SPECIATION AMONG THE T-EVEN BACTERIOPHAGES

Dissertation by

Richard Lawson Russell

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1967

(Submitted September 23, 1966)

ACKNOWLEDGMENTS

I am grateful to Dr. Robert S. Edgar for his valuable guidance and interest throughout my tenure as a graduate student; the guidance was usually instructive and almost never heavy handed, and his genuine interest constituted a considerable stimulus. Probably his most valuable contribution to my development has been his demonstration, by example, of the esthetic and persuasive value of well-chosen experiments. I am deeply indebted to Robert J. Huskey for his considerable contribution to the content of this dissertation; many of the studies on the T2-T4 mixed infection were joint efforts in which we participated equally, and it is only with his permission that they appear below. Jean Weigle, whose earlier work laid the foundations for this dissertation, provided welcome encouragement, a willing ear, and a valuable sense of perspective. To Jon and Flash, for listening, for criticizing, and for generally livening the place up, I owe a great deal.

I would also like to thank the people who have made the Biology Division a pleasant place in which to work; Ray Owen, for his light but effective administrative touch, Geraldine Cranmer, for administering it, Jerry Fling, for financing it, Max Delbrück, for his inspiring pensive perambulations past our lab and for his role as Papa Phage, and the staffs of the Biology Office and the Stockroom, for the efficiency and willingness with which they provide appreciated services. Robbi Hunt deserves special praise for her devotion to the task of typing this dissertation.

To Stephanie go my most heartfelt thanks for her continued support and love, which have contributed greatly to my development. I hope that this

dissertation constitutes some measure of reward for her.

This work has been supported by United States Public Health Service Training Grant 5 T1 GM 86 and stimulated by the Fried Fund.

ABSTRACT

A system of amber mutants has been developed for each of the T-even phages T2, T6, and RB69. In T2 these mutants identify 53 genes, of which 46 are homologous with T4 genes. The order of genes in the T2 map is virtually identical to that of T4, and recombination in T2 strongly resembles that in T4.

In T6 the mutants identify 42 genes, of which 34 are homologous to T4 genes and 3 are homologous to T2 genes not yet identified in T4. The few T6 gene orders which have been determined are the same as in T2 and T4, and T6 recombination resembles that of T2 and T4.

RB69 is judged to be a member of the T-even phage species by its serological properties, its particle morphology, and various physical parameters of the RB69 particle and its contained DNA. RB69 mutants identify 37 genes, and their phenotypes are the same as those of T4 mutants. Recombination in RB69 strongly resembles that in T2 and T4, and the distribution of mutant phenotypes around the RB69 map is very much like that of T4.

T2, T4, and T6 differ most noticeably in the tail fiber region. Genes 37 and 38 are a unique example of a pair of co-adapted genes; T2-T6 combinations of the products of these genes are compatible, whereas T2-T4 and T4-T6 combinations are not. The host range differences between the phages are determined by gene 38. Genes 34 and 35 are genetically much smaller in T2 and T6 than in T4, but there is considerable homology between T2 gene 34 and T4 gene 34. The degree of homology

between T2 and T4 decreases from gene 34 to gene 38.

In T2-T4 mixed infections T4 excludes T2 by acting against localized exclusion sensitivity determinants near early genes in the T2 genome. This action prevents the T2 genome from participating in the formation of the replicating structure, and it prevents the determinants from appearing among the progeny, but it does not prevent T2 genes from functioning. Markers from the T2 genome appear among the progeny by recombination away from these determinants. The specificity of the T4 action is not controlled by any known T4 gene, nor does it depend on differences between T2 and T4 in DNA glucosylation.

The T4-T6 mixed infection is characterized by a depressor effect, and T4 excludes T6 strongly. T6 excludes T2 weakly, and RB69 excludes T2, T4, and T6 very strongly. T4 excludes thirty other newly-isolated T-even phages fairly strongly. A general model has been developed to account for all of these cases of exclusion, and it also accounts for cases of partial exclusion in the T5 and T3-T7 phage species.

From the relationships among the T-even phages and their interactions in mixed infections, an attempt is made to reconstruct the evolution of exclusion as a bacteriophage isolating mechanism. Analogies between exclusion and host-controlled modification as a bacterial isolating mechanism are discussed.

TABLE OF CONTENTS

I. Introduction

Species have always occupied a unique position in the classification of living things. As Dobzhansky has noted,

> There is, however, a systematic category which, in contrast to the others, has withstood the changes in nomenclature with a singular tenacity. This is the species. . . . Modern systematics has vindicated the intuitive conviction which workers in this field always had, and which was expressed concisely by Bateson (1922): 'Though we cannot strictly define species, they yet have properties which varieties have not, and . . . the distinction is not merely a matter of degree.' (1)

The differences between species are usually discontinuous, whereas those between varieties are not (2), and this distinction probably accounts for the tenacity of the species concept.

The basis for the discontinuities between species has been understood in principle for quite some time (3). Within a species, genetic exchange can occur between any two individuals; the genes of all the members of the species constitute, potentially at least, a single gene pool. Although geographically distinct varieties with different genetic compositions can arise within a species, the differences between these groups are always subject to elimination by genetic exchange, and at geographical confrontation points such differences do, in fact, disappear (4). Between members of different species, however, genetic exchange is prevented; the genes of the two species constitute two separate gene pools. Differences which arise between these species cannot be eliminated by genetic exchange, and discontinuities between them appear as these differences accumulate.

Such discontinuities are clearly the result of those so-called "isolating mechanisms" which prevent genetic exchange between species, and the question of the origin of species, which is of paramount importance to all of evolution, is in large part the question of the origin of these isolating mechanisms. Previous studies have revealed a variety of mechanisms which prevent genetic exchange between closely related pairs of existing species (5). Unfortunately, these species have always been those of higher organisms, in which genetic exchange occurs as a result of complicated reproductive processes, and the isolating mechanisms revealed often depend on secondary aspects of these processes. For instance, genetic exchange between some species of Drosophila is prevented by differences in mating behavior (6), and plant species are often genetically isolated by differences in ecological preference or in the season during which gametes are exchanged (7). The very complexity of these isolating mechanisms makes it difficult to investigate their origins.

It seems likely that a study of organisms with somewhat simpler processes for genetic exchange might give a clearer understanding of the origin of isolating mechanisms. Organisms for this study should fulfill two basic requirements; 1) they should have simple processes for genetic exchange, and 2) they should fall into clear-cut species. The microorganisms are an obvious choice, and among them the bacteriophages appear to fulfill the requirements best. Genetic exchange between them apparently involves simply the exchange of segments between naked DNA molecules inside the same cell (8), and several criteria

divide them into clear-cut species (9). (Since the concept of species among the bacteriophages may seem a bit novel, it is worth dwelling for a moment on these criteria; they are 1) serological relationship (9 - 15), 2) similarity of particle morphology (9, 16 - 23), 3) capacity for genetic exchange (9, 24 - 27), and 4) DNA base sequence homology (28). Each of these criteria divides bacteriophages into distinct groups, members of the same group resembling each other rather closely, members of different groups showing little resemblance. Whenever all four criteria have been used to classify the same set of phages, each has divided them into the same groups (28 , 29). The discontinuities between these groups, the consistency of the criteria in defining them, and the fact that three of the four criteria (nos. 2, 3, and 4) are also used to distinguish species among higher organisms, all suggest that these groups are truly analogous to the species of higher organisms.)

Within the T-series of phages active on Escherichia coli, four species can be distinguished; the so-called T-even phages, T2, T4, and T6 constitute one species, T3 and T7 another, T1 a third, and T5 a fourth (9, 28, 29). Mixed infections between phages of different species in this series reveal an isolating mechanism which prevents genetic exchange between them; each mixedly infected bacterium yields phages of only one of the two infecting types, never both, and in general the vast majority of the bacteria yield phages of a particular one of the two types (29 - 31). Since one of the parental phage types is excluded from the progeny, this phenomenon has been called exclusion. It occurs in all mixed infections between phages of different species from the T-series (29).

Mixed infections between T2 and T4, two different members of the T-even species, show a so-called partial exclusion; most of the mixedly infected bacteria yield phages of both infecting types plus various recombinants, but the progeny resemble T4 more than T2 (24, 32, 33). Since genetic exchange between T2 and T4 is possible, the T2 and T4 parental genes enter a common gene pool inside the bacterium, but the genes of the progeny are drawn primarily from the T4 component of this pool. Similar partial exclusion occurs in mixed infections between different members of the T5 species (26, 34) and between different members of the T3-T7 species (27, 35, 36).

Just as exclusion acts as an isolating mechanism between unrelated phages, partial exclusion operates as a partial isolating mechanism between related phages, and it is a central contention of this dissertation that partial exclusion represents one stage in the evolution of exclusion as an isolating mechanism.

The T-even phages offer a unique opportunity for the study of this evolution. First, the best studied case of partial exclusion occurs in T2-T4 mixed infections. Second, the T-even species contains many other members whose interactions may reveal other evolutionary stages (37, 38). Third, the attention focussed on the T-even phages by workers in the schools of Delbrück and Luria has made them the most thoroughly characterized of all phages (39). Fourth, the conditional lethal mutants recently developed in T4 provide a genetic description of that phage which is unrivalled in completeness (40, 41, 42). This dissertation consists of an exploration of the relationships between various members of the T-even

species and an examination of their interactions in mixed infections. Its approach relies heavily upon conditional lethal mutants, and its goal is to arrive at an understanding of some of the stages in the evolution of exclusion as an isolating mechanism among the bacteriophages.

