsuperscript{+}, in the reciprocal case only 4 of the 9 \textit{am} segregants were \textit{imm}~\textsuperscript{−} (Table 1, C). This double mutant was constructed a second time, retested, and found to yield similar results to the first cross: 20 of the 20 \textit{ts} segregants were \textit{imm}~\textsuperscript{+}, but only 9 of the 18 \textit{am} segregants were \textit{imm}~\textsuperscript{−} (Table 1, D). The \textit{ts} segregants in both

\(^1\) am segregants indicates \textit{am}~\textsuperscript{−}-\textit{ts}~\textsuperscript{+}, \textit{ts} segregants indicates \textit{am}~\textsuperscript{+}-\textit{ts}~\textsuperscript{−}. 
Fig 2. Relative Positions of the T4 Mutants in the Gene 41 to Gene 43 Region That Were Used in the Mapping of the imm Gene (not drawn to scale).

All mutants were grown from single plaques and their genotype confirmed by spot test complementation (see Materials and Methods). amE117 was positioned to the left of gene 42 by two-factor crosses with gene 42 and gene 43 mutants. The map order of amNG352(42) and tsL66(42) was determined by three-factor crosses with mutants both to the left and right of gene 42, employing the respective double mutants amE117-tsL66(42) and tsL66(42)-amE4301(43). The remaining gene 42 mutations were ordered by three-factor crosses. The order of the gene 43 mutations was taken from Allen, Albrecht and Drake (1970). The interval tsL66(42)-tsL91(43) is the average of five determinations (2.72, 3.54, 3.56, 3.58, 4.00) from two-factor crosses.
Fig 3. Diagram of the Cross Between the Double Mutant $tsP39(43)-amE4314(43)$ and $imm2$.

The dashed line (----) indicates the $ts-imm^+$ segregants; the solid line (-----) indicates the $am-imm^-$ segregants.
Table 1. Segregant Analysis and Positioning of the imm Gene.

From the crosses of am-ts double mutants to imm, am and ts segregants were selected and tested for their immunity phenotype. The immunity assay is described in the Materials and Methods.

<table>
<thead>
<tr>
<th>DOUBLE MUTANT</th>
<th>CROSS</th>
<th>SEGREGANTS</th>
<th>POSITION OF IMM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% IMM⁺</td>
<td>% IMM⁻</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tsP39(43) - omE431(43)</td>
<td>om</td>
<td>0 (0/10)</td>
<td>100 (0/10)</td>
</tr>
<tr>
<td></td>
<td>ts</td>
<td>100 (0/10)</td>
<td>0 (0/10)</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tsA14 (41) - omNG205(42)</td>
<td>om</td>
<td>85 (6/7)</td>
<td>15 (1/7)</td>
</tr>
<tr>
<td></td>
<td>ts</td>
<td>15 (3/20)</td>
<td>85 (17/20)</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>omNG352(42) - tsL66(42)</td>
<td>om</td>
<td>55 (5/9)</td>
<td>45 (4/9)</td>
</tr>
<tr>
<td></td>
<td>ts</td>
<td>90 (9/10)</td>
<td>10 (1/10)</td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>omNG352(42) - tsL66(42)</td>
<td>om</td>
<td>50 (9/18)</td>
<td>50 (9/18)</td>
</tr>
<tr>
<td></td>
<td>ts</td>
<td>100 (20/20)</td>
<td>0 (0/20)</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>omE117 - tsL66(42)</td>
<td>om</td>
<td>5 (1/20)</td>
<td>95 (19/20)</td>
</tr>
<tr>
<td></td>
<td>ts</td>
<td>90 (18/20)</td>
<td>10 (2/20)</td>
</tr>
</tbody>
</table>
determinations indicated the order am-ts-imm, whereas the am segregants did not give conclusive information. The observation that am segregants are equally likely to be imm\(^+\) as imm\(^-\) may result from an unusual local stimulation of multiple recombination by the am allele. Specific localized effects of an am allele on recombination have been reported in other contexts (Fisher and Bernstein, 1970).

A second am-ts combination, amE117-tsL66(42), was tested to further verify the location of the imm gene with respect to gene 42. The amE117 mutation, as originally received from the California Institute of Technology collection, was assigned to gene 42. However, G. Holmes (unpublished data, this laboratory) has shown that amE117 complements other gene 42 amber mutations. amE117 maps approximately eight map units from the nearest gene 42 mutation, amNG352(42) (see Fig 2). The complementation and mapping data of amE117 by G. Holmes was confirmed and it was further shown that this mutant produces normal levels of the gene 42 product, dCMP-hydroxymethylase (data not shown). Thus, the amE117 mutation may lie outside gene 42 as indicated in Fig 2.

As shown in Table 1, E, 95% of the am segregants from the cross of amE117-tsL66(42) to imm2 had the phenotype of imm\(^-\), while 90% of the ts segregants were imm\(^+\). The position of the imm gene is clearly to the right of amE117. However, on the basis of these results alone, the imm gene could be either to the left or to the right of gene 42 as amE117 is a short distance to the left of this gene. If the first alternative were correct and the imm gene were between amE117 and tsL66(42) (and to the left of gene 42), then the results indicate that more than 90% of
segregants occurred from recombinational events in the *am-imm2* interval; and very few occurred in the interval between *imm2* and *tsL66(42)*. This seems implausible considering that this latter interval would be rather long (see Fig 2). The results are more consistent with positioning the *imm* gene to the right of gene 42.

The combined results obtained in all the different crosses indicates that the *imm* gene resides between genes 42 and 43, (Cornett and Vallée, 1973). Amber mutants defective in genes 42 and 43 have an *imm*\(^+\) phenotype, thus precluding the possibility that the *imm* function is determined by the product of these genes.

Recently, Childs (1973) has isolated a mutant (*imml*) defective in the exclusion of superinfecting phage. Like the *imm2* mutant, Childs' mutant does not protect the host cell against superinfecting ghosts (Table 2). Childs (1973) has designated the mutant *imml* and has located the mutation between genes 42 and 43. Host cells doubly infected with these two *imm*\(^-\) mutants do not develop immunity against superinfecting ghosts (Table 2). This lack of complementation indicates that both mutations affect the same gene. The *imm*\(^-\) mutant isolated by Vallée and Cornett (1972) was designated *imm2* to distinguish it from the *imml* mutant of Childs.

**The *imm* Gene Function Is An "Immediate Early" Function**

The preceding section demonstrates that the *imm* gene is in the "early" region of the T4 map, that is, the region containing most of the pre-replicative gene functions of the phage. This information, along
Table 2. Immunity to Superinfecting Ghosts Developed in Wild Type, \textit{imml} and \textit{imm2} Infected Cells.

\textit{E. coli} B/5, grown in M9G medium (30° C) to a cell density of $4 \times 10^8$ cells/ml, was infected with the indicated phage strains and superinfected with ghosts (MOK = 7) 9 minutes later. Immunity was measured as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Infection</th>
<th>TCA-insoluble $^{3}\text{H-leu cpm}$</th>
<th>% immunity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- ghosts</td>
<td>+ ghosts</td>
</tr>
<tr>
<td>none</td>
<td>21,140</td>
<td>49</td>
</tr>
<tr>
<td>wild type (WT)</td>
<td>3,609</td>
<td>2,422</td>
</tr>
<tr>
<td>\textit{imml}</td>
<td>11,430</td>
<td>1,820</td>
</tr>
<tr>
<td>\textit{imm2}</td>
<td>8,706</td>
<td>467</td>
</tr>
<tr>
<td>WT + \textit{imml}</td>
<td>6,120</td>
<td>4,515</td>
</tr>
<tr>
<td>WT + \textit{imm2}</td>
<td>6,176</td>
<td>3,626</td>
</tr>
<tr>
<td>\textit{imml} + \textit{imm2}</td>
<td>10,480</td>
<td>1,134</td>
</tr>
</tbody>
</table>

\textsuperscript{a} TCA = trichloroacetic acid  
$^{3}\text{H-leu} = ^{3}\text{H-leucine}$  
cpm = counts per minute
with the stronger evidence that the immunity function develops within minutes after infection (Vallée and Cornett, 1972), suggested that the immunity function was an "immediate early" function of T4. The immediate early functions of phage T4 are those transcribed by the host RNA polymerase and whose mRNA transcripts are formed within 1.5 minutes after infection in minimal glucose medium at 30° C (Grasso and Buchanan, 1969; Salser, Bolle and Epstein, 1970; Black and Gold, 1971). To confirm the mapping and physiological data suggesting that the imm gene was of the immediate early class, the following two types of experiments were performed.

The imm Gene Is Transcribed Immediately After Infection

The first experiment utilized rifampicin (RIF) which blocks initiation of transcription (Sipple and Hartman, 1968). The concentration of RIF used in these experiments (400 μg/ml) completely blocks transcription in phage infected cells as measured by protein synthesis when RIF is added 60 seconds before the phage (Fig 4). If the imm gene were immediate early, it should by definition be transcribed within 1.5 minutes after infection (Salser et al., 1970) under standard conditions (minimal glucose medium, 30° C). It then follows that the addition of RIF at various times after infection, but before 1.5 minutes, should allow some imm gene transcripts to be formed. These transcripts can be detected by allowing an additional short period of time after RIF addition for their translation and subsequent development of immunity to superinfecting ghosts (DELAYED ASSAY). The control for this experiment was to superinfect with ghosts at the time of RIF addition to determine
E. coli B/5 was grown in M9G medium (30°C) to a cell density of 4 x 10^8 cells/ml and divided into two portions; one received RIF in methanol (final concentration 400 μg/ml), the other an equal volume of methanol without RIF. Both portions were infected with wild type T4 60 seconds later followed by the addition of 3H-leucine (final concentration 5 x 10^{-5} M, 0.5 μC/ml) 1 minute later. At various times 100 μl samples were pipetted into 2 ml of ice cold 5% TCA and chilled for at least 20 minutes before filtering and measuring the amount of radioactivity as described in the Materials and Methods.
the level of immunity developed at the time the RIF was added (IMME-
DIATE ASSAY). An increase in immunity in the delayed assay compared to
that of the immediate assay demonstrates that imm gene transcripts were
present before the addition of RIF. Since RIF will ultimately decrease
protein synthesis by reducing the amount of gene transcripts (Fig 4),
immunity to superinfecting ghosts was measured by $^3\text{H}$-leucine uptake in
the presence of chloramphenicol (CAM) following the challenge with
ghosts (Duckworth, 1970b; Vallée, Cornett and Bernstein, 1972). The re-
sults in Table 3 show that within 30 seconds after infection, imm gene
transcripts are being formed. These data also demonstrate that immunity
is developing during the first minute after infection by the constant
increase of immunity with time in the immediate assays.

Separate experiments (data not shown) utilizing assays with
times less than 30 seconds (-10, or 0 seconds) were not directly compa-
rable to the experiments presented in Table 3 due to the great variabil-
ity in the absolute amount of uptake of $^3\text{H}$-leucine. These results were
informative, however, if the ratio $\frac{\text{delayed assay}}{\text{immediate assay}}$ is taken as a measure
of immunity development. When RIF and phage are added simultaneously
(0 time assay), there should be no immunity development and this ratio
should be unity. The ratios for the 45 seconds and 30 seconds assays of
Table 3 are 19.9 and 15.4 respectively. The ratios for separate exper-
iments employing 0 seconds and -10 seconds assays were 2.3 and 0.7 re-
spectively. The fact that the ratio is less than unity if RIF is added
10 seconds before the phage indicates that RIF at this concentration
Table 3. Time of the Initiation of Transcription of the imm Gene.

E. coli B/5 was grown in M9G medium (30°C) to a cell density of $4 \times 10^8$ cells/ml. At various times after infection with wild type T4 (MOI = 5), 1.0 ml samples of infected cells were transferred to tubes containing: RIF (final concentration 400 μg/ml) + ghosts (MOK = 7) and CAM (final concentration 75 μg/ml) [IMMEDIATE ASSAY] or; RIF to which ghosts and CAM were added 7.5 minutes later [DELAYED ASSAY]. Each sample received a two minute pulse of $^3$H-leucine (final concentration $2 \times 10^{-5}$ M, 1 μC/ml) 3.5 minutes after the addition of ghosts. Uptake of $^3$H-leucine was stopped by pouring the tube contents into 1-2 ml M9 salts (25°C) and immediately filtering (Millipore 0.45 μ). The tube was quickly rinsed with 1-2 ml M9 salts and poured over the filter followed by two rinses with M9 salts. The filters were dried and counted by liquid scintillation counting. The delayed assay of 120 seconds (ca. 20,000 cpm), arbitrarily taken as 100%, was equal to 65% immunity in the standard assay where immunity was measured by a two minute pulse of $^3$H-leucine following superinfection by ghosts 10 minutes after infection by wild type T4. The values shown are the average of three experiments.

