Fall 1971

STUDIES OF VERY EARLY EVENTS OCCURRING IN BACTERIOPHAGE-SP82G INFECTION

WILLIAM T. MCALLISTER

STUDIES OF VERY EARLY EVENTS OCCURRING IN BACTERIOPHAGE SP82G INFECTION

by

WILLIAM T. McALLISTER

B.A. Lehigh University, 1966

A THESIS

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

Doctor of Philosophy

Graduate School

Department of Biochemistry

October, 1970

This thesis has been examined and approved.

Thesis director, D. MacDonald Green, Prof. of Biochem.

Fred Hickson, Asst. Prof. of Microbiology

Gerald L. Klippenstein, Asst. Prof. of Biochem.

Sam Smith, Assoc. Prof. of Animal Sci.

James A. Stewart, Asst. Prof. of Biochem.

May 28, 1971
Date

To my wife and parents whose patience and understanding were remarkable; and to my Uncle Carl, who told me not to become a dentist.

Acknowledgements

I gratefully acknowledge the expert guidance and support of Dr. D. MacDonald Green. I am also very indebted to Mrs. George Gregory and Miss Margaret Urban for their technical assistance, and to Mrs. William Colby for her efficient kitchen work. I am also grateful to Dr. Stephen F. Goldmann for assistance in computer programming and to Mrs. K.A.C. Gebe and Mr. Gordon Williams for critical reading of the manuscript. This work was supported by NSF grant GB 8110. During part of these studies I was the recipient of an NSF predoctoral traineeship.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

STUDIES OF VERY EARLY EVENTS OCCURRING IN BACTERIOPHAGE SP82G INFECTION

by

WILLIAM T. McALLISTER

PART I

Competent B. subtilis may be infected by exposure to isolated SP82G bacteriophage DNA (5). The fate of genetic markers introduced into the cell by the infective DNA was examined by infecting the DNA-infected cell with intact phage of a different genotype and determining the ability of such complexes to give rise to wild type recombinants. Such marker rescue experiments have shown that there exists within the DNA-infected cell a process that exponentially inactivates and unlinks genetic markers carried by the infective DNA (8). The inactivation process does not normally appear to be active during the course of infection by intact bacteriophage (8, 11) and can be modified by pre-infecting the host cells with marker rescue phage before they are exposed to the infective DNA (8). Modification of intracellular inactivation is termed preinfection protection.

Through the use of appropriate mutant phage in marker rescue experiments it was possible to directly examine the genetic stability of transfecting DNA in preinfected cells under a variety of conditions. These experiments show that preinfection protection is the result of an early phage function which inhibits the intracellular inactivation process and leads to an increased stability of infective DNA during

its time of residence in the cell. The function is maximally expressed by the sixth minute after phage infection and is completely prevented by the addition of chloramphenicol at the time of phage infection. The protective effect extends even to infective DNA which was present in the cell before the time of addition of intact phage. Continued protein synthesis does not appear to be a requirement for the maintenance of the inhibition.

In an analogous situation, if infectious centers resulting from singly infecting phage (multiplicity of infection < 0.1) are exposed to chloramphenicol shortly after the time of infection, an exponential decrease in the survival of infectious centers time held in chloramphenicol is observed. If the addition of chloramphenicol is delayed until the sixth minute after infection the infectious centers are resistant to chloramphenicol. The sensitivity of infectious centers to chloramphenicol at early times after infection is strongly dependent upon the multiplicity of infection and is consistent with a model of multiplicity reactivation. These results support the contention that the inhibition of the intracellular inactivation process is an early phage function which is necessary for the successful establishment of an infectious center.

PART II

One reason for the ability of intact bacteriophage to circumvent the intracellular inactivation process during the course of normal infection might be that under these conditions the phage DNA is introduced into the cell in such a way as to rapidly set up a required series of events. In the experiments reported it was found that the transfer of DNA from bacteriophage SP82G to its host may be halted by

chilling, but is affected little by chloramphenicol, Actinomycin D or sodium cyanide. The order of entry of markers on the phage genome was determined by halting the transfer of DNA at intervals, removing the untransferred DNA by blending, and assaying for the presence of markers in the blended complexes. Markers on the phage genome are transferred in a linear, polar fashion consistent with the previously determined genetic and physical maps (6). Those markers which are concerned with early functions are transferred first, and at 33°C the transfer of the entire genome is complete in 1.4 minutes. These results are contrasted with the kinetics of entry of transfecting DNA molecules.

PART III

The order of entry of markers on the SP82G genome during phage infection is known and proceeds in a linear polar fashion consistent with the genetic and physical maps. The effects of ^{32}P decay on the transfer of the genome may be examined genetically by determining its effects on marker transfer, and physically by examining the transfer of labelled DNA. The results described in this report show that about 42% of all lethal events result in the non-transfer of some portion of the genome. At 4°C double strand breaks probably always prevent the transfer of portions of the genome distal to the break, and while they are in themselves lethal, they do not prevent the rescue of markers on undamaged portions of the genome. All markers that <u>are</u> transferred are transferred at the normal rate.

PART I

THE INVOLVEMENT OF AN EARLY PHAGE FUNCTION IN THE ESTABLISHMENT OF PREINFECTION PROTECTION

Introduction

Under suitable growth conditions B. subtilis cells acquire the ability to take up isolated DNA. (1) In the process known as transfection such competent cells take up DNA isolated from a number of subtilis bacteriophages in such a way that they become infected and give rise to progeny (2). The infective DNA from these phage exhibit behavior during transfection that indicates that there are two modes of transfection which depend upon the bacteriophage from which the DNA was isolated (3). The first mode of transfection requires only single phage DNA molecules to successfully establish an infectious center. Phage DNA's that exhibit this property have been isolated from bacteriophage ϕ 29 and SP02 (3, 4). In the second form of transfection more than one molecule of DNA is required. Bacteriophage which have DNA exhibiting this property are SP82G, SP3, SP50, SP01 and ϕ 1. (5, 6, 2, 7, 4).

By examining the progeny resulting from such infectious centers, it has been found that genetic recombination between two or more infective molecules is required for the establishment of an infectious center. (5, 7) Green (8) has found that the multimolecular requirement for SP82G transfection is the result of an inactivation mechanism that exists within the DNA-infected cell. This process exponentially inactivates and unlinks genetic markers carried by the phage DNA. The reconstitution of a complete phage genome under such conditions has been

found to involve portions of the infective DNA that comprise less than 1/40 of the genetic map. (9)

The integrity of genetic markers introduced into the cell by transfecting DNA can be examined by superinfecting the DNA infected cell with intact phage of a different genotype and measuring the ability of such complexes to give rise to wild type (WT) recombinants. The formation of a WT recombinant indicates that the superinfecting phage has "rescued" the WT gene from the infecting DNA. Such experiments have shown that the rate of inactivation of the transfecting DNA with time of residence in the cell (prior to the addition of marker rescue phage) is proportional to the map distance between marker pairs and that markers on the same DNA molecule can be independently inactivated (8). These results indicate that there is an intracellular factor which randomly damages the infective DNA molecule and that these damages result in a partial inactivation of the genetic potential of the genome.

Of particular interest was the observation that the inactivation process could be modified by preinfecting the cell with marker rescue phage prior to the addition of infective DNA (8). Under these conditions the rescue of marker pairs no longer exhibited the strict dependence on intermarker map distance observed under superinfection marker rescue conditions. This modification, termed preinfection protection, was found to have a maximal effect when the infective DNA was added at the sixth minute after phage infection.

Intracellular inactivation may also be modified by adding U.V. irradiated homologous or heterologous DNA to the competent cell, (10) or by irradiating the competent cells themselves (11) prior to their exposure to infective DNA. Epstein (10, 11) has suggested that this

protective effect is the result of the entrapment of a host nuclease (an inactivating factor) by the U.V. irradiated DNA. In agreement with this proposal is the observation that the effectiveness of various heterologous U.V. irradiated DNA preparations paralleled their adenine plus thymine content and reached a maximum when pyrimidine dimerization was maximized (10).

Similar observations have been made during studies of transformation of the Challis strain of Streptococci. (Transformation is a process in which bacterial DNA is taken up by competent recipients and incorporated into their genome. Successful transformation can be detected by the expression of genetic markers which are introduced into the cell by the transforming DNA.) Chen and Ravin (12) have found that the presence of non-transforming DNA (genetically unmarked DNA) enhances the transformation of the bacteria. The authors proposed that there is an inactivating agent within the Challis bacteria which acts upon DNA fragments and that the agent can be "swamped" by multiple infection of the recipients. A delay in the addition of helping DNA to recipients which had already been exposed to transforming DNA caused a decrease in the yield of transformants, but if the helping DNA was added prior to the transforming DNA a marked increase in transformation resulted.

The observation that inactivating enzymes can be "swamped" by the addition of a number of DNA molecules makes unclear the role of the preinfecting phage in establishing preinfection protection. Epstein has found that the maximum protective effect of U.V. irradiated DNA is obtained when transfecting DNA is added 20 minutes after the U.V. treated DNA (even when U.V. inactivated SP82G bacteriophage were used as donors of irradiated DNA). (10) Green, however, has reported that preinfection protection by intact phage reaches a maximum by six minutes (8).

This indicates that there are two quite different protective mechanisms operating. During the course of normal infection of B. subtilis by intact SP82G bacteriophage there is no evidence to indicate that the phage DNA is inactivated (8, 10). Either injected DNA is not susceptible to intracellular inactivation, or the phage manages to directly overcome the inactivation process. The observation that U.V. irradiated phage can be used as donors of "swamping" DNA suggests that injected DNA is also susceptible to intracellular inactivation.

In experiments reported here it was found that the modification of intracellular inactivation by preinfecting phage is the result of an early phage function which directly inhibits the inactivation process. This function is maximally expressed by the sixth minute after phage infection and is completely prevented by the addition of chloramphenicol at the time of infection. In an analagous situation, if the infecting phage particle is prevented from carrying out the early protective function, its own (injected) DNA is susceptible to a similar type of inactivation.

Materials and Methods

Bacterial strains, growth conditions and phage preparations.

Bacillus subtilis strain SB-1 (13) was the host cell for all experiments. Techniques for the isolation, propagation and detection of bacteriophage SP82G are identical to those of Green (5). Temperature sensitive (ts) mutants of SP82G which will grow at 33°C but not at 47°C have been described previously (9, 14) with the exception of mutant E119 which was isolated by D.M. Green (personal communication). The techniques for scoring wild type (WT) recombinants have also been described before (8).

The primary media for the growth of all phage preparations was Nomura salts (NM) supplemented with 0.5% glucose, 0.2% casein hydrolysate, 2.5×10^{-3} M $MgCl_2$, 0.1% yeast extract, 0.05 mg/ml of DL-tryptophan, 4 mg/ml of arginine and 0.2 mg/ml of L-histidine (15).

Dilution media (DM) was prepared by dissolving 25 g of Casamino acids and 1 g of $MgCl_2$ in 5 liters of water. Occasionally this was supplemented with 0.5% glucose and 0.1% yeast extract and used as a growth media for final lysis of large phage preparations.

Phage lysates grown at 33°C were concentrated by two cycles of high (10,000 x g) low (6,500 x g) centrifugation and stored in 1XNM salts plus $MgCl_2$ (10^{-3}M). Phage DNA was prepared by phenol extraction (16) and stored after dialysis against 1xSSC (SSC contains 8.76 g NaCl and 4.41 g sodium citrate per liter of water). The purity and concentration of DNA preparations were determined from their optical density at 260 and 280 nm. assuming that a solution of 20 ug/ml of DNA has an A_{260}

of 0.4

Competent cells were prepared by a modification of the techniques of Spizizen (1). Overnight slants of SB-1 grown on Tryptose Blood Agar (Difco, Detroit, Mich.) at 37°C were transferred to HS media (1) supplemented with 8 mg/ml arginine and 400 ug/ml histidine and grown for 4.5 hours at 37°C. At that time the media was made 7.5% in dimethyl-sulfoxide and stored at -20°C. On the day of use, samples thawed at 47°C were diluted 1/10 into LS media (1) supplemented with 5×10^{-4} M spermine tetrahydrochloride, 2.5×10^{-3} M $MgCl_2$, and 5×10^{-4} M $CaCl_2$ and grown at 37°C for 90 minutes.

Marker rescue experiments

Transfection experiments varied somewhat and the exact details of each experiment are described in the text and figures. A typical marker rescue experiment not involving preinfected cells would proceed as follows. Competent cells (grown in LS for 90 minutes as above) were exposed to DNA (2-3 ug/ml) isolated from a ts mutant phage for 3 minutes at 33°C. The cells were diluted 1/10 in fresh LS, held for an additional 10 minutes and treated with DNAase (Beef pancreas, Miles Laboratories, Elkhart, Ind). At intervals, genetic markers were "rescued" (8) from phage DNA by superinfecting the cell with other ts mutant phage and plating at a selective temperature (47°C) at which only WT recombinants can give rise to plaques. Most of the experiments described in this report are a variation of this technique in which the cells are also preinfected with another ts mutant phage which is unable to contribute the necessary WT alleles to the progeny. The effect of infection with this phage on the stability of markers introduced by the transfecting DNA is examined under various conditions.

Survival of infectious centers and bacteria in chloramphenicol

The effect of chloramphenicol (CM) on bacterial survival is described in the text. The effect of arresting protein synthesis shortly after phage infection was examined by briefly exposing bacteria to phage and then adding sufficient antisera to inactivate 99.9% of the unadsorbed phage. These rapidly infected complexes were diluted into NM containing chloramphenicol (Sigma, St. Louis, Mo.) and held for various lengths of time until plating.

Results

Inhibition of growth of B. subtilis strain SB-1 by chloramphenicol

The effects of chloramphenicol (CM) and Actinomycin D (Act. D) on protein synthesis and RNA synthesis in B. subtilis strain W23 have been examined by Levinthal who found that the addition of chloramphenicol at a concentration of 100 ug/ml to a growing culture resulted in an immediate cessation of the incorporation of ^{14}C-valine. Similarly, the addition of Actinomycin D at 10 ug/ml immediately stopped RNA synthesis (17).

The effects of chloramphenicol and Actinomycin D (Merck, Sharpe and Dohme Co., Rahway, N.J.) on the growth rates of B. subtilis strain SB-1 were examined by following the change in absorbance at 620 nm of a log growth culture after the addition of the inhibitors. The results (Fig. 1) show that while 5 ug/ml of Actinomycin D was sufficient to stabilize the absorbance, a concentration of 500 ug/ml of chloramphenicol was necessary to prevent an increase in absorbance of the culture.

When the growth rate of SB-1 was followed by viable count, however, (Fig. 2) concentrations of as low as 50 ug/ml of chloramphenicol prevented multiplication in the culture. At concentrations of 500 ug/ml, the number of colony formers in the culture remained stable for one hour (Fig. 3) and the ability of cells to support phage growth after holding in chloramphenicol was not impaired with time. It is known that prolonged incubation in chloramphenicol leads to a thickening in the cell wall of B. subtilis (18) and this may lead to the increased

absorbance in the presence of the inhibitor. For the experiments that follow, concentrations of chloramphenicol in the range of 100-500 ug/ml were used.

Effect of chloramphenicol on preinfection protection

The efficiency of infection of competent B. subtilis by bacteriophage SP82G DNA is such that the incorporation of a single molecule of DNA into the competent cell does not result in the production of phage by that cell (5). The genetic contribution of that molecule can be measured however, by superinfecting the DNA-infected cell with phage particles of a different genotype. Genetic markers carried by the infective phage DNA appear in the progeny of such superinfected cells and these recombinant particles may be detected under suitable conditions. For example, if a competent cell which has taken up wild type (WT) DNA is superinfected by a phage particle carrying a temperature sensitive (ts) mutant gene (or genes) WT recombinant particles may be found among the progeny. These may be detected by plating at a selective temperature at which phage particles having a ts mutation in their genome do not yield progeny. The formation of a WT recombinant indicates that the superinfecting phage has "rescued" the WT gene from the infecting DNA (8).

When DNA-infected cells were superinfected with bacteriophage that carried two ts mutations, Green (8) found that the rescue of marker pairs (i.e. the rescue of both WT alleles from the transfecting DNA) decreased exponentially as a function of the genetic map distance between the markers, and that even the most closely linked markers were rescued with only about one-half the efficiency of single markers. It was shown that this is the result of an inactivation process which both

Figure 1

Inhibition of growth of SB-1 by chloramphenicol (CM)

Actinomycin D. (Act. D)

Log growth SB-1 were diluted into NM containing inhibitors at various concentrations. At intervals the A_{620} was determined in a Spectronic 20.

■ control, no inhibitor, □ 100 ug/ml CM, ▲ 200 ug/ml CM,
△ 300 ug/ml CM, ● 500 ug/ml CM, ○ 5 ug/ml Act. D

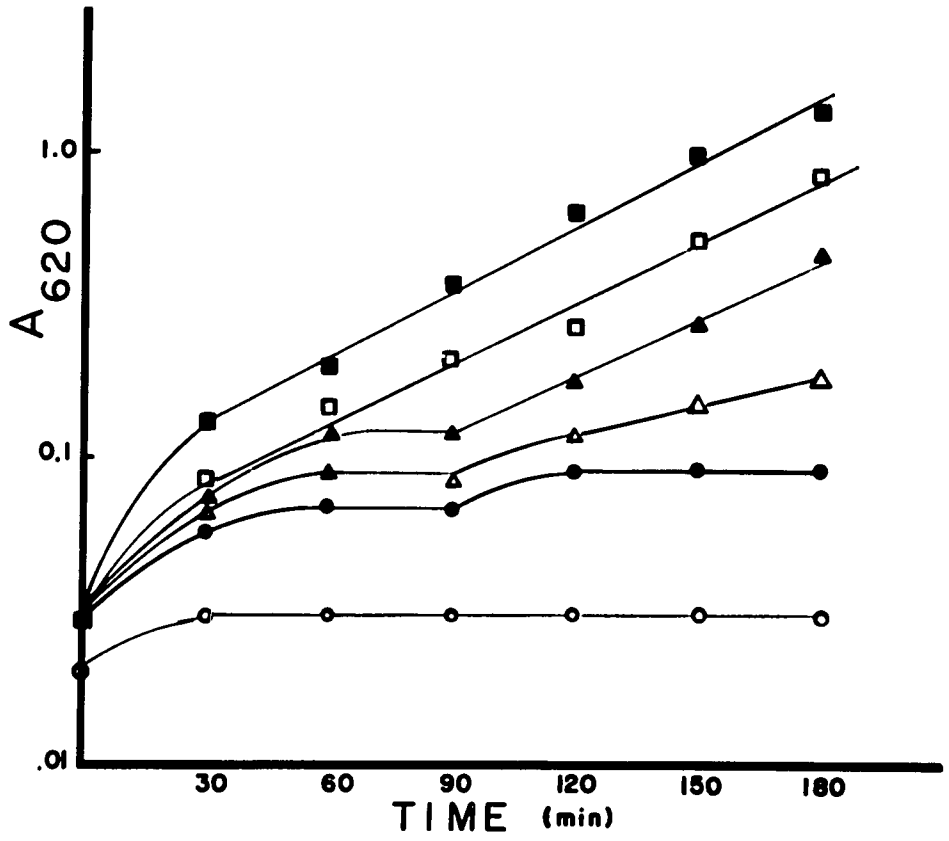


Fig. 1

Figure 2

Inhibition of growth of SB-1 by chloramphenicol

Log growth SB-1 were diluted into NM containing chloramphenicol at various concentrations. At intervals the viable titer of the bacteria was determined by plating dilutions at 33°C . ○-control (no CM), □ -5 ug CM/ml, △-50 ug CM/ml.

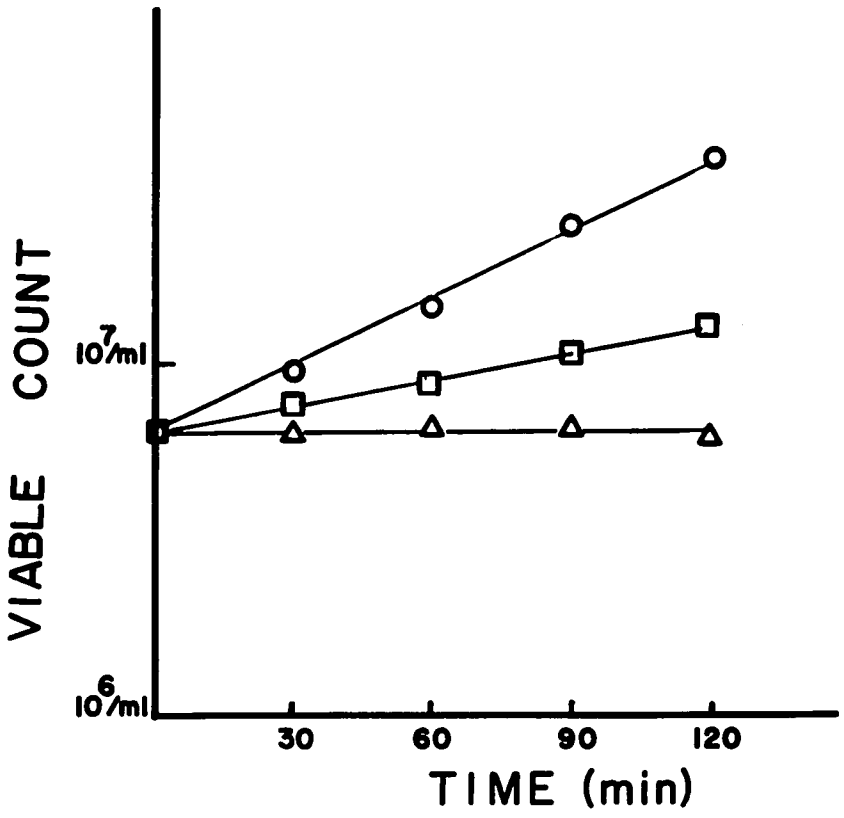


Fig 2.

Figure 3

The effect of incubation in chloramphenicol (500 ug/ml) on colony formation and the ability to support phage growth

Bacteria at 10^8/ml were diluted 1:10 into NM containing chloramphenicol at 33°C. At intervals samples were withdrawn and plated for viable titer. Also at intervals samples were exposed to phage at a multiplicity of infection = 2.0 for 5 minutes. Unadsorbed phage were inactivated by a 3 minute exposure to antisera (k=1.35) and the samples were assayed for plaque forming units. The yield of colony formers (○) and plaque forming units (△) are expressed as the fraction of a control which was never exposed to chloramphenicol.

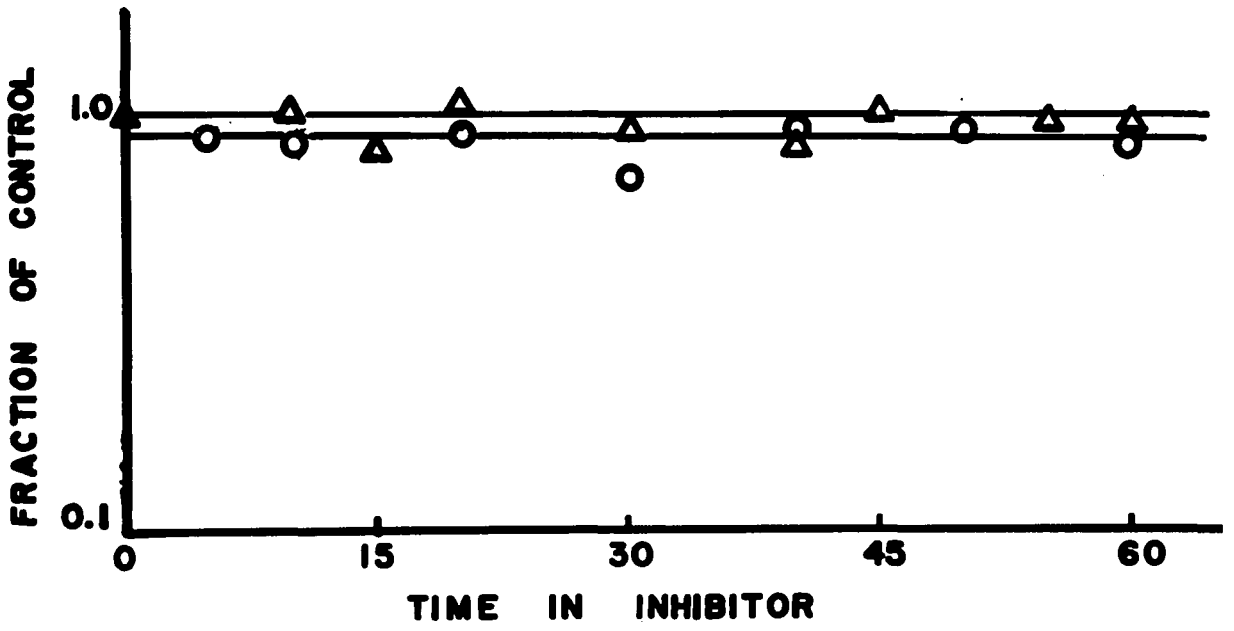


Fig. 3

inactivates and unlinks markers on the transfecting DNA after their introduction into the cell. The rate of inactivation of marker pairs is porportional to the map distance between the markers.

The inactivation process can be modified by exposing the competent cells to the rescuing phage before the addition of phage DNA. Under these conditions (i.e., preinfection marker rescue) the rescue of marker pairs no longer exhibits the severe dependence upon intermarker map distance that is observed under superinfection conditions. This modification of the intracellular inactivation process is called preinfection protection, and reaches a maximum at about the sixth minute after phage infection.

If preinfection protection requires the synthesis of a protein after infection of a cell by bacteriophage, then no modification of intracellular inactivation should occur when cells are preinfected in the presence of chloramphenicol. A marker rescue experiment was performed in which markers introduced into the competent cell by WT phage DNA were rescued by ts mutant phage under three conditions: by preinfecting phage added six minutes before the phage DNA, by preinfecting phage in the presence of chloramphenicol and by superinfecting phage added after the phage DNA. The rescuability of marker pairs in each group was expressed as the percent of the single marker $H362^{+}$ rescued under the same conditions. The results are presented in Figure 4 as the rescuability of double markers as a function of the map distance they subtend. In agreement with the results of Green (8) it is found that under conditions of preinfection the rescuability of double markers does not exhibit the high degree of dependence on inter-marker map distance that is observed under superinfection conditions. If preinfection is carried out in the presence of chloramphenicol however, the rescue of marker pairs shows the inverse dependence on map distance that is characteristic of superinfection marker rescue conditions. In the presence of

chloramphenicol, preinfecting phage do not modify the intracellular inactivation process.

Time course of preinfection protection

By adding phage DNA at intervals after the infection of cells by preinfecting marker rescue phage, Green (8) found that the maximum number of WT recombinants resulted when the phage DNA was added six minutes after phage infection. This defined the concept that by six minutes preinfection protection had reached a maximum and that at this time the intracellular inactivation process was largely inhibited. If the time course of preinfection protection reflects the synthesis and/or the accumulation of a necessary protein, then the cessation of protein synthesis at intervals after the addition of pre-infecting phage should lead to the same time course of protection for markers introduced by phage DNA at some later, fixed time. To test this hypothesis, cells were preinfected at 33°C with a mutant phage carrying three ts mutations (H20-H362-H15). At intervals, protein synthesis in the infected cell was stopped by the addition of chloramphenicol to a concentration of 100 ug/ml. At a later time (nine minutes after infection) all cells were exposed to DNA isolated from the ts mutant phage H15, and after a sufficient period of time to allow penetration of the DNA, were treated with DNAase. The cells were then superinfected with the ts mutant phage H20-H 362, and plated at 47°C. Under these conditions only WT recombinants will yield plaques, and these may only be formed when the wild type genes for $H20^+$ and $H362^+$ contributed by the phage DNA are present intact in the infected cell. The maximum yield of WT recombinants was obtained when protein synthesis was interrupted at the sixth minute after the cells were preinfected. (Fig. 5) The results of Green in which phage

Figure 4

Effect of chloramphenicol on the modification of intracellular inactivation

The ability of a variety of doubly mutant phage to rescue genetic markers from infective DNA was measured under three conditions: ■, superinfection marker rescue (rescuing phage added after DNA) ●, preinfection marker rescue (rescuing phage added before DNA) and △, preinfection marker rescue in the presence of CM. All values are expressed as the percent of rescue obtained using the single mutant H362 under identical conditions and are plotted as a function of the map distance subtend by the marker pairs.