The experimental section of the dissertation is divided into two parts. In the first part, systems of conditional lethal mutants are established for each of the phages T2, T6 and RB69; these mutants are used to compare these phages to one another and to T4. The purpose of this part is to elucidate possible evolutionary relationships among these phages. In the second part, conditional lethal mutants are used to study the interactions of these phages in mixed infections, with particular emphasis on the T2-T4 partial exclusion. The purpose of this part is to elucidate the nature and the evolutionary significance of the partial isolating mechanisms which characterize these phages.

II. Materials and Methods

A. Materials

Phages. T4D wild type, T2L wild type, T6 wild type, and all mutants of T4D were obtained from the collection of Dr. Robert S. Edgar. Phages indicated by the prefix RB followed by a number were obtained from Rosina O. Berry, who isolated them from six Long Island sewage treatment plants during her tenure as an Undergraduate Research Participant at the Cold Spring Harbor Laboratory for Quantitative Biology in the summer of 1964. These phages were selected for their ability to plate on Escherichia coli strain B/5 on LT plates, and were subsequently characterized by their reactions to antisera prepared against T4 and T6. Of the sixty-one phages isolated, thirty-one were judged to be related to the T-even phages by this criterion. T2 gt-1, a mutant of T2 deficient in alpha-glucosyl transferase (43), was obtained from Dr. Stanley Hattman. Amber mutants of T2 with the prefix FS were obtained from Dr. Franklin W. Stahl. All other mutants of T2 and all mutants of T6 and RB69 were isolated in this study and are described below.

Bacteria. The bacterial strains B/4_o of E. coli and Sh/4_o and Sh15 of Shigella dysenteriae were obtained from Dr. Stanley Hattman. The Shigella strains accept T-even phages which lack the normal glucose residues on their DNA, and B/4_o and Sh/4_o produce only such phages. The strain W4597 of E. coli, obtained from Dr. Sydney Brenner, resembles B/4_o in these respects. E. coli strain 704 was obtained from Dr. W. B. Wood, who had shown its ability to accept unglucosylated phages; it was

later shown to be permissive for most T2 and T4 amber mutants. All other bacterial strains were obtained from the collections of Drs. Robert S. Edgar and Jean J. Weigle. The E. coli strain BB from these collections proved to be permissive for most T2 and T4 amber mutants (44), in contrast to the BB strain maintained at the Virus Laboratories of the University of California at Berkeley (45). The origin of this difference is not clear.

Media. Hershey broth contained 8 g. DIFCO Nutrient Broth (dehydrated), 5 g. DIFCO Bacto Peptone, 5 g. NaCl, 1 g. dextrose (glucose), and 1000 ml. distilled H_2O, and was adjusted to pH 7.4 with 4% NaOH before distribution and autoclaving. K medium contained 7 g. Na_2HPO_4, 3 g. KH_2PO_4, 1 g. NH_4Cl, 0.5 g. NaCl, 0.6 g. $MgSO_4$, 2 g. dextrose (glucose), 15 g. DIFCO Casamino Acids, and 1000 ml. H_2O. Enriched Hershey bottom agar (used for most plates, 30 - 35 ml. per plate) contained 10 g. DIFCO Bacto-Agar, 13 g. DIFCO Bacto-Tryptone, 8 g. NaCl, 2 g. sodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), 1.3 g. dextrose (glucose), and 1000 ml. H_2O. Enriched Hershey top agar (used for most plates, 2 ml. per plate) contained 6.5 g. DIFCO Bacto-Agar, 13 g. DIFCO Bacto-Tryptone, 8 g. NaCl, 2 g. sodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), 3 g. dextrose (glucose), and 1000 ml. H_2O. Slant Medium No. 1 contained 18 g. DIFCO Bacto-Agar, 8 g. DIFCO Bacto-Tryptone, 1 g. DIFCO Yeast Extract, 5 g. NaCl, and 1000 ml. H_2O, and was adjusted to pH 7.4 with 4% NaOH before distribution and autoclaving. Tryptone broth contained 10 g. DIFCO Bacto-Tryptone, 5 g. NaCl, and 1000 ml. H_2O, and was adjusted to pH 7.4 before autoclaving. SSC contained 8.77 g. NaCl, 4.41 g. sodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$),

and 1000 ml. H_2O. LT bottom agar contained 10 g. DIFCO Bacto-Agar, 10 g. DIFCO Bacto-Tryptone, 5 g. NaCl and 1000 ml. H_2O. LT top agar (used only on LT bottom agar plates) contained 6.5 g. DIFCO Bacto-Agar, 10 g. DIFCO Bacto-Tryptone, 5 g. NaCl, and 1000 ml. H_2O. Phosphate buffer contained 7 g. Na_2HPO_4, 3 g. KH_2PO_4, 4 g. NaCl, and 1000 ml. H_2O; 2 ml of 1 M $MgSO_4$ was added after autoclaving. Dilution buffer contained 7 g. Na_2HPO_4, 3 g. KH_2PO_4, 4 g. NaCl, 0.5 g. gelatin, and 1000 ml. H_2O; 1 ml. of 1 M $MgSO_4$ was added after autoclaving.

Scintillation fluid contained 15.16 g. 2,5-diphenyloxazole (PPO), 0.19 g. 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), and 8 pt. toluene (Mallinckrodt Analytical Reagent), and was stored in brown bottles.

Chemicals. 5-bromodeoxyuridine (5-BUdR) was obtained from the California Corporation for Biochemical Research (A grade). 5-fluoro-deoxyuridine (5-FUdR) was the gift of Dr. R. Duschinsky of Hoffman-Laroche. PPO and POPOP were obtained from the Packard Instrument Company. CsCl (Optical Grade) was obtained from Harshaw Chemical Company. Redistilled phenol was the gift of L. Wenzel. Tritiated (H^3) thymidine was obtained from the New England Nuclear Corporation. Propylene glycol monolaurate, often used as an anti-foaming agent, was obtained from K & K Laboratories, Inc. All other chemicals were standard reagent grades.

Enzymes. Egg white lysozyme was the Grade 1 (3 x crystallized, dialyzed, and lyophilized) from Sigma Chemical Company. Trypsin was the bovine pancreas type 1 (2 x crystallized, ethanol precipitate) from Sigma Chemical Company. DNAse was the DNAse 1 (Stock no. DN-C, 155,000 units/mg.) from Sigma Chemical Company.

Antisera. The antisera used in this study were gifts from various people, as follows: anti-T2, J. J. Weigle; anti-T4, R. S. Edgar and J.E. Flatgaard; anti-T6, R. S. Edgar.

B. Methods

Maintenance of bacterial strains. Bacteria were maintained on slants of Slant Medium No. 1; transfers to fresh slants were carried out every six to eight weeks, followed by one day of growth at 37°C. and subsequent storage in the cold (4°C.).

Stock cultures of bacteria were made by inoculating about 30 ml. of Hershey broth with a loopful of cells from a slant and aerating for a period of about 16 hr. at 30°C. The final concentration of bacteria in such cultures varies from 2-8 x 10^9/ml., depending on the strain. Stock cultures were stored in the cold (4°C.) and used for 7-10 days.

Plating bacteria were made by diluting a stock culture 100-fold into fresh Hershey broth, aerating for either 2.5 hr. at 30°C. or 1.75 hr. at 37°C., centrifuging the turbid suspension for 10 minutes at 1500 x g in the cold (4°C.), and resuspending the bacterial pellet in one-tenth the original volume of chilled Hershey broth. Bacteria prepared in this manner have a higher efficiency of plating than do saturated cultures, and maintain their higher efficiency for about two days if stored at 4°C. Two drops of this suspension were used for each plate.

Bacteria for infections, including crosses and liquid culture complementation tests, were made by diluting a stock culture 500-fold into fresh Hershey broth, aerating for either 2.5 hr. at 30°C. or 1.75 hr. at 37°C., centrifuging the somewhat turbid suspension for 10 minutes at 1500 x g in the cold (4°C.), and resuspending the bacterial pellet in

one-fiftieth the original volume of chilled Hershey broth. By this procedure, early logarithmic phase bacteria were obtained at a final concentration of 1-2 x 10^9/ml., and could be diluted as desired.

Phage stocks were prepared in either of two ways. In the first method a plaque was picked from a young plate (4-5 hr. at 37°C., 6-8 hr. at 30°C., or 8-10 hr. at 25°C.) and used to inoculate a culture which had been grown for 2.5 hr. at 30°C. after a 500-fold dilution from a stock culture. After inoculation, aeration was continued until the culture cleared and a few drops of chloroform were added to lyse the remaining unlysed cells. (When the stocks were of T2 amber mutants and the bacteria for the stock-making were CR63, it was found best to incubate the inoculated culture at 25°C. Incubation at higher temperatures resulted in lower stock titers and often higher reversion indices.) The resulting stocks were sometimes centrifuged for 10 minutes at 4800 x g to rid them of large bacterial debris, and were then filtered through a Mandler candle filter to complete the cleaning. In the other method of stock-making, about 10^5 phages were plated on each of several plates, and after 12-15 hr. of incubation at 25°C., 0.5 ml. of chloroform was added to the surface of the confluent plate and allowed to evaporate. 5 ml. of Hershey broth was then added to the plate, and 2-3 hr. at room temperature was allowed for the phage to diffuse from the top agar layer into the broth. The broth was then collected (3-4 ml. could be recovered from a normal plate), and the resulting "plate stock" was centrifuged and filtered in the same way as the normal liquid culture stock described above. The Sagik effect (46) did not hamper use of T2 stocks, probably

because a matter of weeks usually elapsed between the making of a stock and its use for experiments in which its titer had to be accurately known.