<table>
<thead>
<tr>
<th>Time of RIF Addition After Wild Type Infection (seconds)</th>
<th>Percent Immunity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Ghost Challenge</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>± 0.2</td>
</tr>
</tbody>
</table>

\[ ^a \text{RIF} = \text{rifampicin} \]

\[ ^b \text{Percent immunity is the percent of} \ ^3\text{H-leucine uptake in cells superinfected with ghosts relative to the amount which is taken up in cells not receiving ghosts. The values are given ± the standard deviation.} \]
acts within 10 seconds and that the values shown in Table 3 are then realistic with respect to the time of action of RIF.

The imm Gene Is Transcribed in the Absence of Phage Protein Synthesis

Inhibition of protein synthesis by chloramphenicol. This experiment utilized chloramphenicol (CAM), an inhibitor of protein synthesis (Hahn, 1967) to block phage gene expression. The addition of CAM (100 μg/ml) prior to phage infection prevents the phage induced alteration of the host RNA polymerase so that transcription is restricted to the early class of phage genes (Salser et al., 1970). If the immunity function is immediate early, then infection in the presence of CAM should allow transcription of the imm gene. As in the previous experiment, detection of these transcripts requires their translation and the subsequent development of immunity to superinfecting ghosts, so RIF was added (400 μg/ml) to prevent further transcription before removing the CAM. The results of such an experiment where immunity was measured by 3H-leucine uptake are shown in Table 4. By comparing the immunity developed in tube 2 (control where CAM was continually present) to tube 3 (where CAM was removed after adding RIF) it can be seen that some imm gene mRNA was formed in the presence of CAM. However, the amount of immunity developed was much less than expected for the time allowed for the translation and expression of this mRNA. The control value of tube 4 without RIF also shows less immunity development than expected. The value of tube 4, where CAM was removed 20 minutes prior to the ghost challenge, should be greater than the value of tube 1 which represents
Table 4. Transcription of the imm Gene in the Presence of Chloramphenicol (CAM).

E. coli B/5 was grown in M9G medium (30° C) to a cell density of 4 x 10^8 cells/ml and divided into two lots. One lot received CAM (final concentration 100 μg/ml), the second receiving an equal volume of M9 salts; both lots were infected with wild type T4 (MOI = 6) 2.5 minutes later. After 5 minutes, 2.0 ml portions of phage infected cells were transferred to tube 1 (-CAM) or to tubes 2, 3, and 4 (+CAM). The phage infected cells were washed by first diluting with 2 volumes of ice cold M9 salts (tube 3 received M9 salts + RIF) and plunging the tubes into ice. The following steps were carried out at 0-4° C: the tubes were centrifuged simultaneously (12,000 x g, 2 minutes at speed); decanted and resuspended in 5.0 ml ice cold M9 salts (tube 3 again receiving RIF); centrifuged as before, decanted and set in ice. The sedimented cells were resuspended in pre-warmed (30° C) M9G medium (tube 3 receiving M9G + RIF) by rapid mixing for 15 seconds and then the tubes were returned to 30° C. RIF, added to a final concentration of 400 μg/ml, was added with the ghosts + CAM to tubes 1, 2, and 4 so that uptake was always measured in the presence of the drug. Uptake of 3H-leucine (final concentration 1 x 10^-4 M, 1 μC/ml) was allowed for 2.0 minutes before filtering the cells (Millipore, 0.45 μ) and washing with three volumes of room temperature M9 salts. The filters were dried and counted by liquid scintillation spectrometry. The values presented are corrected for non-specific adsorption by subtracting the value obtained with phage infected, heat killed bacteria under the same conditions. The values for immunity are the average of two experiments.

<table>
<thead>
<tr>
<th>Time After Infection (minutes)</th>
<th>Tube</th>
<th>CAM</th>
<th>RIF</th>
<th>Wash</th>
<th>Ghosts + CAM (+RIF)</th>
<th>3H-leucine</th>
<th>Filter</th>
<th>Percent Immunity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>10.0</td>
<td>10.5 (+)</td>
<td>14.0</td>
<td>16.0</td>
<td>(100)</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>-</td>
<td>-</td>
<td>10.0</td>
<td>10.5 (+)</td>
<td>14.0</td>
<td>16.0</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
<td>7.5</td>
<td>-</td>
<td>10.0</td>
<td>30.0 (-)</td>
<td>33.5</td>
<td>35.5</td>
<td>1.26 ± 0.69</td>
</tr>
<tr>
<td>4</td>
<td>2.5</td>
<td>-</td>
<td>-</td>
<td>10.0</td>
<td>30.0 (+)</td>
<td>33.5</td>
<td>35.5</td>
<td>24.4 ± 0.75</td>
</tr>
</tbody>
</table>

a The percent immunity is arbitrarily set at 100% for tube 1 equivalent to 10 minutes of immunity development. The values for tubes 3 and 4 represent 15-20 minutes of translation (immunity development). The values are given ± the standard deviation.
10 minutes of immunity development without any CAM. Of the possible explanations for these low values, two are most plausible; (i) the imm mRNA is degraded during the 12 minutes required to centrifuge the cells and remove the CAM even though the entire procedure was performed at 0–4° C; (ii) removal of the CAM by washing the cells does not allow efficient recovery of protein synthesis.

The second possibility was more easily tested. Several experiments were performed to assess the ability of uninfected and T4 infected CAM pre-treated cells to recover protein synthesis after removal of the inhibitor by centrifugation or by the more rapid technique of filtering. The results of such an experiment utilizing centrifugation (Fig 5) indicate that CAM inhibition is not effectively reversed in T4 infected cells but is reversed in uninfected cells. This, of course, does not rule out the first possibility that the imm gene mRNA is labile under these experimental conditions; but since the control experiments with CAM adequately explained the results, this first possibility was not explored. Additional experiments revealed that the ability to reverse CAM inhibition in phage infected cells is dependent upon the time of addition of the CAM (see Appendix 1). Taking into account the poor reversibility of CAM inhibition under these conditions, the results indicate that the immunity gene is transcribed in the absence of phage protein synthesis. Since this type of experiment is unsatisfactory due to the effects of CAM, the experiment was repeated using a second method to block protein synthesis.
Fig 5. Recovery of Protein Synthesis in CAM-Treated Uninfected and T4 Infected E. coli B/5 After Removal of the CAM.

E. coli B/5 was grown in M9G medium (30° C) to a density of $4 \times 10^8$ cells/ml and divided into two portions; one received 100 µg/ml chloramphenicol (CAM), the other an equal volume of M9 salts. Two ml portions of each culture were infected with wild type T4 (MOI = 5) or an equal volume of M9 salts after 5 minutes. Ten minutes later each portion was diluted 4-fold with ice cold M9 salts and immediately centrifuged (12,000 x g, 1.5 minutes) at 4° C; the pellet was resuspended in 5 ml ice cold M9 salts and centrifuged a second time. The pellet was finally resuspended in pre-warmed M9G, returned to 30° C and $^3$H-leucine was added (final concentration $5 \times 10^{-5}$ M, 0.5 µC/ml). At the times indicated, 200 µl samples were pipetted into 100 µl of ice cold 20% TCA and chilled in ice for at least 20 minutes before filtering and determining the radioactivity as described in the Materials and Methods.
Inhibition of protein synthesis by potassium starvation in potassium requiring host cells. *E. coli* B207 is unable to retain intracellular potassium and requires the continual presence of this cation to synthesize protein (Lubin and Kessel, 1960; Ennis, 1970). The results of an experiment (Fig 6) show that when a culture of B207 is washed and resuspended in potassium-free medium, protein synthesis does not begin until the addition of potassium. Upon addition of KCl, linear protein synthesis begins immediately. Fig 6 also shows that protein synthesis in B207 is inhibited upon challenge with ghosts.

*E. coli* B207 was used in an experiment identical to that previously described utilizing CAM except the cells were washed free of potassium and resuspended in potassium-free medium to block protein synthesis prior to infection with T4 phage. The results in Table 5 show that 50% of immunity develops (tube 3) from transcripts formed in the absence of protein synthesis when further transcription is blocked by RIF before allowing these transcripts to be expressed. The actual amount of immunity developed is closer to 40% since approximately 10% of the value represents background as seen by the value of tube 2 where 10% "immunity" develops in the complete absence of protein synthesis. The reason for this background level is unknown, but it does not appear to represent immunity development due to residual protein synthesis in the absence of potassium since an equal amount of background (12%) is seen in tube 5 where RIF was added before infection to prevent transcription and subsequent translation of the *imm* gene (see also Fig 4).
Fig 6. Protein Synthesis in E. coli B207 + Potassium, and After Ghost Infection.

E. coli B207 was grown at 30° C to a cell density of 4 x 10^8 cells/ml in M9G-KD medium (K⁺-deficient M9G where KH₂PO₄ is replaced by an equimolar amount of NaH₂PO₄) supplemented with KCL to 33 mM and casamino acids (0.3%). The cells were washed free of K⁺ by filtration and resuspended in M9G-KD medium containing 0.03% casamino acids and ³H-leucine was added (final concentration 5 x 10⁻⁵ M, 0.5 μC/ml). After 10 minutes the cells were divided; one portion received K⁺ (●) (final concentration 33 mM) and the other an equal volume of distilled water (○). One portion of the cells receiving K⁺ received ghosts (MOK = 7) 15 minutes later (▲). At the indicated times 200 μl samples were pipetted into 100 μl of ice cold 20% TCA and chilled in ice for at least 20 minutes before filtering and determining the radioactivity as described in the Materials and Methods.
Table 5. Transcription of the imm Gene in the Absence of Phage Protein Synthesis in E. coli B207.

E. coli B207 was grown at 30° C to a cell density of 4 x 10^8 cells/ml in M9G-KD (K^+-deficient M9G medium where KH_2PO_4 is replaced by an equimolar amount of NaH_2PO_4) supplemented with KCl to 33 mM and casamino acids (0.3%). The cells were collected by filtration (Millipore, 0.45 μ, 47 mm diameter), washed with three volumes of M9-KD salts, resuspended in an equal volume of pre-warmed (30° C) M9G-KD containing 0.03% casamino acids, and 1.0 ml transferred to each experimental tube. The cells were infected with wild type T4 (MOI = 5) suspended in K^+-free buffer before receiving the following additions at the indicated times: RIF (400 μg/ml); K^+ (33 mM as KCl); ghosts + CAM (MOK = 7, 100 μg/ml respectively); ^3_H-leucine (1 x 10^-4 M, 1 μC/ml). RIF was added with the ghosts + CAM to tubes 1, 2, 4 and 5 so that uptake was always measured in the presence of RIF. Uptake of ^3_H-leucine was stopped after 2.0 minutes by pouring the tube contents into 1-2 ml of room temperature M9 salts and immediately filtering (Millipore, 0.45 μ) followed by five 2.5 ml rinses. The second and third rinses washed the experimental tube and the entire filtering operation required less than 20 seconds. The filters were dried and their radioactivity measured as previously described. The values presented represent the average of three determinations ± the standard deviation.

<table>
<thead>
<tr>
<th>Tube</th>
<th>RIF</th>
<th>K^+</th>
<th>Ghosts + CAM</th>
<th>(+RIF)</th>
<th>^3_H-leuc</th>
<th>Filter</th>
<th>% Immunity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-2.0</td>
<td>10.0</td>
<td>(+)</td>
<td>14.0</td>
<td>16.0</td>
<td>(100)</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>10.0</td>
<td>(+)</td>
<td>14.0</td>
<td>16.0</td>
<td>9.9 ± 7.3</td>
</tr>
<tr>
<td>3</td>
<td>9.25</td>
<td>10.0</td>
<td>20.0</td>
<td>(-)</td>
<td>24.0</td>
<td>26.0</td>
<td>49.6 ± 3.1</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>10.0</td>
<td>20.0</td>
<td>(+)</td>
<td>24.0</td>
<td>26.0</td>
<td>74.2 ± 6.3</td>
</tr>
<tr>
<td>5</td>
<td>-.75</td>
<td>-</td>
<td>10.0</td>
<td>(+)</td>
<td>14.0</td>
<td>16.0</td>
<td>11.6 ±22.1</td>
</tr>
</tbody>
</table>
Both methods of blocking phage directed protein synthesis allowed transcription of the immunity gene. This fact, taken with the first experiment which shows that imm gene transcription initiates before 1.5 minutes under standard conditions (M9G medium, 30° C) shows that the imm gene is an immediate early function of T4. This conclusion is consistent with both the map position of the imm gene and the rapid development of immunity observed after T4 infection (Vallee and Cornett, 1972).