Competent cells were preinfected by exposure to phage at a multiplicity of infection of 10 for 2 minutes and diluted into either LS or LS containing chloramphenicol (100 ug/ml) and held for an additional 6 minutes. These cells and another sample which had not been preinfected were exposed to WT phage DNA at 0.1 ug/ml for 15 minutes and treated with DNAase. Aliquots from the third group (non-pre-infected) were then superinfected with mutant phage and dilutions of all samples were plated at 47°C.

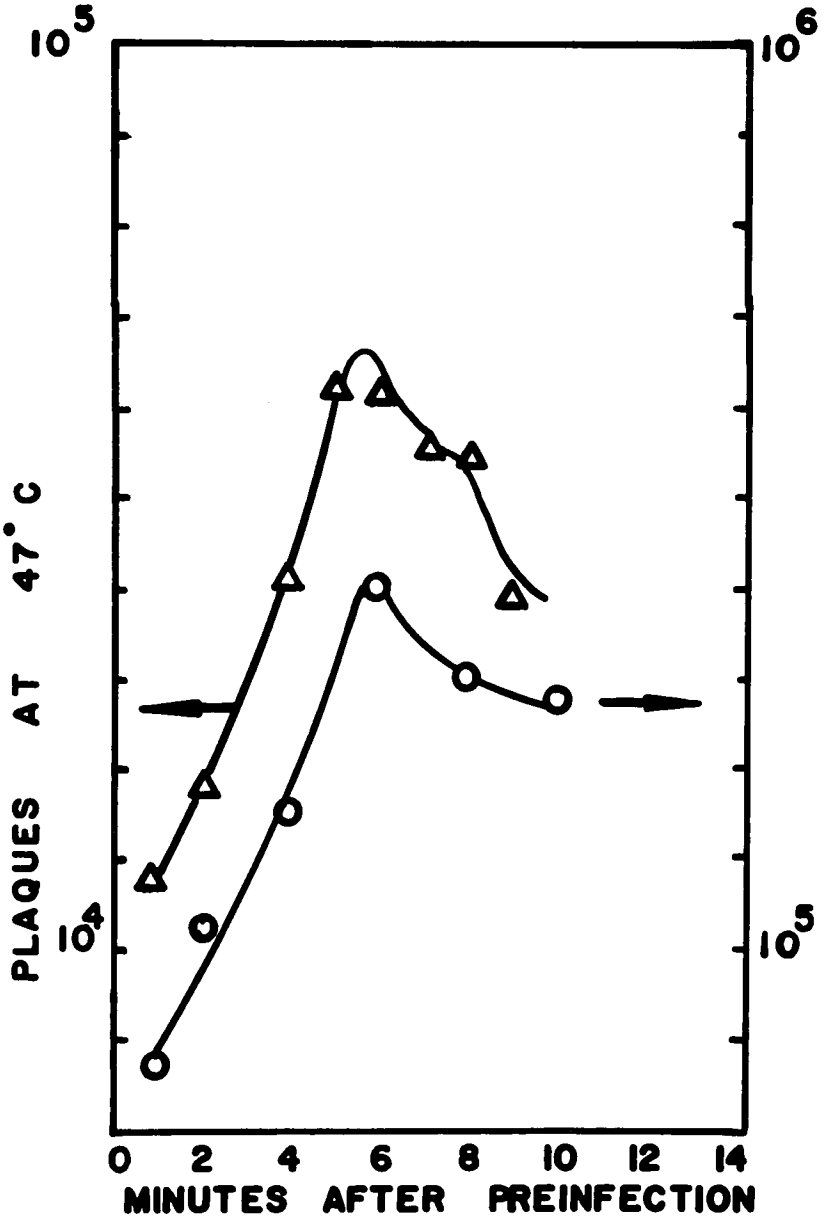

10^5
10^6
PLAQUES AT 47° C
10^4
10^5
0 2 4 6 8 10 12 14
MINUTES AFTER PREINFECTION

Fig. 4

Figure 5

Time course of preinfection protection

Competent cells were exposed to preinfecting ts mutant phage (H20-H362-H15) at a MOI=10. At intervals after phage infection chloramphenicol (CM) was added to samples at a concentration of 100 ug/ml. At the ninth minute after infection all samples were exposed to phage DNA (H15) at 1 ug/ml for 3 minutes, diluted 1:10 into LS containing CM and held for an additional 10 minutes. DNAase was added and the cells were exposed to superinfecting marker rescue phage (H20-H362), diluted and plated at 47°C (△). Also shown are the results of Green (8) who measured the ability of pre-infecting phage alone to rescue markers from infective DNA which was added at subsequent intervals. (○)

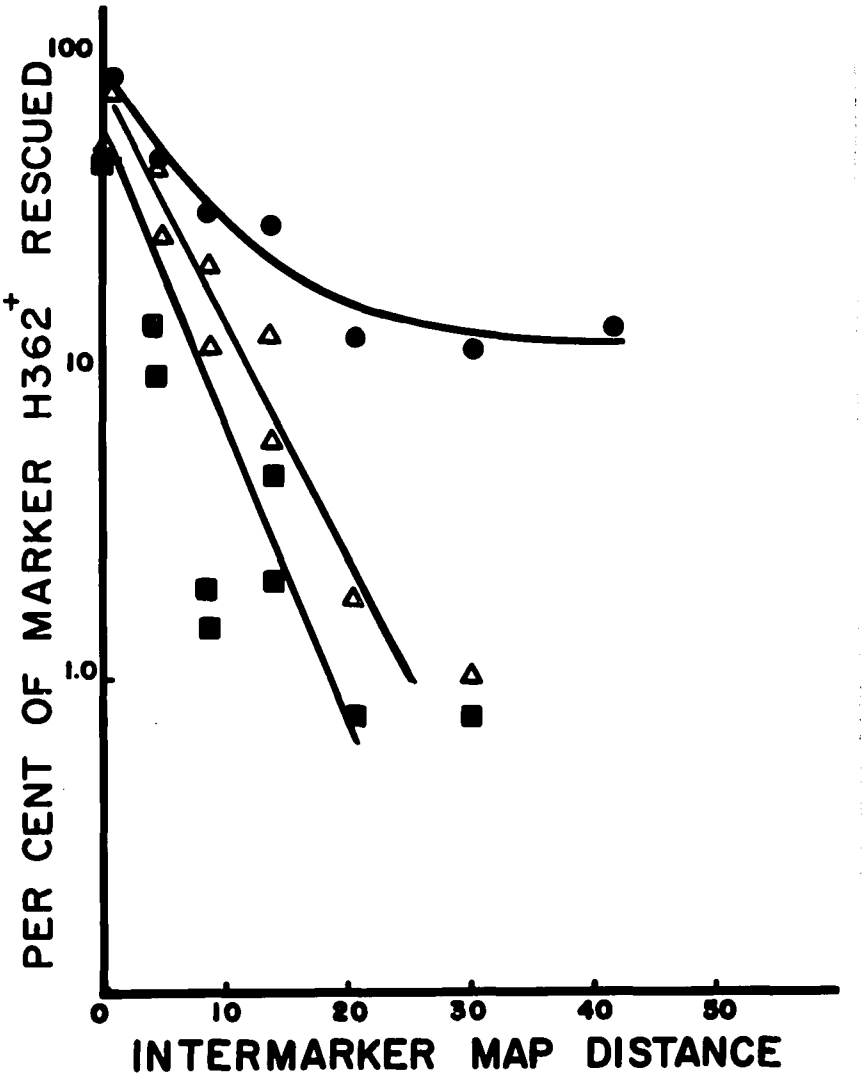

PER CENT OF MARKER H362+ RESCUED
100
10
1.0
0
10
20
30
40
50
INTERMARKER MAP DISTANCE

Fig. 5

DNA was added to cells at intervals after the preinfecting phage are also shown. Under both experimental conditions the maximum yield of WT recombinants occurs at the sixth minute after infection. This indicates that the appearance of preinfection protection with time reflects the synthesis of a protein within the preinfected cell.

Stability of genetic markers in preinfected cells

The peak of preinfection protection observed in Figure 5 presumably reflects the increasing inhibition of intracellular inactivation in the preinfected cell. This interpretation predicts that the stability of markers in the preinfected cell would be greater if protein synthesis were arrested six minutes after the addition of preinfecting phage than if protein synthesis were arrested at the time of preinfection.

To examine the stability of markers introduced by phage DNA into preinfected cells, marker rescue experiments similar to that shown in Figure 5 were performed. Competent cells were preinfected with the triple mutant H20-H362-H15 and after six minutes were permitted to take up H15 phage DNA. At intervals the linkage integrity of markers $H20^+$ and $H362^+$ introduced into the cell by the infective phage DNA was assayed by superinfecting the complexes with phage carrying temperature sensitive mutations at both these sites. The yield of wild type recombinants with time reflects the stability of these markers in the preinfected cell. Protein synthesis was arrested either at the time of preinfection or at six minutes after infection, corresponding to the times at which the lowest and highest yield of WT recombinants were obtained under the conditions described in Figure 5. The stability of markers with the time of residence in non-preinfected cells was also examined by omitting the preinfection step but otherwise following the procedure described above.

Protein synthesis in these non-preinfected cells was arrested either at the time of addition of the phage DNA or was not arrested at all.

The results of this experiment are shown in Figure 6. When non-preinfected cells were used a sharp decline in the number of recombinants with time is observed. This is characteristic of intracellular inactivation (8). Neither the rate of decline nor the yield of recombinants was affected by the addition of CM to non-preinfected cells at the beginning of the experiment. One can conclude from this that the intracellular inactivation phenomenon itself does not require the synthesis of a new protein. Similarly, it follows that the uptake of DNA by competent cells does not require an induced enzyme.

When preinfected cells are examined, it is apparent that the time of the cessation of protein synthesis has a large effect on the stability of markers subsequently introduced by the phage DNA. If CM is added at the same time as the preinfecting phage, the rate of loss of WT recombinants is identical to that observed in cells which never saw preinfecting phage. However, if the addition of CM is postponed until the sixth minute after the addition of the preinfecting phage, a marked improvement in the stability of markers introduced by transfecting DNA is observed. This increased stability of markers is observed for single markers as well as for double markers (Figure 7). In the experiment shown in Figure 8, it was found that if the addition of CM is delayed until the ninth minute after preinfection the rate of loss of recombinants is greater than if protein synthesis is interrupted at the sixth minute after preinfection. Clearly, the peak of preinfection protection observed in Figure 5 reflects the stability of markers in the preinfected cells. The maximum stability is obtained when protein synthesis is arrested at the sixth minute after phage infection.

Figure 6

The effect of arresting protein synthesis on the stability of genetic markers introduced into preinfected or non-preinfected cells

At time zero, competent cells were exposed to the ts mutant phage H20-H362-H15 at a MOI=10. After six minutes these preinfected cells, and cells which had not been preinfected were exposed to phage DNA from the ts mutant H15 at a concentration of 3 ug/ml and held for 3 minutes. The cells were diluted 1:10 into LS and after an additional 10 minutes were treated with DNAase. At intervals thereafter samples were withdrawn, superinfected with the ts mutant phage H20-H362 at a MOI=10 for 4 minutes, and dilutions plated at 47°C. The number of WT recombinants is plotted as a function of the time of addition of the superinfecting phage. Upper curves are for non-preinfected cells which were not exposed to CM (○) or which were exposed to CM (100 ug/ml) from time zero (●). Lower curves are for results obtained with preinfected cells which were exposed to CM from time zero (□) or exposed to CM from the sixth minute on (△).

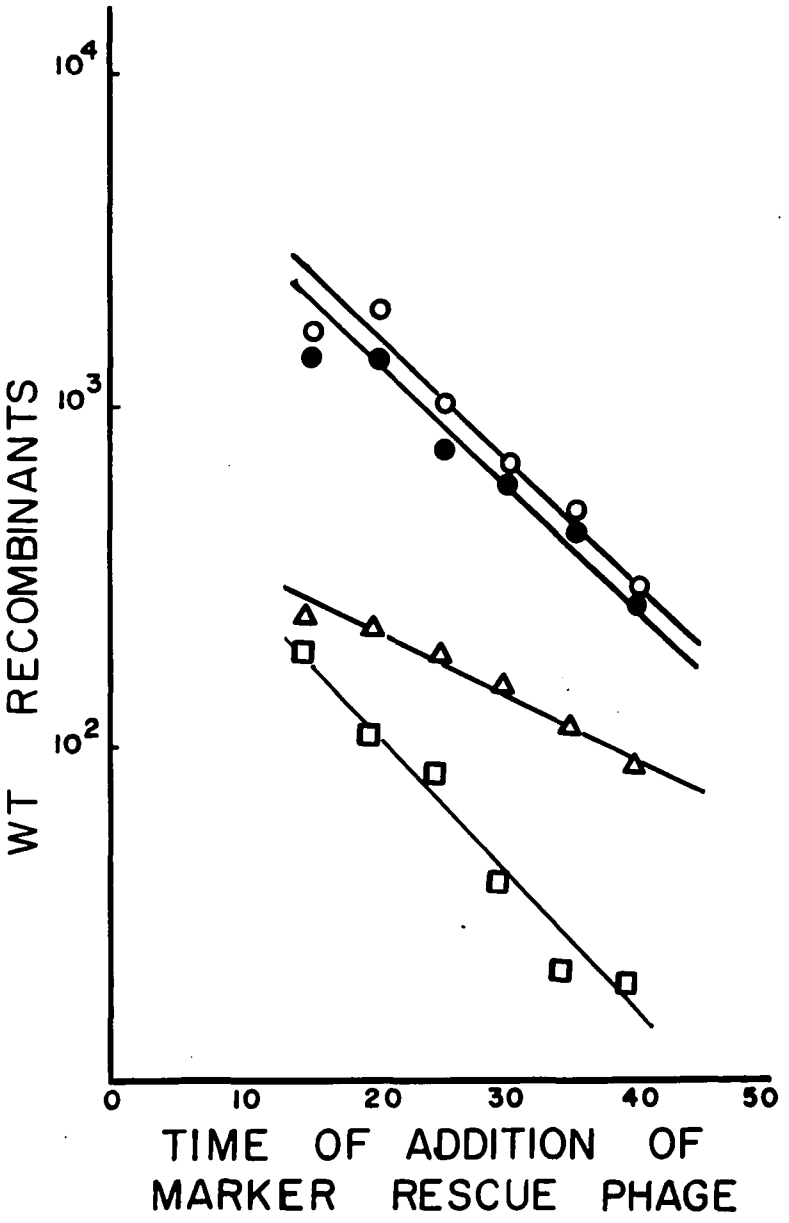

WT RECOMBINANTS
10^4
10^3
10^2
0
10
20
30
40
50
TIME OF ADDITION OF
MARKER RESCUE PHAGE

Fig. 6

Figure 7

Stability of double and single markers in preinfected cells

Competent cells were preinfected with the ts mutant phage H20-H362-H15 and exposed to H15 phage DNA as described in Figure 6. Protein synthesis in the cells was arrested either at the time of phage infection (● ■) or at six minutes thereafter (◐ ◪). Adsorption of DNA was terminated at the nineteenth minute by the addition of DNAase. At intervals thereafter the cells were superinfected with the ts mutant phage H20 (circles) or H20-H362 (squares) and plated at 47°C. The number of WT recombinants is plotted as a function of the time of addition of the superinfecting phage. Dashed lines represent the survival of markers in non-preinfected cells.

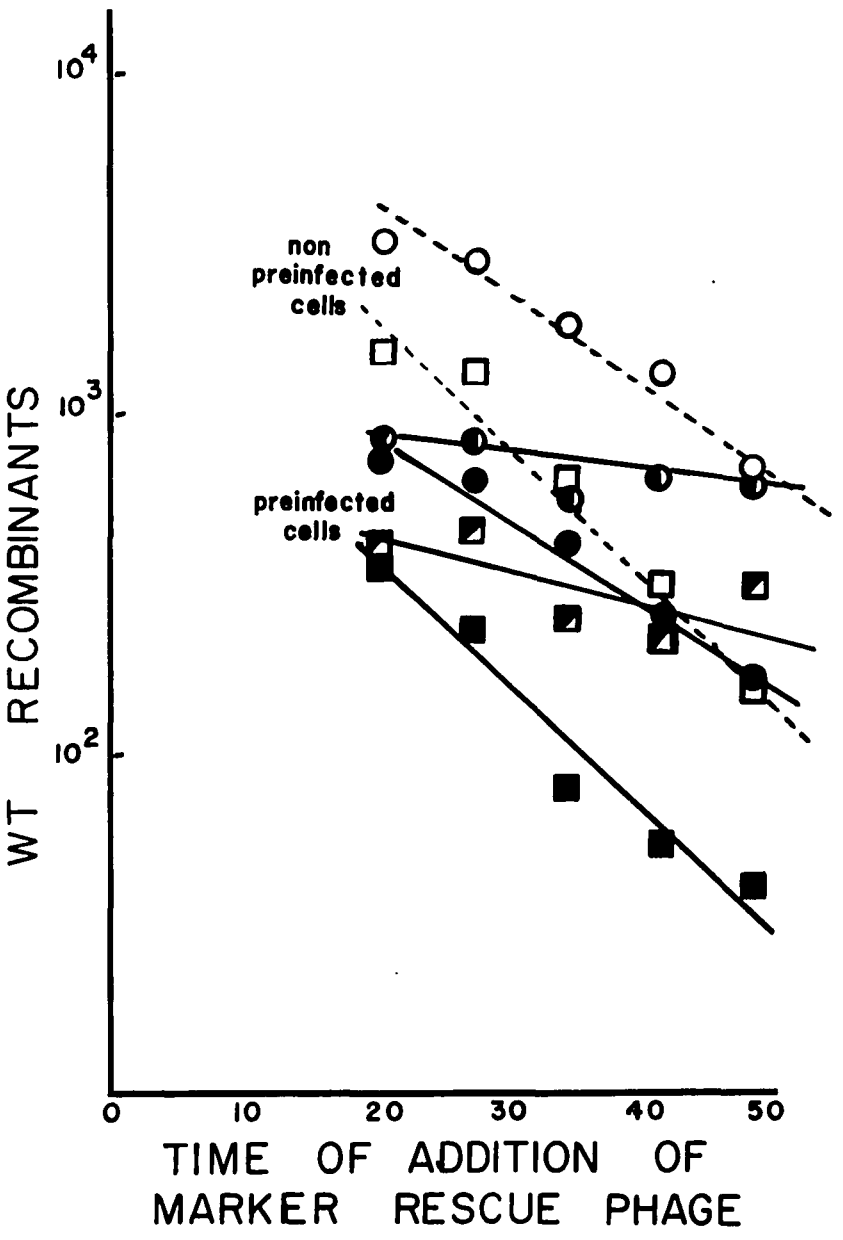

10^4
10^3
10^2
WT RECOMBINANTS
non preinfected cells
preinfected cells
0
10
20
30
40
50
TIME OF ADDITION OF MARKER RESCUE PHAGE

Fig. 7

Figure 8

Stability of genetic markers in cells in which protein synthesis was arrested at six minutes or at nine minutes after pre-infection

At time zero, competent cells were preinfected with the ts mutant phage H20-H362-H15 and exposed to H15 phage DNA as described in Fig. 6. Protein synthesis in the cells was arrested either at the sixth minute (○△) or the ninth minute (●▲) after preinfection by the addition of CM to a concentration of 100 ug/ml. At intervals the cells were superinfected with the ts mutant phage H20 or H20-H362. The number of WT recombinants is plotted as a function of the time of addition of the superinfec$_{H20-H362}$ (▲△). Dashed curves are for values obtained using H20 as the superinfecting phage (●○), solid curves for values obtained using H20-H362 (▲△).

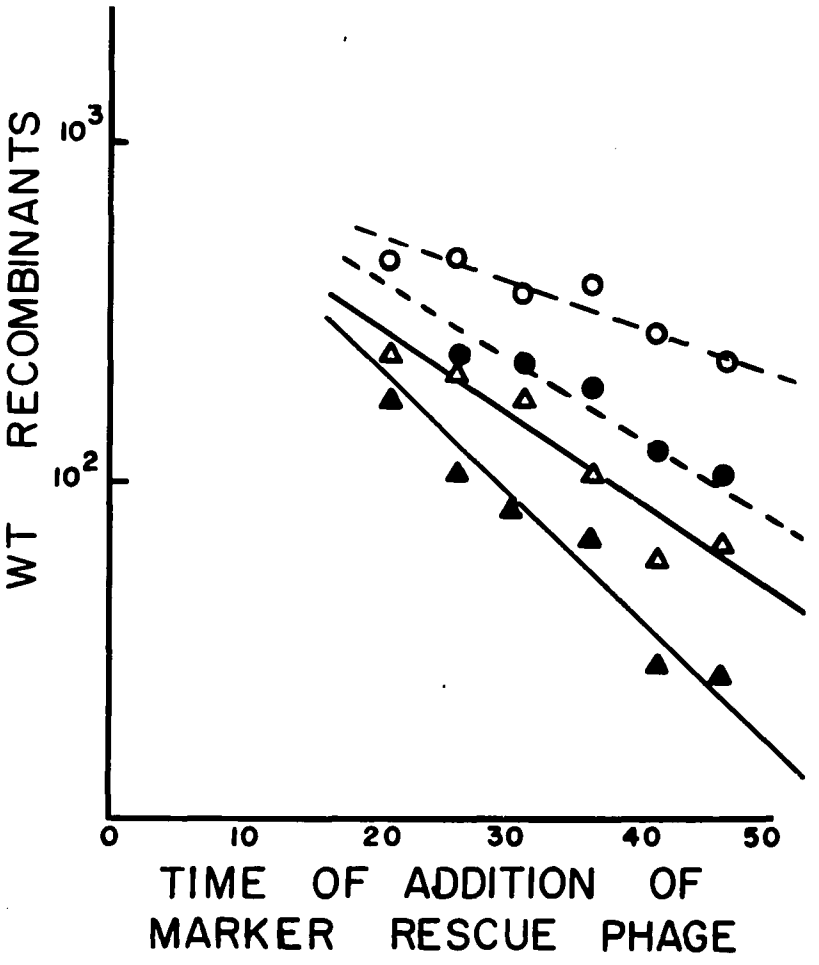

WT RECOMBINANTS
10^3
10^2
0
10
20
30
40
50
TIME OF ADDITION OF
MARKER RESCUE PHAGE

Fig. 8

The addition of chloramphenicol at the time of preinfection completely prevents modification of intracellular inactivation.

Although the maximum inhibition of intracellular inactivation is obtained when protein synthesis is arrested at the sixth minute after preinfection, there is still a slight loss of WT recombinants with time under these conditions. It is possible that continuous protein synthesis is required for the complete inhibition of intracellular inactivation. However, under preinfection marker rescue conditions (where protein synthesis is not arrested) the rescue of double mutants is lower than that of single mutants (Figure 4 and ref. 8). This suggests that even under these conditions intracellular inactivation is not completely turned off.

The reason for the decreased stability of markers in the preinfected cell when protein synthesis is interrupted at the ninth minute after infection is not clear. It may reflect the appearance in the cell of some new function which leads to the decreased rescuability of markers with time, or it may reflect the waning influence of preinfection protection. One way to view such events is that between the sixth and the ninth minute after infection the infectious center has become committed to carrying out a particular function and that the interruption of protein synthesis at this time results in the decreased rescue of markers. Various models for such a decreased rescuability could be proposed. Either the entire genetic input of the transfecting DNA is lost (through the death of the infected cell, immunity to superinfection or destruction of the entire phage genome) or, as in intracellular inactivation, only a partial inactivation of the genome occurs. If the former is the case, then all markers, single or double, should be lost at the same rate. When the stability of double and single markers in

preinfected cells was examined, however (Figure 7) it was found that double markers were lost more rapidly than single markers. Thus, as in intracellular inactivation (8), the loss of markers with time is due to a partial inactivation of the genome.

Even though the stability of markers on the infective DNA is greater in preinfected cells, the absolute recovery of WT recombinants from these cells is less than that obtained from non-preinfected cells. This is true even for cells which were preinfected in the presence of CM. One reason for this might be the presence in the preinfected cell of a large number of ts mutant genes introduced by the preinfecting phage. An increased number of defective genes in the gene pool would lower the probability of rescue of WT genes from the transfecting DNA by the superinfecting phage. Another possibility is that preinfecting phage render some portion of the cell population refractory to attack by superinfecting phage. This does not seem likely since there is no superinfection exclusion observed in the SP82G system (14) and since the same decrease is observed even when protein synthesis is inhibited at the time of addition of preinfecting phage.

Stabilization of markers after inactivation has begun

The results of the previous sections show that when infective DNA is introduced into a cell in which the intracellular inactivation process has been modified, a marked improvement in the stability of the DNA during its time of residence in the cell results. It would be of interest to know if transfecting DNA which is present in the cell before the time of phage infection can also be protected by the inhibition of intracellular inactivation. That is, once the inactivation of DNA has started, can it be stopped?

Competent cells were permitted to take up H15 phage DNA and were treated with DNAase. The cells were infected with the triply mutant phage H20-H362-H15 and were treated with chloramphenicol either immediately or at six minutes after the addition of the phage. The integrity of markers $H20^+$ and $H362^+$ introduced by the transfecting DNA was examined at intervals thereafter by superinfecting the cells with the double mutant H20-H362 and plating at 47°C. The results (Fig. 9) show that when the triply mutant phage added immediately after the termination of DNA uptake was not permitted to direct the synthesis of proteins there was a loss of rescuability of markers with time. The rate of loss was identical to that observed in cells in which intracellular inactivation had never been modified (cells which had never seen the triply mutant phage). If the triply mutant phage was permitted to synthesize protein for six minutes, however, a marked improvement in the stability of markers $H20^+$ and $H362^+$ was observed. These results indicate that the addition of phage to competent cells which have already taken up transfecting DNA results in the inhibition of intracellular inactivation in these cells. This protective mechanism affects the stability of transfecting DNA which was present in the cell before the addition of phage. In short, intracellular inactivation which is in progress may be modified by the infection of the cell with phage particles. This process can be called super-infection protection.

Sensitivity of infectious centers to chloramphenicol

After the infection of B. subtilis by bacteriophage SP82G, the injected phage DNA is not susceptible to intracellular inactivation presumably because the introduction of the phage DNA is sufficiently fast to permit the organized programming of the phage functions necessary

Figure 9

Stability of genetic markers in superinfected cells

At time zero competent cells were exposed to H15 phage DNA at a concentration of 3 ug/ml. After 13 minutes DNAase was added (to 10 ug/ml). The cells were infected with the ts mutant phage H20-H362-H15 and were treated with CM either at the time of infection (□) or six minutes thereafter (△). At intervals, samples were withdrawn and infected with the ts mutant phage H20-H362 and plated at 47°C. The number of WT recombinants is plotted as a function of the time of addition of the second (marker rescue) phage. The survival of markers H20-H362 in non-preinfected cells is also shown. (○).