High titer stocks for examination in the ultracentrifuge and the electron microscope were prepared by differential centrifugation. Large volume liquid culture stocks (1-2 l.) were prepared and centrifuged for 10 minutes at 4800 x g to remove large bacterial debris. They were then filtered through Mandler candle filters and the filtrate was centrifuged for 90 minutes at 23,300 x g to bring down the phage particles. The resulting pellet was gently resuspended in a small volume of 0.1 M tris buffer, pH 7.4, containing 0.01 M $MgSO_4$, and the suspension was centrifuged once again for 10 minutes at 4800 x g to rid it of large aggregates. Usually the resulting supernatant had a marked bluish opalescence and did not demonstrate the grayish turbidity characteristic of bacterial cultures.

5BU-labelled phages were prepared by a modification of the method of Stahl et al. (47). Cells were grown to 10^8/ml. in K medium, and 5-FUdR and 5-BUdR were added to give final concentrations of 10 µg/ml. and 200 µg/ml., respectively. The 5-FUdR prevented the formation of thymidine and the 5BUdR was an incorporable thymidine analog. The bacteria were allowed to double in number (about 2.5 hr. at 30°C.) and then phages were added at a multiplicity of about 3. Aeration was continued for several hours and the cells were then lysed artificially with chloroform. The resulting phages were purified by differential centrifugation and filtration. The burst sizes of viable phages

from such infections were commonly between 30 and 40. The viable titers of the purified phages were about four-fold lower than the bacterial killing titers, suggesting that about three-fourths of the phages had received lethal mutational hits as the result of incorporation of 5-BUdR into their DNA. The incorporation, as measured by the density of the phages in CsCl, and by their sensitivity to inactivation by visible light, was at least as extensive as that achieved by Stahl et al. (47), and appeared to be uniform for over 99% of the population.

Non-glucosylated T2 and T4 phages were prepared by one cycle of growth on either W4597 (for T4) or $B/4_o$ (for T2). The resulting phages plated normally on Shigella strains, but plated with an efficiency of only about 1-4 x 10^{-3} on S/6/5.

Standard phage techniques (48) were used for the dilution, plating and counting of phage samples, with the following restrictions: 1) no more than 0.5 ml. and no less than 0.05 ml. of a phage sample was ever plated, 2) no plates were used later than five days after they had been poured (the use of older plates leads to smaller plaques and an apparent drop in efficiency of plating), 3) no plating bacteria were used later than two days after they had been prepared, and 4) no counts of less than 100 plaques were accepted.

Standard phage terminology used below is explained in the glossary presented by Adams (48). A few relatively new terms require definition here. Conditional lethal mutants are able to grow under one set of conditions (permissive conditions) but not under another (restrictive conditions); wild type is able to grow under both. Amber mutants are

conditional lethals which grow on bacterial strains (such as CR63) which carry certain suppressor genes but not on strains (such as B/5 and S/6/5) which lack them. Temperature sensitive mutants grow at 25°C. but not at 42°C. Early genes make products which are involved in the metabolism of phage DNA; mutants defective in these genes fail to induce phage DNA synthesis. Late genes make products which are involved in the assembly of the phage coat; mutants defective in these genes are blocked at some stage in this assembly. As their names imply, early genes are generally expressed early in the latent period, and late genes late.

Mutagenesis of phages was accomplished as follows. The phage to be treated was grown in a restrictive host, to eliminate any large clones of amber mutants already present by chance. This stock was then used to infect permissive bacteria (CR63, 1-4 x 10^8/ml.) at a multiplicity of 5, and 5BUdR was added to bring the final concentration to 10 ug./ml. (This concentration had previously been found (49) to give a reasonably high frequency of amber mutants in T4.) The infected cells were then aerated vigorously until they lysed, and the progeny were plated as described below.

Selection of amber mutants was carried out by a method based on a suggestion by Dr. Richard H. Epstein. The phage sample plus two drops of normal CR63 plating bacteria were added to 2 ml. of melted top agar, mixed, and poured over 2-3 day old plates. After this initial layer had hardened, a second layer, consisting of 2 ml. of melted top agar plus two drops of a 100-fold dilution of restrictive (B/5 or S/6/5) plating bacteria was poured over the first layer and allowed to harden. After incubation

the bacteria in the upper layer gave rise to clearly separate microcolonies which were easily distinguished from the bacterial lawn below. Over a wild type plaque these microcolonies were missing from an area somewhat larger than the plaque. Over a plaque of an amber mutant, however, the microcolonies were present without reduction in number or size, giving the plaque a characteristic stippled appearance. With T2, T6, and RB69 about 10% of plaques selected for their stippled appearance contained amber mutants.

Stab testing of suspected mutants was carried out by stabbing into a stippled plaque a sterile pin which had been coated with a sterile mixture of vegetable dye and glycerin (to identify the plaque stabbed). The pin was then stabbed successively into defined positions on each of two plates, the first of which had been seeded with restrictive, and the second with permissive bacteria. Tests indicating the presence of an amber mutant were checked by picking the area of lysis from the permissive plate with a sterile glass capillary, transferring it to a few ml. of chloroform-saturated Hershey broth, allowing a few hours for the phage to diffuse out of the agar plug, and then respotting the resulting suspension on two plates seeded, respectively, with restrictive and permissive bacteria.

Complementation spot tests were carried out as follows. Amber mutant phage suspensions were prepared at 10^8/ml. One drop of each of a pair of suspensions was placed in one well of a UV-sterilized, clear plastic disposable tray (Linbro Chemical Corp. 96U-CV). Restrictive plating bacteria were diluted 20-fold into melted top agar held at 45°C., and

1 ml. of the mixture was squirted into the well with an automatic pipette. For each test two controls, one of each mutant alone, were performed. After incubation, tests were scored as negative if the wells contained only about as many plaques as present in the controls and positive if the wells contained a considerably greater number of plaques; controls and negative tests usually contained only a few scattered plaques, whereas in positive tests there was nearly confluent lysis of the bacterial lawn. Rare intermediate tests were repeated or abandoned in favor of liquid culture complementation tests. (The numerous plaques in positive spot tests were due to wild type recombinants formed in mixedly infected bacteria; since the bacteria were restrictive, the formation of these recombinants depended on the ability of the two mutants to complement one another.) Spot tests between mutants of the same phage worked well, whether the phage was T2, T4, T6, or RB69. Spot tests between mutants of different phages worked for phages T2, T4, and T6 in all combinations, but not for combinations of RB69 with any of these three.

Liquid culture complementation tests were used to check the results of spot tests and to clarify situations in which these results were ambiguous. Restrictive bacteria were prepared for infection, at a final concentration of 4×10^8/ml. Just before use, KCN was added to give a final concentration of 0.004 M. A test was performed by mixing together equal volumes of two amber mutant phage suspensions, each at 6×10^9/ml., and then adding to one volume of the mixture an equal volume of bacteria. After 8 minutes of adsorption at 30°C., anti-phage serum was added to give a final neutralization constant (k) of 1 min^{-1}. The antiserum was

allowed 5 minutes to inactivate unadsorbed phages, and the adsorption mixture was then diluted 40,000 fold into Hershey broth at 25°C. Samples were quickly taken for measuring infective centers and residual unadsorbed phages. After 120 minutes of incubation chloroform was added to ensure complete lysis. The progeny phage were then assayed, and the burst size was computed and compared to that of simultaneous controls. These controls were infections with wild type phages at the same total multiplicity (ca. 15) used in the tests, and they were usually inserted at the beginning and at the end of a series of tests. When the tests were between mutants of different phages, the controls were mixed infections between the wild types of the same two phages. The liquid culture tests have the advantage over spot tests that the results do not depend upon the production of recombinants.

Crosses. Bacteria were prepared for infection at a final concentration of 4×10^8/ml., and, just before use, KCN was added to give a final concentration of 0.004 M. Phage were prepared each at 6×10^9/ml. A cross was performed by mixing equal volumes of two amber mutant phage suspensions, each at 6×10^9/ml., and then adding to one volume of the mixture an equal volume of bacteria. Adsorption was allowed to proceed for 8 minutes if unadsorbed phage were to be eliminated with antiserum (occasionally) or 10 minutes if not (usually). Antiserum was used at a final k of 1 min^{-1} for 5 minutes. After adsorption the mixture was diluted 40,000 fold into Hershey broth at 25°C., incubated for 120-150 minutes, and then treated with chloroform to ensure complete lysis. A sufficiently small number of crosses (usually 20) was done with any one

batch of bacteria to ensure that no bacteria had been exposed to KCN for more than 22 minutes (such exposure tends to reduce the burst sizes obtained).

Progeny phages were plated at various dilutions on permissive and restrictive bacteria. The frequency of recombination was calculated by multiplying the titer on the restrictive strain by 2(to compensate for the unscored double mutant recombinants), dividing by the titer on the permissive strain, and multiplying by a correction factor for the inherent differences, if any, between the two strains in efficiency of plating. This correction factor was determined separately for each batch of plating bacteria by plating on them 0.5 ml. of a large volume stock of wild type phage at a titer of 1.2×10^3/ml.; it was often quite significant, usually being, for example, about 0.7 with T2 on CR63 and S/6/5.

Often a given cross was repeated a number of times. In general the agreement between the various values for the frequency of recombination was good, and an average value was taken. Occasionally, however, one or even sometimes two of a series of crosses seemed out of line with the rest of the series. In these cases, an average of the remainder of the values was taken, and the exclusion of some values from the average was noted.