In the above experiments, the level of immunity that developed after removal of the block in protein synthesis was less than that developed in an equal or greater period of time in cells in which protein synthesis was not inhibited. These lower levels of immunity appear to result during infections in which protein synthesis is blocked by CAM, since such phage infected cells do not fully recover the ability to synthesize protein once the block is removed (see Appendix 1). That this reduced level of protein synthesis was clearly detectable under these conditions is due to the fact, as shall be shown later, that immunity development is proportional to the amount of immunity protein formed. This stoichiometric action of the imm protein is distinct from other early phage functions which are catalytic in nature and thus do not show such a marked decrease in activity under similar conditions (see Peterson, Cohen and Ennis, 1972).
The Immunity Function of T4D

The Model

A model, formulated by M. Vallée, for the development of immunity in T4 infected E. coli to superinfecting ghosts and the exclusion of superinfecting phage governed by the imm gene has been presented by Vallée and Cornett (1972). This model was based chiefly on two lines of evidence: (i) electron micrographs of phage infected cells indicated that T4 preferentially adsorbed to areas (receptor sites?) of the cell wall which were contiguous with the cell membrane (Bayer, 1968). We assume that ghosts, acting extracellularly to disrupt the cells, adsorb to these same sites; (ii) the exclusion of superinfecting T4 occurs at the cell periphery since endonuclease I is located in the periplasmic space between the cell inner membrane and the cell wall (Anderson and Eigner, 1971; Anderson, Williamson and Eigner, 1971). On the basis of these observations, it was suggested that the imm gene protein functionally alters the cell wall-membrane adhesions to prevent the passage of superinfecting DNA into the cytoplasm and to prevent the transmission of the disruptive effect of ghost attachment.

The Immunity Function and Colicin K

A means by which to test one aspect of this model was sought through the use of colicin K. Colicins are a class of bacterial proteins produced by gram negative enteric bacteria carrying an autonomously replicating plasmid which is the genetic determinant for colicin production. The colicins exert their lethal effect extracellularly by
attaching to specific receptor sites on the cell surface and as such their actions resemble very closely those of T4 ghosts; this is especially true for colicins E1 and K which cause a general metabolic disruption of their target cells (Nomura, 1967). Colicin K was chosen for study since its receptor site appears to share a common component with the receptor site for phage T6; bacteria which mutate to T6 resistance are often found to have acquired a concomitant resistance to colicin K (Fredericq and Gratia, 1950). If the imm protein blocked or neutralized transmission of the disruptive effect of ghost attachment, it seemed likely that it would also negate the lethal effects following adsorption of colicin K to the cell surface. The results of an experiment to test this hypothesis, (Table 6), show that both T4 and T6 infections immunize the cell against superinfecting T4 ghosts, as measured by the formation of infective centers and progeny phage, but fail to neutralize the lethal effects of colicin K. These results suggest that the immunization action of the imm protein is not a general effect but most likely specific to those sites or transmission channels through which phage or phage ghosts gain access to the cell membrane. It should be noted that T4 is able to inhibit DNA degradation caused by colicin E2 but that this inhibition occurs intracellularly since the inhibition is observed if T4 infection follows that of the colicin (Swift and Wiberg, 1971).

The Immunity Function is Non-Catalytic

The following experiment, designed by and performed in collaboration with M. Vallée, was carried out to determine if the imm
Table 6. The Immunity Function and Colicin K.

E. coli B/5 was grown in H broth (37° C) to approximately 3 x 10^8 cells/ml, infected with T4 or T6, and superinfected 4 minutes later with either T4 ghosts or colicin K. Eight minutes after the addition of ghosts or colicin K, one sample was diluted 100-fold into ice cold buffer and (i) plated for infective centers with S/6 or B/5 as indicators (for the T4 and T6 experiments respectively); (ii) treated with CHCl3 and plated as above to determine the number of unadsorbed phage which was subtracted from the values given for infective centers in the table. Another sample was diluted 10,000-fold into 30° C H broth and phage growth was allowed for an additional 90 minutes before termination with CHCl3 and plating for the progeny titer. The values given as burst size represent the number of progeny produced per bacterium.

<table>
<thead>
<tr>
<th>Infection</th>
<th>MOK^a</th>
<th>Infective Centers (x 10^8/ml)</th>
<th>Burst Size^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4 ghosts</td>
<td>5.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>colicin K</td>
<td>8.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T4 phage</td>
<td>6.1</td>
<td>1.6</td>
<td>71</td>
</tr>
<tr>
<td>T4 phage + colicin K</td>
<td>10.9</td>
<td>0.05</td>
<td>0.03^c</td>
</tr>
<tr>
<td>T4 phage + T4 ghosts</td>
<td>7.3</td>
<td>1.5</td>
<td>92.0</td>
</tr>
<tr>
<td>T4 ghosts</td>
<td>6.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>colicin K</td>
<td>7.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T6 phage</td>
<td>5.8</td>
<td>2.1</td>
<td>60</td>
</tr>
<tr>
<td>T6 phage + colicin K</td>
<td>13</td>
<td>0.05</td>
<td>0.03^c</td>
</tr>
<tr>
<td>T6 phage + T4 ghosts</td>
<td>9.1</td>
<td>1.2</td>
<td>68</td>
</tr>
</tbody>
</table>

^a The MOK (multiplicity of killing) was calculated from the number of survivors by the zero term of the Poisson equation.

^b The burst size is expressed as plaque forming units/ml.

^c Represents a value equal to the titer of unadsorbed phage.
protein acted in a catalytic or stoichiometric fashion to immunize the phage infected cell to superinfecting ghosts. Duplicate samples of wild type T4 infected cells were treated with inhibitors of protein synthesis at various times after infection when only partial immunity had developed. Either chloramphenicol (CAM) or puromycin (PM) was used to inhibit protein synthesis. One sample received ghosts simultaneously with the inhibitor (IMMEDIATE ASSAY) and the level of immunity at this time was measured by the uptake of $^3$H-leucine. The second sample was challenged with ghosts 7.5 minutes after the addition of CAM or PM (DELAYED ASSAY) and immunity was again measured by $^3$H-leucine uptake. If the imm protein acted catalytically, immunity would continue to develop after the addition of the inhibitor and the delayed assay would show a greater level than the immediate assay. The results of such an experiment shown in Fig 7 demonstrate that the levels of immunity in both the immediate and the delayed assays did not differ significantly. These results show that the imm protein acts in a non-catalytic fashion to immunize the host cell against the superinfecting ghosts. This conclusion was substantiated by an almost identical experiment (data not shown) which measured immunity by the ability to exclude superinfecting phage in similar immediate or delayed assays (Vallée and Cornett, 1973).

The preceding results suggest that the imm protein may act to neutralize those portions of the cell membrane that are presumably associated with phage receptor sites (ca. 300 per cell; Watson, 1950) and does so in a stoichiometric manner. Since a single ghost is
Fig 7. Development of Immunity in Wild Type T4 Infected E. coli B/5 as a Function of the Time of Addition of Chloramphenicol (CAM) or Puromycin (PM).

E. coli B/5, grown in M9G medium to a cell density of $1 \times 10^8$ cells/ml at $37^\circ$ C was shifted to $30^\circ$ C for two generations ($4 \times 10^8$ cells/ml) from which duplicate one ml portions were infected with wild type T4 (MOI = 5). At various times after infection, one portion received ghosts (MOK = 7) plus the inhibitor (IMMEDIATE ASSAY; open symbols); at the same time the second portion received only the inhibitor followed by ghosts 7.5 minutes later (DELAYED ASSAY; closed symbols). Both portions received $^3$H-leucine (final concentration, $5 \times 10^{-5}$ M, 1 µC/ml) 3.5 minutes after the addition of ghosts. Two minutes later the cells were filtered (Millipore, 0.45 µ) and washed with room temperature M9 salts. The filters were dried and their radioactivity measured as previously described. The control (leucine uptake in the absence of ghosts = 100%) consisted of cells receiving the inhibitor 5 minutes after T4 infection and leucine uptake was measured 3.5 minutes later. The inhibitors were added to yield a final concentration of 75 µg/ml and 1.25 mg/ml for CAM and PM, respectively. The different symbols correspond to different experiments.
Fig 7. Development of Immunity in Wild Type T4 Infected E. coli B/5 as a Function of the Time of Addition of Chloramphenicol (CAM) or Puromycin (PM).
capable of killing, then the probability of a phage infected cell exhibiting the immune state depends upon the probability of the ghosts adsorbing to a site not neutralized by the \textit{imm} protein. According to this view, at any given time after phage infection, the probability of killing will increase with increasing MOI of superinfecting ghosts. This hypothesis predicts that the level of immunity at any time after infection should decrease as the MOI of the superinfecting ghosts increases. The results of an experiment, performed in collaboration with M. Vallée, which confirm this hypothesis are shown in Fig 8. If these same results are replotted as log percent immunity versus MOI of superinfecting ghosts (Fig 9), the straight line plots indicate that at any time after infection the probability of a ghost to kill is always described by single hit kinetics, that is one ghost can kill; but that the probability of killing decreases as time (and the number of neutralized sites) increases.

Fig 8 shows that even as late as 15 minutes after infection by wild type T4 phage, the maximum level of immunity decreases with increasing MOI of superinfecting ghosts. This could result from (i) lysis of the cells since ghosts cause LFW at multiplicities as low as 10 which is less than required for LFW by intact phage (cf Duckworth, 1970a); (ii) the very early synthesis of the \textit{imm} protein is shut off as infection proceeds (Salser et al., 1970) so that complete neutralization of all the sites is never achieved. In an attempt to distinguish between these alternatives, the immunity to high MOI of superinfecting ghosts was determined in the presence of 25 mM Mg$^{++}$
Fig 8. Development of Immunity as a Function of Time at Varying MOI's of Superinfecting Ghosts.

E. coli B/5 was grown in M9G medium (30° C) to a cell density of 4 x 10^8 cells/ml. Sets of 4 tubes, each containing 1 ml of bacteria, were infected with wild type T4 (MOI = 7) and at various times after infection ghosts were added at MOI's of 0, 5, 10 or 20. Each tube received 3H-leucine 3.5 minutes after the ghosts (final concentration 5 x 10^-5 M, 0.5 μC/ml) followed by one-half volume of ice cold 20% TCA 4 minutes later. The tubes were chilled in ice for at least 20 minutes before filtering (Millipore, 0.45 μ). The filters were rinsed twice with 5% TCA, dried, and their radioactivity measured as previously described. The experimental points represent average values of 2 to 5 separate experiments.
Fig 9. Development of Immunity as a Function of the MOI of Superinfecting Ghosts at Varying Times After Wild Type T4 Infection of E. coli B/5.

The experiment is the same as presented in Fig 8.
which prevents LFW (Barlow, 1954). In the same experiment a T4 mutant (SP62) was included which overproduces several early phage proteins when in combination with a DNA-negative mutation such as amB22(43) (J. Wiberg, personal communication).

The results in Table 7 show that the addition of Mg$^{++}$ (25 mM) increased the level of immunity in wild type infected cells to high MOI's of superinfecting ghosts (MOI = 20) added at 12 or 15 minutes after infection. This result indicates that the reduced immunity at high MOI's of ghosts is due to LFW by the ghosts. This result must be interpreted with caution since Mg$^{++}$ also reduces the killing effect of ghosts (Barlow, 1954). The levels of immunity developed under conditions of overproduction of early proteins [SP62-amB22(43)] are not much greater than the control infections [SP62 and amB22(43) alone]. However, the levels are greater than those developed in the wild type infections. These results imply that SP62 need not be coupled with a DNA-negative mutant [amB22(43)] to overproduce the imm protein. As it is known that DNA-negative mutants of T4 do overproduce early proteins (Hosoda and Levinthal, 1968; Cascino and Geiduschek, 1971), the fact that the immunity values are greater than wild type in all three cases [SP62, amB22(43) and SP62-amB22(43)] implies that immunity levels are increased through extended production of early phage proteins.

The conclusion from these experiments is that the reduced immunity in wild type T4 infected cells at a greater MOI of
Table 7. Immunity to High MOI of Superinfecting Ghosts in E. coli B Infected with SP62–amB22(43) and Wild Type + Mg++. 

E. coli B, grown in M9G medium (30° C) to a cell density of 4 x 10^8 cells/ml, was infected with phage (MOI = 5) and superinfected with ghosts (MOK = 20) at 12 or 15 minutes later. The measurement of immunity by ^3H-leucine incorporation is described in Materials and Methods. M9G medium contains 1 mM Mg++. 

<table>
<thead>
<tr>
<th>Infection</th>
<th>12 minutes % immunity</th>
<th>15 minutes % immunity</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>89.0</td>
<td>77.7</td>
</tr>
<tr>
<td>wild type + Mg++ (25 mM)</td>
<td>96.5</td>
<td>88.6</td>
</tr>
<tr>
<td>SP62</td>
<td>91.2</td>
<td>96.5</td>
</tr>
<tr>
<td>amB22(43)</td>
<td>91.6</td>
<td>92.4</td>
</tr>
<tr>
<td>SP62–amB22(43)</td>
<td>92.9</td>
<td>97.7</td>
</tr>
</tbody>
</table>
superinfecting ghosts is due to both LFW and the failure to neutralize all the sites through which superinfecting ghosts transmit their disruptive effects.

**Other Phage Functions Affecting Immunity**

In their initial report of the immunity gene of T4, Vallée and Cornett (1972) noted that if sufficient time was allowed after infection by T4 imm\(^{-}\) (imm2), approximately 60% levels of immunity could be reached. This background level is approximately 10% in the standard immunity assay where immunity development is allowed for 9-10 minutes before the ghost challenge, a time sufficient for wild type (imm\(^{+}\)) infected cells to develop >90% immunity. This observation offered three possible explanations; (i) the imm2 mutant makes a partially functional imm protein and as such imm2 infected cells were able to develop immunity but at a slower rate than WT (imm\(^{+}\)), (ii) immunity could result from the injection of phage DNA and attendant internal proteins, since the adsorption of infectious phage does not result in a ghost-like effect upon the cell; (iii) gene(s) other than the imm gene also govern immunity development.