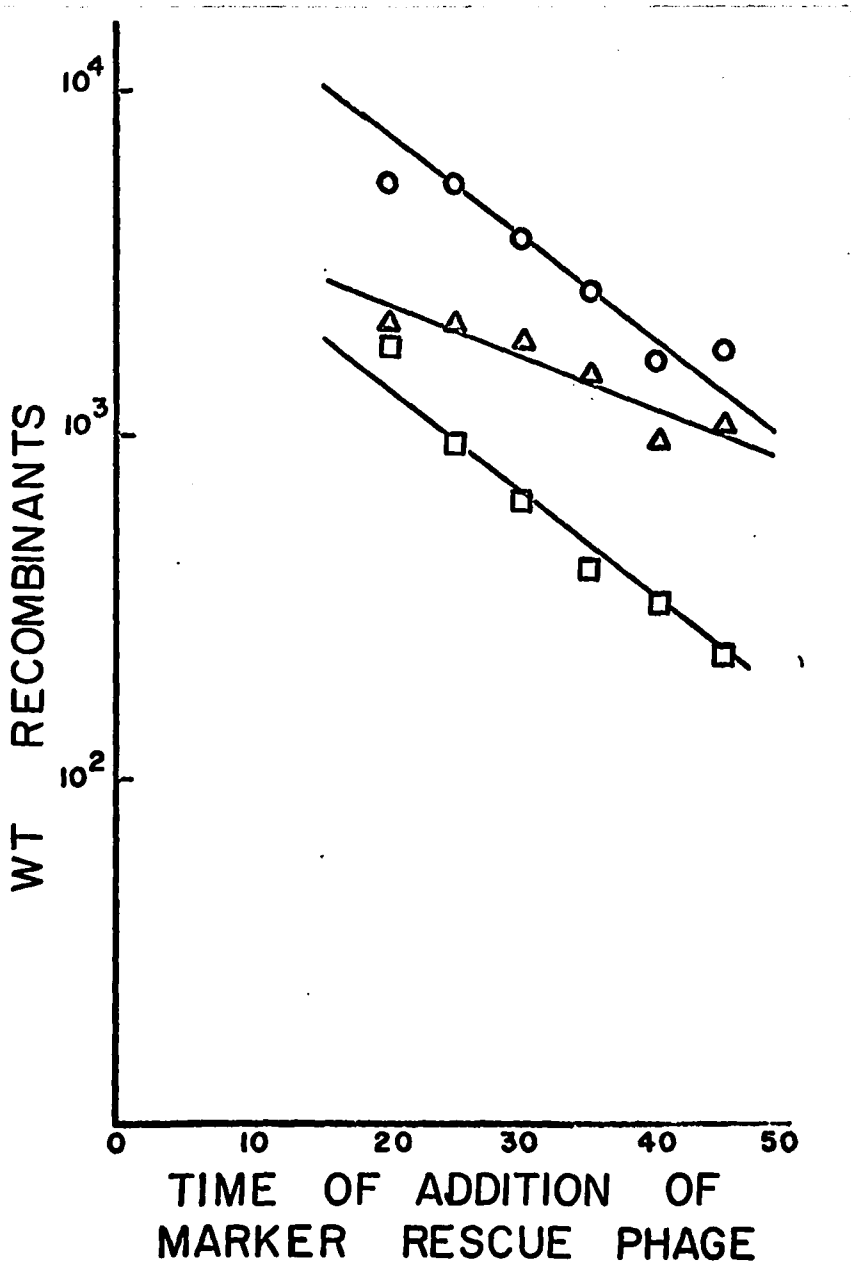

WT RECOMBINANTS
10^4
10^3
10^2
0
10
20
30
40
50
TIME OF ADDITION OF
MARKER RESCUE PHAGE

Fig. 9

to overcome the inactivation mechanism (8). Since protein synthesis is required for the modification of intracellular inactivation, the arrest of protein synthesis shortly after phage infection might result in the inactivation of injected DNA as well. If this were the case, the survival of infectious centers established by phage infection should be sensitive to the effects of chloramphenical up to the time at which the inactivation mechanism has been modified. To examine this possibility, bacteria at 10^8/ml were exposed to phage at a multiplicity of infection (MOI) of 0.1 for 15 seconds, and adsorption was terminated by the addition of antisera. At intervals, samples were removed and diluted in NM containing chloramphenicol. After 30 minutes in the presence of the inhibitor, the surviving infectious centers were assayed by dilution and plating at 33°C. The results (Fig. 10) indicate that at early times after infection, infectious centers established by SP82G are sensitive to CM, but that by the fifth to the sixth minute after infection they have become resistant to the effects of inhibition of protein synthesis.

To examine the sensitivity of infectious centers more closely, bacteria were briefly exposed to a low multiplicity of phage as before and after two minutes were diluted into NM containing chloramphenicol. After various lengths of time in the inhibitor, samples were withdrawn and assayed for surviving infectious centers (Fig. 11). Under these conditions the number of infectious centers decreased with single hit kinetics and reached an e^{-1} survival in 32 minutes. The same results were obtained if the inhibitor was added prior to phage adsorption.

While these results suggest that the inability to modify intracellular inactivation results in the inactivation of injected phage DNA, the sensitivity of infectious centers to CM might also be due to the

Figure 10

Sensitivity of infectious centers to chloramphenicol

Bacteria at 10^8/ml were exposed to phage at a MOI=0.1 for 15 seconds and the adsorption was terminated by the addition of phage specific antisera. At intervals samples were diluted into NM containing chloramphenicol (500 ug/ml) and held for 30 minutes at 33°C. The surviving infectious centers were assayed by plating at 33°C and are expressed as the percent of a control in which protein synthesis was not arrested.

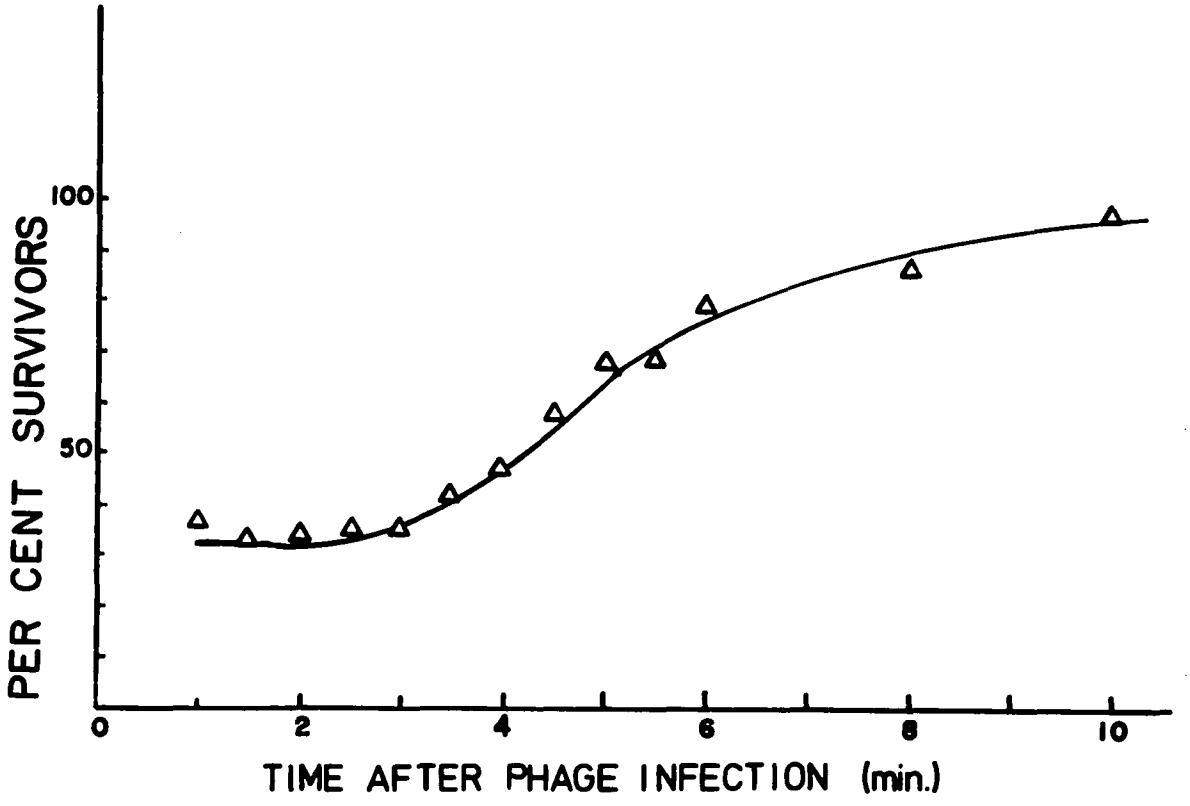

PER CENT SURVIVORS
100
50
0
2
4
6
8
10
TIME AFTER PHAGE INFECTION (min.)

Fig. 10

inability of the infected cell to repair damages caused by the phage during infection. If this were the case, then increasing the multiplicity of infection should increase the number of damages sustained by each cell, and lead to a decreased survival in chloramphenicol. On the other hand, if the intracellular inactivation of injected DNA were responsible for the loss of infectious centers in CM, then the presence of more than one phage genome in the cell should increase the chance of survival of an infectious center. For, under these conditions, undamaged portions of the phage genome could cooperate in the successful establishment of an infectious center.

Bacteria which had been multiply infected by a brief exposure to varying concentrations of phage were diluted into NM containing chloramphenicol. At intervals the survival of infectious centers was assayed by dilution and plating at 33°C. As shown in Figure 12 as the multiplicity of infection increased, the survival of the infected bacteria in CM improved. This demonstrates that the loss of infectious centers under these conditions does not result from damages in the host cell caused by phage during infection.

The increased survival of multiply infected complexes suggests that there is some form of multiplicity reactivation operating in these cells. Multiplicity reactivation was first observed by Luria (19) who found that cells multiply infected with U.V. irradiated phages showed a much greater ability to produce progeny than singly infected cells. To account for these observations a model was proposed (20) which assumed that the phage genome consisted of a number (n) of different subunits that could be inactivated independently. The inactivation of any one subunit would be lethal in a singly infected cell. However, in a multicomplex, successful infection could take place as long as a complete

Figure 11

Inactivation of infectious centers in chloramphenicol

Bacteria were briefly exposed to phage at a MOI=0.1 as in Figure 10 and after two minutes were diluted into NM containing chloramphenicol (500 ug/ml). At intervals samples were withdrawn and assayed for surviving infectious centers.

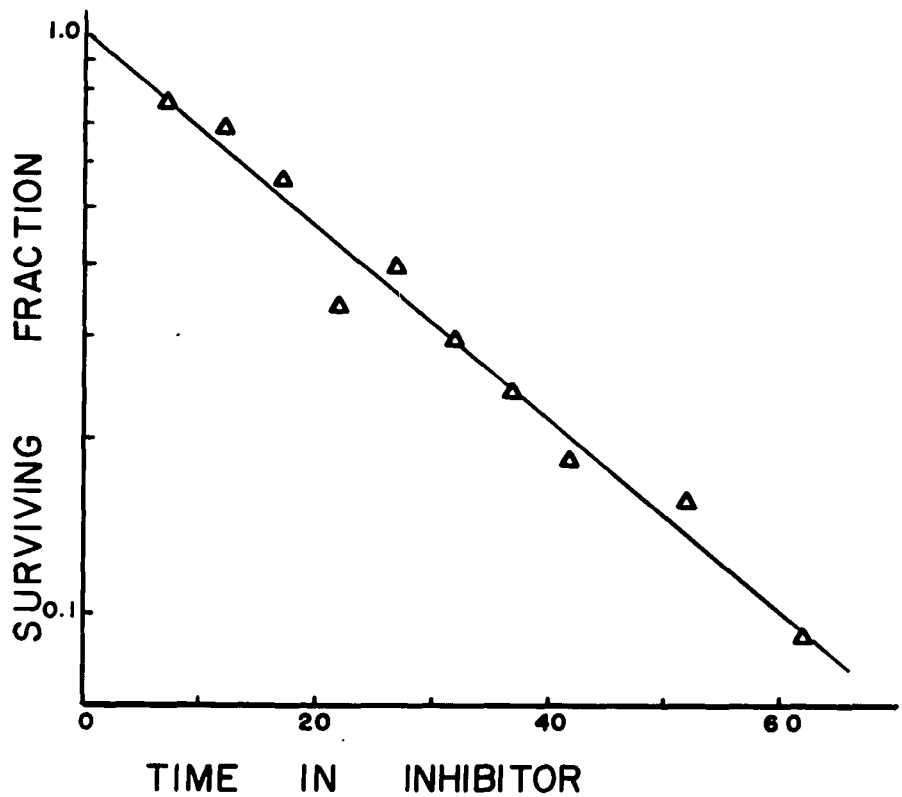


Fig. 11

Figure 12

Effect of the multiplicity of infection on the survival of infectious centers in chloramphenicol

Bacteria at 10^8/ml were briefly exposed to phage at various multiplicities and the adsorption terminated by the addition of phage specific antisera. After two minutes the samples were diluted into NM containing chloramphenicol (500 ug/ml). At intervals the survival of the infectious centers was assayed by plating. The measured multiplicities of infection were: △ 5.1, ● 3.0, □ 1.7, ○ 0.13.

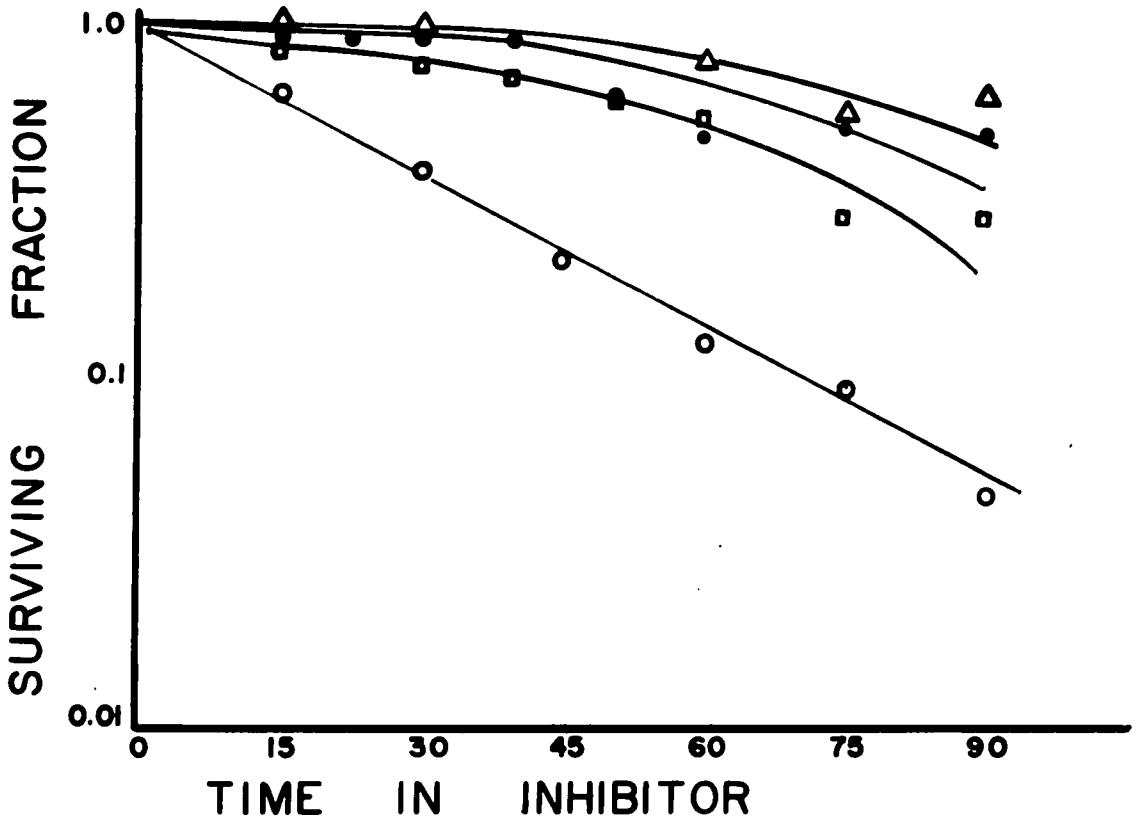

SURVIVING FRACTION
1.0
0.1
0.01
0
15
30
45
60
75
90
TIME IN INHIBITOR

Fig. 12

set of subunits could be reassembled from the undamaged subunits of the infecting phages. While this model proved to be an oversimplification (21) it does permit a rudimentary mathematical analysis of the curves presented in Figure 12.

When singly infected cells are placed in CM they are inactivated as potential infectious centers with exponential, single hit kinetics approaching e^{-1} survival in 32 minutes. Their survival function is thus given by

$$e^{-kt}$$

where t is the time in CM and k is the inactivation constant. This gives a value for k of 0.0313 and an inactivation function of

$$1 - e^{-0.0313t}$$

If the phage genome is composed of n subunits of equal sensitivity the inactivation function for one of these is

$$1 - e^{-kt/n}$$

and thus in complexes infected with exactly K phages the probability that at least one copy of a given subunit remains intact is

$$1 - (1 - e^{-kt/n})^K$$

The probability that one of <u>each</u> subunits is intact is given by

$$[1 - (1 - e^{-kt/n})^K]^n$$

When the observed (average) multiplicity of infection is m, the probability of a host cell becoming infected with exactly K phages is

$$p(K) = \frac{m^K e^{-m}}{K!}$$

From this, the fraction of surviving infectious centers relative to the total number of bacteria is

$$\sum_{K=1}^{\infty} \frac{m^K e^{-m}}{K!} \quad [1 - (1 - e^{-kt/n})^K]^n$$

and the fraction of surviving infectious centers relative to the total

number of infected bacteria is

$$\sum_{K=1}^{\infty} \frac{\frac{m^K e^{-m}}{K!} \left[1 - (1 - e^{-kt/n})^K\right]^n}{1 - e^{-m}}$$

This formula provides a theoretical expectation for the experimental observation S/S_o or the surviving fraction of infectious centers for given values of m, k and n. The value of k is known (0.0313) and m is the measured multiplicity of infection. The predicted curves for the survival of infectious centers, which are depicted in Figure 13, were generated by assigning values of n from 1 to 5 to the function above. The best fit to the data presented in Figure 12 was obtained for values of n from 3 to 5.

The true significance of curves obtained in this manner is unclear. The formulas presented above are at best a crude mathematical representation of the complex events taking place within the cell. Certain tentative conclusions can be drawn however. The value of n is assumed to represent the number of equally sensitive subunits that may be inactivated independently. The observation that n>1 indicates that the events leading to the loss of infectious centers are the result of a partial inactivation of the genome, and are not due to the inactivation of the entire genome (i.e. there is more than one sensitive unit in the genome). This is consistent with the observation by Green (8) that intracellular inactivation is due to the partial inactivation of the phage genome and suggests that events occuring in CM treated infectious centers are not dissimilar.

Luria and Dulbecco (20) found that values of n=15 to 30 provided the best fit for data obtained in a study of the multiplicity reactivation of U.V. irradiated T4. There are about 24 linkage groups in the T4 genome and Green (personal communication) has estimated that there

Figure 13

Predicted and observed survival of multicomplexes in chloramphenicol

The predicted survival of infectious centers at the average multiplicities of infection shown here were calculated as described in the text. The theoretical number of sensitive subunits in the genome is denoted by n. The observed survival of multicomplexes (O) are from the experiment described in Figure 12.

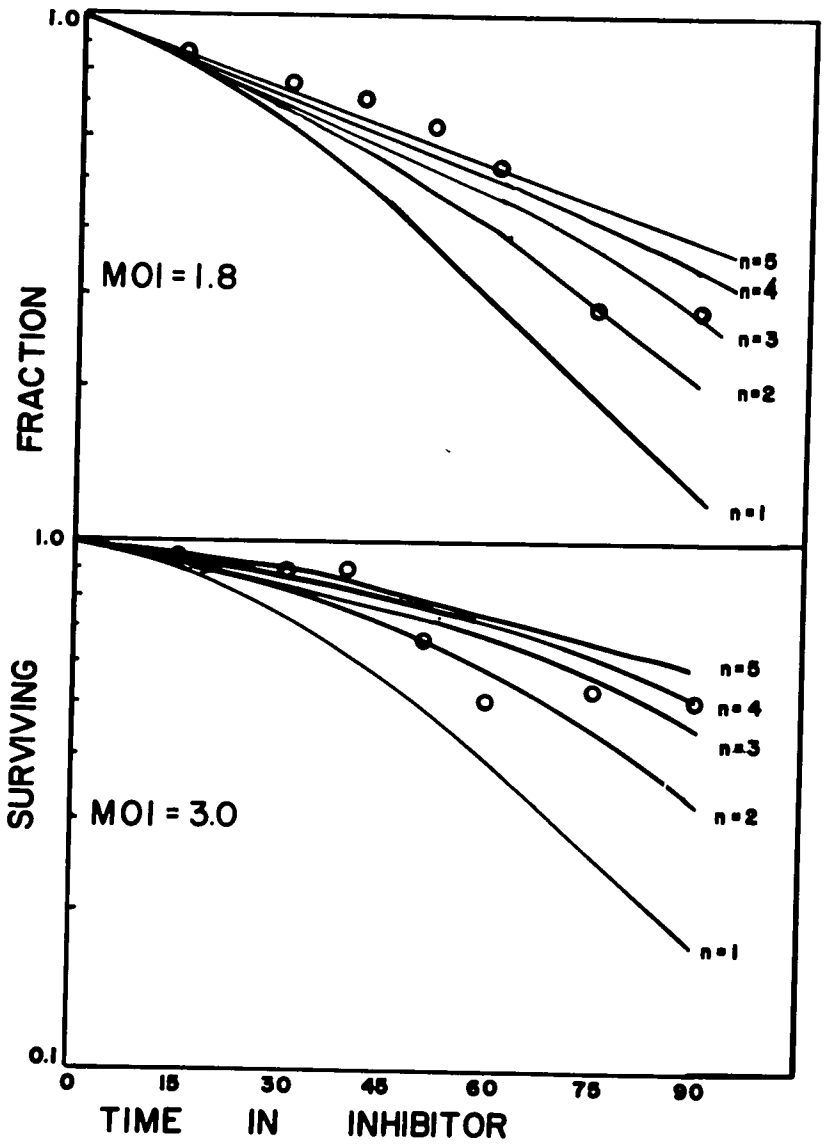

1.0
FRACTION
SURVIVING
MOI = 1.8
n=5
n=4
n=3
n=2
n=1
1.0
MOI = 3.0
n=5
n=4
n=3
n=2
n=1
0.1
0
15
30
45
60
75
90
TIME IN INHIBITOR

Fig. 13

are about 4 linkage groups in the SP82G genome. The similarities of these values to the values obtained for n suggest that perhaps for multiplicity reactivation to occur the inactivated portions of the genome must be randomly assorted.

Discussion

The experiments described here demonstrate that the modification of the intracellular inactivation phenomenon which takes place after phage infection is due to the synthesis and accumulation of a protein in the infected cell. While it is not proven that this is a phage specific protein, it is highly unlikely that it is a host enzyme. Aside from the obvious observation that it would be improbable for the host to countermand its own cellular defense mechanisms, it is known that infection of B. subtilis with virulent bacteriophage leads to a sharp reduction in host protein synthesis (23, 24). For these reasons I conclude that the modification of intracellular inactivation is due to the expression of an early (before 6 minutes) phage function.

The question as to why infecting phage are able to overcome the inactivation mechanism while transfecting DNA is not, is not answered in this study. Green (8) has suggested as one possibility that the rapid entry of phage DNA during phage infection permits a more organized programming of the necessary phage functions. This possibility and others are examined in a later part of this thesis.

If the phage is prevented from carrying out the modification function, its DNA is susceptible to a similar type of inactivation phenomenon. However, the sensitivity of injected DNA appears to be less than that of transfecting DNA. Green (8) has estimated that the rate of inactivation of a transfecting DNA molecule is about 0.3 lethal events/minute/ 10^8 daltons of DNA. This would result in an e^{-1} survival of the phage genome in about 3 minutes. In contrast, infectious centers resulting from singly infected bacteria attain an e^{-1} survival in about 32 minutes

when held in CM. Since phage may infect cells which are not in a state of competency these very different sensitivities might be due to a much lower activity of the inactivation enzyme in non-competent cells. On the other hand, if the activity of the enzyme were the same in both cells, the different sensitivities might reflect structural differences in the DNA substrates as a result of their mode of entry, or might reflect differences in the mode of entry itself (e.g. the physical location of the DNA after its introduction into the cell). Some of these possibilities will be explored in the next section of this thesis.

As a brief summary of the observations made here, the following conclusions were reached.

1. Preinfection protection is the result of the expression of an early phage function which inhibits intracellular inactivation.
2. This function is maximally expressed by the sixth minute after infection and is completely prevented by the addition of chloramphenicol at the time of infection. However, continued protein synthesis is not a requirement for the inhibition of intracellular inactivation.
3. The inhibition of intracellular inactivation leads to an increased stability of transfecting DNA during its time of residence in the cell. This protective effect extends even to transfecting DNA which was present in the cell before phage infection began.
4. If an infecting phage is prevented from carrying out the early protective function by the addition of CM at the time of infection, the injected DNA is susceptible to a similar type of inactivation phenomenon.

References

1. Spizizen, J., Genetic activity of deoxyribonucleic acid in the reconstitution of biosynthetic pathways. Fed. Proc. 18, 957 (1959).
2. Földes, J. and Trautner, T.A., Infectious DNA from a newly isolated B. subtilis phage. Z. Vererb., 95, 57 (1964).
3. Okubo, S. and Romig, W.R., Comparison of Ultraviolet Sensitivity of B. subtilis bacteriophage SP02 and its infectious DNA. J. Mol. Biol. 14, (1965).
4. Reilly, W. and Spizizen, J., Bacteriophage deoxyribonucleate infection of competent B. subtilis. J. Bact. 89, 782 (1965).
5. Green, D.M., Infectivity of DNA isolated from B. subtilis bacteriophage SP82. J. Mol. Biol. 10, 438 (1964).
6. Romig, W.R., Infection of B. subtilis with phenol extracted bacteriophages. Virology, 16, 452 (1962).
7. Okubo, S., Strauss, B. and Stodolsky, M., The possible role of recombination in the infection of competent B. subtilis by bacteriophage deoxyribonucleic acid. Virology, 24, 552 (1964).
8. Green, D.M., Intracellular inactivation of infective SP82 bacteriophage DNA. J. Mol. Biol., 22, 1 (1966).
9. Green, D.M., Gene dislinkage in transfection of SP82G phage DNA. Genetics, 60, 673 (1968).
10. Epstein, H.T. and Mahler, I., Mechanisms of enhancement of SP82 transfection. J. Virol., 2, 710 (1968).
11. Epstein, H.T., Transfection enchancement by ultraviolet light. Biochem. Biophys. Res. Comm., 27, 258 (1967).
12. Chen, K. and Ravin, A.W., Mechanism of the deoxyribonucleic acid

helping effect during transformation. J. Mol. Biol., 33, 873 (1968).

13. Nester, E.W., Schafer, M. and Lederberg, J., Gene linkage in DNA transfer in a cluster of genes concerned with aromatic biosynthesis in B. subtilis. Genetics, 48, (1963).

14. Kahan, E., A genetic study of temperature sensitive mutants of the subtilis phage SP82. Virology 30, 650 (1966).

15. Nomura, M.K., Matsubara, K., Okamoto, K. and Fujimara, R., Inhibition of host nucleic acid and protein synthesis by bacteriophage T4: Its relation to the physical and functional integrity of the host chromosome. J. Mol. Biol., 5, 535 (1962).

16. Mandell, J.D. and Hershey, A.D., A fractionating column for analysis of nucleic acids. Anal. Biochem., 1, 66 (1960).

17. Levinthal, C., Messenger RNA turnover and protein biosynthesis in B. subtilis inhibited by Actinomycin D. Proc. Nat. Acad. Sci. (U.S.), 48, 1,631 (1962).