Exclusion experiments were performed in much the same way as crosses and liquid culture complementation tests. The choice of permissive or restrictive bacteria depended on the purposes of the experiment, and in general the experiments were mixed infections between the wild type of one phage and a mutant of another. Mixed antiserum was almost always

used to eliminate unadsorbed phages. Progeny were assayed as in crosses, but instead of the frequency of recombination, the fraction of progeny carrying a marker from the excluded phage was calculated.

Measurements of lysis were performed with RB69 amber mutants as follows. Restrictive bacteria were prepared for infection at a final concentration of 4×10^9. Phages were prepared at 2×10^{10}/ml. Equal volumes of phage and bacteria were mixed and phage were allowed to adsorb for 5 minutes at 30°C. (During the adsorption period, the high bacterial concentration leads to rapid establishment of anaerobic conditions, and development of the complexes is prevented.) The contents of the adsorption tube were then diluted 10-fold by the addition of 9 volumes of 30°C. Hershey broth. Vigorous aeration was begun and the optical density at 450 mμ was measured at 5 minute intervals in a Bausch and Lomb Spectronic 20 Colorimeter. Measurements were continued for 70 minutes or until lysis was evident from a drop in the optical density.

Measurements of DNA synthesis were carried out in two ways. The first of these was a simplified method devised in collaboration with Dr. Charles M. Steinberg for making qualitative comparisons of DNA synthesis in restrictive bacteria infected with amber mutants. Whatman 3MM filter paper squares 2 cm. on a side were impregnated with 0.1 ml. of a solution containing tritiated (H^3) thymidine at a concentration of 5 μg/ml. and a specific activity of 0.5 μc/μg. The squares were then dried and stored at room temperature until used. Restrictive bacteria were prepared in K medium for infection at a final concentration of 4×10^9/ml. Phages were prepared at 2×10^{10}/ml. Equal volumes of phage and bacteria were mixed,

and adsorption was allowed to proceed for 5 minutes. The adsorption mixture was then diluted 20-fold into K medium, and three drops of this dilution were delivered from a sterile Pasteur pipette onto each of five prepared squares. One square was immediately transferred to a beaker containing 5% trichloroacetic acid (TCA) and the other four were transferred at 15 minute intervals. Each square was then transferred through two further washes of 5% TCA, two washes with ethanol, and one wash with diethyl ether, standing for 10 minutes in each wash. After the final wash the squares were dried and counted in a liquid scintillation counter. Controls indicated that unincorporated thymidine was efficiently washed out of the squares.

The second method was the same except that dilution from the adsorption tube was 20-fold into K medium which contained tritiated (H^3) thymidine at a concentration of 5 μg./ml. and a specific activity of 0.1 μc./μg., this dilution was aerated, and 0.5 ml. samples were withdrawn at five minute intervals and transferred to tubes containing 0.05 ml. of 1.0 M NaN_3 at ice temperature. 2.5 ml. of 6% TCA was then added and the tubes were allowed to stand for 15 minutes on ice, after which time the contents of the tube were filtered through Millipore filters (0.45 μ pore size). The filters were washed three times with 10 ml. of 5% TCA, dried, and counted in a liquid scintillation counter. This method, although more time-consuming than the first one, was more reproducible, and it was used when the rate of DNA synthesis was to be determined accurately.

DNA extractions were performed by two methods, depending on whether the source was phages or infected bacteria. For extractions from phages, a phage suspension at about 2 x 10^{11}/ml. in 0.1 M tris buffer, pH 7.4, containing 0.01 M $MgSO_4$, was added to an equal volume of redistilled phenol previously equilibrated with the same buffer. The two phases were mixed very gently by slow hand rotation, and then the tube was centrifuged at low speed to separate them. The aqueous phase was withdrawn gently, using the broad end of a 1 ml. pipette, and the extraction was repeated with a second volume of phenol. The second aqueous phase was then dialyzed against the same buffer and used without further purification. For extractions from infected cells a modification of the method of Tomizawa and Anraku (50) was used. Cell samples were chilled by mixing with an equal volume of ice-cold distilled water, concentrated by centrifugation for 7 minutes at 4800 x g, and resuspended in one-twentieth the original volume of SSC. One-tenth volume each of a solution of egg-white lysozyme (5 mg./ml.) and 0.1 M EDTA were added, and the mixture was frozen and thawed three times by alternate immersion in a dry ice-acetone bath and a 30°C. water bath. One-tenth volume of a solution of trypsin (1 mg./ml.) was then added, and the mixture was incubated for 30 minutes at 37°C. Finally, one-fiftieth volume of 20% sodium dodecyl sulfate was added and the mixture was incubated a further 10 minutes at 37°C. The resulting clear, viscous preparation was added to an equal volume of redistilled phenol previously equilibrated with SSC. The extraction was then carried out as described for the phage DNA samples, with a final dialysis against SSC, rather than 0.1 M tris buffer.

Analytical ultracentrifugation was performed according to standard techniques, using a Spinco Model E centrifuge equipped with ultraviolet optics. Density gradient "equilibrium" sedimentation was often carried out for only 12-13 hours at 44,770 rpm, since longer periods of centrifugation did not produce detectable shifts in the position of bands. In experiments involving phage particles, the suspension of phages in CsCl was usually preheated for 2 hr. at 48°C. to prevent the appearance of double bands (see Figure 11). Band or zone centrifugation was carried out with a Type III banding cell (51), using a supporting CsCl solution of density 1.336 g/ml. Tracings of ultraviolet photographs were made with the Joyce-Loebl Double-Beam Recording Microdensitometer (52). When the areas under peaks were to be determined, care was taken to ensure that the entire peak fell within the linear range of the film; the relative areas under various peaks were determined by cutting the peaks out of tracings on standard K&E graph paper and weighing them on an analytical balance.

Electron microscopy. Grids were prepared as follows. One drop of a 1.4% solution of parlodion in amyl acetate was dropped onto the surface of a water trough to create a thin parlodion film, and this film was lowered onto several submerged copper grids (200 mesh). The film was allowed to dry on the grids, strengthened by carbonization, and ionized. A drop of the material to be examined was applied to the grid, allowed to stand for about 30 seconds, and drawn off with a filter paper wick. If desired, the grid was washed with a few drops of distilled water. A drop of stain (usually 1% uranyl acetate) was added and the excess drawn

off with another wick. After drying, the grids were examined in a Phillips EM 200 electron microscope (53).

III. Results

A. Genetic Characterization of Some T-even Phages

1. Introduction

The phages of the T-even species are the most thoroughly characterized of all bacteriophages, thanks in large part to the attention focussed on them by workers in the schools of Delbrück and Luria. The general features of their life cycles are well known, and need not be reviewed here. Of importance for this study, however, are the extent to which these phages have been shown to resemble one another and the nature of any known differences between them.

The most striking evidence for the close relationship among the T-even phages is provided by the electron microscope (18, 19, 54, 55, and see Figures 7-10). All three phages, T2, T4, and T6, have polygonal heads about 1060 Å long and about 810 Å wide, which are probably prolate icosahedra (56). The tails of all three phages are composed of striated sheaths, about 930 Å long and 170 Å wide, central cores which are slightly longer than the sheaths and fit inside them, hexagonal endplates with six short spikes, and six tail fibers, about 1300 Å long with a characteristic kink in the middle, which are attached to the endplates. Short neck regions, in which the core appears not to be surrounded with sheath, occur at the joints between the heads and the tails. The only apparent difference between the three phages is the presence of a thin collar in the neck regions of T4 and T6 and its absence in T2 (57, 58 and see Figures 7-9).

Antisera prepared against any one of the phages can inactivate the other two, but at slower rates (29, 59); thus the phages must have both serological similarities and serological differences. (Unrelated phages such as T1 or T3 show no serological cross reaction.)

All three phages have been shown to contain DNA's of about 66% A-T content (60,61), and these differ from all other known DNA's in containing the unusual base 5-hydroxymethyl cytosine, or HMC (62), to which glucose moieties are attached through the hydroxymethyl group (63-65). The three phages differ, however, in the extent to which the HMC residues are glucosylated and in the nature of the linkage between the glucose moieties and the hydroxymethyl group (66), as shown in Table 1. The glucosylating enzymes responsible for these differences have been characterized and partially purified (67, 68), and their specificities are at least partially understood (69, 70, 71).

The sizes of the DNA's from the three phages, as determined by sedimentation velocity measurements, are the same within experimental error (72). Considerable base sequence homology between the DNA's is indicated by their ability to form artificial hybrids upon heating and renaturing, but slight differences between them are indicated by the amounts of hybrid formed (28).

T2 and T4 (and probably T6) require monovalent cations for adsorption, the optimum concentration being 0.1 M (73). Cofactor effects on adsorption differ, some strains of T4 showing a requirement for tryptophan or an analog (54, 74-76), and some strains of T2 showing a sensitivity to indole (77). [The strain-to-strain differences and one step mutations which

TABLE 1

GLUCOSYLATION OF T-EVEN PHAGE DNA'S

PHAGE	PERCENTAGE OF HMC RESIDUES CARRYING			
	NO GLUCOSE	ALPHA GLUCOSE	BETA GLUCOSE	TWO GLUCOSES
T2	25	70	0	5
T4	0	70	30	0
T6	25	3	0	72

Data are drawn from Lehmann and Pratt (66). In each case the glucose moieties are attached to the hydroxymethyl group of HMC. In HMC residues which carry two glucoses, the second glucose is attached to the first to form gentiobiose.

create or destroy cofactor adsorption requirements (77, 78), suggest that these differences between the phages are not profound.] Different receptors on the bacterial surface are responsible for adsorbing the three different phages, since bacterial mutants can be found which are selectively resistant to each of them (79). The receptors for T2 and T6 reside in the lipoprotein layer of the cell wall, whereas those for T4 reside in the lipopolysaccharide layer (80).