**Does the imm2 Mutant Make a Partially Functional Protein**

If the imm2 mutation originated by an amino acid substitution in the wild type protein, this mutation would be of the missense class and could be leaky due to residual activity of the missense protein. An indirect indication of whether imm2 made a partially functional imm protein was to determine if another imm\(^{-}\) mutant also exhibited the same
phenotype. As mentioned previously, Childs (1973) had isolated an immunity negative mutant (imm1), which was allelic to imm2. As shown in Table 2, a significant amount of background immunity was developed in the independently isolated imm1 mutant (15.9%), compared to the level (5.4%) obtained with imm2 in these assays. This observation merely indicates that both the imm1 and imm2 phage mutants develop background levels of immunity but does not determine if the background immunity level results from partial gene function in both mutants. An attempt to determine if the imm2 mutation resulted from a nonsense mutation which would yield a protein fragment (and presumably be non-leaky) was not successful since the ghosts were not effective in inhibiting protein synthesis in the K12 (Su+) strains.

Can Immunity Develop as a Mechanical Effect of Primary Phage Infection

If infection is carried out in the presence of CAM where there is no phage genetic expression, the phage should behave, in principle, like the genetically inert, DNA-less, phage ghosts. However, even under this condition the effects of phage and phage ghosts on host cell metabolism are quite dissimilar (Duckworth, 1971b; Winkler and Duckworth, 1971). Duckworth (1971b) has proposed that injection of the "germinal substance" of the infecting phage (DNA plus any other injected substance) may be able to counteract the possibly disruptive effects of phage coat attachment and penetration. If this hypothesis were correct, then the injection of phage germinal substance may cause the observed background immunity. The experiments presented in the
following sections show that divalent cations found within the phage head do provide protection against ghosts in phage infected and uninfected cells, but do not account for the background level of immunity.

A Reduction in Killing by Ghosts Due to Putrescine. There are several cationic species within the phage head which purportedly serve to neutralize the negatively charged phage DNA molecule (Ames and Dubin, 1960). Approximately 30% of the cationic charge is due to the divalent cation, 1-4 diaminobutane or putrescine (Ames and Dubin, 1960). If the introduction of putrescine during injection of phage DNA reversed the disruptive effect of phage attachment and penetration, then the exogenous addition of putrescine may raise the background level of immunity. The results of an experiment to test this hypothesis are presented in Table 8 where various concentrations of putrescine were added to cells prior to infection with T4 imm2 and subsequent challenge with ghosts to determine the levels of immunity. The results show that all concentrations of putrescine increased the level of immunity in the imm2 infected cells, with an optimal effect at 50 mM. Similar protection against ghosts by putrescine in uninfected cells is seen in Table 9. Since higher concentrations of putrescine (approximately 100 mM) are known to inhibit phage multiplication (Shalitin, 1967) the remaining experiments were conducted with putrescine at 20 mM.

At first glance it might seem that putrescine could be inactivating the lethal effects of the ghosts. This does not appear to be true as seen from the results of Table 10, which show that ghosts
Table 8. Increased Immunity to Superinfecting Ghosts in \textit{imm2} Infected \textit{E. coli} B/5 Due to Exogenous Putrescine.

\textit{E. coli} B/5 was grown to a cell density of $4 \times 10^8$ cells/ml in M9G medium at 29° C at which time 0.80 ml portions were transferred to duplicate sets of tubes containing 100 µl of T4D \textit{imm2} (MOI = 1.4) and 100 µl of putrescine (pH 7.0) to give the final concentration indicated. One tube received 20 λ of ghosts (MOK = 7) 9 minutes after phage addition and both tubes received $^3$H-leucine (final concentration $5 \times 10^{-5}$ M, 0.5 μC/ml) five minutes later. Incorporation of $^3$H-leucine and the calculation of the percent immunity is described in Materials and Methods.

<table>
<thead>
<tr>
<th>Infection</th>
<th>Putrescine (mM)</th>
<th>TCA-insoluble $^3$H-leu cpm$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>0</td>
<td>10,273</td>
</tr>
<tr>
<td>none</td>
<td>20</td>
<td>10,490</td>
</tr>
<tr>
<td>\textit{imm}</td>
<td>0</td>
<td>5,905</td>
</tr>
<tr>
<td>\textit{imm}</td>
<td>5</td>
<td>5,936</td>
</tr>
<tr>
<td>\textit{imm}</td>
<td>10</td>
<td>7,695</td>
</tr>
<tr>
<td>\textit{imm}</td>
<td>20</td>
<td>8,841</td>
</tr>
<tr>
<td>\textit{imm}</td>
<td>50</td>
<td>8,873</td>
</tr>
<tr>
<td>\textit{imm}</td>
<td>100</td>
<td>8,190</td>
</tr>
<tr>
<td>\textit{imm}</td>
<td>200</td>
<td>7,318</td>
</tr>
</tbody>
</table>

\[
^a \text{TCA} = \text{trichloroacetic acid}
\]
\[
^3\text{H-leu} = \text{$^3$H-leucine}
\]
\[
cpm = \text{counts per minute}
\]
Table 9. Increased Survivors to Ghost Infection Due to Putrescine.

E. coli B/5 was grown in M9G medium (30°C) to a cell density of \(4 \times 10^8\) cells/ml at which time 0.9 ml portions were added to tubes containing various concentrations of putrescine (pH 7.0) in 100 µl. Ghosts (MOK = 7) were added after 4 minutes and allowed 4 minutes for adsorption before samples were diluted in ice cold M9G medium and plated for survivors. The ghost preparation was titered on cells grown in broth which are more sensitive to killing by ghosts than cells grown in M9G medium. The apparent MOK was calculated from the number of survivors employing the zero term of the Poisson equation as described in the Materials and Methods.

<table>
<thead>
<tr>
<th>Infection</th>
<th>Putrescine (mM)</th>
<th>Survivors/ml</th>
<th>apparent MOK</th>
</tr>
</thead>
<tbody>
<tr>
<td>uninfected</td>
<td>0</td>
<td>(3.6 \times 10^8)</td>
<td>-</td>
</tr>
<tr>
<td>uninfected</td>
<td>200</td>
<td>(3.5 \times 10^8)</td>
<td>-</td>
</tr>
<tr>
<td>ghosts</td>
<td>0</td>
<td>(2.7 \times 10^5)</td>
<td>3.1</td>
</tr>
<tr>
<td>ghosts</td>
<td>10</td>
<td>(2.5 \times 10^5)</td>
<td>3.1</td>
</tr>
<tr>
<td>ghosts</td>
<td>20</td>
<td>(7.5 \times 10^5)</td>
<td>2.7</td>
</tr>
<tr>
<td>ghosts</td>
<td>50</td>
<td>(13 \times 10^5)</td>
<td>2.4</td>
</tr>
<tr>
<td>ghosts</td>
<td>100</td>
<td>(20 \times 10^5)</td>
<td>2.2</td>
</tr>
<tr>
<td>ghosts</td>
<td>200</td>
<td>(52 \times 10^5)</td>
<td>1.8</td>
</tr>
</tbody>
</table>
incubated in 20 mM putrescine are not inactivated, or at least not in an irreversible manner. It could yet be argued that putrescine interferes with the attachment and thus the lethal action of ghosts. Since intact T4 and T4 ghosts utilize the same mode of attachment, the effect of putrescine on infection of intact T4 was determined. Table II shows that putrescine at a concentration as high as 50 mM does not interfere with the adsorption of intact phage nor does it retard the infective process as measured by infective center formation or burst size.

If the putrescine content of phage were able to retard the lethal action of ghosts, then phage deprived of this polyamine might be unable to counteract the disruptive effect of their own protein coats and thus act as ghosts or "inactivated" phage. Such phage would retain the ability to kill their host but lose the capacity to form infective centers. An experiment to test this hypothesis utilized the permeable head mutant T4B01 from which putrescine may be removed by dialysis against 10 mM Mg++; buffer (Ames and Dubin, 1960). Phage T4B01 was dialyzed against Tris buffer (pH 7.2) or Tris buffer containing putrescine (10 mM) or Mg++; (10 mM) and each dialyzed preparation was tested for its ability to kill E. coli and to form infective centers. These assays showed no significant difference between the variously treated phage. The most plausible explanation of these results is that putrescine is removed from the phage head by virtue of exchange with Mg++; since the total cation content of the phage must be conserved to neutralize the polyanionic DNA molecule. The effects of putrescine in increasing immunity are most probably analogous to the effect
Table 10. Effect of Putrescine Incubation With T4 Ghosts Used to Superinfect imm2 Infected E. coli B/5.

E. coli B/5 was grown in M9G medium (30° C) to a cell density of $4 \times 10^8$ cells/ml at which time 0.90 ml portions was transferred to duplicate sets of tubes. Each set of tubes received putrescine as indicated in the Table. Superinfecting ghosts were added 9 minutes after addition of phage to one tube and both tubes received $^3$H-leucine (final concentration $5 \times 10^{-5}$ M, 0.5 µC/ml) 5 minutes later. Cold TCA was added to both tubes after 5 minutes (final concentration 5%). The percent immunity was calculated as described in the Materials and Methods.

<table>
<thead>
<tr>
<th>Infection</th>
<th>Conditions</th>
<th>$^3$H-leu cpm\textsuperscript{a}</th>
<th>percent immunity</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td></td>
<td>12,526</td>
<td>89</td>
</tr>
<tr>
<td>imm2</td>
<td></td>
<td>5,346</td>
<td>608</td>
</tr>
<tr>
<td>imm2</td>
<td>ghosts incubated in 20 mM putrescine; final concentration = 0.04 mM</td>
<td>6,074</td>
<td>816</td>
</tr>
<tr>
<td>imm2</td>
<td>putrescine added simultaneously with ghosts to 0.04 mM</td>
<td>6,069</td>
<td>762</td>
</tr>
<tr>
<td>imm2</td>
<td>putrescine added with phage to 20 mM</td>
<td>6,691</td>
<td>5,108</td>
</tr>
<tr>
<td>imm2</td>
<td>putrescine added with ghosts to 20 mM</td>
<td>6,262</td>
<td>4,585</td>
</tr>
</tbody>
</table>

\textsuperscript{a} counts per minute of trichloroacetic acid insoluble $^3$H-material
Table 11. Effect of Putrescine (50 mM) on Adsorption and Growth of Wild Type T4D.

E. coli B/5 was grown in M9G medium (30°C) to a cell density of 4 x 10^8 cells/ml and infected with wild type T4D (MOI = 6) in the absence or presence of putrescine (50 mM). After 10 minutes: one portion of the infected cells was plated to determine the number of infective centers; a second portion was treated with CHCl₃ and titered to determine the percentage of unadsorbed phage; a third portion was diluted 100-fold into M9G medium + putrescine (50 mM) for further multiplication of the phage for 2 hours before adding CHCl₃ and titering for number of phage progeny produced.

<table>
<thead>
<tr>
<th>Adsorption Without Putrescine</th>
<th>Adsorption With Putrescine</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Adsorption</td>
<td>99.0</td>
</tr>
<tr>
<td>Infective Centers/ml</td>
<td>9.3 x 10⁷</td>
</tr>
<tr>
<td>Burst Size^a</td>
<td>68.0</td>
</tr>
</tbody>
</table>

^a The burst size was determined as the total number of progeny per ml divided by the infective centers per ml. The value for infective centers was corrected for unadsorbed phage.
produced by the general content of divalent cations in the phage head and not specifically those of putrescine. Indeed, Barlow (1954) had previously shown that Mg$^{++}$ could reduce killing and completely prevent LFW by T2 ghosts while Buller and Astrachan (1968) showed a reduction in LFW by ghosts due to spermidine$^{+++}$. However, Barlow's results did indicate that killing and LFW were separate effects of ghost infection. If putrescine (or possibly Mg$^{++}$) could truly prevent the lethal effects of ghosts on bacterial membranes irrespective of LFW, then it should be possible to show this same effect on other bactericidal agents which act extracellularly like ghosts, but do not cause lysis of the target cells. Such an agent is the bacterial colicin K (Nomura, 1967) whose lethal properties are similar to those of ghosts (Winkler and Duckworth, 1971). The experiment presented in Table 12 shows that the addition of 20 mM putrescine to bacterial cells increases the number of survivors or lowers the apparent MOK of colicin K treatment. The effect of putrescine on the adsorption or possible inactivation of colicin K was not determined. Thus putrescine (or possibly Mg$^{++}$) is able to reduce the lethal effect of both ghosts and colicin K; the latter indicating that putrescine does not protect the cells simply by preventing LFW in agreement with the results obtained by Barlow (1954) with Mg$^{++}$. The removal (or exchange) of putrescine in the phage head did not reduce the lethal action of intact phage coats attaching to the cell, whereas exogenous addition of putrescine did protect the cells from ghosts or colicin K.
Table 12. Increased Survivors to Colicin K Due to Putrescine (50 mM).