18. Miller, I.L., Zsigray, R.M. and Landman, O., The formation of protoplasts and quasi-spheroplasts in normal and chloramphenicol preinfected B. subtilis. J. Gen. Microbiol., 49, 513 (1967).

19. Luria, S., Reactivation of irradiated bacteriophage by transfer of self reproducing units. Proc. Nat. Acad. Sci. (U.S.), 33, 253 (1947).

20. Luria, S. and Dulbecco, R., Genetic recombinations leading to production of active bacteriophage from ultraviolet inactivated bacteriophage particles. Genetics, 34, 93 (1949).

21. Dulbecco, R., A critical test for the recombination theory of multiplicity reactivation. J. Bact., 63, 199 (1952).

22. Osowitz, B.M., Of sterile origin. (submitted for publication).

23. Pene, J., Host macromolecular synthesis in bacteriophage infected B. subtilis. Bact. Rev., 32, 379 (1968).

24. Levinthal, C., Hosoda, J. and Shub, D., The control of protein synthesis after phage infection. In The Molecular Biology of Viruses (J.S. Colter and W. Paranchych, eds.) Academic Press, N.Y., pp. 71-91 (1967).

PART II

THE KINETICS OF ENTRY OF DNA DURING SP82G BACTERIOPHAGE INFECTION

Introduction

After normal infection of B. subtilis by bacteriophage SP82G the injected phage DNA is not susceptible to intracellular inactivation (1, part I of this thesis). Green (1) has suggested as one possible explanation that injected DNA (as opposed to transfecting DNA) escapes the inactivation process because the introduction of phage DNA into the cell is sufficiently fast to permit the organized programming of the required phage functions. The introduction of markers on transfecting DNA, measured by the time of their acquisition of DNAase resistance, requires about 8 minutes for the slowest marker (1) while the establishment of a DNAase resistant infectious center by transfecting DNA requires 13 minutes (2). Since protection against intracellular inactivation (pre-infection protection) is set up by the sixth minute after phage infection (1, part I of this thesis) the above hypothesis has obvious merit.

The rapid entry of the DNA molecule might not be the only requirement for the successful infection of the cell, however. The order of entry of genes on the DNA might also play an important role in determining the proper programming of phage functions. Various investigators have studied the entry of genes on transforming DNA by examining the time at which genetic markers become resistant to the effects of DNAase and have provided evidence that markers enter in a sequential pattern. Strauss has found that during the uptake of transforming DNA by competent

B. subtilis there is a correlation between the intermarker map distance and the length of time required for pairs of genetic markers to acquire DNAase resistance (3, 4). This indicated a linear mode of entry of the DNA molecule and permitted an estimate of the rate of uptake of DNA of approximately 55 base pairs per second at 28°C. In the Pneumoccal transformation system, Gabor and Hotchkiss (5) have found that three different markers become DNAase resistant in the same order as their positions on the genetic map, suggesting that in this case the uptake of DNA is not only linear but also proceeds with a definite polarity.

The DNA of bacteriophage SP82G has a linear, non-permuted structure which is co-linear with the genetic map (6). An analysis of the function of temperature sensitive (ts) mutants has shown that there is good correlation between the time of function of a gene and its map position. The known markers at the left end of the map are largely concerned with early phage functions, while those at the right end are concerned with late functions (7; Laman and Green, personal communication). Green (personal communication) has examined the time of acquisition of DNAase resistance by markers on transfecting SP82G DNA and found that there is a linear and polar entry of the molecule. Those markers on the right end of the genetic map become DNAase resistant before those on the left end of the map. Thus, markers concerned with early phage functions enter the cell last. This suggests that the proper programming of the cell might require that all genetic information be introduced into the cell before any phage functions could be expressed. Alegria (8) has also reported a polar entry of markers on the SP82G genome but concluded that those on the left end of the map are present in the transfected cell with a higher frequency at early times than markers on the right end of the map. The validity of the

latter results is questionable however, since the markers were rescued 45 minutes after the addition of the phage DNA and at this late time the frequency of markers present could also reflect the participation of some other selective phenomenon.

In light of the above discussion, it was considered appropriate to study the kinetics of transfer of DNA from the bacteriophage to its host during the course of infection (injection), and to compare the events occurring during this mode of transfer with those occurring during transfection and transformation.

The transfer of DNA from phage to host was first examined by Hershey and Chase in their classical "blendor experiment" (9). These investigations demonstrated that after bacteriophage T2 had infected its host, the protein envelope of the phage particle remained at the cell surface where it could be removed by the shearing forces generated in a blendor. However, the phage DNA was transferred to the cell and could no longer be removed by blending. This experiment offered one of the first proofs that DNA was the carrier of genetic information and since that time, very little investigation of the events occurring immediately after the adsorption of the bacteriophage to the cell has been done. Lanni, investigating the infection of E. coli by bacteriophage T5, found that the transfer of phage DNA proceeded in two steps. About 8% of the phage genome (the first step transfer or FST-DNA) entered the cell first, (10, 11) and only after protein synthesis directed by markers on the FST-DNA was the remainder of the genome transferred (12). This mode of infection would have obvious advantages in the SP82G system, for the transfer of the majority of the genome might not occur until the proper conditions for its survival had been arranged.

The fate of the DNA after its transfer from the phage to a

blendor resistant state is not clear. Elegant electron microscopy by Simon and Anderson (13) has suggested that the tail cores of bacteriophage T2 and T4 penetrate the cell wall of E. coli but do not penetrate the cell membrane. This could mean that phage DNA is injected into the periplasmic space between the cell wall and the cell membrane and would then have at least one of the barriers to complete penetration in common with transfecting DNA. On the other hand, Bayer (14) has presented equally compelling electron micrographs showing that during infection of E. coli by bacteriophage T3 and T5 the phage are adsorbed almost exclusively to areas where the cell membrane is attached to the cell wall. This would suggest that at least for these phage, the receptor sites for phage adsorption are arranged so that the phage DNA is injected closest to the protoplasmic contents of the cell.

Similarly, the location of transfecting (or transforming) DNA after it has attained DNAase resistance is not clear. Some evidence has been presented that the DNA spends at least some of its time exterior to the cell membrane. For example, even after transforming DNA has become resistant to DNAase, transformation can still be inhibited by antisera prepared against single stranded DNA (15, 16). This presumably occurs because the donor molecules have combined with sites on the surface of the plasma membrane where they are insensitive to DNAase but still accessible to antisera. In support of this it was observed that after solubilization of the cell wall with lysozyme the majority of the DNA (about 67%) and the anti-DNA antisera were still associated with the cell in a lysozyme resistant state (17).

Using more rigorous lysozyme treatment, Miller and his associates found that practically all of the transforming DNA that was associated with the cell in a DNAase resistant state could be removed (18). If the

cell walls of competent B. subtilis were removed 20 minutes after the addition of transforming DNA, an 80% loss of transformants resulted, even though the resulting protoplasts show 90-100% survival as L-colonies (19). If the removal of the cell wall was delayed for 120 minutes, however, no great loss in transformants was observed. While these results do not necessarily prove that the DNA is located in the periplasmic space, they do show that the DNA is held outside the cell membrane for a considerable time.

If both injected and transfecting DNA share this fate, it would seem that the main benefits which would accrue to injected DNA would be a safe physical passage through the cell wall. The physical condition of the injected DNA, or its location in the cell (near a DNA uptake site in the cell membrane) might then account for its more efficient transport across the final barrier. An estimate of the time of residence in this space has not been made in either case. However, the rapid and early transfer of genes which are necessary for the proper programming of the cell would obviously give injected DNA a distinct advantage in decreasing its time of residence in an unfavorable environment.

Since the DNA of SP82G has a linear, non-permuted structure which is co-linear with the genetic map, (6) it was reasonable to expect that this phage might transfer its DNA in a unique manner during infection. In the experiments described below, the kinetics of entry of markers along the entire phage genome were examined by interrupting the transfer of DNA at intervals by chilling and removing the untransferred DNA by blending. The fact that there is no super-infection exclusion phenomenon in this system (7) made it possible to assay for the presence of genetic markers in the blended complexes by a super-infection marker rescue technique. The results show that during phage infection the genome of SP82G

is transferred much more rapidly than transfecting DNA, and in the opposite direction. In addition, it was observed that transfer of the DNA occurs in the absence of protein synthesis, and that, unlike transfecting DNA, practically all of the injected DNA is bound in a lysozyme resistant state shortly after infection.

Materials and Methods

Bacterial strains, growth conditions and phage preparations

Bacillus subtilis strain SB-1 was the host cell for all experiments. Techniques for the isolation, propagation, and assay of bacteriophage SP82G are identical to those of Green (1). Nomura salts media (NM) supplemented with 0.5% glucose, 0.2% casein hydrolysate, 2.5 X 10^{-3} M MgCl, 0.1% yeast extract, 0.05 mg/ml of DL-tryptophan, 4 mg/ml of arginine, and 0.2 mg/ml L-histidine was used for growth of phage (20).

Procedures for preparing bacteriophage DNA and for obtaining competent cells have been described before (part I of this thesis) and are essentially those of Green (1). The techniques used to determine competence by transfection and pre-infection marker rescue have also been described before (1, part I of this thesis).

Radioactively labelled phage were obtained by high-low centrifugation of appropriate lysates. For ^{32}P-labelled phage, $H_3{}^{32}PO_4$ was added to the growth media at 5 μc/ml along with the phage inoculum. For ^{14}C-labelled cultures, lysates were grown on a minimal medium similar to NM but having no casein hydrolysate and only one-quarter the usual amounts of DL-tryptophan, L-histidine, and yeast extract. ^{14}C-lysine was added to the cultures to a final concentration of 0.01 μc/ml 25 minutes after the addition of phage. Lysates were further purified on a preformed CsCl gradient extending from 1.2 to 1.7g/cm^3. Under these conditions, only 10% of the label appeared in the DNA of the phage as measured by phenol extraction (21).

Blendor experiments

To synchronize as nearly as possible the infection of a

bacterial culture, bacteria were concentrated to $4x10^9$ cells/ml and were only briefly exposed to bacteriophage. The measured multiplicity of infection (MOI) was 7 to 8 for radioisotope experiments, and 0.1 for marker entry studies. Adsorption was terminated either by dilution and centrifugation away from unadsorbed phage (radioisotope experiments) or by a 45-sec exposure to phage-specific antisera sufficient to inactivate 99.9% of the unadsorbed phage (marker entry studies). After adsorption, the infected bacteria were diluted in NM at the appropriate temperature to 10^8 cells/ml. After a suitable holding period in the warm growth media, samples were removed and rapidly chilled by dilution into equal amounts of cold NM in an ice bath. Samples of 20 ml were blended in an ice-jacketed Omni-Mixer (Ivan Sorvall, Inc., Norwalk, Conn.) at 11,500 rev/min. The duration of blending was 1.5 min. unless otherwise specified. The blended samples were assayed for surviving infectious centers, and then spun at 7,000 X g. Bacterial pellets were resuspended in water, transferred to planchets, and dried, and their radioactivity was assayed either in a gas-flow detector (nuclear-Chicago Corp., Des Plaines, Ill.) or by a liquid scintillation counter (Omniflour Toluene counting solution, New England Nuclear Corp., Boston, Mass.; Nuclear Chicago scintillation detector, Nuclear Chicago, Des Plaines, Ill.).

Renografin gradients

Renografin-76 (methyl-glucamine N,N'-diacetyl-3,5-diamino-2,4,6-triiodobenzoate, E.R. Squibb and Sons, New York) was obtained as a 76% solution containing in addition, 0.32% sodium citrate, 0.04% EDTA, 0.01% methyl-p-hydroxybenzoate and 0.03% propyl-p-hydroxybenzoate. This solution was diluted with water to give solutions of the appropriate densities, determined by their refractive index at 25°C using a Bausch &

Lomb refractometer. To prepare a linear gradient, nine 0.5 ml portions of solutions ranging in density from 1.12 to 1.16 g/cc. were carefully layered in a Siliclad (Clay Adams, Parsippany, N.J.) cellulose nitrate tube (Beckman Instruments, Palo Alto, Calif.). Samples were applied to this gradient, centrifuged at 20°C in a Spinco SW65 rotor, and collected as described in the text.

Lysozyme experiments

Bacteria concentrated to $4x10^9$ cells/ml were exposed to radioactively labelled phage at a MOI = 1 for 2 minutes, diluted 1:40 into NM at 33°C and held for an additional 6 minutes. Chloramphenicol (100 ug/ml) or sodiumcyanide (0.005M) was added to the cells either prior to the addition of phage, or at the time of dilution. Thereafter, all operations were carried out in the presence of the inhibitor. The diluted cells were washed twice by centrifugation at room temperature and resuspended in 10 ml of warm media. The cells were finally resuspended in isotonic NM containing sucrose (0.5M). Lysozyme (Sigma, St. Louis, Mo.) was added to a concentration of 200 ug/ml and the mixture incubated at 37°C for 20 minutes. In some cases DNAase (beef pancreas, Miles Laboratories, Elkhart, Ind.) was added to a concentration of 10 ug/ml. The protoplasts were separated by centrifugation (12,000 x g for 10 minutes) and the supernatant and pellet were assayed for ^{32}P activity.

In experiments involving competent cells, preinfected or non-preinfected cells (part I of this thesis) were exposed to phage DNA at a concentration of 0.2 ug/ml at 33°C. After 20 minutes, DNAase (to 10 ug/ml) was added and the cells were held for an additional 5 minutes. The cells were washed thoroughly, treated with lysozyme and DNAase, and examined for ^{32}P activity associated with the protoplasts as

described above. Chloramphenicol was added to the cells as described in the text.

Marker rescue studies

Temperature-sensitive (ts) mutants of SP82G which will grow at 33°C but not at 47°C were originally isolated and described by Kahan (7); other ts mutants were subsequently isolated by D.M. Green (1, personal communication). In marker rescue experiments, a sample of a temperature-sensitive mutant phage was rapidly adsorbed to bacteria for 45 seconds, and adsorption was terminated by antisera. The cells were diluted into growth media at the appropriate temperature, held for various lengths of time and exposed to the chilling and blending regimen described above. Subsequently, portions of blended samples and non-blended controls were exposed to a different ts mutant at a MOI of 15 to 20 for 4 minutes. Suitable dilutions were plated at 47°C to determine the frequency of wild-type recombinants.

Results

Effect of blending

To determine whether phage protein could be removed from the surface of the bacterium and separated from its DNA after bacteriophage SP82G infection, a blendor experiment similar to that of Hershey and Chase (9) was performed. Concentrated bacteria were exposed to radioactively labelled phage, spun free of unadsorbed phage and resuspended in growth media (NM) at 33°C. After 12 minutes the mixture was chilled and then blended for various lengths of time. Samples were assayed for surviving bacteria and for radioisotope still associated with the bacteria after centrifugation (Figure 14). Blending had little effect upon the survival of bacteria, infected or uninfected. However, free phage were quite sensitive to the effects of blending and were reduced to 30% survival in two minutes. While no more than 22% of the ^{32}P could be removed from the bacteria, up to 50% of the ^{14}C could be removed by blending (approximately 90% of the ^{14}C label was in the protein of the phage). This demonstrates the transfer of DNA from phage to host during infection.

Effect of chilling on DNA transfer

If the transfer of the phage genome were interrupted and the phage was sheared from the surface of the bacterium by blending before the transfer of its DNA to the host was completed, the resulting complex should not be able to give rise to an infectious center. The effect of chilling on blendor sensitivity was examined by chilling the phage-host

complexes at early and late times after adsorption, and then blending (Table 1). Chilling at an early time after adsorption resulted in a phage-host complex of low blendor resistance, but complexes chilled at later times were resistant to blending. When the sensitive complexes chilled at an early time were reheated for a further 10 minutes, the high blendor resistance characteristic of complexes chilled at later times was acheived.

These results indicate that chilling causes an interruption in the transfer of DNA and that any DNA which has not been transferred by the time of chilling is removed during the blending operation. The transfer of DNA in the chilled unblended complexes is resumed when they are warmed to 33°C. This interpretation would predict that the process of DNA transfer could be interrupted at any time by chilling, and the untransferred DNA removed by blending. This was tested by monitoring the transfer of ^{32}P from the phage to host at various times after adsorption, and by assaying for the viability of the blended complexes as infectious centers. The results of this experiment are shown in Figure 15.

The shapes of the curves for ^{32}P transfer and blendor resistance are different, but reach saturation levels at the same time. The ^{32}P transfer curve is consistent with the prediction that by chilling and blending the complexes at various times after adsorption, the transfer of DNA may be interrupted at any point. The delayed appearance of blendor resistant infectious centers relative to the ^{32}P uptake is expected. For, although transfer starts at an early time after adsorption and proceeds towards saturation, the first appearance of blendor resistant infectious centers in the population can occur only when transfer is complete in any given complex. The transfer of DNA in the

Figure 14

The effects of blending

Bacteria at 4 x 10^9 cells/ml were exposed to radioactively labelled phage for 2 minutes and diluted 1:100. Unadsorbed phage were removed by centrifugation and the infected bacteria were resuspended in NM at 33°C to 10^8/ml. After 12 minutes the suspension was chilled and aliquots were blended for the lengths of time indicated. Samples were assayed for surviving infectious centers (■) uninfected bacteria (○) and free phage (□) and for the amount of radioactivity still associated with the bacterial pellet after spinning at 7,000 x g for 10 minutes. (● ^{14}C labelled phage, ▲ ^{32}P labelled phage).

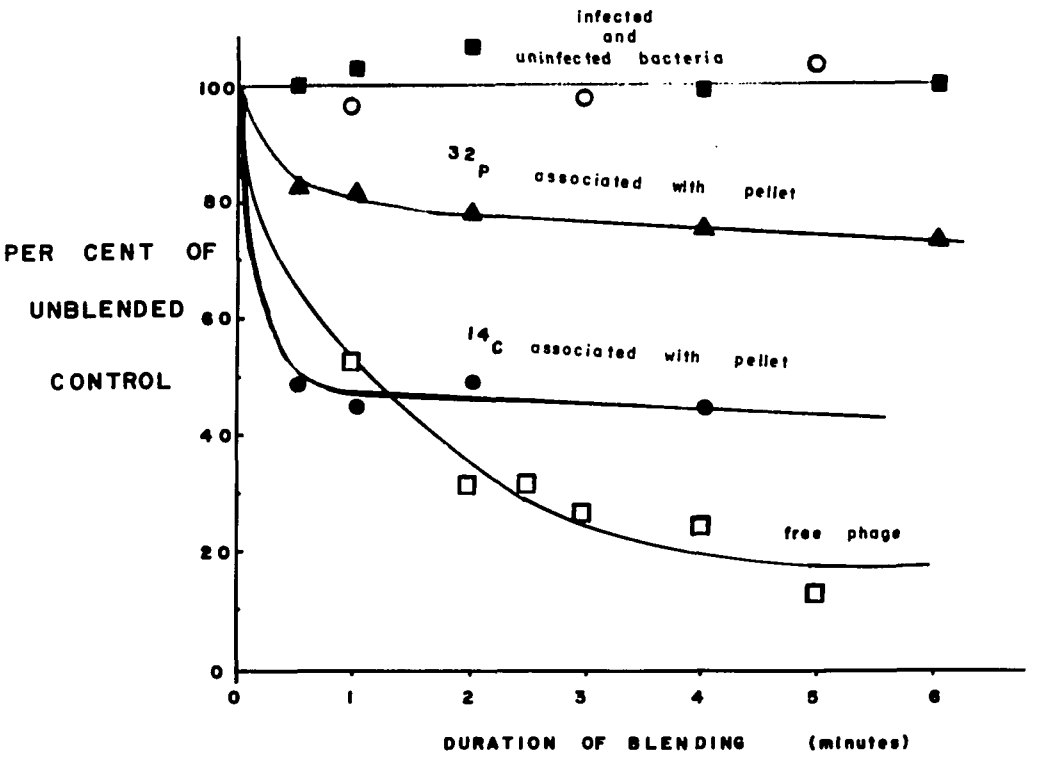

infected and uninfected bacteria
32P associated with pellet
14C associated with pellet
free phage
PER CENT OF UNBLENDED CONTROL
100
80
60
40
20
0
0
1
2
3
4
5
6
DURATION OF BLENDING (minutes)

Fig. 14

Table 1. Effect of chilling on blendor sensitivity.

TIME OF CHILLING AFTER PHAGE ADSORPTION	PER CENT OF INFECTIOUS CENTERS RESISTANT TO BLENDING	
	Chilled to 15°C, blended.	Reheated to 33°C for 10 minutes, chilled, blended.
2 min.	2%	100%
10 min.	85%	80%

Figure 15

Transfer of DNA and blendor resistance

Concentrated bacteria were exposed to radioactively labelled phage for 45 seconds. Adsorption was terminated by a 45 second exposure to antisera, and the bacteria were diluted to 10^8/ml in NM at 33°C. At the times indicated aliquots were removed, chilled and blended. Samples were assayed for surviving infectious centers and the amount of radioactivity still associated with the bacterial pellet after centrifugation.

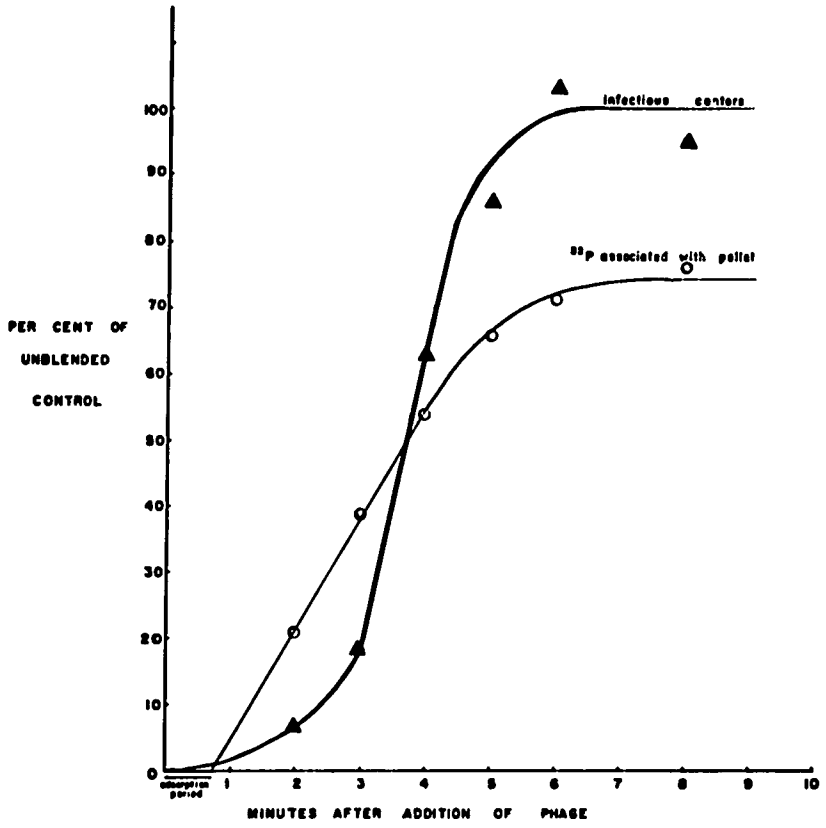

PER CENT OF
UNBLENDED
CONTROL
100
90
80
70
60
50
40
30
20
10
0
infectious centers
^{32}P associated with pellet
1
2
3
4
5
6
7
8
9
10
MINUTES AFTER ADDITION OF PHAGE

Fig. 15

population is complete by about 6 minutes, which coincides with the complete blendor resistance of the population. Identical experiments conducted at 28°C show that complete blendor resistance requires 10-12 minutes at this temperature.

Effect of metabolic inhibitors

Since Lanni (10) has demonstrated the necessity for protein synthesis in achieving blendor resistance in T5, the effect of various metabolic inhibitors on SP82G blendor resistance was examined. (Table 2). Chloramphenicol, Actinomycin D, and cyanide had little effect on the achievement of blendor resistance whether added shortly after adsorption, or prior to adsorption. Thus, the transfer of DNA from SP82G to its host does not require synthesis of a phage protein.

DNA transfer in heat killed cells

The transfer of DNA to heat killed cells was examined by determining the amount of radiophosphorous label that was transferred to cells which had been inactivated at various temperatures (Table 3). Heating to temperatures of 60°C and above did not affect the adsorption of phage to host, but did result in a marked depression in the amount of label associated with the cells after blending. The decrease in cell-associated ^{32}P after blending is not due to any increased permeability or fragility of heat killed cells, since cells which were heat treated after phage infection took place retained the same amount of label as untreated cells, even after blending. Clearly, heating to 60°C does not affect the adsorption of the phage to the cell but does inhibit the transfer of DNA from phage to host.

Table 2. Effect of metabolic inhibitors on blendor resistance

INHIBITOR	PER CENT OF INFECTIOUS CENTERS RESISTANT TO BLENDING	
	Inhibitor added prior to phage adsorption.	Inhibitor added after phage adsorption.*
Chilled to 15°C	---	5.3%
Chloramphenicol (200 ug/ml)	77.0%	73.4%
NaCN (0.0025 M)	92.5%	87.0%
Actinomycin D (10 ug/ml)	100.0%	100.0%

* Bacteria at 4 x 10^9 cell/ml were briefly exposed to bacteriophage and adsorption terminated as described in Figure 15. The bacteria were diluted either into chilled media, or into media containing the inhibitors indicated.

Table 3. Transfer of DNA to heat killed cells.

Treatment for 5 minutes	%Survivors	^{32}P associated with unblended pellet	^{32}P associated with blended pellet	% ^{32}P adsorbed and not removed by blending
I Cells exposed to heat before phage adsorption				
None	100%	15,400 CPM	13,500 CPM	88%
50°C	75%	13,200	12,900	98%
60°C	0%	16,600	7,000	43%
70°C	0%	14,200	5,270	37%
80°C	0%	17,700	5,460	31%
II Cells exposed to heat after phage injection				
None		11,700 CPM	9,400	81%
50°C		11,200	9,400	84%
60°C		10,600	9,300	90%
70°C		11,200	8,800	78%
80°C		10,600	8,800	85%

Kinetics of DNA transfer in competent cell populations

Alegria (8) has studied the induction of deoxyuridine menophosphate hydroxymethylase (a phage specific enzyme) after the infection of B. subtilis by bacteriophage SP82G and found that in a population of competent cells there is about an eight minute lag in the appearance of the enzyme compared to the time of appearance in a non-competent (logarithmic phase) population. He suggests that changes which occur in the composition of the cell wall when B. subtilis become competent (22, 23) might influence either the attachment of the phage or the injection of its DNA.

The time required for injection by SP82G adsorbed to a population of competent cells was compared to the time required by phage adsorbed to a population of cells in logarithmic phase. Cells which had been grown in a competence regime (in LS media) and cells in logarithmic growth in NM media were concentrated to 4 x 10^9 cells/ml and rapidly infected with phage. At intervals, samples were chilled and blended as before and assayed for surviving infectious centers (Fig. 16). There is no significant difference in the appearance of blendor resistant infectious centers in the two populations.

Since only a small portion (2-10%) of the cells in a competent population are truly competent, that is, able to take up DNA (24, 25, 26) the possibility exists that the majority of cells requires a normal time for injection, while the minority might require a much longer time. If this were the case the longer injection time required by the minority of cells might be obscured in the entire population, and not detected under the conditions described above. The minor fraction of cells that is

Figure 16

Blendor resistance of infected complexes from competent and logarithmic phase cell populations

A culture of competent cells (grown in LS media) and logarithmic phase cells (grown in NM) were concentrated to 4 x 10^9 cells/ml, briefly exposed to phage, and chilled and blended as described in Fig. 15. The surviving infectious centers are expressed as the per cent of an unblended control. O infected competent cells, △ infected logarithmic phase cells.