The intracellular growth cycles of all three phages are remarkably similar. The times of onset of DNA synthesis, the eclipse periods, the latent periods and the burst sizes of all three phages are nearly identical on B strains of E. coli (81). On certain K strains, however, T2 can be distinguished from the other two phages by its low burst size (33, 81). The latent periods of all three phages can be prolonged by superinfection of an infected cell (82), and all three phages are capable of mutating to forms ("r" or "rapid lysis" mutants) in which this capacity for "lysis inhibition" is lost (82). Frequent recombination events characterize T2 and T4 infections (35-37), and are probably responsible for the efficient multiplicity reactivation which is seen with all three phages (83).

Mixed infections between the phages reveal some other aspects of their relationships. Recombinants between them occur with detectable frequencies (24), and the progeny of T2-T4 mixed infections include "phenotypically mixed" phages with T2 genes but a T4-determined coat (84, 85).

T2 and T6 demonstrate their relationship to T4 by their ability to induce lysis inhibition in a T4-infected cell (82); superinfection with other phages of the T-series does not induce lysis inhibition.

The information presented above provides a reasonably detailed comparison of the T-even phages, and it is clear that they show both strong overall similarities and some clear, but probably minor, differences. It will become apparent below, however, that considerable new information on the relationships among these phages can be provided by a genetic comparison using conditional lethal mutants.

Of the two systems of conditional lethal mutants exploited in T4, the amber mutant system was chosen for the other T-even phages. The choice was based on several considerations. The nature of the amber mutation is well understood (it consists of an alteration in a normal coding triplet to produce the nonsense codon UAG (86,87)), and the physiological basis for suppression of amber mutants is also reasonably well understood (suppression requires the presence of a transfer RNA which can recognize the codon UAG and translate it into an acceptable amino acid (88)). Amber mutants are, in general, less leaky than temperature sensitive mutants under restrictive conditions (21), and are thus easier to work with. Since intragenic complementation between amber mutants does not occur (42), they are more suitable for identifying complementation groups than are temperature sensitive mutants. Finally, an efficient selective plating method for detecting amber mutants was available, as described in Materials and Methods.

In this section of the Results numerous amber mutants have been isolated in each of the phages T2, T6, and RB69; their characterization has provided detailed genetic descriptions of these phages which permit new comparisons between them.

2. Characterization of Mutants

a. T2

1. Complementation Testing

Since T2 was the first T-even phage to be analyzed genetically (89-92), a partial genetic description of it already existed before this study (93, 94). Many of the types of mutants found in T4 had also been found in T2, including rI and rII mutants, minute- and turbid-plaque mutants, and host range mutants (89-98). Maps had been constructed for both T2L and T2H (two laboratory strains of T2), and frequencies of recombination in T2 were known to resemble those of T4 (93, 94).

This genetic description of T2, however, was not nearly so complete as the description of T4 constructed with conditional lethal mutants (21, 38, 29), and it was clear that only a more complete description of T2 would permit detailed comparisons of the two phages. Consequently a set of amber mutants was developed for T2.

From a stock of T2L, grown in the presence of 5-bromodeoxyuridine as described in Materials and Methods, 118 amber mutants were isolated. On the assumption that the genetic description of T2 would resemble that of T4, an attempt was made to characterize these mutants by their behavior

in complementation spot tests with T4 amber mutants; it was reasoned that if T2 and T4 were sufficiently similar, T2 mutants should behave like T4 mutants in these tests. This was the case, as judged by the following characteristics of the tests; 1) each T2 mutant, with a very small number of easily explainable exceptions, complemented all T4 mutants except those with defects in one particular gene, 2) with a few exceptions, all the T2 mutants which failed to complement a given T4 mutant failed to complement one another, indicating that they carried defects in the same T2 gene, and 3) a set of representative T2 mutants, whose behavior in tests against T4 mutants implied that each one should carry a defect in a different T2 gene, complemented one another in all possible combinations, indicating that each of these genes was, in fact, distinct from all the others. Accordingly, the T2 mutants were characterized by a stepwise series of complementation spot tests, as follows.

Each T2 mutant was first tested against a series of seven multiple mutants of T4; these multiple mutants, between them, contained mutations in 24 of the 70 T4 genes. About half of the T2 mutants failed to complement one or another of these T4 multiple mutants, and they were subsequently tested against the T4 single mutant constituents of the relevant multiple mutant; with rare exceptions, they failed to complement one or another of these single T4 mutants. The remaining T2 mutants were spot tested against a series of double and single mutants of T4 which, between them, contained mutations in all the remaining T4 genes, and all but seven of them failed to complement one or another of these T4 mutants. Thus the mutant defects in all but seven of the T2 mutants could be

assigned to T2 genes homologous with genes already identified in T^4; these seven mutants were tested against one another in all possible combinations, and their defects proved to fall into three genes. The characterizations of all T2 mutants were checked by spot tests against one another, as described above.

During the spot testing occasional exceptional results were observed. Some of these exceptions were due to T2 double mutants, others were due to some interesting features of the T2-T^4 mixed infection which are treated in Section B.2 of the Results, and still others were due to a particular T2-T^4 gene product incompatibility which is treated in Section A.3.a of the Results. These were the only exceptions observed.

After the complementation testing of the T2 mutants had been completed, I learned that Dr. Franklin W. Stahl of the University of Oregon was engaged in a similar study. In order to have as complete a set of T2 mutants as possible, I obtained some of Stahl's T2 mutants and tested them in the fashion described above. A summary of the spot test results is presented in Table 2. A total of 53 genes have been identified in T2; 46 are homologous to genes already identified in T^4, while 7 are apparently new. The defects of 137 single mutants and 12 double mutants have been assigned to these genes.

The results in Table 2 support the notion of a close relationship between T2 and T^4 in several ways. First, and perhaps least obvious, the very success of the T2-T^4 complementation spot tests, which depends on the production of viable wild-type T2-T^4 recombinants, implies that a T2-T^4 hybrid with virtually any combination of T2 and T^4 genes is viable;

TABLE 2

GENE DEFECTS OF T2 AMBER MUTANTS

Gene Number	Mutants
1	153, 99a, 166a
57	15
2	163
64	FS109
4	FS114
53	72
5	38, 127, 144
6	106, 160, FS63, 30a, 154a
7	14, 27(L), 77(L)
8	37, 140
12	60, FS13
14	60, 93(L), 167, FS62
16	FS15
17	16, 98a
20	108
21	111, 124
22	99b
23	94, 104, 113, 138, 157, 148a
25	11
26	28, 56
51	FS82, 148b
27	156, FS57, 30b
29	7, 65, 81 FS29
54	95, FS66
30	FS5
63	43, 54, FS51, 152a
32	8, 51, 145
33	96, 130, FS44
34	12, 67, 73, 74, 75, 78, 79, 135, FS40, 25a, 110a
35	FS6
36	21, 24, 129, FS23
37	123, 129, FS4, 25b
38	35, 91, 125, FS64
52	32
39	33, 70, 71, 84, 114

TABLE 2 (Continued)

Gene Number	Mutants
56	13, 61, 64, 87, 102, 133, 165, FS2, FS18, 154b
58	100
41	82, 86, 137, FS99, 98b
42	3, 6, 38, 136, FS85, 76a, 110b
43	83, 105, 141, 146, 151(L), 162, 164, FS115,76b, 147a
44	FS8
45	FS98
46	31, 59, 115, 122, 155, FS32, 132a, 152b
47	4(L), 80, 92, FS7, FS45
55	17, 150, FS56, 147b
49	2, FS87
	10, 117, 159, 132b, 166b
	26, 36, 109
	89, 5a
	FS19
	FS48
	FS78
	5b

Mutants are listed with the genes in which their defects lie; single mutants are listed once, and double mutants twice, once with the suffix "a" and once with the suffix "b". Mutant 5 has been resolved into two single mutants, 5a and 5b, by crosses with T2 wild type. Each gene is given the number of its T4 homolog and the numbered genes are presented in the order in which they occur in the T4 map. Genes not yet identified in T4 are not numbered. Mutants isolated by Dr. F.W. Stahl are given the prefix FS, followed by his isolation number. All other mutants were isolated in this study and have been given sequential isolation numbers. In cases where spot tests were inadequate to decide the complementation properties of mutants, liquid culture complementation tests were used. Assignments made on the basis of such tests have been denoted by (L). Identification of the T2 homologs of genes 37 and 38 of T4 was inferred from mapping experiments described below (See Sections A.2.a.2. and A.3.a. of the Results).

even relatively minor differences between the phages might have vitiated this conclusion. In fact, the one detected exception to this rule, the case of genes 37 and 38, appears to be the result of such a difference; it will be discussed further in Section A.3.a of the Results.

Second, a relatively limited number of T2 mutants has sufficed to establish that at least two-thirds of the genes already identified in T4 have homologs in T2.

Third, T2 must not contain very many genes which T4 lacks, since over 90% of the T2 mutant defects occurred in genes already identified in T4.