E. coli B/5 was grown in M9G medium (30° C) to 4 x 10^8 cells/ml at which time 0.90 ml portions were added to tubes containing in equal volumes: colicin K + putrescine (50 mM) or T4D ghosts + putrescine (50 mM). After 10 minutes for adsorption, portions were diluted into ice cold M9 salts and plated for survivors. The apparent multiplicity of killing (MOK) was calculated from the number of survivors employing the zero term of the Poisson equation as described in the Materials and Methods.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Putrescine</th>
<th>Survivors/ml</th>
<th>Apparent MOK</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>-</td>
<td>4.1 x 10^8</td>
<td>-</td>
</tr>
<tr>
<td>none</td>
<td>+</td>
<td>3.9 x 10^8</td>
<td>-</td>
</tr>
<tr>
<td>colicin K</td>
<td>-</td>
<td>3.9 x 10^6</td>
<td>4.6</td>
</tr>
<tr>
<td>colicin K</td>
<td>+</td>
<td>8.5 x 10^7</td>
<td>1.5</td>
</tr>
<tr>
<td>ghosts</td>
<td>-</td>
<td>1.1 x 10^5</td>
<td>8.2</td>
</tr>
<tr>
<td>ghosts</td>
<td>+</td>
<td>8.0 x 10^6</td>
<td>3.9</td>
</tr>
</tbody>
</table>
The possibility still remained that increasing the amount of germinal substance injected into the cell might increase the background levels of immunity to superinfecting ghosts. The rationale of the following experiment was to infect cells in the presence of RIF or CAM to prevent phage gene expression and determine if immunity to superinfecting ghosts increased with increasing MOI of phage (and internal protein or associated divalent cations). The results shown in Table 13 indicate that increased MOI did not increase immunity (background immunity) to superinfecting ghosts by measuring $^3$H-leucine uptake. Table 13 also shows that in the absence of phage gene expression (+ CAM), increasing the MOI of phage decreases the ability of the cells to take up the $^3$H-leucine. This effect will be referred to later during the discussion of superinfection of imm2 infected cells by high MOI's of phage. The measurement of immunity under these conditions is technically impossible by these means, since the greater MOI decreases the uptake ability with or without superinfecting ghosts. These results measuring uptake of $^3$H-leucine are in agreement with other studies showing that, in the absence of phage gene expression, the disruptive effect of the infectious phage attachment is proportional to the MOI employed (Nomura et al., 1966; Duckworth, 1971b).

It can be concluded from the results reported here that putrescine can reduce the lethal effect of two extracellularly acting bactericidal agents, T4 ghosts and colicin K; and that this protective effect does not appear to be specific for LFW, but of a more general nature in preventing membrane disruption. In effecting this protection,
Table 13. Immunity to Superinfecting Ghosts in the Absence of Phage Expression.

E. coli B/5 was grown to $4 \times 10^8$ cells/ml in M9G medium (30° C) and pretreated with CAM (100 μg/ml) prior to infection with wild type T4 at the MOI indicated. Superinfecting ghosts (MOK = 7) were added 10 minutes after infection of the CAM pretreated cells, and 3 minutes after infection of the RIF pretreated cells. $^3$H-leucine (final concentration $1 \times 10^{-4}$ M, 1 μC/ml) was added 3-4 minutes after the ghosts and uptake for 2.0 minutes was measured as described in Table 3.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Phage MOI</th>
<th>- ghosts</th>
<th>+ ghosts</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAMb</td>
<td>0</td>
<td>60,841</td>
<td>472</td>
</tr>
<tr>
<td>CAM</td>
<td>5</td>
<td>1,220</td>
<td>472</td>
</tr>
<tr>
<td>CAM</td>
<td>20</td>
<td>313</td>
<td>225</td>
</tr>
<tr>
<td>CAM</td>
<td>40</td>
<td>202</td>
<td>182</td>
</tr>
<tr>
<td>RIFc</td>
<td>0</td>
<td>-</td>
<td>150</td>
</tr>
<tr>
<td>RIF</td>
<td>5</td>
<td>-</td>
<td>115</td>
</tr>
<tr>
<td>RIF</td>
<td>10</td>
<td>-</td>
<td>105</td>
</tr>
<tr>
<td>RIF</td>
<td>15</td>
<td>-</td>
<td>102</td>
</tr>
</tbody>
</table>

a Values are presented as counts per minute
b CAM = chloramphenicol
c RIF = rifampicin
putrescine most probably acts in the role of a divalent cation. The removal (or exchange) of putrescine in the phage head did not reduce the lethal action of infectious phage coats attaching to the cell, nor did increasing the MOI of the phage (and the amount of putrescine injected) in the absence of phage gene expression increase immunity. These results, as well as those appearing in the literature (see Winkler and Duckworth, 1971); do not provide information on whether the phage germinal substance is responsible for background levels of immunity.

Since it was not possible to determine if the background level of immunity was due to residual function of the imm1 or imm2 mutant gene products, the third possibility, that gene(s) other than imm control immunity to superinfecting ghosts, was explored.

Other Phage Genes Affecting Immunity

Most early mutants, including all of those which had no assigned function, had been screened for the immunity defect without success (Vallée, Cornett and Bernstein, 1972). In addition the isolation procedure of Childs, specific for exclusion negative mutants, had shown only one region to contain the exclusion function and had yielded an exclusion negative mutant allelic to imm2. Thus it seemed that if other gene functions were involved in the development of immunity, they were probably undetectable in the presence of the imm+ gene. It seemed possible that such gene functions contributing to immunity development could be detected, however, in the absence of imm gene functions. Thus,
mutants of two early T4 genes, \text{rII} and spackle (s), which appear to affect the host membrane, were assayed for immunity development singly and when coupled with the \text{imm2} mutation.

\textbf{Immunity and the rII Gene.} Phage carrying mutations in the \text{rII} gene of T4 were among the earliest phage mutants used in genetic studies due to their clearly distinguishable plaque morphology. The \text{rII}^-\text{mutants also provided a conditionally lethal phage system since a mutational lesion in either of the \text{rII} cistrons results in abortive infection of host strains harboring a lambda prophage. Numerous studies on the physiological defects occurring in abortive \text{rII}^- infections have implicated the \text{rII} gene as defective in a membrane function (Buller and Astrachan, 1968; Furrow and Pizer, 1968), notably its response to the divalent cations and monocations in the infection medium (Garen, 1961; Sekiguchi, 1966) and the reports detecting the \text{rII}B protein in the membrane of phage infected cells (Peterson, Kievitt and Ennis, 1972; Weintraub and Frankel, 1972). Since the \text{rII} region is transcribed early in infection (Schmidt et al., 1970) it seemed possible that it might influence immunity to superinfecting ghosts when coupled with the \text{imm2} mutation, even though \text{rII}^-\text{mutants alone have no effect on immunity development (Vallée, Cornett and Bernstein, 1972).}

The \text{imm2} and \text{rII}^-\text{mutations were combined by crossing (see Materials and Methods) the \text{rII}^-\text{mutant with the double mutant amNG205(42) -imm2 (see Fig 10) from which progeny were selected to contain the \text{rII}^- and am(42) markers. It is expected that most of these recombinants will also carry the unselected \text{imm2} mutation (an exception is seen with}
Fig 10. Diagram of the Cross to Construct the rII⁻-imm⁻ Double Mutant.

A logarithmically growing culture of E. coli BB (Su⁺UAG) was concentrated by centrifugation to 4 x 10⁸ cells/ml and simultaneously infected in the presence of 2 mM KCN with rA105 and amNG205(42)-imm2 in H broth at 30°C. After 10 minutes for adsorption, the phage infected cells were diluted 4 x 10⁶ into pre-warmed H broth and aerated for 90 minutes before termination of growth with CHCl₃. The progeny phage were first plated on strain BB (Su⁺UAG) from which individual plaques were stabbed with sterile toothpicks and transferred to plates seeded with strains S/6 (Su⁻), CR63(λ) (Su⁺UAG) and CR63 (Su⁺UAG). Those phage which grew only on the CR63 plate were picked from the plate and replated for single plaques from which a stock was prepared for use in the immunity assays. The same protocol was followed for constructing the double mutant rED19-imm2 except that the imm2 mutation was coupled with amE4314(43) (see Fig 2). The dashed line (----) indicates the rII⁻-am⁻-imm⁻ recombinants.
am(42)-imm2-rA105 isolate 1). These rII-imm phage were assayed for their immunity character in a (λ−) host, permissive for rII mutants, as shown in Table 14. These results show that the presence of the rII− mutation does not significantly change the background level of immunity seen with the imm2 mutation alone. Also, the fact that the immunity development in the complete absence of the rII products (the rA105 mutation deletes both the A and B cistrons) appears no different than the missense mutant (rED19), implies that the rII proteins are not interacting with the imm protein.

Phage Induced Immunity and Spackle (s) Gene. The spackle (s) mutant of T4 originated spontaneously from a lysozyme deficient strain of T4 and was detected by its ability to lyse the infected cell even in the absence of the phage lysozyme (Emrich, 1968). This phenotype implicated the s− phage as defective in a function which normally strengthens or maintains the integrity of the cell envelope after infection. This view appeared justified from the unique inability of s− infected cells to develop resistance to LFW (Emrich, 1968). Since the s gene involves changes in the host cell envelope and has been identified as an immediate early function of T4 (Peterson, Cohen and Ennis, 1972). Therefore the s− mutant was tested for its ability to confer immunity to superinfecting ghosts. Table 15 shows that the spackle mutant does not develop full immunity (ca. 60%) to superinfecting ghosts under standard conditions where wild type develops approximately 90% and imm− approximately 10% levels of immunity. The partial defect
Table 14. Immunity to Superinfecting Ghosts in imm2-rII Infected E. coli B/5.

Immunity to superinfecting ghosts was determined as described in Materials and Methods using E. coli B/5 for rED19 phage and E. coli B for rA105 phage. Assays were carried out at 30°C in M9G medium (plus 100 µg/ml tryptophan for all but the rED19 assays) allowing 10 minutes for immunity development and 5 minutes for ghost action. Spot tests to determine the markers amNG205(42), rED19 (rII frameshift mutant) and rA105 (rII A and B deletion mutant) (see Materials and Methods) are scored: (-) not determined, (0) no growth, (+) growth of the phage.

<table>
<thead>
<tr>
<th>Phage</th>
<th>Spot Tests</th>
<th>TCA-insol. $^3$H-leu (cpm)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Su$^+\lambda$</td>
<td>Su$^-\lambda$</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>wild type</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>imm2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>am(42)-imm2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>rA105</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>am(42)-imm2-rED19</td>
<td>isolate 1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>isolate 2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>isolate 3</td>
<td>-</td>
</tr>
<tr>
<td>am(42)-imm2-rA105</td>
<td>isolate 1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>isolate 2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>isolate 3</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ TCA = Trichloroacetic acid, $^3$H-leu = $^3$H-leucine, cpm = counts per minute
Table 15. Immunity to Superinfecting Ghosts in $s^-$ and imm2 Infected E. coli B/5.

E. coli B/5, grown in M9G medium (30° C) to 4 x 10^8 cells/ml, was infected with the indicated T4 strains (MOI = 5) and superinfected with ghosts (MOK = 7) 10 minutes later. Triple mutants $s^-$-am(42)-imm2 were constructed by the crosses (see Materials and Methods) diagrammed in Fig 8A; backcrosses to segregate the imm2 and $s^-$ mutations are diagrammed in Fig 8B. The genotypes of the backcross segregants were determined from their levels of immunity development relative to the single imm2 and $s^-$ mutants. The values of immunity for imm2 and $s^-$ shown here are the averages of seven determinations.

<table>
<thead>
<tr>
<th>Phage</th>
<th>Percent Immunity</th>
<th>Genotype</th>
<th>Frequency</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>0.18</td>
<td>- -</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>wild type</td>
<td>89.4</td>
<td>+ +</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>imm2</td>
<td>8.3</td>
<td>- +</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$s^-$</td>
<td>60.2</td>
<td>+ -</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$s^-$-am(42)-imm2 iso 1</td>
<td>1.8</td>
<td>- -</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td>am+ revertant</td>
<td>1.0</td>
<td>- -</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td>backcross</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>recombinants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$s^-$</td>
<td>70.1</td>
<td>+ -</td>
<td>1/3</td>
<td></td>
</tr>
<tr>
<td>$ts(40)$-am(42)-imm2</td>
<td>9.1</td>
<td>- +</td>
<td>1/3</td>
<td></td>
</tr>
<tr>
<td>$s^-$-am(42)-imm2 iso 2</td>
<td>3.4</td>
<td>- -</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td>am+ revertant</td>
<td>1.4</td>
<td>- -</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td>backcross</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>recombinants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$s^-$</td>
<td>62.8</td>
<td>+ -</td>
<td>2/3</td>
<td></td>
</tr>
<tr>
<td>$ts(40)$-am(42)-imm2</td>
<td>11.5</td>
<td>- +</td>
<td>1/3</td>
<td></td>
</tr>
</tbody>
</table>

a Immunity to superinfecting ghosts was determined as described in Materials and Methods.

b Frequency is equal to the number of segregants exhibiting the indicated genotype divided by the total number of segregants isolated.
in immunity to superinfecting ghosts seen in \( s^- \) infections indicated that the \( s \) gene may be responsible for the background immunity in \( \text{imm}2 \) infections when the ghost challenge is quite late in infection (Vallée and Cornett, 1972). Accordingly, the double mutant \( s^-\text{-imm}^- \) was constructed to determine the immunity levels to superinfecting ghosts developed in cells infected with phage lacking both gene functions.