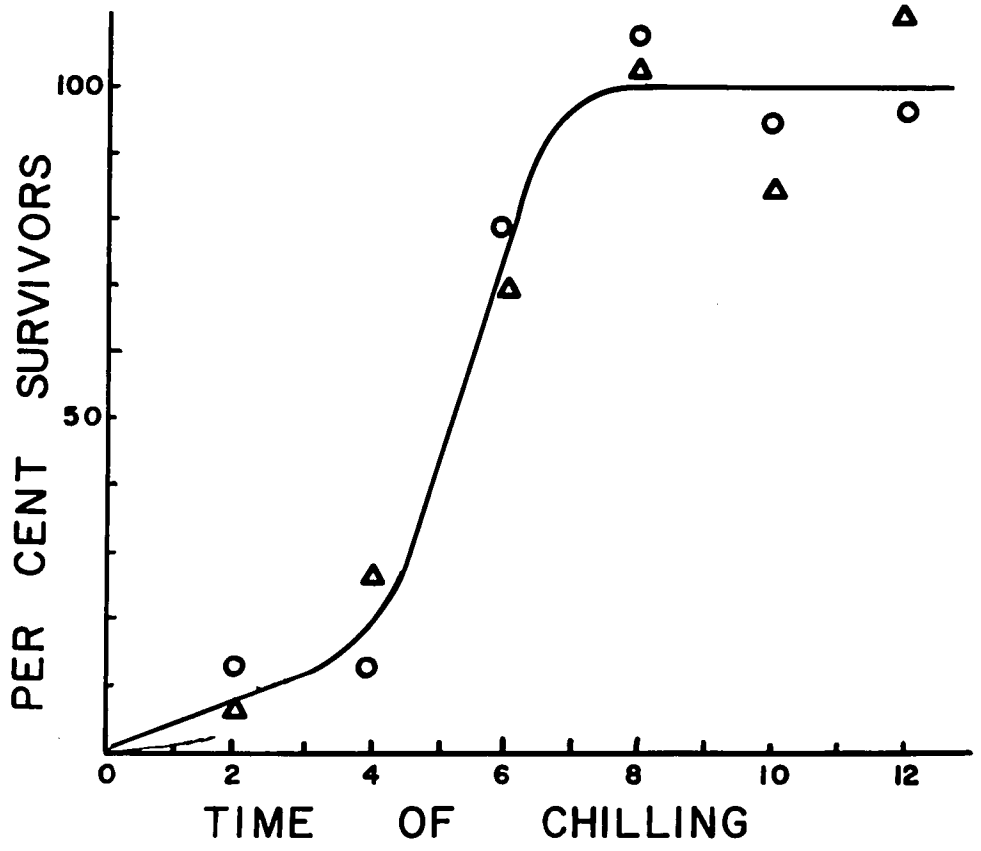


Fig. 16

competent has different properties that permit its separation from the bulk of the population by zonal centrifugation (25, 27) or by isopycnic separation on a linear gradient of Renografin (24, 28). The ability to fractionate a competent population and to enrich for the minority, competent portion, permits an examination of the injection time required by phage adsorbed to this minor fraction.

To determine the characteristic densities of the two fractions in a population of competent B. subtilis strain SB-1, a linear gradient of Renografin extending from a density of 1.12 g/cc. to 1.16 g/cc. was prepared in a cellulose nitrate tube and overlayered with 0.25 ml of a competent cell culture that had been exposed to WT phage DNA (2.3 ug/ml) for 20 minutes. After centrifugation at 37,000 x g for 20 minutes, 15 drop fractions were collected by bottom puncture. Each fraction was assayed directly for colony formers and infectious centers (Figure 17, panel A). The density of alternate fractions (Figure 17, panel B) was determined from the refractive index at 25°C using the conversion figures of Cahn and Fox (24). Colony formers showed a sharp peak at a density of 1.145 g/cc., while transfectants were found in a broader band centered at about 1.1325 g/cc. Using B. subtilis strain SB-25 grown under slightly different conditions Cahn and Fox found that the lighter bacteria banded at ρ=1.110 and ρ=1.131 g/cc. The differences in the densities of the light and heavy bands may reflect differences in the strains used or may result from the different growth conditions.

To fractionate the large quantities of competent cells required for blendor experiments, a discontinuous gradient was prepared in a 1.5 x 10 cm Corex tube. Using Renografin solutions diluted to appropriate densities, the gradient was arranged so that a separating layer of a density intermediate between the heavy and light bacteria would maximize

Figure 17

Fractionation of a competent cell population on a linear gradient of Renografin

A linear gradient of Renografin was overlayered with 0.25 ml of competent cells that had been exposed to infective phage DNA. After centrifugation fractions of 15 drops were collected and assayed directly for colony formers (○) infectious centers (△) and their refractive index (η_o^{25}) (▲, panel B).

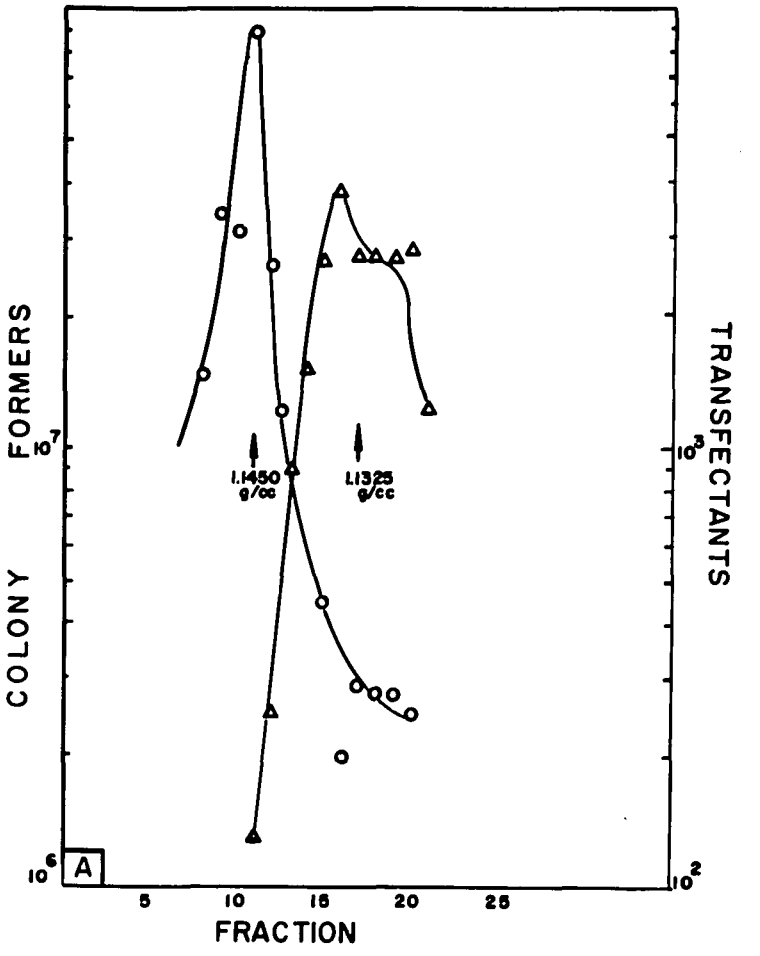


Fig. 17

A

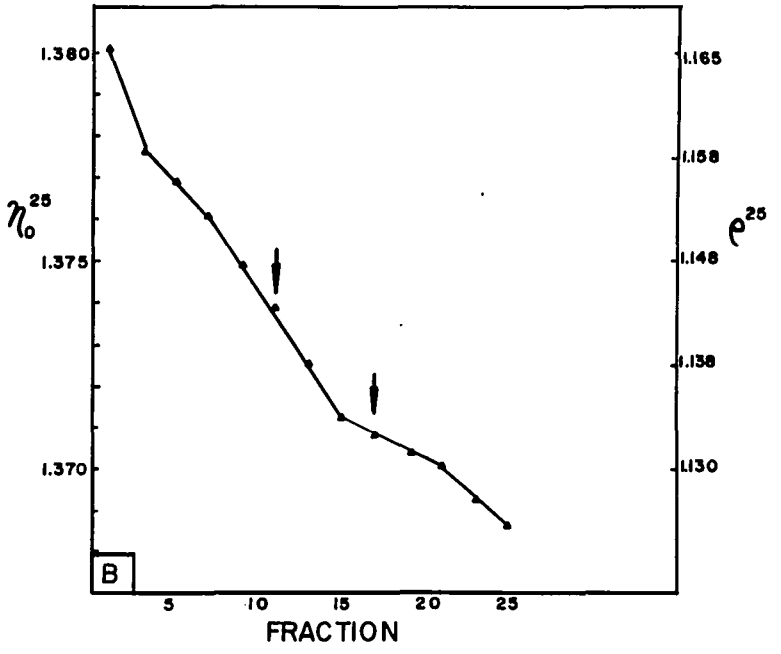


Fig. 17

B

their separation. The gradient contained three layers: a 2 ml layer of ρ=1.40 g/cc used as a cushion on the bottom, a 6 ml layer of ρ=1.138 g/cc. used as a separating layer, and a 2 ml layer of ρ=1.100 g/cc. as the top layer. The gradient was overlayed with 1 ml of a competent cell culture concentrated to 2 x 10^9 cells/ml and centrifuged at 18,500 RPM in a Sorvall SS-34 rotor at room temperature (Ivan Sorvall, Norwalk, Conn.) The separated layers were collected by aspiration, washed with LS on Millipore filters (HA, 50 u) and resuspended in LS. Dilutions were plated for the total number of bacteria in each band, and the competence of the cells was determined by the pre-infection marker rescue technique (Table 4). Under these conditions about 80% of the viable bacteria that were applied to the gradient were recovered. Although the overall yield of transfectants is low there is an obvious enrichment for competent cells in the top band; the frequency of transfection in this band is 75 times greater than that of the bottom band and 5 times greater than that of the overall population.

It is not known if all of the cells found in this band are competent. The frequency of transfection observed in this band (1.5%) may indicate that during the time of experimental exposure to DNA only 1.5% of the cells were in a state of competency. Cahn and Fox (24) report that only cells found in the top band are able to take up transforming DNA and the results shown here indicate that very few of the competent cells are found in the bottom band. If competent cells require a longer time for infection by bacteriophage, then phage-infected cells from the top band should show a much different blendor resistance pattern than cells from the bottom band.

A competent cell culture concentrated to 2 x 10^9 cells/ml was applied to a didiscontinuous gradient and fractionated as described above. The top and bottom bands were washed in NM, rapidly infected with phage

Table 4. Fractionation of competent cells on a discontinuous gradient of Renografin

	Top Band	Bottom Band	Total
Colony formers	3.28×10^8	1.25×10^9	1.58×10^9
% of total bacteria	16%	63%	79%
Transfectants*	5.0×10^6	2.89×10^5	5.3×10^6
frequency of transfection	1.5%	0.023%	0.3%
% of total transfectants	94%	6%	

*Measured by preinfection marker rescue after fractionation

Figure 18

Blendor resistance of infected complexes from a fractionated competent cell population

The separated fractions of a competent cell population corresponding to the top and bottom bands described in Table 4 were rapidly infected with phage and chilled and blended as described in Fig. 2. The surviving infectious centers are expressed as the per cent of a non-blended control. ○ infected bottom band cells, □ infected top band cells.

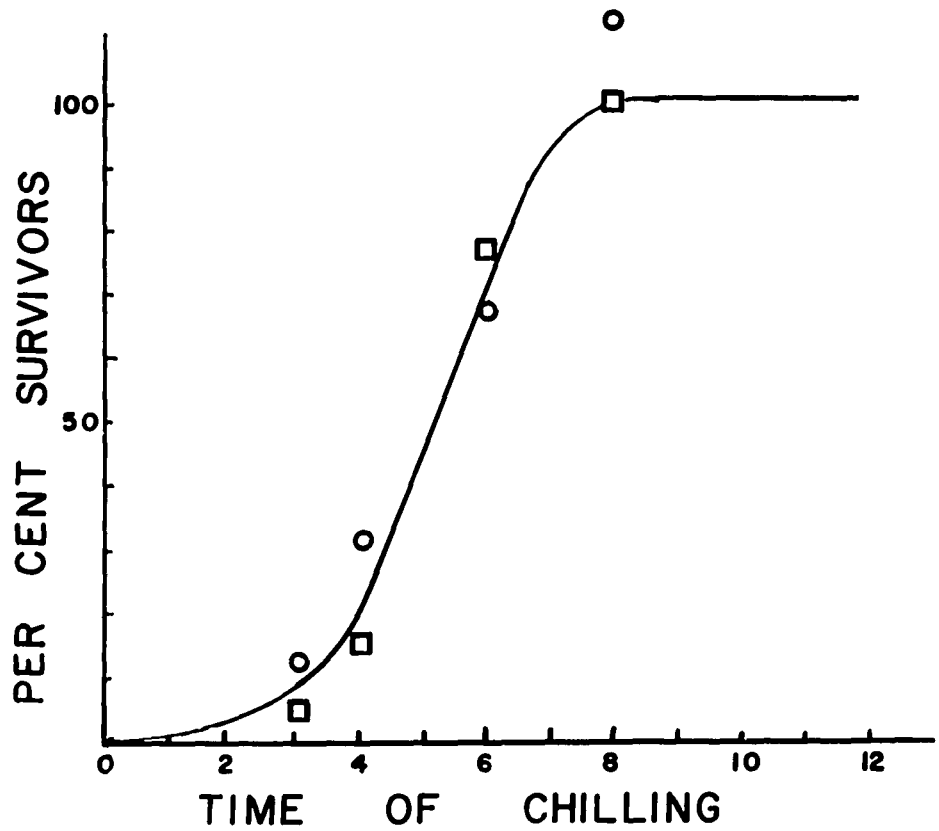


Fig. 18

and chilled and blended as before. The simultaneous appearance of blendor resistant infectious centers (Figure 18) indicates that there is no significant difference in the injection times required for cells from the top or bottom bands. All cells in the competent population require the same amount of time for the transfer of the phage genome, and this is the same amount of time required for infection of logarithmically growing cells (Figure 3). There is no evidence here to support the hypothesis that phage adsorbed to competent cells require a longer time for the transfer of their genome. A more likely explanation for the lag observed in the induction of phage enzymes (8) is that competent cells are known to be in a state of biosynthetic latency during which cellular polymers are synthesized at less than the usual rate (29).

Fate of DNA after transfer

Work by Miller et al and others has indicated that even after transforming DNA has acquired DNAase resistance it resides outside the cell membrane and may be freed from the cell by removing the cell wall with lysozyme (18, 19). If injected DNA shared the same fate, it might be expected that interference with the protein synthesis or energy production of the cell would prevent the transfer of the DNA across the cell membrane. To determine the fate of injected phage, ^{32}P labelled phage were adsorbed to cells under various conditions and permitted to inject their DNA. The infected cells were washed thoroughly and resuspended in an isotonic (0.5M) sucrose media. The cell walls were removed by treatment with lysozyme (200 ug/ml for 15 minutes at 37°C) and the protoplasts were separated from the media by centrifugation. In some cases DNAase was added to the media to determine if any DNA still associated with the protoplasts was sensitive to the enzyme. The results (Table 5,

part I) show that even in the presence of NaCN (0.005M) and chloramphenicol (100 ug/ml) practically all of the ^{32}P label is associated with the protoplasts. This is observed regardless of whether the inhibitors were added at the time of infection or six minutes later. Very little (perhaps 10%) of the cell associated label is still sensitive to DNAase. The small amount of DNA that is lost from the cells during protoplast formation is not due to leakage caused by non-repaired phage damage since cells which are superinfected with unlabelled phage at a multiplicity of infection = 10 retain the same amount of label as non-superinfected cells.

The observation that nearly all of the injected DNA is bound to the cell in a lysozyme resistant state is quite different than the results obtained by Miller et al using transforming DNA. Since binding of the DNA might play a role in the acquisition of DNAase resistance (17) it was considered appropriate to re-examine the fate of transfecting DNA, particularly under conditions in which pre-infection protection had been set up within the infected cell (part I of this thesis). Competent cells were pre-infected and exposed to CM either at the time of addition of the preinfecting phage or six minutes thereafter. These cells, and cells which had not been preinfected, were exposed to ^{32}P labelled phage DNA at a concentration of 0.2 ug/ml. After 20 minutes the cells were treated with DNAase, held for an additional five minutes, and washed thoroughly. The cells were treated with lysozyme as before, and the amount of label still associated with the protoplasts was determined. As seen in Table 5, part II the amount of DNA remaining with the protoplasts was nearly the same under all conditions tested, and never exceeded 61% of the label associated with the cells before the removal of the cell wall. Thus, unlike injected phage DNA, a large fraction of the

Table 5. Association of ^{32}P Labelled DNA with Lysozyme Treated Cells

PART I	AFTER INFECTION WITH ^{32}P LABELLED BACTERIOPHAGE					
		Distribution of ^{32}P Label				
Treatment of Cells*	DNAase added	Supernatant CPM	%total	Pellet (protoplasts) CPM	%total	Total CPM
NaCN (.005M) at 0 min.	no	4,460	10%	39,500	90%	43,960
NaCN at 6 min.	no	2,440	5%	43,000	95%	45,440
NaCN at 6 min. superinfected at 8 min. **	no	32,000	14%	37,300	86%	43,500
chloramphenicol (CM) at 0 min. (100 ug/ml)	no	1,040	5%	20,100	95%	21,150
	yes	4,750	16%	18,700	84%	23,500
CM at 6 min.	no	1,065	4%	28,100	96%	29,165
	yes	3,700	14%	23,100	86%	26,850
CM at 6 min. superinfected at min. **	no	1,440	5%	27,000	95%	28,440
	yes	5,150	19%	21,500	81%	26,650

* Bacteriophage are added to the cells at time = 0 min.

** Cells were superinfected with unlabelled phage at a MOI = 10-20.

Table 5. Association of ^{32}P Labelled DNA with Lysozyme Treated Cells

PART II	AFTER TRANSFECTION WITH ^{32}P LABELLED DNA					
		Distribution of ^{32}P Label				
Treatment of Cells	DNAase added	Supernatant		Pellet (protoplasts)		Total
		CPM	%total	CPM	%total	CPM
no preinfection no CM	no	525	46%	605	54%	1,130
	yes	550	52%	505	48%	1,055
no preinfection CM added (100 ug/ml.)	no	440	40%	656	60%	1,096
	yes	475	43%	625	57%	1,100
cells preinfected * with unlabelled phage. CM at 0 min.	no	338	40%	496	60%	834
	yes	343	40%	520	60%	863
cells preinfected * with unlabelled phage. CM at 6 min.	no	370	39%	570	61%	940
	yes	370	41%	537	59%	907

* Cells were preinfected with unlabelled phage at time 0. Labelled DNA was added at 6 min.

transfecting DNA which is resistant to externally added DNAase may be freed from the cells by removing the cell wall. The location of this fraction is not clear - it may be associated with the cell wall itself, or it may be located in the periplasmic space. It is apparent however, that preinfection of the competent cell does not increase the amount of DNA bound in a lysozyme resistant state, nor does it increase the total amount of DNA taken up by the cell in a DNAase resistant fashion.

Even though the lysozyme treatment used in these experiments is similar to that of Miller et al and is sufficient to give 90% conversion to protoplasts (30) the fraction of DNA found in a lysozyme resistant state is larger than that observed by these investigators (although nearly the same as that observed by Erickson et al) (17). Raising the lysozyme concentration to 400 ug/ml or lowering it to 50 ug/ml had no effect on the results. The reason for the different results in these experiments is not known.

Marker entry studies

The ability to interrupt DNA transfer in a stepwise fashion by chilling and blending, and the absence of a superinfection-exclusion phenomenon in SP82G (7) permits a study of the transfer of various markers on the phage genome. Temperature sensitive mutant phage which will not form plaques at 47°C but which will grow normally at 33°C were used in this study. A sample of a temperature sensitive mutant phage was rapidly adsorbed to bacteria, and the transfer of DNA from phage to host was interrupted at intervals by blending. Blended complexes were then super-infected with a different mutant phage and plated at 47°C. At this selective temperature only wild-type (WT) recombinants are able to form plaques and these could only be formed

when the superinfecting phage was able to "rescue" the necessary genes from the blended complex. Clearly, only genes transferred prior to the time of blending can be rescued from such blended complexes. The time at which a marker is able to be rescued from a blended complex reflects the time of transfer of that marker from phage to host.

One such marker rescue experiment is shown in Figure 19. The ts mutant phage H167-H362 was rapidly adsorbed to bacteria and at intervals the mixture was chilled and blended as described before. Separate samples of the blended complexes were exposed to three different superinfecting phage: H177, E119, and the double mutant H177-E119. They were subsequently plated at 47°C to determine recombinants. The number of complexes able to give rise to recombinants at any time was expressed as the per cent of an unblended control. Also shown is the appearance of blendor resistant infectious centers detected by plating the blended complexes alone, without exposure to super-infecting phage, at the permissive (33°C) temperature. This indicates the transfer of all information necessary for plaque formation.

The results indicate that marker H177 is transferred at an early time, and E119 at a later time. This polarity of entry was verified by the use of the double mutant H177-E119. In this case both markers must be rescued and the appearance of blendor resistance coincides with the entry of the latest marker to be transferred - E119. The complete transfer of all information necessary for sucessful infection follows shortly thereafter.

Another marker rescue experiment showing entry time for closely linked markers at three different locations on the phage genome is presented in Figure 20. Closely linked double mutants were used in this study for the purpose of lowering the "leakiness" and reversion

Figure 19

Rescue of genetic markers from blended complexes

Bacteria were infected with a ts mutant phage at a MOI = 0.1. The transfer of DNA was interrupted at intervals by chilling and blending. Blended complexes were superinfected with the ts mutants indicated and plated for recombinants at 47°C. Blendor resistant infectious centers were assayed by plating blended complexes alone at 33°C.

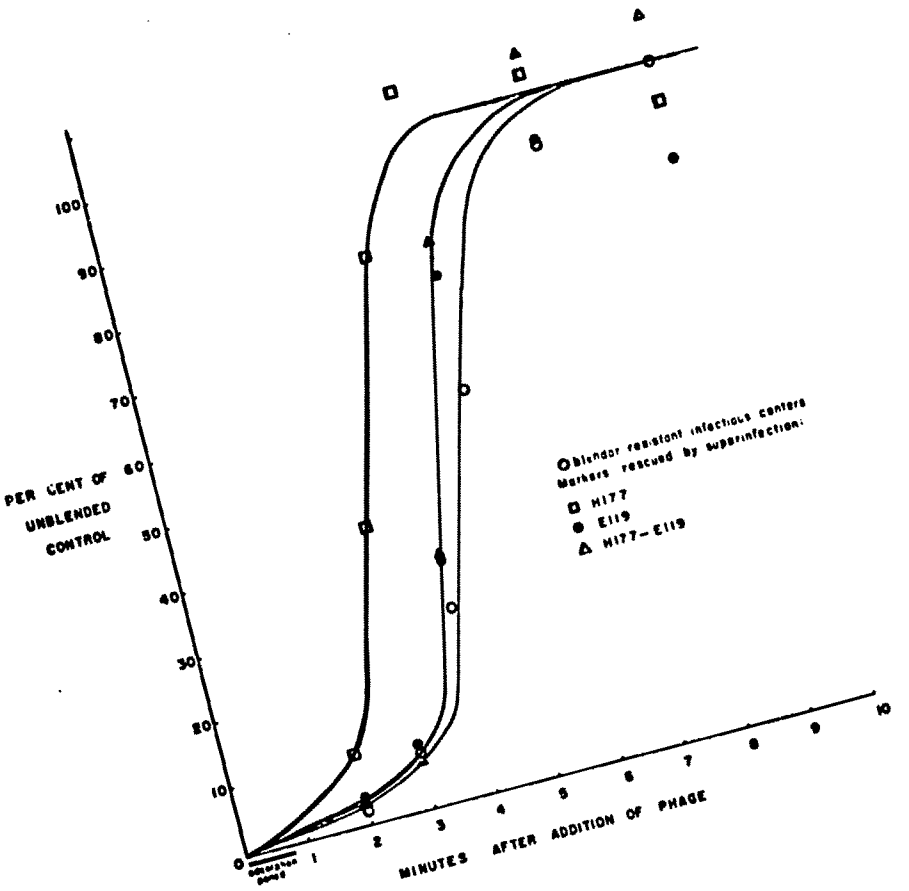

PER CENT OF UNBLENDED CONTROL
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
MINUTES AFTER ADDITION OF PHAGE
Blender resistant infectious centers
Markers rescued by superinfection:
H177
E119
H177-E119

Fig. 19

Figure 20

Rescue of markers from blended complexes

Bacteria were rapidly infected with a ts mutant phage (H362-H180) at a MOI = 0.1 and a blendor experiment carried out as described in Figure 15. Blended complexes were superinfected with the ts mutants indicated and plated for recombinants at 47°C. Blendor resistant infectious centers were assayed by plating blended complexes alone at 33°C.

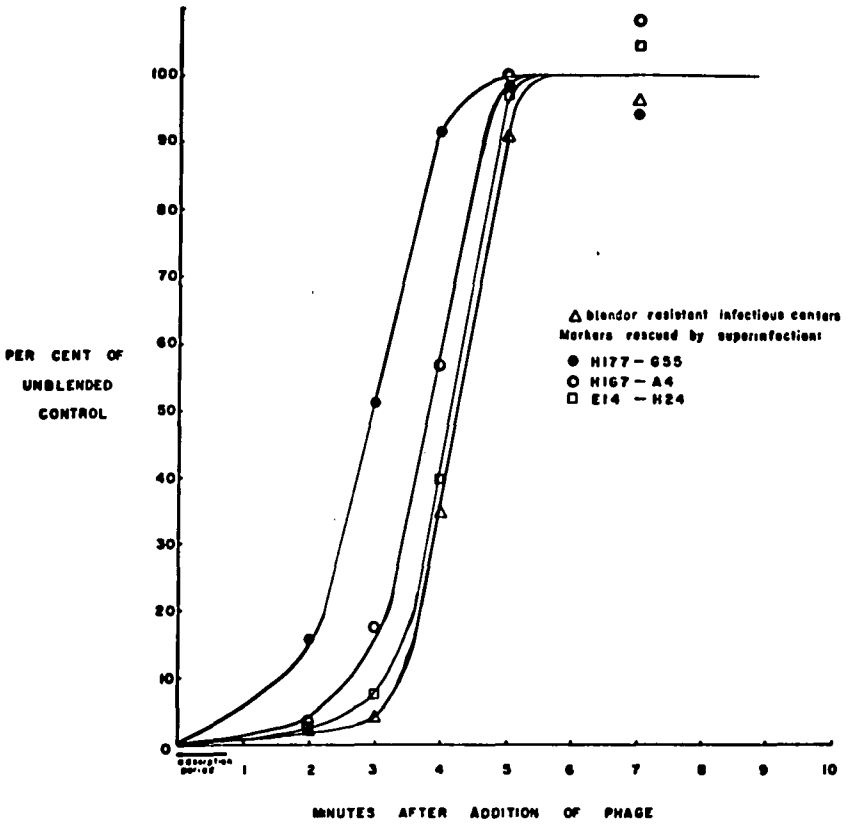

PER CENT OF
UNBLENDED
CONTROL
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
MINUTES AFTER ADDITION OF PHAGE
△ blendor resistant infectious centers
Markers rescued by superinfection
● H177 – G55
○ H167 – A4
□ E14 – H24

Fig. 20

rate sometimes associated with single markers. The map positions of the mutants used in this study are shown in Figure 21. (31)

The results of this experiment indicate a polar entry of markers, that is, markers on the left end of the map (H177-G55) enter first, followed by markers in the middle of the map (H167-A4) and subsequently those on the right end of the map (E14-H24). The same polarity of entry, and the same times of entry were observed in all experiments regardless of the position of the marker carried by the pre-infecting phage. (Three different pre-infection phage were tested in various experiments: H20, H362-H180, and H27-H326).