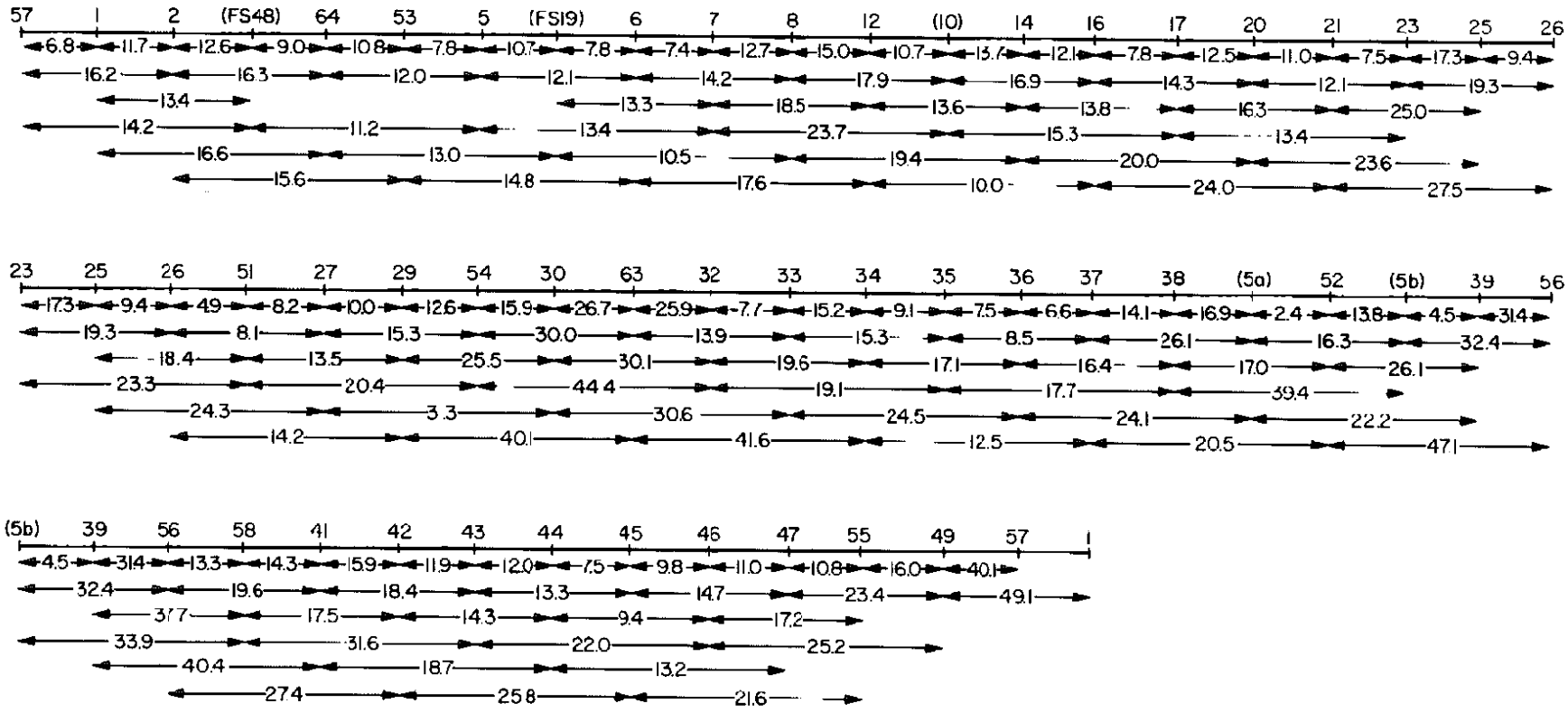
2. Mapping

a. Gene order

The relationship between the gene orders of closely related organisms can reveal important evolutionary relationships between the organisms themselves (99), especially since chromosomal rearrangements often play important roles in isolating mechanisms among higher organisms (100). Consequently it was of interest to determine whether the gene order in T2 resembled that in T4.

The problem of mapping the T2 mutants was approached by asking whether the order of genes in the T4 map could successfully be used to predict the order in the T2 map. One mutant was selected to represent each T2 gene and was crossed first with its three predicted nearest neighbors on either side. The data from these crosses, presented in Figure 1,

FIGURE 1. RECOMBINATION DATA FROM T2 CROSSES. Each number in the body of the figure is the average percentage of recombination observed in crosses between selected mutants with defects in the genes at the ends of the arrow. The calculation of percentages of recombination includes a correction for plating efficiency differences of the permissive and restrictive strains used, as described in Materials and Methods. Each percentage is the average of from one to seven separate determinations. The average number of determinations per number in the figure is 1.8.


57 1 2 (FS48) 64 53 5 (FS19) 6 7 8 12 (10) 14 16 17 20 21 23 25 26
6.8 11.7 12.6 9.0 10.8 7.8 10.7 7.8 7.4 12.7 15.0 10.7 13.7 12.1 7.8 12.5 11.0 7.5 17.3 9.4
16.2 16.3 12.0 12.1 14.2 17.9 16.9 14.3 12.1 19.3
13.4 13.3 18.5 13.6 13.8 16.3 25.0
14.2 11.2 13.4 23.7 15.3 13.4
16.6 13.0 10.5 19.4 20.0 23.6
15.6 14.8 17.6 10.0 24.0 27.5
23 25 26 51 27 29 54 30 63 32 33 34 35 36 37 38 (5a) 52 (5b) 39 56
17.3 9.4 4.9 8.2 10.0 12.6 15.9 26.7 25.9 7.7 15.2 9.1 7.5 6.6 14.1 16.9 2.4 13.8 4.5 31.4
19.3 8.1 15.3 30.0 13.9 15.3 8.5 26.1 16.3 32.4
18.4 13.5 25.5 30.1 19.6 17.1 16.4 17.0 26.1
23.3 20.4 44.4 19.1 17.7 39.4
24.3 3.3 30.6 24.5 24.1 22.2
14.2 40.1 41.6 12.5 20.5 47.1
(5b) 39 56 58 41 42 43 44 45 46 47 55 49 57 1
4.5 31.4 13.3 14.3 15.9 11.9 12.0 7.5 9.8 11.0 10.8 16.0 40.1
32.4 19.6 18.4 13.3 14.7 23.4 49.1
37.7 17.5 14.3 9.4 17.2
33.9 31.6 22.0 25.2
40.4 18.7 13.2
27.4 25.8 21.6

were examined for consistency with the predicted ordering. As can be seen by comparing the figure with the T4 map in Figure 5, the data conflict with the T4 order in only one case; the genes 1, 57, and 2 have been given the order 1-57-2 in T4, whereas the data seem to indicate the order 57-1-2 in T2. These genes occur at the end of a well mapped region in T2, and consequently their ascribed order depends on the results of fewer crosses than if they had occurred in the middle of a well mapped region. For this reason their order is less definite than the order of genes within the well mapped regions, and the apparent discrepancy with the T4 order may not be real. It is also worth noting that the order of these genes in T4 has been determined only by two-factor crosses and thus is subject to the same criticisms. The three-factor crosses needed to determine the orders rigorously have not been performed in either T2 or T4.

Although the data from the crosses between T2 mutants are consistent with the predicted order for the T2 genes (with the single possible exception cited above), they are not in themselves sufficient to rule out some other possible orders. In short, because of the high recombination frequencies which are observed between some pairs of neighboring mutants, a rigorous treatment of the data only permits the genes to be divided into three well mapped groups, one consisting of genes 57 through 30 on the map, the second of genes 32 through 39, and the third of genes 56 through 49 (the "early region"); within each of these groups, the data apparently establish a unique order for the mutants, but the evidence for the relative orientation of these groups with respect to one another

is weak, and there is no evidence for the circularity of the T2 map as a whole. The weak linkage detected between gene 63 and genes 30 and 32 suggests that the relative orientation of the first and second groups is the same as in T4, and the data, as presented, suggest linkage between genes 39 and 56, but this linkage is weak enough so that it could still be claimed that the orientation of the third group with respect to the other two is the opposite of that in T4. To investigate this possibility, mutants with defects in genes falling at the ends of well-mapped groups were crossed together in all possible combinations; if the relative orientations of the groups were different in T2, such crosses might have revealed linkage between genes which are essentially unlinked in T4. No such linkage was detected. Other investigators have provided evidence for the circularity of the T2 map (94).

In summary, the gene order in T2 is very probably nearly identical to that in T4. One apparent discrepancy between the two has not been rigorously tested and the possibility of an inversion of the whole early region has not been rigorously ruled out.

b. Properties of Recombination

Negative interference was observed in the T2 crosses, and was quantitatively similar to that of T4; often the frequency of recombination between the outside markers of a trio, while greater than the recombination frequencies between the middle marker and the end markers, was considerably less than their sum. The generality of this

discrepancy is easily seen in Figure 2; in the absence of negative interference, the data should have scattered about the line drawn in the figure.

The problem of negative interference makes it difficult to use recombination data in constructing a map in which the distances between mutations represent true physical distances. In T4, an attempt to circumvent this difficulty has been made by Stahl, Edgar, and Steinberg (101), who designed mapping functions for converting measured recombination frequencies to distances in such a way that the resulting distances would demonstrate the additivity which the recombination frequencies themselves so clearly lack. They devised several alternative functions in which a proper adjustment of parameters gave the desired additivity, and their success has led to the assumption that the derived distances are more accurate reflections of true physical distances between mutations than are the recombination frequencies themselves. Consequently, these distances have been used in the construction of the T4 map (101).