Construction of an \( s^-\text{-imm}^- \) phage, containing two non-essential genes and thus no selective plating condition, was carried out as follows. The \( s^- \) parent, detectable as forming "rII-like" plaques (sharp borders) on \( E. \text{coli} \) B at 37° C (Emrich, 1968), was crossed to an \( \text{imm}^- \) mutant carrying an amber (am) lesion near the \( \text{imm}2 \) mutation (see Fig 11A). The progeny of this cross were replica plated on \( S/6 \) (\( \text{Su}^- \)) and \( BB \) (\( \text{Su}^+\text{UAG} \)) and plaques showing clear borders on \( BB \) (\( s^- \) phenotype) and which failed to grow on \( S/6 \) (\( am^- \) phenotype) were selected as putative \( s^-\text{-am}^-\text{-imm}^- \) phage. From seven such isolates, two were retained for further testing (iso 1 and iso 2). That the \( s^- \) mutation was present in these putative \( s^-\text{-am}^-\text{-imm}^- \) phage was suggested by their inability to become lysis inhibited by homologous superinfection (Fig 12). The presence of the \( \text{imm}2 \) marker was suggested by the lack of immunity to superinfecting ghosts (Table 15). To insure that these \( s^-\text{-am}^-\text{-imm}^- \) phage did contain the \( s^- \) and \( \text{imm}^- \) alleles, each isolate was also back-crossed (see Fig 11B) to recover the respective single mutants which were identified by their characteristic levels of immunity development. The results of these assays (Fig 12 and Table 15) demonstrate that both isolates did contain the \( s^- \) and \( \text{imm}^- \) alleles. The levels of immunity
Fig 11. Diagram of the Hypothetical Crosses Occurring During Construction and Confirmation of the Genotype of the *s*-am(42)-imm2 Double Mutants.

Mixed infections of *E. coli* BB (*Su*+UAG) were carried out as described in the legend to Fig 8. The position of the spackle gene was taken from Emrich (1968).

(A) The *s*-am(42)-imm2 recombinants (dashed line, ----) were selected as large sharp edged plaques when grown on strain BB (*Su*+UAG) at 40°C [*s* phenotype] which failed to grow on strain S/6 (*Su*-) [am(42) phenotype].

(B) The *s* recombinants (solid line, ---) were selected as large sharp edged plaques on strain S/6 (*Su*). The *ts*(40)-am(42)-imm2 recombinants (dashed line, ----) were selected as plaques which grew on strain BB (*Su*+UAG) at 25°C but which failed to grow at 42°C, or on strain S/6 at either temperature. All recombinant phage stocks were grown from single plaque isolates.
Fig 12. Lysis Inhibition by Homologous Superinfection in 
T4 Infected E. coli BB.

A logarithmically growing culture of E. coli BB (Su^UAG) in H broth 
(37° C) was concentrated to 2 x 10^9 cells/ml by centrifugation and 
infected with the indicated T4 strains (MOI = 5). Five minutes after 
the primary infection, the phage-bacteria mixtures were diluted with 
4 volumes of pre-warmed (37° C) H broth and superinfected with the 
same phage type 5 minutes later (MOI = 5). Lysis of the superinfected 
cultures was determined by a decrease in turbidity measured by a 
Klett-Summerson colorimetric photometer (520-580 nm). The exper­
imental cultures were maintained in a rotary shaker bath at 37° C 
in 125 ml Erlenmeyer flasks fitted with side arm tubes. The tur­
bidity was measured by tilting the flask to transfer the culture 
into the side arm tube which was inserted into the colorimeter.
developed by the s^-am^-imm^- phage were much lower than that of the singly mutated imm^- (s^+) phage (Table 15) which indicated that the background level of immunity seen in imm2 infected cells is due to the presence of the s gene product. This hypothesis was confirmed by the experiment illustrated in Fig 13 where immunity development was measured with time after infection by imm^- and s^-imm^- mutants. The s^-imm^- double mutant used here and in the subsequent experiments was selected as a spontaneous am^+ revertant of an s^-am^-imm^- isolate.

Comparison of the imm and s Genes

Genetic Exclusion of Superinfecting Phage

The finding that s^- phage are partially deficient in developing immunity to superinfecting ghosts, a function primarily governed by the imm gene, prompted the following experiment to determine what role the spackle gene might play in a second imm gene function; the genetic exclusion of superinfecting phage. The results presented in Table 16 show that s^- phage are able to exclude only about 40% of the superinfecting phage under conditions where wild type excludes 90% and imm^- phage fail to exclude at all. As in the case of development of immunity to superinfecting ghosts, the s gene appears to play a lesser role in developing a function controlled mainly by the imm gene.

Insofar as the s^- phenotype parallels at a reduced level that of imm^-, the imm2 phage was then tested for those properties characteristic of the spackle mutant; (i) the failure to exhibit lysis
Fig 13. The Development of Immunity to Superinfecting Ghosts in T4 Infected *E. coli* B/5.

*E. coli* B/5 was grown in M9G medium (37°C) to a cell density of $4 \times 10^8$ cells/ml. Duplicate portions of cells were infected with the indicated T4 strains (MOI = 5). One portion was superinfected with ghosts (MOK = 7) at the times indicated to determine the level of immunity present, relative to the control portion not receiving ghosts. The measurement of immunity by incorporation of $^3$H-leucine is described in the Materials and Methods.
Table 16. Exclusion of Superinfecting Wild Type T4 Phage by imm2 and s~ Infected E. coli B/5.

*E. coli B/5 (Su~)*, grown in H broth (30° C) to a cell density of about 4 x 10^8 cells/ml, was concentrated by centrifugation (10,000 x g, 5 minutes, 25° C) to a density of 2 x 10^9 cells/ml. To 1.0 ml portions of bacteria were added: 100 λ M9 salts followed in 15 seconds by 50 λ of the primary phage (MOI = 5), and superinfected with 50 λ of wild type phage (MOI = 5) 8 minutes later or; 100 λ of CAM (final concentration 100 μg/ml) followed in 15 seconds by 100 λ of a mixture of primary phage and wild type, (MOI of each = 5). Measurement of infective centers was taken 9.5 minutes after simultaneous infection, or 5 minutes after superinfection, by diluting the phage-bacteria mixture 20-fold into H broth containing T4 antiserum (K = 4) and incubated for 2 minutes (30° C) before diluting 100-fold into ice cold broth and plating with *E. coli S/6 (Su~)* as indicator. Adsorption of the primary phage was >98% in all experiments as determined by removing a sample just before superinfection and shaking with CHCl3 before plating on *E. coli BB (Su~)*. The percent exclusion is equal to: 100 - (N x 100/ N₀) where N₀ is the number of infective centers formed in the simultaneous infection (+CAM) and N is the number of infective centers formed after superinfection.

<table>
<thead>
<tr>
<th>Primary Phage <em>a</em></th>
<th>Exp</th>
<th>Superinfected Cell Cultures</th>
<th>Simultaneous Infection of Cell Cultures</th>
<th>% Exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>am(42)</td>
<td>1</td>
<td>3.0 x 10^7</td>
<td>2.9 x 10^8</td>
<td>90</td>
</tr>
<tr>
<td>am(42)</td>
<td>2</td>
<td>2.6 x 10^7</td>
<td>4.9 x 10^8</td>
<td>95</td>
</tr>
<tr>
<td>am(42)</td>
<td>3</td>
<td>3.7 x 10^7</td>
<td>3.4 x 10^8</td>
<td>90</td>
</tr>
<tr>
<td>am(42)-imm2</td>
<td>1</td>
<td>3.6 x 10^8</td>
<td>1.3 x 10^8</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>am(42)-imm2</td>
<td>2</td>
<td>4.1 x 10^8</td>
<td>3.4 x 10^8</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>am(42)-imm2</td>
<td>3</td>
<td>3.7 x 10^8</td>
<td>3.0 x 10^8</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>am(42)-s~</td>
<td>1</td>
<td>2.0 x 10^8</td>
<td>3.3 x 10^8</td>
<td>39</td>
</tr>
<tr>
<td>am(42)-s~</td>
<td>2</td>
<td>2.2 x 10^8</td>
<td>4.8 x 10^8</td>
<td>53</td>
</tr>
<tr>
<td>am(42)-s~</td>
<td>3</td>
<td>2.5 x 10^8</td>
<td>3.7 x 10^8</td>
<td>33</td>
</tr>
</tbody>
</table>

* The am(42) mutation is amNG205(42).
inhibition and (ii) the failure to protect the infected cell from lysis from without (LFW) resulting from high MOI's of superinfecting phage.

**Lysis Inhibition**

Cells infected with s^ phage phenotypically resemble rII^- infections in that these cells are not lysis inhibited by superinfecting homologous phage (Emrich, 1968). The rII^- and s^- mutant types are distinguishable, however, by the inability of rII^- mutants to produce progeny in host cells carrying the prophage lambda, which does not restrict growth of s^- phage or imm^- phage. As shown in Fig 12, the imm^- mutant in contrast to the s^- strain, is lysis inhibited by superinfection. This property provides the one clear distinction between these two mutant types.

**Resistance to Lysis From Without**

The second phenotype of the s^- mutant which is unique to this strain is its inability to develop resistance to lysis from without (LFW), (Emrich, 1968). The ability of cells infected with s^- or imm^- mutants to resist LFW by superinfecting wild type phage was measured by the turbidity of the superinfected cultures. The results (Fig 14) show that with respect to LFW, the imm^- infected cells are intermediate between wild type and s^- . The turbidity measurements used here merely reflect the integrity of the superinfected cells, but do not measure their viability (ability to form an infective center). In a separate, but similar, experiment the viability of the infected cells was measured before and after superinfection by plating for infective centers.
Fig 14. Lysis From Without (LFW) by Superinfecting Wild Type T4 in T4 Infected E. coli B.

E. coli B, grown in H broth (37° C) to a cell density of 4 x 10⁸ cells per ml, was primarily infected with the indicated phage strains (MOI = 5) and superinfected with wild type T4 (MOI = 120) 7 minutes later. Lysis of the superinfected cultures was followed by turbidity measurements as described in the legend to Fig 12. The percent lysis, determined as the percent turbidity at 100 minutes relative to the turbidity at 4.5 minutes equal to 100%, was 90%, 42%, and 21% for the cultures primarily infected with wild type, imm2 and s⁻ respectively.
after treatment with T4 antiserum to inactivate the unadsorbed phage. The viability of the superinfected cells in general reflected the turbidity measurements for wild type and s^- infections but was greatly reduced for the imm^- infections (cf Fig 14 and Table 17). The turbidity measurements seen with imm^- superinfected cells indicate that superinfecting phage did not cause extensive LFW in these cells but appeared to "traumatize," in some manner, the immunity-negative cells, thereby reducing their ability to form infective centers. The measurements of both turbidity and infective center formation after superinfection were combined in the following experiments performed in M9G medium (30° C) rather than in broth (37° C) which was used for the first experiments. These conditions are analogous to the standard immunity assay for superinfecting ghosts in measuring the amount of protein synthesis by ^3H-leucine incorporation after superinfection. This same measurement is now used as an index of the degree of traumatization of the phage infected cells after superinfection by phage. The results of these experiments, using varying MOI of superinfecting phage, are presented in Table 18. The same relationship between turbidity and viability is seen here as before; the measurements of turbidity (cell lysis), infective center formation, and protein synthesis are very similar for wild type and s^- infections, while in imm^- infections the superinfecting phage severely damage the cells (low levels of infective center and protein synthesis) but do not cause a concomitant decrease in turbidity (LFW).
Table 17. Infective Center Formation in Wild Type, \textit{imm}^{-} and \textit{s}^{-} Infected Cells After Superinfection by Phage.

\textit{E. coli} B was grown in H broth (37° C) to a cell density of $4 \times 10^8$ cells/ml and infected with primary phage as indicated at MOI = 5. Seven minutes after infection, one portion was diluted 2000-fold into H broth, containing T4 antiserum (K = 4) and incubated for 4 minutes before subsequent diluting and plating with \textit{E. coli} S/6. A second portion was removed at the same time for superinfection with wild type (MOI = 170). The superinfecting phage were adsorbed for 3 minutes before dilution into antiserum and plating as above.