By plotting the mean time of entry for the markers tested (the time at which a 50% level of blendor resistance is achieved) against the map distance from the left hand origin of the map, the relationship between genetic map distance and entry time can be seen (Fig. 22). The transfer of markers on the phage genome to the recipient bacterium proceeds in a linear, polar fashion consistent with the genetic and physical maps of SP82G. Markers on the left end of the map enter first.

The slopes and positions of the curves in Figure 22 indicate that both the rate of transfer of the genome and the time of initiation of transfer are temperature dependent. The mean time required for the transfer of the phage genome can be obtained from Figure 22 by calculating the interval between the time of entry of the origin (map units=0) and the time of entry of the terminus (map units=52). The values obtained in this manner are: 1.4 minutes at 33°C, 2.3 minutes at 28°C, and 4.0 minutes at 25°C. An independent estimate of the time required for the transfer of the genome may be calculated from Figure 15. The time at which ^{32}P transfer begins and the first appearance of blendor resistant infectious centers are calculated by extrapolating the linear

Figure 21

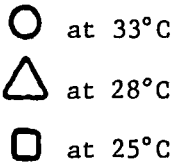
Genetic map of the ts mutants used in this study. The known terminal markers (NG14 and H201) are also shown. (Green, personal communication and ref. 31).


H177
NG14
2.2
G55
2.1
9.7
H20
18.4
H180
H362
1.3
A4
2.1
H167
1.0
H2
4.9
H326
H42
4.0
H27
1.5
E19
2.3
E14
1.3
H24
1.7
H201

Fig. 21

Figure 22

The mean time of entry of genetic markers versus their map distance from the left end of the map.



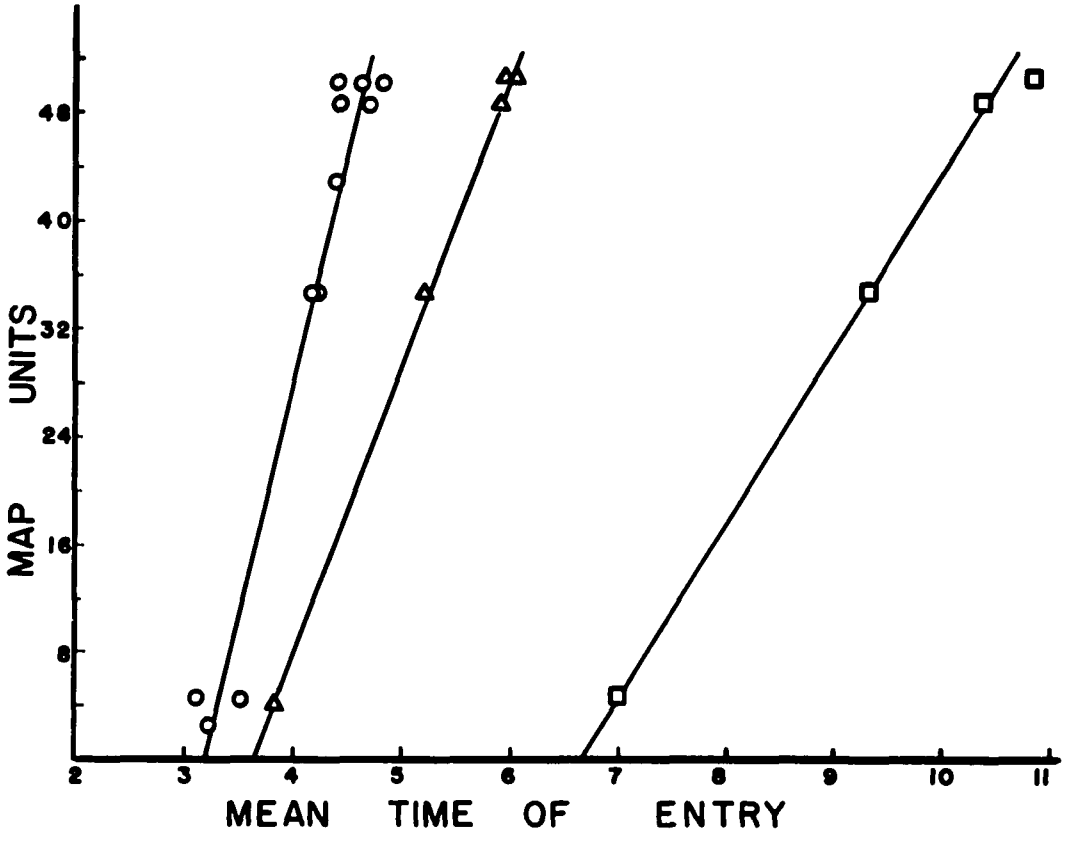


Fig. 22

portions for the curves to the base line. The difference between these values (1.7 minutes) is the time required for the complete transfer of the genome and is in good agreement with the mean time of transfer calculated above.

The rates of transfer of the phage DNA at various temperatures may be calculated from the known length of the SP82G DNA molecule. The contour length of SP82G DNA measured by electron microscopy is 52.9 μm (D. M. Green, personal communication). Using this value, the mean rate of transfer is 1,850 base pairs/second at 33°C, 1130 base pairs/sec at 28°C, and 650 base pairs/sec. at 25°C. These rates may be fitted to an Arrhenius plot which is linear in this range (Figure 23) and which gives a value for the energy of activation of 18.76 kcal/mole of base pairs and for a pre-exponential factor (A) of 3.3×10^3.

The mean time of initiation of DNA transfer (the intercepts of the curves in Figure 22 with the base line) are also temperature dependent, but do not give a linear Arrhenius plot (Fig. 23). This probably indicates that more than one reaction or reaction mechanism is involved in this process.

Figure 23

An Arrhenius plot of the rate of transfer of the phage genome and the time of initiation of DNA transfer

The logarithm of the rate of transfer of phage DNA (○) and the time of initiation of DNA transfer (△) is plotted as a function of the inverse temperature.

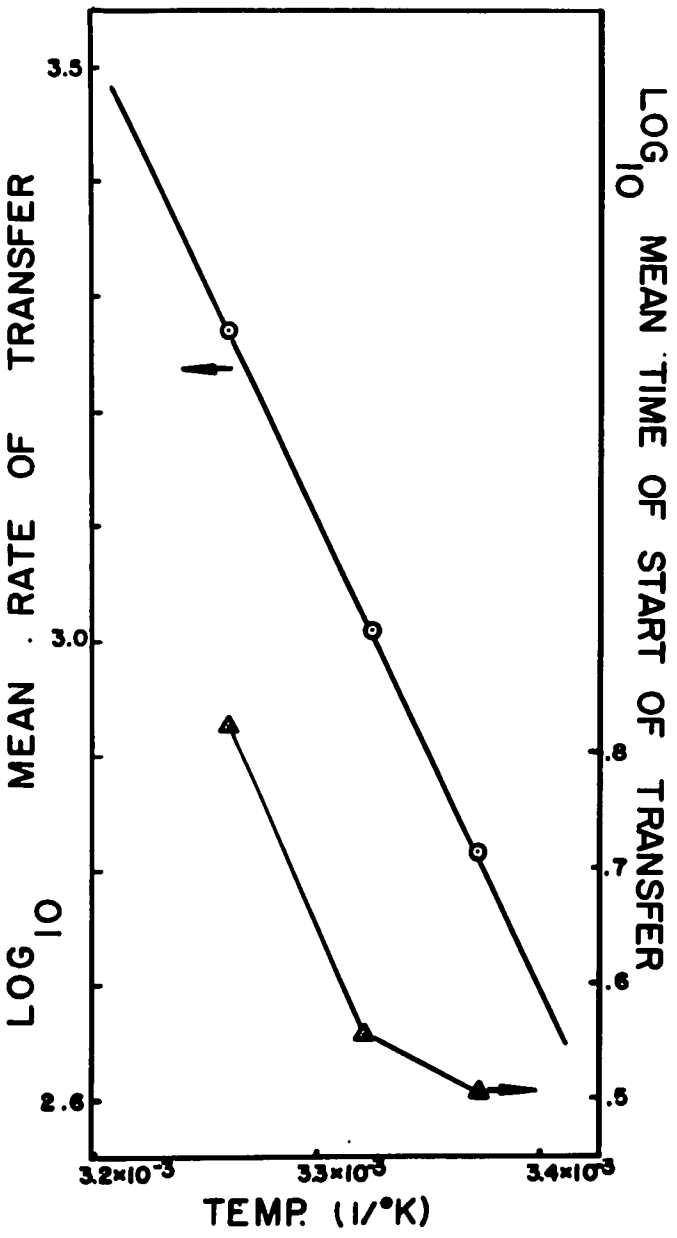

LOG10 MEAN RATE OF TRANSFER
LOG10 MEAN TIME OF START OF TRANSFER
3.5
3.0
2.6
.8
.7
.6
.5
3.2×10⁻³
3.3×10⁻³
3.4×10⁻³
TEMP. (1/°K)

Fig. 23

Discussion

These experiments indicate that the transfer of DNA from phage to host in SP82G infection proceeds in an orderly fashion. Those genes which are concerned with early phage functions are transferred first, while genes concerned with late functions are transferred at a later time. The polarity of entry of the phage genome suggests that it is advantageous to the phage to have those genes which are to function first transferred first. The entry of markers on infective DNA during transfection (Green, personal communication) exhibits the opposite polarity to that observed during bacteriophage infection. Thus any advantage which phage DNA might have enjoyed as a result of its mode of entry during injection is not available to transfecting DNA. One consideration is that the gene (s) which enable the phage to overcome intracellular inactivation (part I of this thesis) are located in such a position that their early transfer ensures the survival of the phage genome. Under transfection conditions these genes would be the last to enter the cell.

A rapid entry of the phage genome into the cell might also be important in the successful establishment of an infectious center. Strauss (3) has estimated that transforming DNA is taken up by B. subtilis at the rate of 55 base pairs/sec. at 28°C. By measuring the time at which markers on transfecting SP82G DNA become DNAase resistant Green (personal communication) has calculated that entry of the phage DNA proceeds at about 660 base pairs/sec. at 33°C. Both of these measurements are considerably less than the rate of entry of injected phage DNA (1130 base pairs/sec at 28°C, 1850 base pairs/sec at 33°C).

It is apparent that during phage infection DNA is introduced into the cell much more rapidly than when it is taken up by a competent cell.

Work by other investigators (17, 18, 19) has suggested that transforming DNA which has been taken up by the cell remains outside or bound to the cell membrane for some time after acquiring resistance to externally added DNAase. Since it is known that there are nucleases associated with the cell wall-membrane fractions of B. subtilis (32) this could be considered an unfavorable environment for infecting DNA. A rapid and early binding of the DNA in a form resistant to these enzymes would be of advantage to the phage. In the experiments reported here it was observed that practically all DNA introduced into the cell by phage infection is bound to the cell in a lysozyme, DNAase resistant form. In contrast, a significant portion of transfecting DNA taken up by the cell in a form resistant to externally added DNAase can be removed by the addition of lysozyme. Inhibition of protein synthesis or energy production does not affect the binding of injected DNA, nor does the preinfection of competent cells affect the binding of transfecting DNA. The location of the fraction of transfecting DNA that is lysozyme sensitive is not clear. It may be that it is bound to the cell wall itself in a DNAase resistant form and was never destined to enter the cell. If this were the case, transfecting DNA that was successfully transferred across the cell wall might be bound to the cell in a lysozyme resistant form just as efficiently as injected DNA. Due to this uncertainty it is not possible to estimate the relative efficiency of binding of the DNA under the two conditions, however, the fact that pre-infection of the competent cell does not alter the fraction of transfecting DNA which becomes lysozyme resistant indicates that this is not the mechanism of preinfection protection.

To briefly enumerate the results of the work described here, the following observations were made.

1. During phage infection the genome of SP82G is transferred in a linear, polar fashion consistent with the genetic and physical map. The polarity of entry is opposite to that observed during the entry of transfecting DNA, and the rate of transfer is much more rapid than in that process. These differences may be of advantage to the phage in overcoming protection mechanisms known to be operating within the cell.

2. The rate of transfer of the DNA is temperature dependent, and may be halted by chilling.

3. Metabolic inhibitors such as cyanide and chloramphenicol have little effect on the transfer of the genome.

4. Unlike transfecting DNA, nearly all of the injected DNA is bound to the cell in a lysozyme resistant form. However, the efficiency of binding of transfecting DNA is not altered in pre-infected cells, and thus this cannot be the mechanism of pre-infection protection.

5. The time required for the transfer of the phage genome is the same for the infection of cells grown in a competence regime as it is for log growth cells.

References

1. Green, D.M., Intracellular inactivation of infective SP82 Bacteriophage DNA. J. Mol. Biol., 22, 1 (1966).
2. Green, D.M., Infectivity of DNA isolated from Bacillus subtilis Bacteriophage SP82. J. Mol. Biol., 10, 438 (1964).
3. Strauss, N., Configuration of transforming DNA during entry into B. subtilis. J. Bact., 89, 288 (1965).
4. Strauss, N., Further evidence concerning the configuration of transforming DNA during entry into B. subtilis. J. Bact., 91, 702 (1966).
5. Gabor, M. and Hotchkiss, R.D., Manifestation of linear organization in molecules of Pneumococcal transforming DNA. Proc. Nat. Acad. Sci. (U.S.), 56, 1441 (1966).
6. Green, D.M., Physical and genetic characterization of sheared infective SP82 bacteriophage DNA. J. Mol. Biol., 22, 15 (1966).
7. Kahan, E., A genetic study of temperature-sensitive mutants of the subtilis phage SP82. Virology, 30, 650 (1966).
8. Alegria, A., Ph.D. Thesis, Dept. of Chemistry, Harvard University (1968).
9. Hershey, A.D. and Chase, M.A., Independent functions of viral protein and nucleic acid in growth of bacteriophage. J. Gen. Physiol. 36, 39 (1952).
10. Lanni, Y.T., DNA transfer from phage T5 to host cells: Dependence on intercurrent protein synthesis. Proc. Nat. Acad. Sci. (U.S.) 53, 969 (1965).
11. Lanni, Y.T., First step transfer DNA of bacteriophage T5. Bact.

Rev. 35, 227 (1968).

12. Lanni, Y.T., Functions of two genes in the first-step transfer DNA of bacteriophage T5. J. Mol. Biol., 44, 173 (1969).

13. Simon, L. and Anderson, T.F., The infection of E. coli by T2 and T4 bacteriophages as seen in the electron microscope. I. Attachment and penetration. Virology, 32, 298 (1967).

14. Bayer, M.E., Adsorption of phage to adhesions between the cell wall and membrane of E. coli. J. Virol. 2, 346 (1968).

15. Braun, W.O., Plescia, O., Kohoutava, M. and Grellner, J., Proc. Symp. in the Mutational Process, Prague, Academia, Prague (1965).

16. Erickson, R.J. and Braun, W.O., Apparent dependence of transformation on the stage of DNA replication of recipient cells. Bact. Rev. 32, 291 (1968).

17. Erickson, R.J., Young, F.E., and Braun, W., Binding of rabbit gamma globulin by competent Bacillus subtilis cultures. J. Bact. 99, 125 (1969).

18. Miller, I.L. and Landeman, O.E., On the mode of entry of transforming DNA into B. subtilis. In Symp. Proc. on the mutational process, M. Kohovtava and J. Hubacek, eds., Prague Academia, 187-195 (1965).

19. Miller, I.L. and Landeman, O.E., Obstacles to transformation presented by bacterial anatomy. Bact. Proc., 34 (1963).

20. Nomura, M., Matsubara, K., Okamoto, K and Fujmara, R., Inhibition of host nucleic acid protein synthesis by bacteriophage T4: its relation to the physical and functional integrity of the host chromosome. J. Mol. Biol., 5, 535 (1962).

21. Mandell, J.D. and Hershey, A.D., A fractionating column for analysis of nucleic acids. Anal. Biochem. 1, 66 (1960).

22. Young, F.E. and Spizizen, J., Biochemical aspects of competence in

the B. subtilis transformation system. II. Autolylic enzyme activity of cell walls. J. Biol. Chem., 238, 3,126 (1963).

23. Pene, J., A sensitive assay for egg white and bacteriophage induced lysozyme. Biochem. Biophys. Res. Comm. 28, 365 (1967).

24. Cahn, F.H. and Fox, M.S., Fractionation of transformable bacteria from competent cultures of B. subtilis on Renografin gradients. J. Bact. 95, 867 (1968).

25. Singh, R.N. and Pitale, M.D. Enrichment of B. subtilis tranformants by Zonal Centrifugation. Nature, 213, 1262 (1967).

26. Nester, E.W., Penicillin resistance of competent cells in DNA transformation of B. subtilis. J. Bact., 87, 867 (1964).

27. Richardson, A.G. and Leach, F.R., Fractionation of competent B. subtilis cells by sucrose density gradient centrifugation, Anal. Biochem. 27, 175 (1969).

28. Hadden, C. and Nester, E.W., Purification of the competent cells in the B. subtilis transformation system. J. Bact. 95, 876 (1968),

29. Nester, E.W. and Stocker, B.A., Biosynthetic latency in early stages of DNA transformation in B. subtilis transformation system. J. Bact. 86, 785 (1963).

30. Landeman, O.E. and Hall, S. Enzymically and physicallv induced inheritance changes in B. subtilis. J. Mol. Biol., 7, 721 (1963).

31. Green, D.M., Gene dislinkage in transfection of SP82G phage DNA. Genetics 60, 673 (1968).

32. Birnboim, H.C., Cellular site in B. subtilis of a nuclease which preferentially degrades single stranded DNA. J. Bact. 91, 1004 (1966).

PART III

THE EFFECTS OF THE DECAY OF INCORPORATED RADIOACTIVE PHOSPHOROUS ON THE SURVIVAL OF BACTERIOPHAGE SP82G

Introduction

The observation that the decay of ^{32}P atoms incorporated into the genome of bacterial viruses leads to their inactivation was first made by Hershey et al (1) who concluded that this was probably the result of damages to the phage DNA. Stent and Fuerst (2) examined this "^{32}P suicide" in bacteriophage T2 and found that only a fraction (about 1/10) of the ^{32}P disintegrations occurring in phages stored at 4°C resulted in lethal events. This low efficiency of inactivation (α, in lethal hits/ ^{32}P decay) has proved to be a characteristic of most bacteriophages having genomes of double stranded DNA (3). To explain this phenomenon Stent and Fuerst proposed that the transmutation of the ^{32}P atom could result in two types of damages to the DNA helix: those which break only one strand, and those which break both strands, and that only damages of the latter type are lethal.

The presence of both kinds of breaks in the DNA has been demonstrated by direct measurement of the molecular weight of DNA extracted from phage after ^{32}P decay (4, 5) however these experiments were not sufficiently quantitative to verify the relationship between strand breakage and lethal events predicted by Stent and Fuerst. More recently Tomizawa and Ogawa (6, 7) have performed elegant experiments in which they measured the frequency of strand breaks resulting from ^{32}P decay by determining the change in the molecular configuration of phage λ DNA. Intracellular phage λ DNA is known to form a closed twisted circular

structure (species I) in which both polynucleotide strands are unbroken. A break in one of the polynucleotide strands leads to the formation of an untwisted, though still circular form (species II) which sediments more slowly than species I in a sucrose gradient. A break in both strands results in a linear molecule (species III) which has the slowest sedimentation rate of all three forms. The ability to directly measure the frequency of single and double strand breaks led to the conclusion that double strand breaks can account for only about 39% of the lethal damages occurring in phage stored at 4°C, while at -79°C, 83% of the lethal events can be attributed to double strand breaks. It was also found that there is a repair mechanism operating in the infected cell that rapidly and efficiently repairs single strand breaks in the damaged DNA. The authors concluded that the lethal events that are not due to double strand breaks are due to single strand breaks that are not repaired in time to permit the successful replication of the genome.

Studies of the functional survival of cistrons on the phage genome after ^{32}P decay have led to a further interpretation of the nature of lethal events. (8). Non-permissive host bacteria were jointly infected with either a non-radioactive rII or amber (am) mutant of bacteriophage T4 and a highly labelled $T4^{+}$ wild-type phage. The infected complexes were frozen and stored. At intervals the ability of the radioactive wild-type phage to complement the growth of the conditionally lethal mutant was examined by thawing the cells and plating them on a sensitive, permissive indicator strain. Complementation leading to the production of progeny phage by the jointly infected cell could only occur when the wild-type phage was able to perform the function not being performed by the unlabelled mutant phage. Using these techniques Harriman and Stent (8) found that the function of several different cistrons of T4 is in-

activated at about 1/2 the rate of inactivation of the whole phage. This could be interpreted as meaning that each lethal ^{32}P decay results in the functional inactivation of half of the viral genome. However, the observation that the functional inactivation of a variety of double mutants proceeds at a rate only slightly higher than the rate of inactivation of single cistrons (and never at twice the rate) indicates that this is not the case. Nevertheless, as more and more cistrons were involved it was found that the sensitivity of multiple cistron function did increase until, with six cistrons involved, their joint function was inactivated at about 0.8 the rate of inactivation of whole phage. To explain these observations Harriman and Stent proposed that there are two kinds of damages which are lethal to the phage: long range hits that result in the functional inactivation of the entire genome, and short range hits that inactivate only a portion of the genome. About 41% of all lethal events are of the long range type and were presumed to be due to double strand breaks. That is, the macromolecular continuity of the genome is a necessary condition for the expression of any of its parts. The observation that the function of any given cistrons was lost at 1/2 the rate of inactivation of the entire phage genome could thus be attributed largely to the contribution of long range lethal events to phage death. The remainder of the lethal events were attributed to lesions in the phage DNA induced by free radical reactions.

By appropriate analysis of the data Harriman and Stent were able to consider the inactivation of cistrons resulting solely from hits of the short range type. The fraction of all short range hits occurring in the genome that result in the inactivation of a given cistron corresponds to the "cistron target size" for short range ^{32}P hits. The values obtained were in good agreement with the relative sensitivities to U.V.

inactivation determined for some of the same cistrons, and were observed to vary over a 10 fold range.

The functional inactivation of individual cistrons by U.V. irradiation was first examined by Krieg (9) who found that the ratio of the sensitivities of the rIIA and rIIB cistrons of phage T4 agreed well with the ratio of the known genetic lengths of the cistrons. These observations fitted the "target theory" in that each cistron apparently presented a U.V. target in proportion to its total length. However, the sensitivity of the cistrons relative to the sensitivity of the entire phage was much higher than would be expected from the length of the total genome which the cistrons occupied. To explain the high sensitivities of the rII cistrons Krieg proposed that the U.V. inactivation of some cistrons might still allow the duplication of the genome and that "duplication could circumvent or erase the damage if the action of the damaged gene were not required for duplication." Damages to genes which function after duplication could be repaired, and such sub-lethal damages would not contribute to the overall sensitivity of the entire phage. Damages occurring in genes whose function <u>was</u> required for duplication would then result in an apparently disproportionately higher sensitivity for these genes. While this "critical cistron" hypothesis could account for the disproportionate sensitivity of early genes, it could not explain the even greater target sizes subsequently observed for other, late cistrons under both ^{32}P and U.V. inactivation conditions (8). To explain the latter observations Harriman and Stent proposed a modification of Krieg's model which assumed that "sub-lethal" damages are generally repaired in the infected cell, but that once the genome has sustained a lethal hit as well, repair cannot occur. In the case of ^{32}P inactivation only the effect of short range lethal damages is important here (since the cistron

target size is only meaningful in terms of short range hits). Thus, for a cistron to survive functionally the "phage must have sustained neither a long range hit, nor both a sub-lethal damage in that cistron and a short range hit." (8) The fact that lethal events resulting in the inactivation of a cistron do not have to occur within the confines of that cistron would increase the relative target size of the cistron and make it appear disproportionately large.

The above experiments dealt with the survival of the function of a cistron after ^{32}P decay. It has also been possible to examine the genetic survival of markers by means of marker rescue or cross reactivation experiments. (8) In this case it is the ability of phage to contribute genetic markers to the progeny of a mixed infection which is measured. Permissive bacteria jointly infected with a highly radioactive wild-type $T4^+$ bacteriophage and unlabelled T4 am mutant phage were frozen and stored. At intervals the ability of the labelled phage to contribute the wild-type am^+ allele to the progeny was assayed by thawing the cells and plating on a strain of bacteria (E. coli B) which is non-permissive for am mutants. It is also possible to examine the joint genetic and functional survival of cistrons by mixedly infecting non-permissive bacteria with the same class of mutants and plating on a non-permissive indicator strain as before. Under these conditions the function of the wild-type allele (contributed by the labelled phage) is required for the rescue of the allele to take place. Using these techniques Harriman and Stent measured the genetic survival of various cistrons of phage T4 as well as their functional and joint genetic and functional survival and found that all three parameters showed the same sensitivity to ^{32}P decay. This indicated that the same lethal ^{32}P disintegration that abolishes the function of the cistron destroys its genetic capabilities

as well.

While these experiments showed that the functional and genetic potential of a cistron have similar sensitivities to ^{32}P decay, they could not answer the question of whether the genetic survival of a marker is also subject to the differential effects of long and short range hits. (8) The results of experiments performed by Stahl (10) indicate that both types of damages may be critical to genetic survival. By following the genetic potential of three markers on the bacteriophage T4 genome after ^{32}P decay it was found that about one half of the lethal disintegrations inactivate all markers simultaneously (which suggests the action of long range hits) while in phage that were still able to contribute some genetic markers the surviving markers tended to be inactivated together if they were closely linked (suggesting the action of short range hits). Stent (11) has examined the genetic survival of markers on the T2 bacteriophage genome but found that in this system markers were inactivated independently, indicating that in this case short range hits were the predominant cause of genetic inactivation.

The physical effects of ^{32}P decay on the transfer of the phage genome have been examined by Hershey et al (13) and Tomizawa (14) who found that populations of ^{32}P inactivated phage showed a decreased ability to transfer their DNA to the bacterial population during infection. Similar effects on the transfer of the genome in X-ray inactivated phage were found to be consistent with a "fractional injection" model. That is, the genome of the inactivated phage is fragmented and only a fraction is injected. (15)

The order of entry of markers on the SP82G genome during phage infection is known (16, part II of this thesis) and proceeds in a linear polar fashion consistent with the genetic and physical maps. The effects

of ^{32}P decay on the transfer of the genome may be examined genetically by determining its effects on marker transfer, and physically by examining the transfer of labelled DNA. The results described in this report show that about 42% of all lethal events result in the non-transfer of some portion of the phage genome. At 4°C double strand breaks probably always prevent the transfer of portions of the genome distal to the break, and while they are in themselves lethal, they do not prevent the rescue of markers on undamaged portions of the genome but rather contribute to damages of the "short range" type. All markers that are transferred are transferred at the normal rate.

Materials and Methods

Preparation of samples

The bacterial strain, bacteriophage, and growth media used have been described previously (17). Radioactive phage was prepared by adding $H_3{}^{32}PO_4$ to the bacterial culture [grown in Nomura media (NM) lacking NaH_2PO_4] along with the phage inoculum. An unlabelled control lysate was prepared from the same unlabelled culture. Radioactive and non-radioactive control stocks were stored at a 10^{-3} dilution in 1X NM salts (18) plus $MgCl_2$ (10^{-3} M) at 4°C or in 1X NM plus $MgCl_2$ (10^{-3} M) plus 10% dimethyl sulfoxide (DMAO) at -20°C. Under these conditions the control lysates were stable even in the presence of an equal amount of nonincorporated ^{32}P.