In an attempt to circumvent the problem of negative interference in T2, an average of the two best T4 mapping functions, the 4-Parameter Switch Function and the 4-Parameter Modified Bernstein Function (101), was applied to the T2 recombination data. Distances were computed for each observed recombination frequency and the additivity of these distances was examined as a check on the validity of this approach. The results of these calculations are presented in Figure 3, in which the computed distances have been treated exactly as the raw recombination percentages were treated in Figure 2. The additivity of the derived distances is clearly better than that of the recombination percentages

FIGURE 2. ADDITIVITY OF T2 RECOMBINATION PERCENTAGES. The ordinate of each point is the observed percentage of recombination between two non-adjacent mutations; the abscissa is the sum of the recombination percentages for the shortest intervals lying between the same two mutations. The line drawn is for the case in which the observed and summed percentages are equal. The distribution of lengths among the shortest intervals used is indicated by the histogram on the abscissa. Both the ordinate and the abscissa scales are logarithmic.

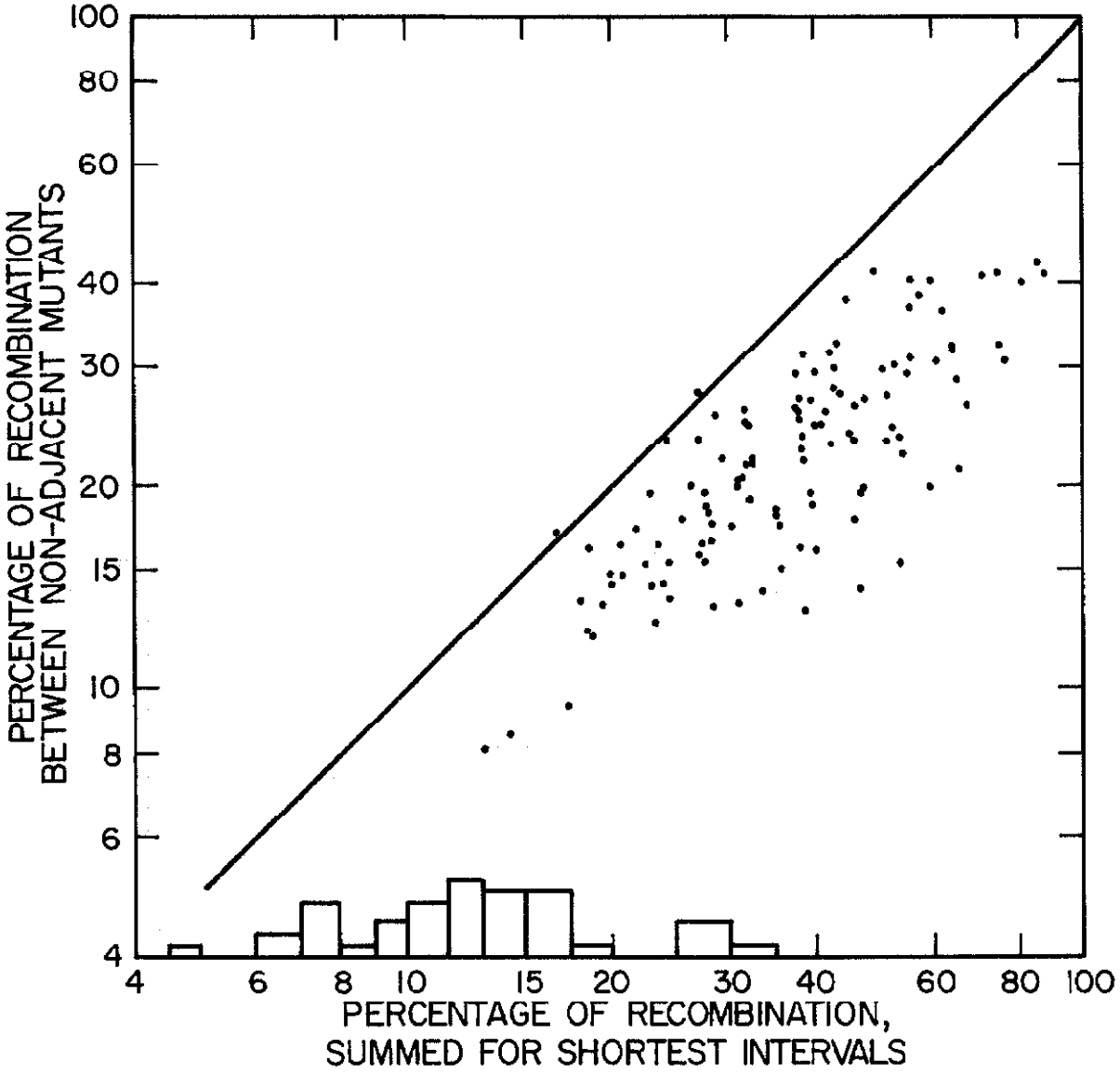
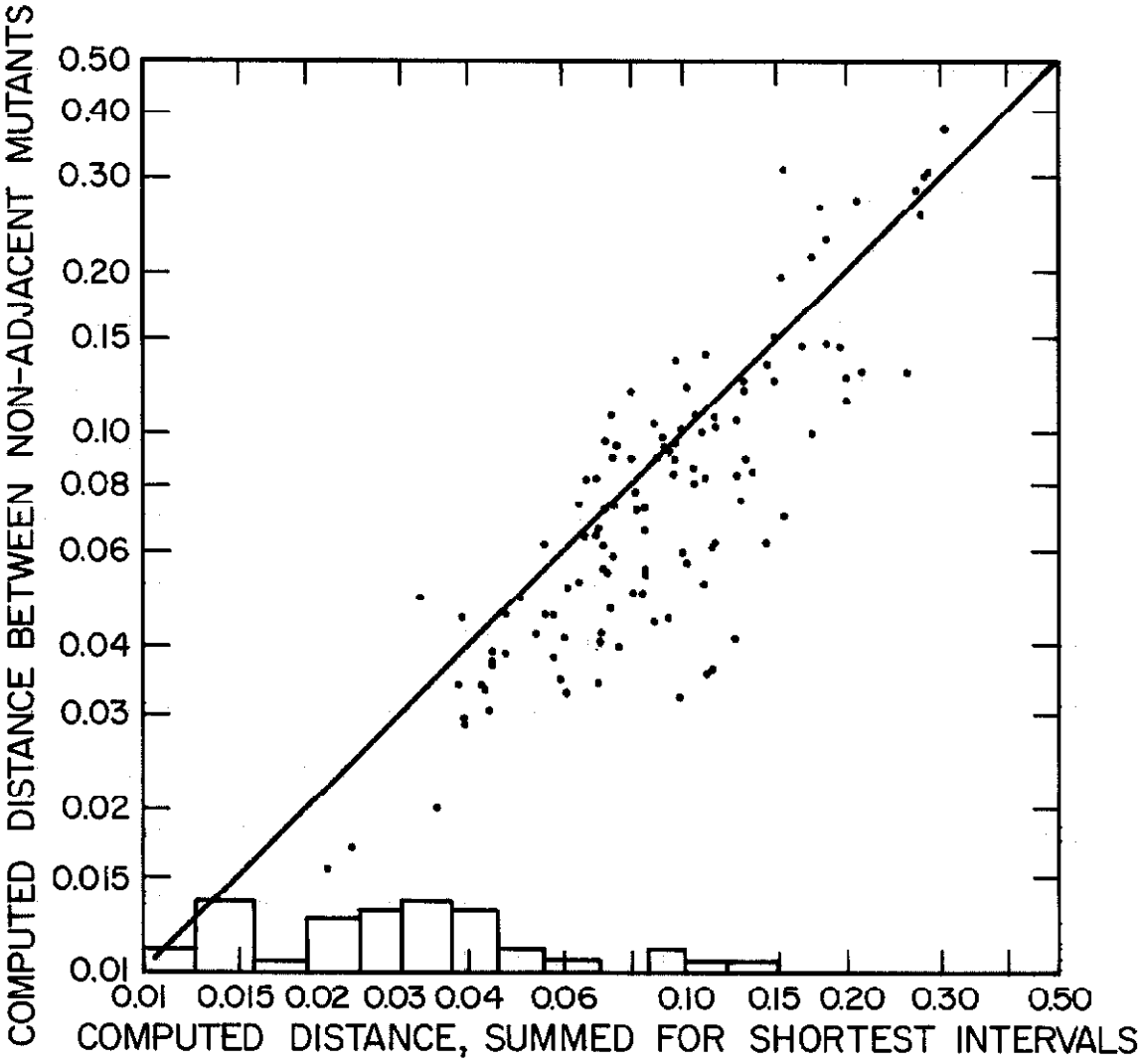

PERCENTAGE OF RECOMBINATION BETWEEN NON-ADJACENT MUTANTS
100
80
60
40
30
20
15
10
8
6
4
4
6
8
10
15
20
30
40
60
80
100
PERCENTAGE OF RECOMBINATION, SUMMED FOR SHORTEST INTERVALS

FIGURE 3. ADDITIVITY OF T2 COMPUTED DISTANCES. All distances are expressed as fractions of the total map length. The ordinate of each point is the distance between two non-adjacent mutations, computed from the recombination percentage between them using the T4 mapping function described in the text; the abscissa is the sum of the corresponding distances for the shortest intervals which lie between the same two mutations. The line drawn is for the case where the distance between the mutations is the same as the sum of the distances of the shortest intervals. The distribution of lengths among the shortest intervals used is indicated by the histogram on the abscissa. Both the ordinate and the abscissa scales are logarithmic.


COMPUTED DISTANCE BETWEEN NON-ADJACENT MUTANTS
0.50
0.40
0.30
0.20
0.15
0.10
0.08
0.06
0.04
0.03
0.02
0.015
0.01
0.01 0.015 0.02 0.03 0.04 0.06 0.10 0.15 0.20 0.30 0.50
COMPUTED DISTANCE, SUMMED FOR SHORTEST INTERVALS

themselves, but it is still not as good as that of T4 distances derived with the same mapping function (101).