<table>
<thead>
<tr>
<th></th>
<th>Infective Centers/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary Phage</td>
</tr>
<tr>
<td></td>
<td>A Before Superinfection</td>
</tr>
<tr>
<td>Wild Type</td>
<td>$2.6 \times 10^8$</td>
</tr>
<tr>
<td>\textit{imm}^{-}</td>
<td>$2.8 \times 10^8$</td>
</tr>
<tr>
<td>\textit{s}^{-}</td>
<td>$2.6 \times 10^8$</td>
</tr>
</tbody>
</table>
Table 18. Lysis From Without, Infective Center Formation, and Protein Synthesis in Phage Infected E. coli B After Superinfection With Wild Type Phage.

E. coli B, grown in M9G medium (30° C) to a cell density of $4 \times 10^8$ cells/ml, was transferred to a Klett flask and infected with primary phage (MOI = 4-6). Superinfecting wild type T4 was added to the flask 10 minutes later at the multiplicities indicated. At 6 minutes after the primary infection, and 3 minutes after superinfection, portions from the flask were diluted 20-fold into M9G medium containing T4 antiserum (K ≈ 4) and incubated for 2.5 minutes before subsequent dilution in ice cold M9G medium and plating for infective centers. Protein synthesis was measured with 0.5 ml portions of the flask cultures during a 5 minute pulse of $^3$H-leucine incorporation (final concentration $5 \times 10^{-5}$ M, 0.5 µC/ml) into ice cold TCA-insoluble material from 15.75 to 20.75 minutes after the primary infection. Turbidity measurements were taken at various times with the flask culture using a Klett-Summerson colorimeter at 520-580 nm wavelength. The lowest possible value for turbidity was approximately 16% which represents complete lysis from without under these conditions. All values are single determinations.

<table>
<thead>
<tr>
<th>MOI of Super-infecting phage</th>
<th>50</th>
<th>70</th>
<th>87</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary Phage</td>
<td>T&lt;sup&gt;a&lt;/sup&gt;</td>
<td>IC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>PS&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wild Type</td>
<td>82</td>
<td>54</td>
<td>80</td>
</tr>
<tr>
<td>s−</td>
<td>49</td>
<td>44</td>
<td>51</td>
</tr>
<tr>
<td>imm2</td>
<td>66</td>
<td>0.4</td>
<td>1.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Turbidity (T) measurements are expressed as percent turbidity at 90 minutes after superinfection relative to the turbidity 1 minute before superinfection taken as 100%.

<sup>b</sup> Infective Centers (IC) per ml (after superinfection) are expressed as percent of infective centers per ml without superinfection.

<sup>c</sup> Protein Synthesis (PS) in the superinfected cultures is expressed as percent of $^3$H-leucine incorporation in the control cultures not receiving the superinfecting phage.
Immunity to Superinfecting Ghosts

The same series of experiments was performed, omitting the turbidity measurement, using superinfecting ghosts rather than phage. These experiments are then identical to the standard immunity assay with varying MOI of superinfecting ghosts. The results (Table 19) indicate that: (i) the traumatization (protein synthesis measurements) caused by superinfecting ghosts is generally more severe than their lethal action (infective center measurements) and that this effect is most pronounced when the phage lacks the imm gene function; (ii) the absence of the s gene function is not significant except at the higher MOI of superinfecting ghosts or when the phage also lacks the imm function.

These results suggest a difference in the function of the s and imm genes in their role in developing immunity to superinfecting ghosts. It has been shown that s− infected cells are sensitive to the disruptive effects of superinfecting ghosts only at higher MOI's of ghosts, whereas imm− infected cells are sensitive at all MOI's. The role of the s gene in determining immunity to superinfecting ghosts may not be one of neutralizing the infected cell membrane, as postulated for the imm gene product, but rather one of preventing lysis by the superinfecting ghosts. Thus s− infected cells may exhibit reduced immunity (reduced protein synthesis) after challenge with ghosts due to lysis of a portion of the superinfected culture.
Table 19. Infective Center Formation and Protein Synthesis
("immunity") in Phage Infected _E. coli_ B After Superinfection With Ghosts.

_E. coli_ B was grown in M9G medium (30° C) to a cell density of 4 x 10^8 cells/ml from which duplicate 1.0 ml samples were infected with the primary phage indicated (MOI = 5); one of which was superinfected 10 minutes later with ghosts at the indicated MOI. Portions of both samples were diluted 20-fold into M9G medium containing T4 antiserum (K = 4) 13 minutes after the primary infection and incubated for 4.5 minutes before subsequent dilution into ice cold M9G medium and plating for infective centers. Protein synthesis in both samples was determined from a 5 minute pulse of ^3H-leucine (final concentration 5 x 10^-5 M, 0.5 μC/ml) into ice cold TCA-insoluble material from 15.5 to 20.5 minutes after the primary infection. The measurements presented are those of the sample receiving superinfecting ghosts expressed as percent of the sample not receiving ghosts. The values represent the average of two determinations.

<table>
<thead>
<tr>
<th>MOI of super-infecting ghosts</th>
<th>4</th>
<th>8</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary Phage</td>
<td>IC^a</td>
<td>PS^b</td>
<td>IC</td>
</tr>
<tr>
<td>wild type</td>
<td>88</td>
<td>98</td>
<td>81</td>
</tr>
<tr>
<td><em>s^-</em></td>
<td>92</td>
<td>95</td>
<td>89</td>
</tr>
<tr>
<td>imm2</td>
<td>54</td>
<td>28</td>
<td>21</td>
</tr>
<tr>
<td>_s^-^-imm2</td>
<td>2.6</td>
<td>5.5</td>
<td>4.2</td>
</tr>
</tbody>
</table>

^a_ Infective centers/ml
^b_ Protein synthesis
DISCUSSION

The Map Position and Time of Transcription of the imm Gene

Much of the discussion has been presented with the experimental results; thus many points will be mentioned only in summary form. The immunity (imm) gene of bacteriophage T4 resides between genes 42 (dCMP hydroxymethylase) and 43 (DNA polymerase) on the standard T4 genetic map. Consistent with this map location, the data show that the imm gene belongs to the earliest class of phage functions since: (i) the initiation of imm gene transcription occurs within 1.5 minutes after infection under standard conditions (M9G medium, 30° C) (Table 3); (ii) the initiation occurs in the absence of phage protein synthesis (Tables 4 and 5).

Mode of Action of the imm Gene Product

Tests of a model, formulated by M. Vallée (Vallée and Cornett, 1972), for the function of the imm gene showed that the imm protein acts stoichiometrically to immunize the phage infected cell against superinfecting ghosts (Fig 7), most probably by neutralizing the sites through which the ghosts transmit their disruptive effect. The level of immunity in the phage infected cells did not reach 100% when higher MOI's of superinfecting ghosts (>10 ghosts/cell) are used to challenge the cells (Fig 8). This less than maximum immunity appears to result from two processes: (i) LFW by the higher MOI of ghosts since the
addition of $\text{Mg}^{++}$, which prevents LFW (Barlow, 1954; Puck, 1954), increased the immunity level (Table 7); and (ii) failure of the imm gene product to neutralize all the possible sites at the cell surface. The latter process conceivably reflects the shutoff of synthesis of the early imm gene protein as the infectious cycle proceeds. The immunity function of phage T6 was effective against T4 ghosts but unable to immunize the cell against colicin K whose receptor site is closely related to T6 (Table 6). Thus, immunity developed by the primary phage appeared to act specifically against superinfecting phage and did not generally secure the membrane from the disrupting effect of colicin K.

**Background Immunity**

**Partial Function of the imm2 Mutant Protein**

The imm2 infected cells developed a "background" level of immunity to ghosts if sufficient time was allowed after phage infection and before challenge with ghosts (Fig 13). The possibility that partially functional imm protein might be produced by the imm2 mutation, accounting for the background immunity, could not be ruled out.

**Immunity as the Result of Phage Injected Divalent Cations**

The possibility that the background immunity resulted from injection of the phage germinal substance was first examined by the exogenous addition of putrescine, a divalent cation that accounts for approximately 30% of the positively charged species found in the phage head. The ability of putrescine to reduce the disruptive effect of ghosts in both phage infected (Table 8) and uninfected (Table 9) cells
was demonstrated. It was further shown that putrescine did not inactivate ghosts, at least not irreversibly (Table 10), nor did it prevent adsorption and growth of intact phage (Table 11). Since the cations Mg
subscript ++
 (Barlow, 1954; Puck, 1954) and spermidine
subscript +++
 (Buller and Astrachan, 1968) are known to prevent LFW by ghosts, the ability of putrescine to protect cells from killing by colicin K (which does not cause cell lysis) was determined (Table 12). Putrescine was also effective in reducing the lethal action of colicin K.

It has been shown that colicin K causes a change in the permeability of the cell membrane leading to an efflux of cellular Mg
subscript ++
 (Lusk and Nelson, 1972). It is also known that following T4 penetration there is a transient leakage of Mg
subscript ++
 and potassium which may be suppressed by the exogenous addition of 25 mM Mg
subscript ++
 (Silver, Levine and Spielman, 1968). For both colicin K and phage, the leakage does not occur at lower temperatures (12° C and 4° C respectively). E. coli cells are known to vary the composition of saturated or unsaturated fatty acids into the growing cell membrane in response to their incubation temperature (Esfahani, Barnes and Wakil, 1969). This response presumably allows the cell to maintain the proper degree of fluidity in the membrane under various temperatures of growth. The lower temperatures imposed on the cells (presumably grown at higher temperatures) in these assays may restrict the fluidity of the membrane and thus restrict its normal response to the disruptive effects of the colicins or ghosts. In accord with this view, the binding of divalent
cations to the membrane may also reflect a restriction on the otherwise normal reaction of the membrane to adsorption of ghosts. It is of some interest that abortive infections by $T_4 r_{III}^-$ (purportedly a membrane related gene function) in $K$ (A) host cells may be rescued by the exogenous addition of putrescine or $Mg^{++}$ (Brock, 1965; Ferro Luzzi Ames and Ames, 1965). Additional studies with $T_4$ phage carrying the $r_{II}$ defect were made by Sekiguchi (1966) who demonstrated that it is the concentration of monovalent cations in the medium which is inhibitory, and that normally abortive infections of $r_{II}$ mutants become productive infections if the monovalent cations are omitted. Sekiguchi (1966) also noted that monovalent cations inhibit the binding of divalent cations to the cell surface which implies that the high levels of $Mg^{++}$ which compensate for the $r_{II}$ defect may do so by overcoming the inhibition to binding that is established by monovalent cations. Once again, the effect of divalent cations may be to stabilize the membrane.

The role of divalent cations in reducing the lethal effects of $T_4$ ghosts and colicin K is unclear but the results reported here show that the protective effect of putrescine is not due solely to preventing LFW but of a more general nature in preventing disruption of the cell membrane. The same conclusion was reached by Barlow (1954) for the protective effect of $Mg^{++}$ which exhibited quite different values of concentration dependence in protecting cells from either LFW or death by $T_2$ ghosts, as well as the time in which $Mg^{++}$ could effect reversal of these two properties of ghosts.
The conclusion from these studies is that injection of the phage germinal substance does not account for the background levels of immunity seen in \textit{imm}^- infected cells. As pointed out by Winkler and Duckworth (1971), if the phage germinal substance is able to counteract the disruptive effect of phage coat attachment, it does so stoichiometrically since simultaneous challenge of cells with phage and ghosts results in an abortive infection and cell death.

**Immunity Resulting from Other Phage Gene Functions**

The possibility that the background immunity resulted from gene functions other than the \textit{imm} gene was tested by combining the \textit{imm2} mutation with mutations in two early function genes which purportedly affected the cell envelope. The background level of immunity was not reduced when the \textit{imm2} mutation was coupled with either an \textit{rIIA} missence mutation or with a deletion covering both the \textit{rIIA} and \textit{B} cistrons (Table 14).

The studies with the spackle (\textit{s}) gene proved quite different. The \textit{s}^- phage developed only a partial immunity to superinfecting ghosts which approximated the background level of immunity seen with the \textit{imm}^- infections (Table 15 and Fig 13). Phage containing mutations in both the \textit{imm} and \textit{s} genes did not develop background immunity suggesting that the \textit{s} gene is responsible for this effect (Fig 13).

**The Phenotypes of \textit{s}^- and \textit{imm}^- Phage**

It was found that \textit{s}^- phage were partially deficient in excluding superinfecting phage while \textit{imm}^- phage were totally lacking this
ability (Table 16). The imm phage showed normal lysis inhibition and were therefore distinct from the s- mutants (Fig 12). However, they were intermediate between s- and s+ (wild type) phage when assayed for resistance to LFW by turbidity measurements (Fig 14). The LFW experiments yielded an unexpected phenomenon; the imm infected cells were not lysed by the superinfecting phage to a degree proportional to the loss in viability (Table 18). Further experiments revealed that the imm infected cells were "traumatized" by the superinfecting phage or ghosts as shown by a marked decrease in protein synthesis and infective center formation following superinfection (Tables 18 and 19). The term "traumatized" is used with respect to superinfection by phage since phage do not immediately kill their host in contrast to ghosts which cause immediate metabolic disruption upon infection. Under the same conditions, the s- phage infected cells exhibited approximately proportional decreases in cell integrity, viability and protein synthesis; while s- imm infected cells exhibited the greatest degree of sensitivity to superinfection. These observations with s- phage infected cells are critical to the difference between the role of the s- and imm genes. It was shown that when s- phage infected cells were superinfected with wild type phage, the reduction in percent of infective centers formed approximated the percent of cell lysis (Table 18). When the s- phage infected cells were superinfected with ghosts, the loss in infective centers (lysis of the cells) paralleled the reduction in protein synthesis (immunity measurement) (Table 19). These results imply that s- phage infected cells exhibit a decrease in
immunity by virtue of cell lysis and not through a lack of immunity per se (i.e., neutralization of the cell to the lethal effects of ghosts).