Total Phosphorous was determined by the King modification of the Fiske-Subbarow technique (19, 20). Radioactivity was assayed in an end-window gas-flow detector.

To examine the effect of ^{32}P after infection, bacteria that had been concentrated to 4×10^9 cells/ml were exposed to phage at a multiplicity of infection (MOI) of 0.1 for 1 minute in the presence of 100 ug/ml of chloramphenicol (CM). The adsorption mixture was diluted into NM containing CM and held for 8 minutes. This is sufficient time to allow the penetration of the entire phage genome (16). An equal portion of ice cold media was added and the infected cells were spun free of unadsorbed phage (7,000 x g for 5 min.) resuspended in ice cold NM plus DMSO (10%) to 10^8 cells/ml and frozen at -20°C. Under these conditions infective centers resulting from unlabelled phage were stable for 25 days.

Phage assays and marker rescue experiments

Temperature sensitive (ts) mutants of SP82G which will grow at 33°C but not at 47°C were used in this study. These mutants, and the techniques for the assay of phage, infectious centers and wild-type (WT) recombinants have been described before (21, 22).

In marker rescue experiments involving stored phage, samples of the stock solution containing temperature sensitive mutant phage at about 10^7/ml were diluted 1:10 into bacteria at 10^8/ml. The mixture was held for 4 minutes at 33°C. Samples of the infected bacteria were then exposed to a different ts mutant phage at a MOI = 5 - 10 for 6 minutes, diluted, and plated at 47°C. Under these conditions only WT recombinants yield plaques.

In experiments involving marker rescue in stored infected bacteria, frozen samples were thawed at 47°C and super-infected as described above.

In all marker rescue experiments the fractional survival of genetic markers was determined by comparison to results obtained with an unlabelled stock preparation stored under identical conditions.

Blendor experiments

The techniques used in blendor experiments have been described before (16) and were followed here unless otherwise noted.

Results

Effects of ^{32}P decay on marker survival in free phage

Previous investigations (13, 14) have shown that populations of ^{32}P inactivated phage have a decreased ability to transfer their DNA to the bacterial population during infection. Hershey et al (13) found that when bacteriophage T2 was inactivated to less than 1% survival, the inactivated particles adsorbed normally but transferred only 20-50% of their ^{32}P label to the bacteria compared to 80% for non-inactivated phage. These observations actually indicate very little, however, about the manner of distribution of DNA fragments in the population of infected complexes. Two possibilities can be envisaged: (i) a fraction of the particles injects all of its DNA while the remainder injects none (all or none injection) or (ii) the genome of extracellularly inactivated phage is fragmented and a fraction of each genome is injected (fractional injection).

The transfer of markers on the phage genome during infection by bacteriophage SP82G proceeds in a linear polar fashion consistent with the genetic map (16, part II of this thesis). Those markers on the left end of the map are transferred first. If the decay of a ^{32}P atom fragments the genome and prevents the transfer of a portion of the genome (fractional injection) then one would expect that those markers which are transferred last and are most distal to the starting end of the genome would be the most sensitive to the effects of the transmutation.

The effect of the decay of incorporated ^{32}P on the survival of markers on the phage genome was examined by measuring the ability of a

superinfecting phage to "rescue" a marker from infectious center. This is possible since super-infection exclusion is not observed in the SP82G system (22). A highly radioactive lysate of the double ts mutant H-167-A4 (see Fig. 25) was prepared and stored at 4°C. At intervals the viable titer of this suspension was determined by plating dilutions at 33°C. At the same time, bacteria were infected at a MOI < 0.1 with this stock and subsequently super-infected with different ts mutants. These complexes were plated at 47°C. At this temperature only WT recombinants will yield plaques and these can only be formed when the super-infecting phage can "rescue" the necessary genes from the complex. If a ^{32}P decay has inactivated a necessary portion of the phage genome, no rescue can take place. The survival of a marker (its ability to be rescued) as a function of the time of storage is an indication of its sensitivity to ^{32}P decay.

The results of such an experiment are presented in Figure 24, in which the logarithm of the fraction of survivors (and the logarithm of the fractional survival of WT recombinants) is plotted as a function of the fraction of ^{32}P atoms decayed ($1-e^{-\lambda t}$). Clearly, not all markers show the same sensitivity to ^{32}P decay. Those markers located on the left end of the genetic map (Fig. 25) are less sensitive than those located at the right end of the map.

In Figure 26 the results of a similar experiment involving the rescue of closely linked double mutants is also shown. The same relationship is apparent - double mutants located at the left end of the map are less sensitive to ^{32}P decay than those located at the right end of the map.

The kinetics of inactivation of a population of radioactive phage labelled with a specific activity of A_o and each containing N atoms of phosphorous can be described as

$$\log_{10} S = 1.48 \times 10^{6} A_o \alpha_T N (1-e^{-\lambda t})$$

where S is the fraction of survivors and λ the fractional disintegration of ^{32}P per day (t). (2). A plot of $\log_{10} S$ against $(1-e^{-\lambda t})$ will have a slope proportional to $A_o \alpha_T N$. From the slopes of the survival curves of free phage plotted in Figures 24 and 26, one can obtain the efficiency of killing, α_T, by the decay of an incorporated ^{32}P atom. This has the dimensions of lethal hits/^{32}P disintegration. The value of N used, 3.4×10^{5}, was derived from the known molecular weight of SP82G DNA, 108×10^{6} daltons (Green, personal communication). Similarly, the slopes of the survival curves for marker rescue give values for α_i, the efficiency of inactivation of the i^{th} genetic marker (8).

In Table 6 the results of three experiments carried out at 4°C are summarized. The average efficiency of killing for SP82G at 4°C is 0.1157 lethal hits/disintegration. This is in fairly good agreement with results published in the literature for other double strand DNA bacteriophages. (3) The efficiency of inactivation of markers, α_i, ranges from 0.0193 to 0.0739 marker inactivations/disintegration.

In Figure 27 the data of Table 6 is plotted in the form of the efficiency of inactivation of markers versus the map distance from the left end of the map. For double mutants the distance to the most distal marker (the farthest to the right) was used. Clearly, markers towards the left end of the map are less sensitive to radioactive decay than those toward the right end of the map. In Figure 28 the efficiency of inactivation of double mutants is plotted against the distance subtended by the double mutant. There does not seem to be any correlation between the two. Values of α_i for closely linked doubles (map units < 2.5) range from 0.023 to 0.069. While there may be a slight skew in favor of higher sensitivity for less closely linked

Figure 24

Sensitivity of genetic markers to ^{32}P decay at 4°C

Radioactive temperature sensitive mutant phage (specific activity = 75.6 mc/mg) stored at 4°C were removed at intervals and adsorbed to bacteria. Samples of the infected bacteria were superinfected with a different ts mutant phage and plated at 47°C. The fractional survival of WT recombinants and of free phage (○) is plotted as a function of the fraction of ^{32}P decayed ($1 - e^{\lambda t}$), Superinfecting phage used were : ■ H177, ▲ H20, □ H362, △ H2, ● E119.

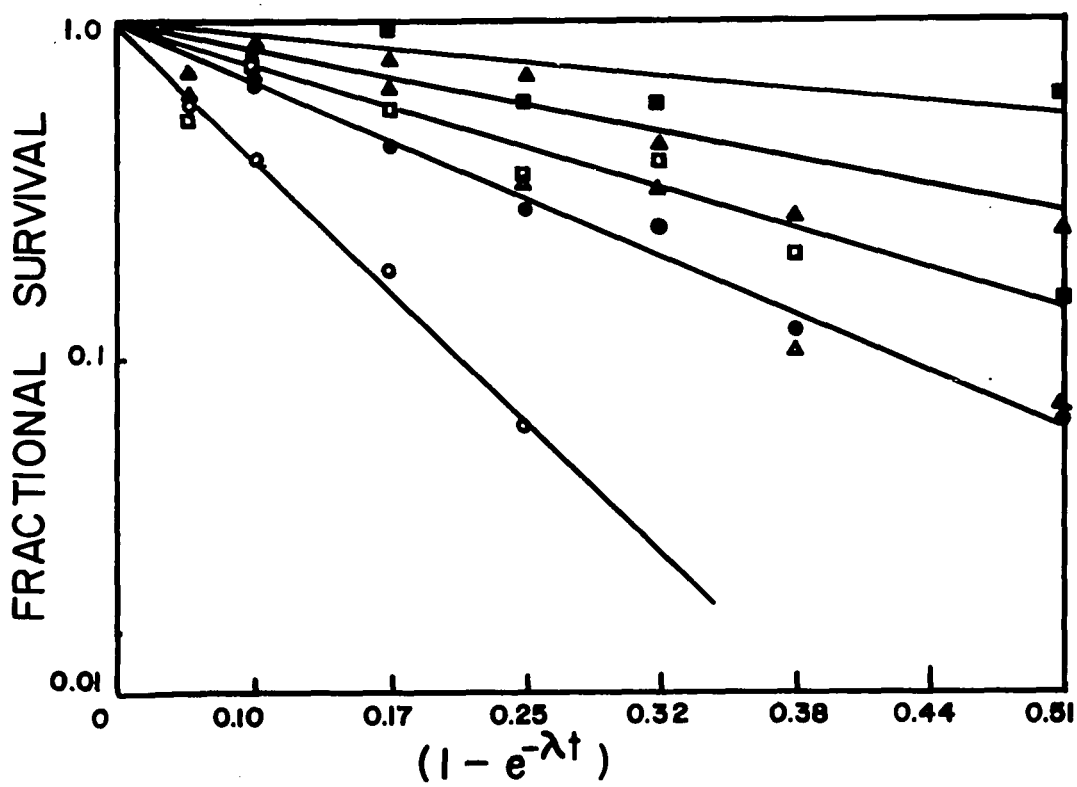

FRACTIONAL SURVIVAL
1.0
0.1
0.01
0
0.10
0.17
0.25
0.32
0.38
0.44
0.51
(1 − e^−λt)

Fig. 24

Figure 25

Genetic map of temperature sensitive mutants of SP82G used in this study

The known terminal markers (NG14 and H201) are also shown (Green, ref. 21 and personal communication).

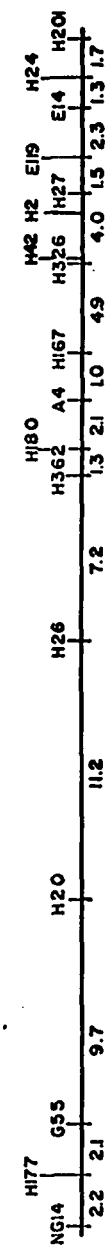

NG14
H177
G55
H20
H26
H180
H362
A4
H167
H326
H42
H2
H27
E119
E14
H24
H20N
2.2
2.1
9.7
11.2
7.2
1.3
2.1
1.0
4.9
4.0
1.5
2.3
1.3
1.7

Fig. 25

Figure 26

Sensitivity of double markers to ^{32}P decay at 4°C

Radioactive temperature sensitive mutant phage (specific activity = 75.6 mc/mg) stored at 4°C were removed at intervals and adsorbed to bacteria. Samples of the infected bacteria were superinfected with a different ts mutant phage and plated at 47°C. The fractional survival of WT recombinants and of free phage (○) is plotted as a function of the fraction of ^{32}P decayed ($1-e^{\lambda t}$). Superinfecting phage used were: ■ H177-G55, △ H362-H180, □ H42-H27, ● E14-H24.

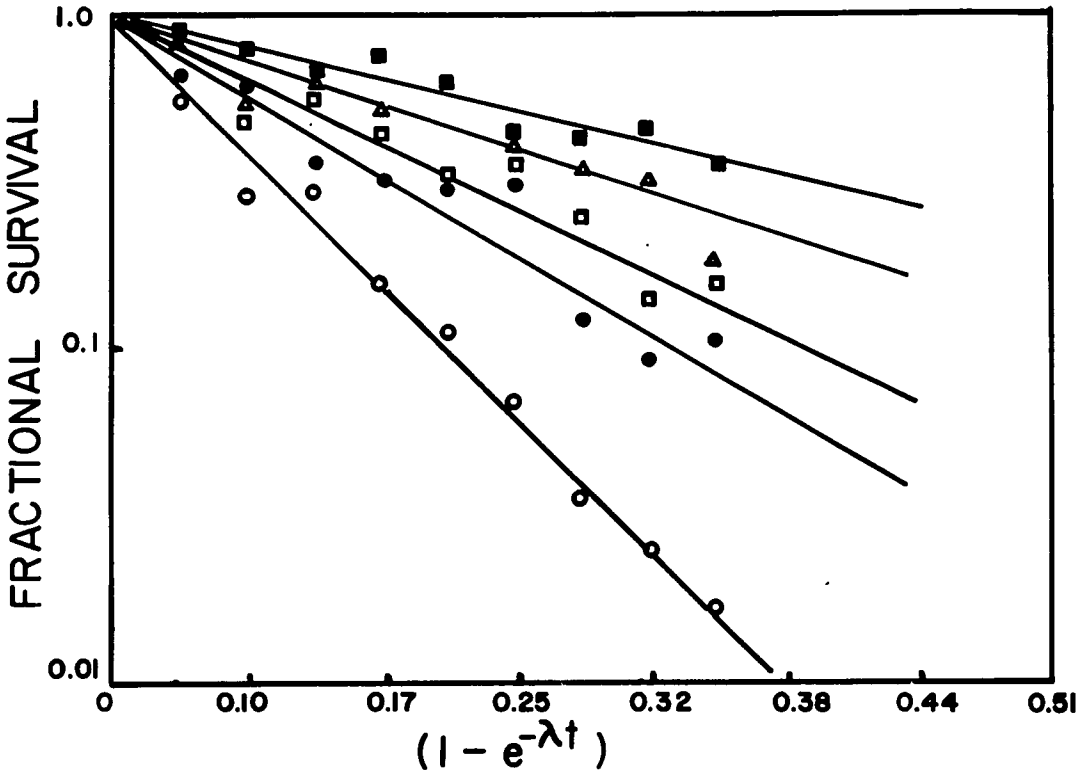

1.0
0.1
0.01
FRACTIONAL SURVIVAL
0
0.10
0.17
0.25
0.32
0.38
0.44
0.51
(1 − e^−λt)

Fig. 26

doubles, this is expected, since widely separated double mutants must have their most distal marker located in a more sensitive map region. Note that the largest double mutant, H177-H26 which spans 23 map units but which is located in the left arm of the map, has a sensitivity which ranks only fifth out of fifteen. Clearly, the position of the marker pair on the map is a more significant parameter in determining sensitivity to ^{32}P decay than the distance it subtends.

In Figure 27, three linear regression curves are drawn for the data: one for values obtained with single mutants, one for values obtained for double mutants, and a curve for all data. It is important to know if double mutants are more sensitive to ^{32}P than single mutants. The data were therefore subjected to statistical analysis to determine if one curve could be used to describe all the data - or if two curves were required. (See appendix and reference 23). The results showed that the data could best be described by two parallel lines with a slope of 0.00082. The efficiency of inactivation of double mutants is slightly higher, having a Y intercept of α_i = 0.0223 compared to 0.0170 for single mutants.

The increased sensitivity of markers located at the distal end (the right end) of the map supports (but does not prove) the hypothesis that ^{32}P disintegrations in the phage DNA lead to a fractional injection of the genome. If this hypothesis is valid, the efficiency of inactivation of markers located at the least sensitive end of the map (the value of α_i at map units = 0, or α_o) could be interpreted as an estimate of the intrinsic sensitivity of markers regardless of their map positions. The concept of intrinsic sensitivity supposes that genetic markers can be made unavailable for marker rescue by two processes: direct inactivation of the particular marker by a ^{32}P "hit", or the prevention of the

TABLE 6

The efficiency of inactivation of single and double mutants at 4°C.

Marker	Map distance from left end	Distance subtended by double	α_i	α_T
Singles				
H177	2.2		.0193	
H20	14.0		.0311	
H362	32.2		.0335	
H2	44.0		.0606	
E119	47.0		.0533	
				.1151
Doubles				
H177-G55	4.3	2.1	.0228	
H362-H180	33.5	1.3	.0605	
H42-H27	45.5	4.0	.0671	
E14-H24	50.6	1.8	.0624	
G55-H20	14.0	9.7	.0371	
H177-H26	25.2	23.0	.0424	
H27-H24	50.6	5.1	.0570	
H362-H15	47.0	14.8	.0605	
H26-H27	45.5	20.3	.0739	
H177-G55	4.3	2.1	.0310	
H362-H180	33.5	1.3	.0450	.1223
H42-H27	45.5	4.0	.0547	
E14-H24	50.6	1.8	.0693	
H177-G55	4.3	2.1	.0252	
H362-H180	33.5	1.3	.0431	.1067
H42-H27	45.5	4.0	.0672	
E14-H24	50.6	1.8	.0583	

Av. α_T = .1157

Figure 27

The efficiency of inactivation of markers, α_i, versus their cumulative map distance from the left end of the map

For double markers the distance to the most distal marker (the farthest right) was used. Three linear regression curves are plotted for the data: the upper one for double markers (△), the lower one for single markers (●), and the middle one (-----) for all data.

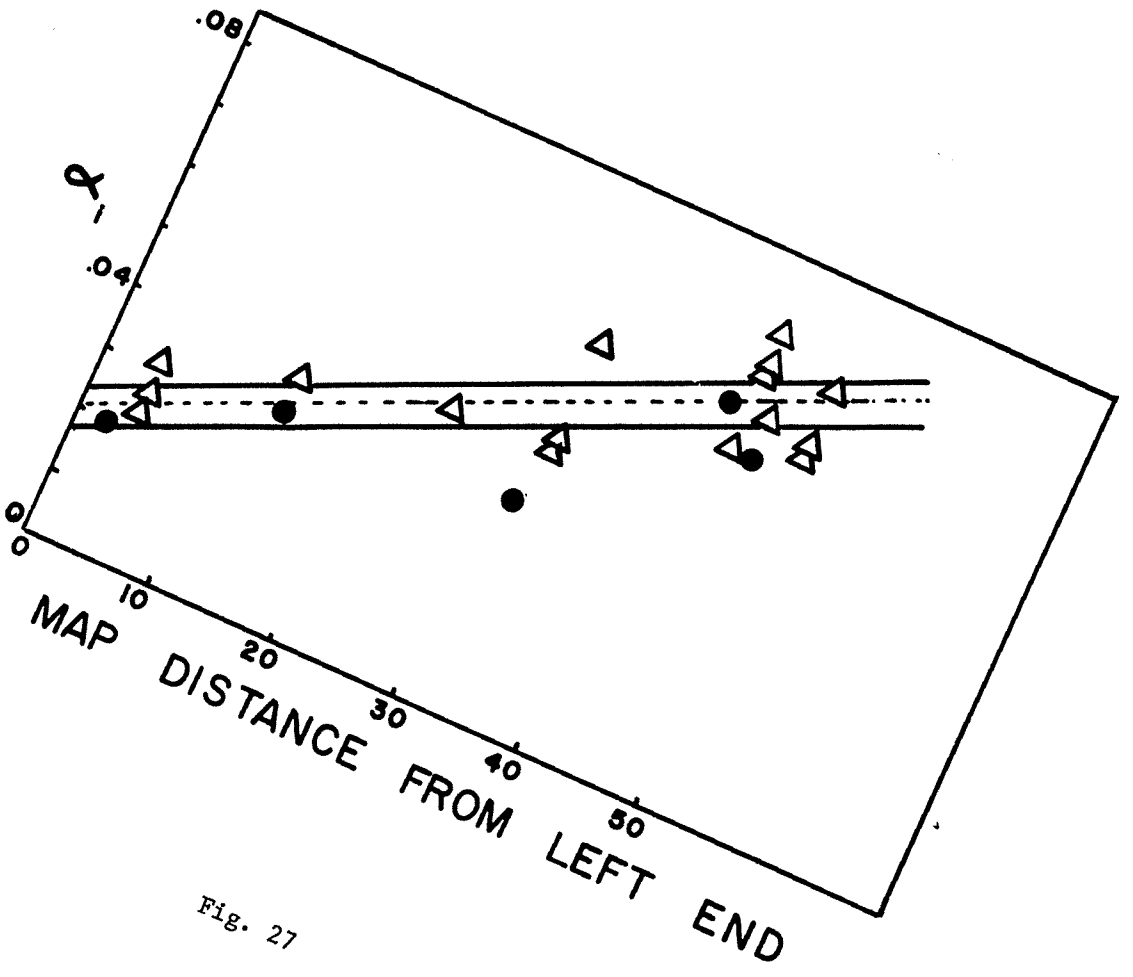


Fig. 27

Figure 28

The efficiency of inactivation of double markers versus the map distance they subtend.

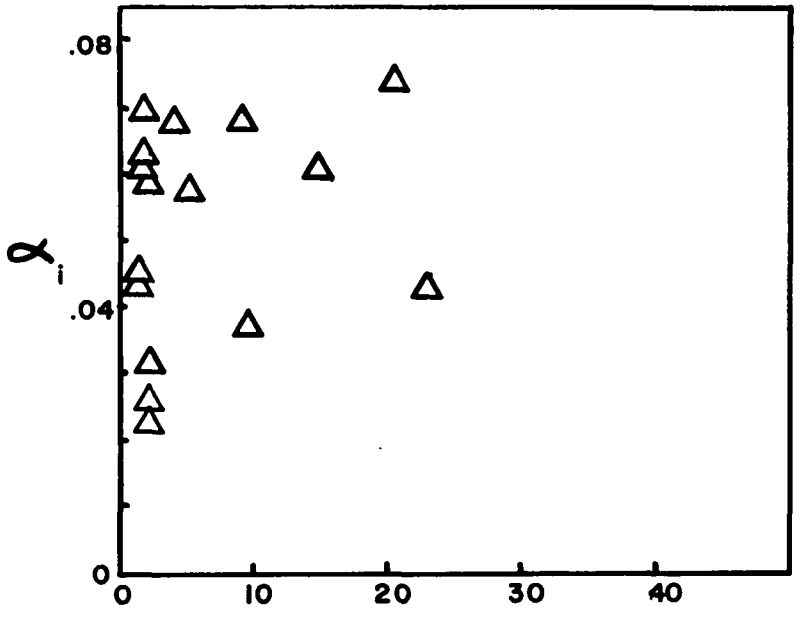


Fig. 28

transfer of that marker to the cell during infection as a result of the fragmentation of the genome. Only events of the former type contribute to the intrinsic sensitivity of the marker and the sensitivities of all markers to these events should be approximately the same. However, the sensitivity of markers to events of the second type would vary with their position on the genome, and would be superimposed upon their intrinsic sensitivities.

If the fractional injection model was not correct, and ^{32}P damages in fact led to an "all or none" injection of the phage genome, then the increased sensitivity of markers at the right end of the map could only be explained by assuming that these markers are intrinsically more sensitive to the effects of ^{32}P decay. Such a bias in the sensitivity of different markers would be observed not only during extracellular inactivation (in free phage) but also during intracellular inactivation (in infectious centers) where ^{32}P decay proceeds after the transfer of the genome to the cell is complete.

Effect of ^{32}P decay on marker survival in infected bacteria

To examine the effect of ^{32}P decay after infection, bacteria were rapidly infected with highly radioactive ts mutant phage (H167-A4) or a non-radioactive control and diluted into growth media. After a sufficient length of time to allow the penetration of the entire phage genome, the bacteria were centrifuged free of unadsorbed phage, resuspended and frozen at -20°C. It is known that shortly after the onset of phage growth the sensitivity of the phage to ^{32}P decay decreases (2, 12). However, this stabilization process does not occur in the absence of protein synthesis (24). To prevent stabilization, all steps prior to freezing were carried out in the presence of chloramphenicol

(100 ug/ml). At intervals, samples were withdrawn, thawed at 47°C, super-infected with a different ts mutant and assayed for WT recombinants. The efficiency of inactivation for infectious centers and for each marker (or marker pair) was calculated as before from the slope of the survival curves. At the same time experiments similar to those described in the previous section were used to determine the effect of ^{32}P decay on the marker sensitivity and survival of free phage stored at -20°C.

The results are summarized in Table 7. The survival curves for free phage and for infectious centers stored at -20°C are shown in Figure 29. There is no difference between the two. The efficiency of inactivation of markers under the same conditions plotted as a function of their cumulative map distance from the left end of the map are shown in Figure 30. In free phage stored at -20°C the dependence of marker sensitivity on map position is still apparent. However, in infectious centers stored at -20°C no dependence of marker sensitivity on map distance is observed. Linear regression analysis of the data in Figure 30 shows that the Y intercept of both curves is nearly identical (0.0054 for free phage, 0.0051 for infected bacteria).

These results are critical for they verify both the hypothesis of fractional injection, and the concept of intrinsic sensitivity. When phage are inactivated by the decay of incorporated ^{32}P under extracellular (free phage) conditions there is an obvious dependency of marker sensitivity on map position. However, when ^{32}P decay takes place after the genome has been transferred to the cell, the sensitivity of all markers, regardless of map position, is approximately the same and coincides with the intrinsic sensitivity of markers calculated as α_o, the sensitivity of markers on free phage when located at the most proximal position of the genetic map.

TABLE 7

The efficiency of inactivation of markers at -20°C under extracellular and intracellular conditions.

Marker	Map distance from left end	α_i	α_T
Extracellular conditions			
H177	2.2	.0024	
H20	14.0	.0038	
H362	32.2	.0164	
H2	44.0	.0282	.0506
E119	47.0	.0218	
H177-G55	4.3	.0128	
H362-H180	33.5	.0216	
H42-H27	45.5	.0088	
E14-H24	50.6	.0191	
Intracellular conditions			
H177	2.2	.0007	
H20	14.0	.0092	.0506
H362	32.2	.0052	
H2	44.0	.0104	
E119	47.0	.0002	

Figure 29

Sensitivity of free phage and infectious centers to ^{32}P decay at -20°C

The ability of highly labelled phage (specific activity = 42 mc/mg) and of bacteria infected with these phage to give rise to infectious centers after storage at -20° is plotted as a function of the fraction of ^{32}P decayed ($1 - e^{\lambda t}$). Free phage △, infected bacteria ○.