Comparison of the s and imm Gene Functions

Rather than consider two sites for the s and imm gene products, both products will be assumed to act at the same site since this view is less complicated and is consistent with the evidence that ghosts and phage attach to the same receptor sites.

The Process of Phage and Ghost Tail Sheath Contraction

Adsorption and penetration by phage can be separated into two processes on the basis of sensitivity of each process to salt concentration and temperature. Adsorption of phage to the cell surface requires the presence of cations presumably to neutralize the mutually repulsive negative charges on both the phage and bacterial surfaces (Puck, 1953). In the presence of low concentrations of salt (20 mM NaCl), phage are reversibly adsorbed to the cell and may be eluted from the cell surface by dilution, leaving both the phage and the cell intact (Puck and Lee, 1954). If the salt concentration is raised to 100 mM NaCl, the attachment becomes irreversible. Once the phage is irreversibly adsorbed, the subsequent penetration process is temperature dependent since neither leakage of cellular material nor LFW occur at 0° C (Puck and Lee, 1954; Silver et al. 1968). It is assumed that the low temperature prevents contraction of the phage sheath and thus penetration by the virus. Similarly, the observation that ghosts are
unable to inhibit the uptake of thiomethylgalactoside at 10° C, but do so at 25° C implies that sheath contraction by ghosts is required to exert their disruptive effect (Winkler and Duckworth, 1971). From these observations it may be tentatively assumed that adsorption of superinfecting phage and ghosts is not sufficient in itself but that sheath contraction must occur to produce their respective effects on the phage infected cell. This assumption allows a possible distinction between the role of the s and imm gene products.

The Role of the imm Gene Function

Evidence has been presented which indicates that the imm gene product functions to neutralize the points of contact for superinfecting phage or ghosts. This neutralization action affects the cell membrane such that the ghosts can no longer transmit their lethal effect (Vallée and Cornett, 1972) and superinfecting phage DNA is retained at the periplasmic level where it is subsequently degraded by endonuclease I (Anderson and Eigner, 1971; Anderson, Williamson and Eigner, 1971).

The Role of the s Gene Function

The primary function of the s gene product has been hypothesized by Emrich (1968) as maintenance of the integrity of the cellular envelope after infection such that in the absence of the s gene function, the phage infected cell is not resistant to LFW. I would add now to Emrich's hypothesis that the means through which the s gene product resists LFW is in part by preventing the sheath contraction and
penetration process of the superinfecting phage. This view is consistent with the observation of several workers (Hershey et al., 1954; Anderson and Eigner, 1971; Sauri and Earhart, 1971) who noted that the ability of superinfecting phage to inject their DNA is greatly reduced. It may be the $s$ gene product of the primary infecting phage which inhibits the injection of the superinfecting phage, thus preventing LFW.

A Modified Model of Immunity to Superinfecting Ghosts

Development of immunity to superinfecting ghosts in wild type $(s^+\text{-imm}^+)$ infected cells is then viewed to result from two processes; (i) the neutralization of the membrane by the imm gene product and (ii) the $s$ gene product acting to reduce the ability of the ghosts to undergo the process of tail sheath contraction and thus reduce their lethal effect. In accord with this hypothesis, the imm$^-$ $(s^+)$ infected cells exhibit an increased background level of immunity with time after infection (Fig 13) due to the development of the $s$ gene function which prevents injection by the ghosts under these conditions. Even though the sites are not neutralized, in the imm$^-$ $(s^+)$ infected cell, the ghosts are ineffective since they are unable to undergo sheath contraction. The reaction of the $s$ protein, like that of imm, is most probably stoichiometric since the assays to detect catalytic action with the imm protein were carried out with $s^+\text{-imm}^+$ phage and the same reasoning applies to the $s$ function. Thus, the growing level of (background) immunity seen with imm$^-$ $(s^+)$ infected cells represents the development of the $s$ gene function to prevent injection of the superinfecting ghosts concomitant with the development of a partial
resistance to LFW (Fig 14). Cells infected with $s^-$ ($imm^+$) phage are able to neutralize the sites against the lethal effect of ghosts by virtue of the $imm^+$ gene function, but cannot retard their contraction and thus are unable to resist LFW (Fig 14) and show a decrease in protein synthesis (immunity) proportional to cell lysis (loss of infective centers) (Tables 18 and 19). The ability of the $s$ function to protect the cell from superinfecting ghosts is not then a true immunization (i.e., neutralization of the membrane), but rather a secondary function resulting from retarding the injection process of the ghosts.

**Superinfection Exclusion**

In regard to genetic exclusion, even wild type ($s^+imm^+$) phage allow some superinfecting phage to enter (Anderson and Eigner, 1971; Table 16). This escape from exclusion no doubt occurs when the superinfecting phage adsorb to a site not affected by the $imm$ or $s$ proteins (Vallée and Cornett, 1973) and are able to inject their DNA. As in the case of immunity, the $imm$ gene function is the major determinant such that in its absence virtually no superinfecting phage are excluded (Table 16). In the absence of the $s$ gene function, the escape from exclusion was increased compared to wild type (Table 16) since a greater number of superinfecting phage were able to inject their DNA, increasing the probability of injection of DNA at a site not yet neutralized by the $imm$ protein.
Lysis From Without

The studies reported here on LFW show that with wild type T4
\((s^+imm^+)\) infected cells, the superinfecting phage probably could not
inject efficiently and thus could not weaken the cell envelope (cause
LFW). In the absence of the \(s\) gene function the superinfecting phage
did inject and by penetrating the cell wall lysed the cells in a manner
which approximated the extent of cell death (loss of infective cen­
ters) (Table 18). The \(imm^-\) phage infected cells were able to partially
retard the LFW effect of the superinfecting phage by virtue of the \(s\)
gene function (Fig 14) but were severely traumatized (exhibited reduced
protein synthesis and infective center formation) (Table 18). In this
case the secondary phage genomes which did enter the cell caused a
traumatization such as seen in primary infections with high MOI (see
Table 13).

Lysis Inhibition

In regard to lysis inhibition, no conclusions can be drawn
since the nature of this phenomenon is not understood. It is of in­
terest, though, since this property most easily distinguishes \(s^-\) and
\(imm^-\) phage. This distinction does not simply reflect the difference
between these two gene functions, however, since \(rII^-\) mutants are not
lysis inhibited but unlike \(s^-\) phage show no defects in genetic exclu­
sion or immunity to superinfecting ghosts.

In summary, it has been shown that both the immunity (\(imm\)) and
spackle (\(s\)) genes of phage T4 determine the response of the phage in­
fected cell to superinfecting phage and ghosts. Both genes are
transcribed almost immediately after infection (Peterson, Cohen and Ennis, 1972; this report). The phenotype of both \textit{imm} and \textit{s} T4 mutants indicate that their gene products normally act upon the cellular envelope. A model, (Vallée and Cornett, 1972), for the function of the \textit{imm} gene protein was discussed. Evidence was presented which indicated the \textit{imm} gene protein acts stoichiometrically to neutralize the sites through which superinfecting ghosts transmit their lethal effects to the cell. In addition to this neutralization process, it is shown that immunization of phage infected cells against superinfecting ghosts also results from the spackle (\textit{s}) gene functions. The function of the \textit{s} gene product is viewed to be primarily concerned with maintaining the integrity of the cellular envelope after infection as first hypothesized by Emrich (1968). The evidence presented here suggests that the means by which the \textit{s} gene product achieves this goal is to retard the penetration of superinfecting phage or ghosts, possibly by preventing contraction of the tail sheath.

The postulated roles of the \textit{imm} and \textit{s} genes require that the immunity to superinfecting ghosts be viewed at two levels: (i) neutralization of the cell membrane by the \textit{imm} gene function (immunity \textit{per se}) and (ii) inhibiting the tail sheath contraction of the superinfecting ghosts by the \textit{s} gene function. It is only through the action of both gene products that full immunity is achieved. This hypothesis is consistent with the phenotypes of both mutant types with regard to immunity, superinfection exclusion, and lysis from without.
Infection of *E. coli* by phage T4 causes a rapid cessation of host protein synthesis (Kaempfer and Magasanik, 1967; Fabricant and Kennell, 1970). Although host transcription continues at a decreasing rate for several minutes following T4 infection, the transcripts formed are excluded from the infected cell ribosomes (Kennell, 1970). Several reports have described alterations in the host cell translational apparatus following infection by phage T4 (Klem, Hsu and Weiss, 1970; Dube and Rudland, 1970; Ihler and Nakada, 1971). At least one alteration has been shown to require phage protein synthesis (Hsu and Weiss, 1969). A second alteration occurs much earlier after T4 infection which suggests that inhibition of host protein synthesis might result from attachment of the phage to the cell surface (Kennell, 1968) such as postulated for the inhibitory actions of phage ghosts (Duckworth, 1970a, 1970b). The inhibition of protein synthesis in cells challenged with phage ghosts has been reported to halt the movement of ribosomes during translation (Fukama and Kaji, 1972). The inhibition appears to result from an alteration of the ribosomes due to ghost attachment to the surface of the sensitive cell (Nugent and Kennell, 1972).
It was observed during the course of experiments with chloramphenicol (CAM), an inhibitor of protein synthesis (Hahn, 1967), that the recovery of protein synthesis in phage infected cells was not efficient after removal of the inhibitor (Fig 5). This situation resulted only in cells infected with T4 (the only phage tested) since uninfected cells did recover the ability to synthesize protein after the removal of the CAM (Fig 5). In every case, the host cells were first treated with CAM before receiving the phage, so that infections were carried out in the absence of phage gene expression.

It seemed possible that this phenomena might reflect damage to host ribosomes incurred by the adsorption of the phage which was normally repaired or prevented by an early phage function. To test this hypothesis, host cells were treated with CAM before and after infection by phage T4. Several minutes after infection, the cells were sedimented by centrifugation and resuspended in fresh medium without CAM. Protein synthesis was monitored in these washed, infected, cells by the incorporation of $^3$H-leucine into cold TCA-insoluble material. The results (Fig 15) show that if CAM is added (final concentration 100 µg/ml) before or simultaneously with the phage, protein synthesis is greatly reduced compared to the control infection not receiving the CAM. Fig 15 also shows that when CAM is added 5 minutes after the phage, protein synthesis resumes at a rate which parallels that of the control infection without CAM. The rates of protein synthesis for each of the conditions shown in Fig 15 are presented in Table 20.
Fig 15. Recovery of Protein Synthesis After the Removal of Chloramphenicol (CAM) in Uninfected and T4 Infected E. coli B/5 Treated with CAM Before and After Infection.

E. coli B/5 grown in M9G medium (30° C) to a cell density of 4 x 10^8 cells/ml, was divided into 2 ml portions and infected with wild type T4 (MOI = 5) or an equal volume of M9 salts. The phage infected cells received chloramphenicol (CAM) at -1, 0, or 5 minutes after infection as indicated in the figure (final concentration 100 μg/ml). Uninfected cells received CAM at 0 time. Ten minutes after infection, the cells were washed free of CAM by centrifugation and protein synthesis was measured by the incorporation of 3H-leucine into TCA insoluble material as described in the legend to Fig 5.
These results suggest that phage infection in the presence of CAM causes some unknown damage to the host cell's ability to synthesize protein. This damage does not occur if phage gene expression is allowed for several minutes before adding CAM. This effect could result from either damage to the ribosomes or to the leucine uptake system of the infected cells. No attempt was made to distinguish between these alternative explanations.

Table 20. Protein Synthesis in Wild Type T4 Infected E. coli B/5 ± Chloramphenicol.

<table>
<thead>
<tr>
<th>Time of CAM(^a) Addition with Respect to Phage Addition (minutes)</th>
<th>No CAM</th>
<th>-1</th>
<th>0</th>
<th>+5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of Protein Synthesis(^b)</td>
<td>69.7</td>
<td>21.0</td>
<td>25.0</td>
<td>62.5</td>
</tr>
</tbody>
</table>

\(^a\) CAM = chloramphenicol

\(^b\) The rates of protein synthesis are given as cpm of TCA-insoluble \(^3\)H-leucine material formed per minute between 15 and 45 minutes after the addition of \(^3\)H-leucine. The values given have been corrected for non-specific adsorption of radioactivity and are normalized with respect to the number of infected cells present as determined by visual counting in a Petroff-Hauser counting chamber using a phase light microscope.
LITERATURE CITED