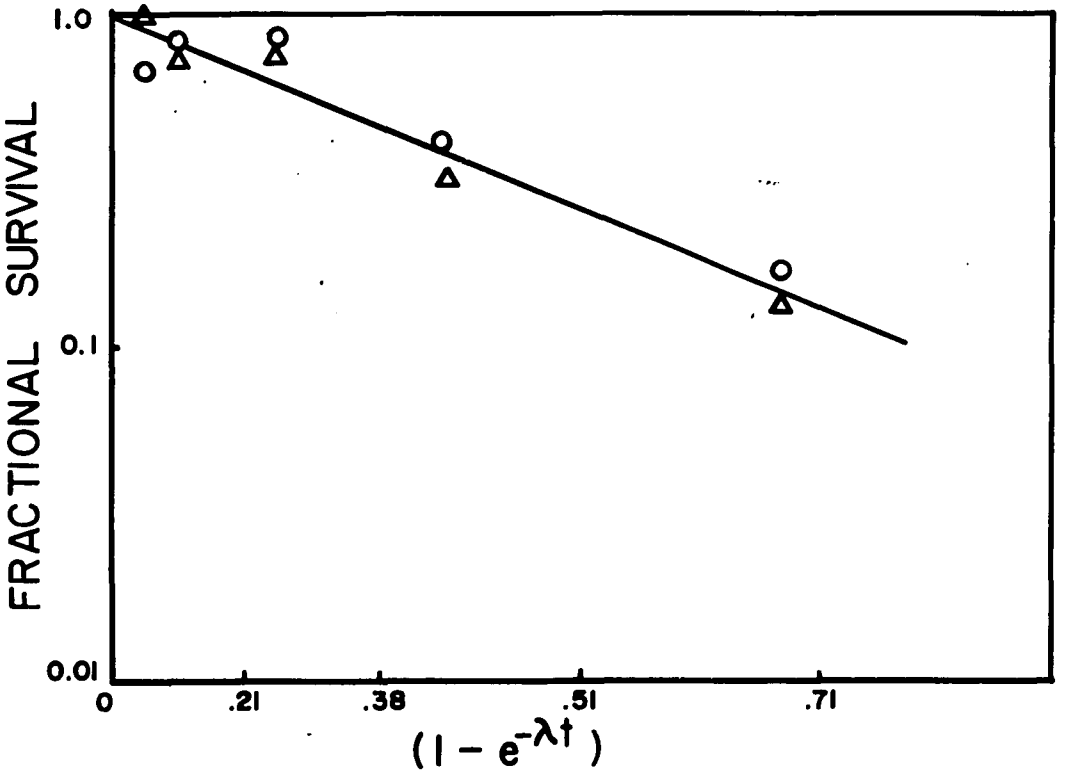


Fig. 29

Figure 30

The efficiency of inactivation of markers at -20°C versus their distance from the left end of the map

The data in Table 7 is plotted in the form of α_i versus the distance of the genetic marker from the left end of the map: △ values of α_i in free phage, ○ values of α_i in infected bacteria, --- values of α_i in free phage stored at 4°C (from Fig. 27).

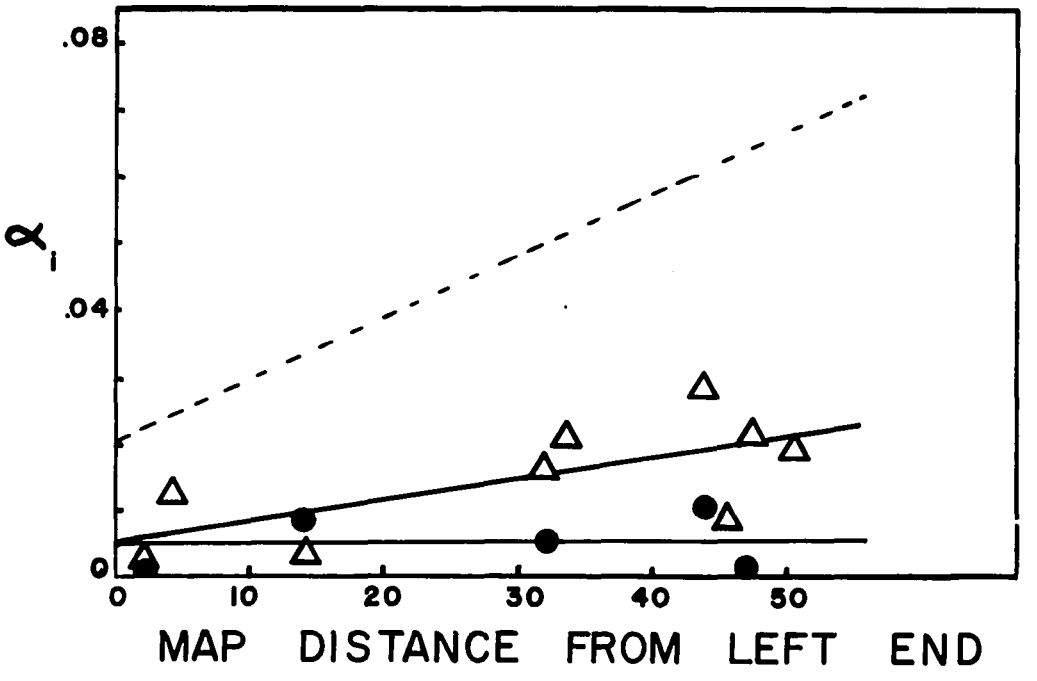


Fig. 30

Blendor experiments

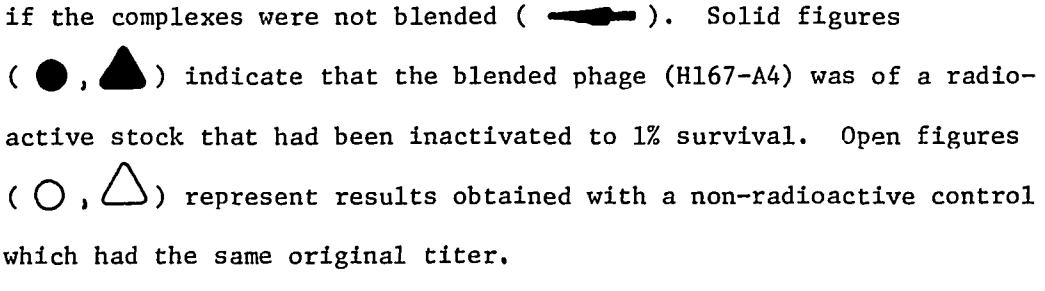
To examine the transfer of DNA from ^{32}P inactivated phage, bacteria were exposed to highly labelled bacteriophage and diluted into growth media at 33°C for 12 minutes. The complexes were chilled and blended and the bacterial pellets were examined for associated radioactivity. When labelled bacteriophage that had been inactivated by ^{32}P decay to a survival level of 1% were used the amount of ^{32}P still associated with the pellet after blending ranged from 28-53% compared with 78% for non-inactivated phage. This is in excellent agreement with the results of Hershey et al (13).

The time of entry of markers on the phage genome may be examined by halting the transfer of DNA, removing the untransferred DNA by blending, and assaying for the presence of markers in the blended complexes (16). Bacteria were rapidly infected with a ts mutant phage (H167-A4) and at intervals the complexes were chilled and blended. To assay for the presence of markers that are transferred at early times (H177-G55) or at late times (E14-H24) the complexes were superinfected with the ts mutants indicated and plated at 47°C. WT recombinants can only be formed when the necessary information has been transferred to the complex prior to the time of blending. This technique permits a comparison of the rate of transfer of markers on a genome which has been damaged by ^{32}P decay to the rate of transfer on an undamaged genome. Two preparations of donor phage (H167-A4) were used: a radioactive lysate that had been inactivated to the 1% level of survival, and a non-radioactive control which had the same original titer. The results are shown in Figure 31 where the number of WT recombinants is plotted against

the time of chilling. The linear portion of each curve is extrapolated to intersect with the yield of WT recombinants from a control that was never blended. This is an indication of the time at which the transfer of each marker has been completed in the entire population. Markers that are transferred on a ^{32}P damaged genome (inactivated to 1% survival) attain saturation levels at the same time as those on undamaged genomes regardless of whether they are transferred at an early time or at a late time. Thus those markers which <u>are</u> transferred on a damaged genome are transferred at the same rate as those on an undamaged genome. Note however the absolute rescuability of markers. At the 1% survival level the rescue of the double mutant H177-G55 is 33% that observed in the non-radioactive control. However, the rescue of the double mutant E14-H24 is 5% that observed in the control. This difference reflects the increased sensitivity of markers at the right end of the map to the effects of ^{32}P decay.

Figure 31

Effect of ^{32}P decay on time of transfer of genetic markers during phage infection



Bacteria were briefly exposed to the ts mutant phage H167-A4 and at intervals the complexes were chilled. DNA which had not yet been transferred to the host bacterium was removed by blending. The presence of markers H177-G55 and E14-H24 in the blended complexes was assayed by superinfecting with these phage and plating for WT recombinants at 47°. The linear portion of each curve has been extrapolated to intersect with the yield of recombinants obtained if the complexes were not blended (▬). Solid figures (● , ▲) indicate that the blended phage (H167-A4) was of a radioactive stock that had been inactivated to 1% survival. Open figures (○ , △) represent results obtained with a non-radioactive control which had the same original titer.

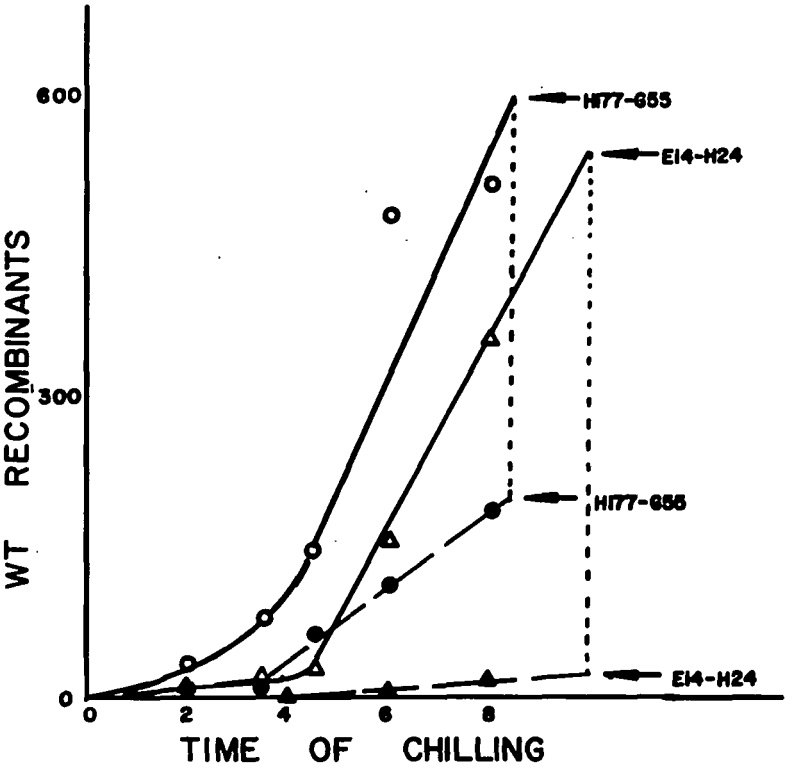

600
300
0
WT RECOMBINANTS
0
2
4
6
8
TIME OF CHILLING
HI77-655
EI4-H24
HI77-655
EI4-H24

Fig. 31

Discussion

These experiments show that the disintegration of incorporated ^{32}P can prevent the rescue of genetic markers on the bacteriophage SP82G genome by two different processes. In the first process, ^{32}P disintegrations fragment the genome and prevent the efficient transfer of markers that are distal to the break. In the second process a direct "hit" by a ^{32}P decay inactivates the genetic potential of the marker even if it is transferred. Events of the latter type (direct hits) contribute to the "intrinsic sensitivity" of the marker and this is approximately the same for all markers tested regardless of their map positions. The intrinsic sensitivity of markers can thus be estimated either as the efficiency of inactivation of markers after their transfer to the cell (in frozen stored infectious centers) or, in free phage as the sensitivity of markers located at the origin of the genetic map. The intrinsic sensitivity of markers α_o, at 4°C is 0.0170 marker inactivation events/^{32}P decay, while at -20°C α_o = 0.0054 marker inactivations/^{32}P decay.

Since the failure to transfer any necessary genetic information would result in the inactivation of the phage, it might be expected that damages leading to the non-transfer of a portion of the genome could exert a lethal effect only under extracellular conditions. If these damages were a special class of lethal events that could occur only in free phage, and were not in themselves lethal under all conditions, then the sensitivity of free phage to ^{32}P decay should be substantially higher than that of intracellular phage. An estimate of just how large a difference could be expected may be obtained from Figure 27. The most distal

gene known (and the most sensitive) is located at about 52 map units from the left end of the map and would have an efficiency of inactivation of $\alpha_{i_{max}} = 0.065$. By extrapolation of the curve to map units = 0, one can obtain the efficiency of inactivation of a marker located at the least sensitive end of the map. This is the intrinsic sensitivity of single markers and at 4°C has a value of $\alpha_o = 0.0170$. The contribution of damages which lead to the non-transfer of a portion of the genome to the total efficiency of killing, α_T, would be the difference between these values, or $\alpha_{i_{max}} - \alpha_o = 0.048$. The efficiency of killing of free phage at 4°C is $\alpha_T = 0.1157$ and thus non-transfer could account for 0.048/0.1157 or 42% of all lethal events. If these damages were lethal in extracellular phage but not in intracellular phage the sensitivity of free phage to ^{32}P decay should be 42% higher than that of intracellular phage. However, the efficiency of killing phage under intracellular conditions at -20°C is no different than that of free phage at the same temperature (Figure 29). Clearly, damages which lead to non-transfer of the phage genome result from events that are in themselves lethal even when injection proceeds normally. This is expected, for it is difficult to conceive of a damage which leads to the fragmentation of the genome and is not lethal. It is noted however, (Fig. 31) that the portion of the genome that <u>is</u> transferred is transferred at the same rate as an undamaged genome.

Single strand breaks probably have little, if any, role in preventing the transfer of genetic material since Green has observed that there are 1.3 naturally occurring randomly placed single strand breaks on the SP82G genome (Green and Hall, personal communication) which apparently have no effect on phage viability. By direct measurement, Tomizawa and Ogawa (7) have found that double strand breaks

account for about 39% of all lethal events occurring in bacteriophage λ at 4°C. Similarly Harriman and Stent (8) have estimated that long range lethal events (presumably resulting from double strand breaks) account for about 41% of all lethal events in bacteriophage T4. The similarities of these values and the observation that in SP82G non-transfer of some portion of the genome occurs in about 42% of all lethal events at 4°C suggests that at this temperature all double strand breaks prevent the transfer of the portion of the genome distal to the break. This conclusion gains some support from the observation that at the 1% survival level, phage transfer only about 28-53% of their ^{32}P label to the host. At this level of survival there have occurred 4.6 lethal hits/phage ($e^{-4.6}=0.01$) and of these lethal events 42% or 1.9 hits/phage should be due to double strand breaks. The mean molecular weight of the phage DNA after sustaining 1.9 double strand breaks is given by:

$$\frac{MW_t}{MW_o} = \frac{2\ (e^{-p} + p - 1)}{p2}$$

where MW_o and MW_t are the mean molecular weights before and after fragmentation, respectively, and p=the total number of hits/molecule. (25). Insertion of p = 1.9 gives a value of $MW_t/MW_o = 0.57$. In an undamaged phage population, 78% of the ^{32}P label is transferred. At the 1% level of survival these calculations would predict that if all double strand breaks prevented the transfer of the distal portion of the genome, only (0.57) (78%) or 44% of the label would be transferred. The actual values observed, though having a wide range (28-53%) are not inconsistent with this prediction.

Harriman and Stent (8) have proposed that in bacteriophage T4 double strand breaks result in long range lethal events which cause the inactivation of the genetic potential of the entire genome. Contrary to

the expectations of the Harriman and Stent model, however, double strand breaks do not always inactivate the genetic potential of the entire genome of SP82G. If they did, then no polarity of marker sensitivity with map position would be noted - any double strand break would result in the inactivation of the entire genome - including that portion that was transferred. That the macromolecular continuity of the genome is not necessary for marker rescue also follows from the blendor experiments in which markers are rescued even after the untransferred DNA has been removed from the infected complexes by blending (16, part II of this thesis). Furthermore, there is some evidence to indicate that this might also be the case in bacteriophage T4. Harm (15) has observed that even though the inactivation of extracellular bacteriophage T4 by X-ray irradiation results in a fractional injection of the genome, genetic markers may be rescued from bacteria infected with these particles. The observation that all markers are rescued with similar efficiencies is due to the fact that T4 has a circularly permuted genetic map, and thus the order of transfer of markers from a T4 population is random.

Although double strand breaks do not inactivate the genetic potential of the entire SP82G genome, whether the same is true of the functional potential of the genome is an unanswered question. This possibility could be tested, however, since the function of gene G55 is known and is readily assayed. (26) If double strand breaks resulted in the functional inactivation of the entire genome, then the sensitivity of function of gene G55 to ^{32}P decay should be substantially higher than its genetic sensitivity measured by marker rescue.

While it is possible that the intrinsic sensitivity of markers (the sensitivity of markers to ^{32}P decay regardless of map position) is

due to some minor fraction of double strand breaks that do inactivate the genetic potential of the entire genome, this does not seem likely, since the intrinsic sensitivity of double mutants is greater than that of single mutants (Figure 30). It is more probable that the intrinsic sensitivity of markers results from damages of a short range type affecting only a portion of the genome. The relative contributions of single and double strand breaks to damages to this type cannot be determined from the data at hand. However, the contribution of single strand breaks to genetic inactivation could be determined by use of another unstable isotope of phosphorous, ^{33}P. This isotope has a similar decay scheme to ^{32}P, but its recoil energy is much less than that of ^{32}P and is not sufficient to cause double strand breaks (27, 28). A comparison of the intrinsic sensitivity of markers to ^{32}P and ^{33}P decay should provide the necessary data.

The sensitivity of genetic markers to ^{32}P damages is about 1/7 to 1/9 that of the whole phage (α_o/α_T = 1/7 at 4°C, 1/9 at -20°C). Since there are twenty seven known cistrons on the SP82G genome (Green, personal communication) each marker must present a target size which is larger than its actual physical contribution to the genome. Thus the effect of a short range hit is not limited to the cistron in which it occurred but may affect the rescue of markers at some distance from the damage. To account for such observations in bacteriophage T4 Harriman and Stent proposed that the functional inactivation of a cistron occurred when (i) a sublethal (single strand break) had occurred within the cistron and (ii) a short range lethal hit had occurred elsewhere in the genome. Short range lethal hits were presumed to be lesions in the DNA caused by free-radical reactions; these damages prevented the repair of the single strand breaks present

in the cistron. To account for the disproportionately large target size of markers in SP82G it is only necessary to assume that ^{32}P disintegrations do not produce localized damages, but have a spreading effect which may affect markers as far away as 1/14 to 1/18 of the genome on either side of the damage.

To briefly enumerate the results of the experiments presented here, the following conclusions have been reached:

1. The disintegration of incorporated ^{32}P can prevent the rescue of genetic markers on the bacteriophage SP82G genome by two different processes. In the first process ^{32}P disintegrations fragment the genome and prevent the efficient transfer of markers that are distal to the break. In the second process damages resulting from ^{32}P decay prevent the rescue of genetic markers even if they are transferred. Events of the latter type contribute to the "intrinsic sensitivity" of the marker and this is approximately the same for all single markers regardless of their map positions.
2. Non-transfer of some portion of the phage genome occurs in 42% of all lethal events at 4°C.
3. At 4°C double strand breaks probably always prevent the transfer of portions of the genome distal to the break, and for this reason are lethal to the phage.
4. Double strand breaks are also lethal if they occur after the transfer of DNA has taken place.
5. Double strand breaks do not prevent the rescue of genetic markers on undamaged portions of the genome but rather contribute to damages of a "short range" nature.

Appendix

The regression curves for single and double mutants are described by:

$$Y = a_i + b_i x \qquad (23)$$

where a_i is the Y intercept and b_i the slope of the curve. For single mutants i=1 and for double mutants i=2. If the lines are identical they will have a common variance, s^2, and the ratio of their individual variances, s_1^2 will be distributed as F with n_1-2 degrees of freedom. For the data presented in Table 1A, $s_1^2/s_2^2 = 1.26$ which is acceptable at the 5% confidence level [$F_{.95}(3,15)=3.29$]. The slopes of the curves are given by b_1=.00081 and b_2=.00083. A test for whether the curves share a common slope is given by dividing (b_1-b_2) by the standard deviation of (b_1-b_2) or $(b_1-b_2)/0.0002=-0.0751$. This is distributed as $t(n_1+n_2-4)$ or t(18) and is not significant [$-1.734 < t_{.95}(18) < 1.734$]. Thus we accept the null hypothesis that the lines are parallel. The joint slope is b = 0.000822 and the joint variance is s^2=0.00005.

If the lines are concident, the statistic

$$\frac{(a_1-a_2) - b(\bar{x}_1-\bar{x}_2)}{s\left[1/n_1 + 1/n_2 + (\bar{x}_1-\bar{x}_2)^2 \Big/ \sum_{1}^{2} \sum_{v}^{n_i} (x_i v-\bar{x}_i)^2\right] 1/2}$$

will be distributed as t with $n_1 + n_2$-3 or 19 degrees of freedom where n_i is the number of observations in the i^{th} set and v is the number of observations of Y_i for each x_i. Evaluation of this with data from Table 1A gives a value of -3.53 which is not acceptable at the 5% level of confidence ($-1.729 < t_{.95} < 1.729$). Thus the regression curves are

parallel, but cannot be regarded as coincident.

TABLE I A(1)

Statistical analysis of data presented in Table 1*

Single mutants

x	2.2	14.0	32.2	44.0	47.0	m
i	1	1	1	1	1	Σ
j	1	2	3	4	5	j
y_{ij}	0.0193	0.0311	0.0335	0.0606	0.0533	
n_j	1	1	1	1	1	5
Σy	0.0193	0.0311	0.0335	0.0606	0.0533	0.1978
Σy^2	0.004	0.0010	0.0011	0.0037	0.0028	0.0090
Σx	2.2	14.0	32.2	44.0	47.0	139.4
Σx^2	4.84	196.0	1036.84	1936.0	2209.0	5382.68
Σxy	0.0425	0.4354	1.0787	2.6664	2.5051	6.7281
$\bar{x}$	27.88					
$\bar{y}$	0.0396					

* The following notations are used:

x = distance of single markers (i=1) or farthest double marker (i=2) from left end of map.

y = observed sensitivity of marker or marker pairs to ^{32}P inactivation.

j = the number of values of x for which values of y were measured.

n = the total number of observation in each set of data.

TABLE I A(2)

Statistical analysis of data presented in Table 1*

Double mutants

x	4.3	33.5	45.5	50.6	14.0	25.2	47.0	m
i	2	2	2	2	2	2	2	Σ^2
j	1	2	3	4	5	6	7	j
y_{ij}	0.0028	0.0605	0.0671	0.0624	0.0371	0.0424	0.0605	
	0.0310	0.0450	0.0739	0.0570				
	0.0252	0.0431	0.0547	0.0693				
			0.0672	0.0583				
n_j	3	3	4	4	1	1	1	17
Σy	0.0790	0.1486	0.2629	0.2470	0.0371	0.0424	0.0605	.8775
Σy^2	0.0021	0.0076	0.0175	0.0153	0.0014	0.0018	0.0037	.0494
Σx	12.9	100.5	182.0	202.4	14.0	25.2	47.0	584.0
Σx^2	55.47	3366.75	8281.0	10241.44	196.0	635.04	2209.0	24984.7
Σxy	0.3397	4.9781	11.9620	12.4982	0.5194	1.0685	2.8435	34.2094
$\bar{x}_2$	34.3529							
$\bar{y}_2$	0.0516							

* The following notations are used:

x = distance of single markers (i=1) or farthest double marker (i=2) from left end of map.

y = observed sensitivity of marker or marker pairs to ^{32}P inactivation.

j = the number of values of x for which values of y were measured.

n = the total number of observation in each set of data.

References

1. Hersey, A.D., Kammen, M.D.,Kennedy, J.W. and Best, H., The Mortality of Bacteriophage Containing Assimilated Radioactive Phosphorous. J. Gen. Physiol., 34, 305 (1957).
2. Stent, G.S., and Fuerst, C.P., Inactivation of Bacteriophages by Decay of Incorporated Radioactive Phosphorous. J. Gen. Physiol. 38, 441 (1955).
3. Stent, G.S., Mating in the Reproduction of Bacterial Viruses. In Advances in Virus Research, 5, K.A. Smith and M. Lauffer, eds., Academic Press, N.Y. pp. 95-149 (1958).
4. Thomas, C.A., The Release and Stability of the Large Subunit of DNA from T2 and T4 Bacteriophage. J. Gen. Physiol. 42, 503 (1959).
5. Davidson, P.F., Freifelder, D., Hede, R. and Levinthal, C., The Structural Unity of the DNA of T2 Bacteriophage. Proc. Nat. Acad. Sci. (U.S.) 47, 1123 (1961).
6. Ogawa, H. and Tomizawa, J.I., Breakage of Polynucleotide Strands by Disintegration of Radiophosphorous Atoms in DNA Molecules and their Repair. I Single Strand Breakage by Transmutation. J. Mol. Biol. 30, 1 (1967).
7. Tomizawa, J.I. and Ogawa, H., Breakage of Polynucleotide Strands by Disintegration of Radiophosphorous Atoms in DNA Molecules and their Repair. II Simultaneous Breakage of both Strands. J. Mol. Biol. 30, 7 (1967).
8. Harriman, P.P. and Stent, G.S., The Effect of Radiophosphorous Decay on Cistron Functions in Bacteriophage T4. I. Long-range and short-range hits. J.Mol. Biol., 10 488 (1964).

9. Krieg, D., A Study of Gene Action in UV irradiated Bacteriophage T4. Virology, 8, 80 (1959).

10. Stahl, F.W., The Effects of the Decay of Incorporated Radioactive Phosphorous on the Genome of Bacteriophage T4. Virology, 2, 206 (1956).

11. Stent, G.S., Cross Reactivation of Genetic Loci of T2 Bacteriophage after Decay of Incorporated Radioactive Phosphorous. Proc. Nat. Acad. Sci. (U.S.) 39, 1234 (1953).

12. Stahl, F.W., Radiobiology of Bacteriophage. In The Viruses, II, F.M. Burnet and W.M. Stanley, eds., Academic Press, N.Y. pp. 353 (1959).

13. Hershey, A.D., Garen, A., Fraser, D.K. and Hudis, J.D., Growth and Inheritance in Bacteriophage. Carnegie Institute of Washington Yearbook, 53, 210 (1954).

14. Tomizawa, J.I., (1959), quoted by F.W. Stahl ref. 12.

15. Harm, W., Multiplicity Reactivation Marker Rescue and Genetic Recombination in Bacteriophage T4 following X-ray Inactivation. Virology, 5, 337 (1958).

16. McAllister, W.T., Bacteriophage Infection: Which end of the SP82G Genome goes in First? J. Virology, 5, 194 (1969).

17. Green, D.M., Intracellular Inactivation of Infective SP82 Bacteriophage DNA. J. Mol. Biol. 22, 1 (1966).

18. Nomura, M.K., Matsubara, K., Okamoto, K. and Fujimara, R., Inhibition of Host Nucleic Acid and Protein Synthesis by bacteriophage T4: Its Relation to the Physical and Functional Integrity of the Host Chromosome. J. Mol. Biol., 5, 535 (1962).

19. King, E.J., The Colorimetric Determination of Phosphorous. Biochem. J., 26, 292 (1932).

20. Fiske, C.H. and Subbarow, Y.J. The Colorimetric Determination of Phosphorous. J. Biol. Chem., 66, 375 (1925).

21. Green, D.M., Gene Dislinkage in Transfection of SP82G Phage DNA. Genetics, 60, 673 (1968).

22. Kahan, E., A Genetic Study of Temperature Sensitive Mutants of the Subtilis Phage SP82. Virology 30, 650 (1966).

23. Brownlee, K.A., Statistical Theory and Methodology In Science and Engineering. Wiley and Sons, N.Y. 334-397 (1965).

24. Uchida, H. and Stent, G.S., Protein Synthesis and the onset of Intracellular Bacteriophage Growth. I. Stabilization of the Infecting Phage to ^{32}P decay. J. Mol. Biol., 2, 251 (1960).

25. Charlesby, A., Molecular Weight Changes in the Degradation of Long-Chain Polymers. Proc. Roy. Soc. London, Ser. A. 224, 120 (1954).

26. Alegria, A., Ph.D. Thesis, Dept. of Chemistry, Harvard University (1968).

27. Apelgot, S., and Laterjet, R., Comparison des 'suicides' d'une bacterie marquee par les Phosphores Radioactifs 32 et 33. Int. J. Rad. Biol. 10, 165 (1966).

28. Steinberg, C., Mechanism of Killing by Decay of Radioactive Phosphorous in Bacteriophage T4. Science, 154, 429 (1966).