Since the mapping function converts recombination frequencies into distances expressed as fractions of the total map length, a second check on the applicability of the T4 mapping function is possible. If the mapping function applied perfectly, and if the T2 map were really circular, the sum of all the computed distances for the shortest intervals around the map should approximate one map length. In T4, the function only roughly fulfills this condition of applicability; the sum of these distances is 1.44 map lengths. In T2, the sum of the distances is 1.77 map lengths, again demonstrating that the T4 mapping function does not apply as well to T2 recombination data as it does to T4 recombination data. Despite this failure of the T4 mapping function to provide a completely satisfactory solution to the problem of negative interference in T2, the distances computed with its help have been used in constructing a T2 map.

c. The Map

The T2 map is presented in Figure 4, and the T4 map constructed with the same mapping function is presented in Figure 5 for comparison. In constructing the T2 map, it has been assumed that the map is circular and that the relative orientations of the well-mapped groups of genes are the same as in T4, although the data presented in this study are insufficient to establish either point rigorously.

FIGURE 4. THE MAP OF T2. Distances separating genes have been computed using the T4 mapping function, as described in the text. The position of each newly identified gene is indicated by an arrow with parentheses containing the number of the representative mutant defective in that gene. Since slight fluctuations in recombination frequency strongly influence the sizes of the unmapped gaps in the map, their true sizes are necessarily somewhat uncertain.

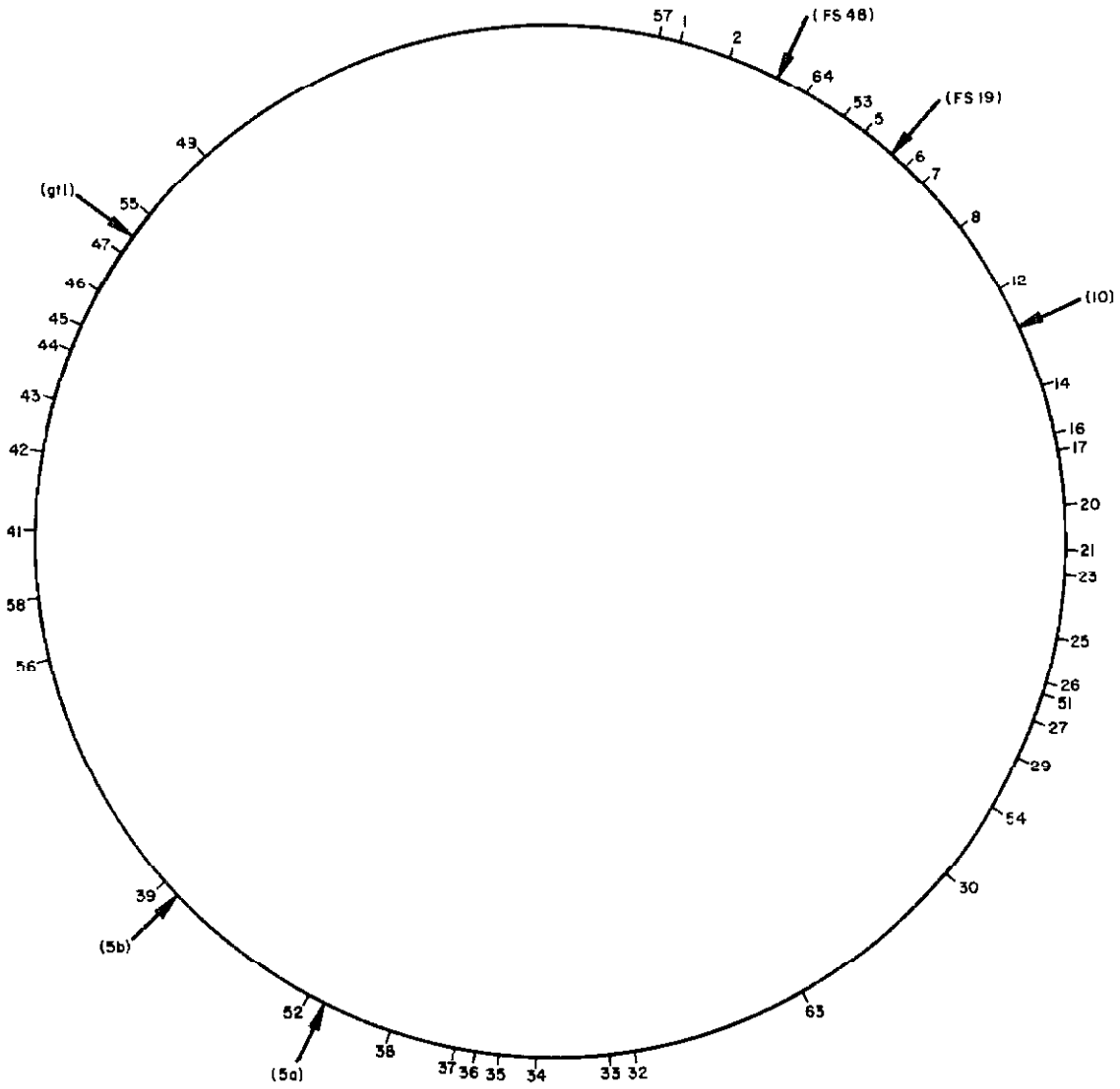
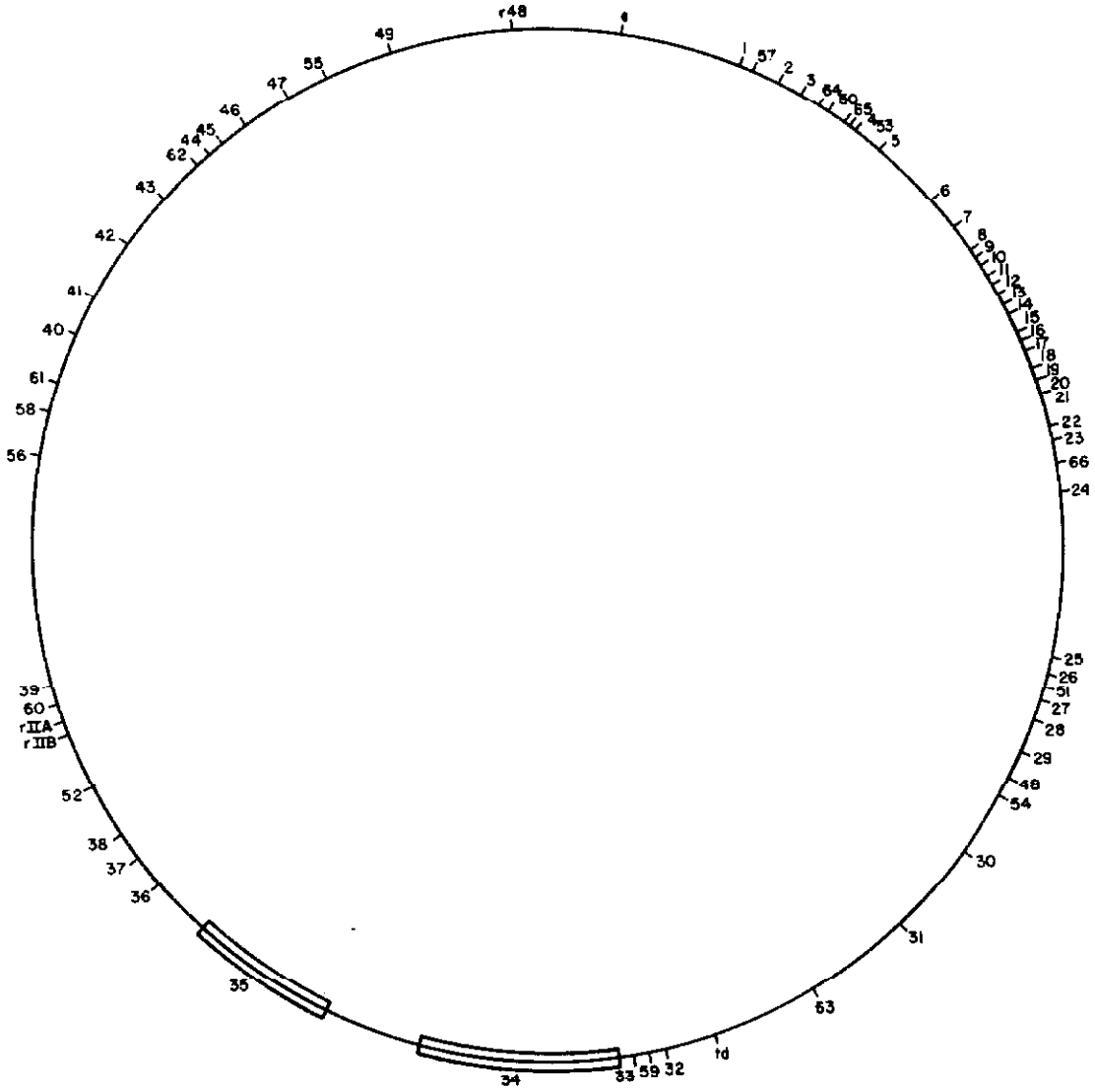

57
1
2
(FS 48)
64
53
5
(FS 19)
6
7
8
12
(10)
14
16
17
20
21
23
25
26
51
27
29
54
30
65
33
32
34
35
36
37
38
52
(5a)
39
(5b)
41
58
56
42
43
44
45
46
47
55
(g11)
49

FIGURE 5. THE MAP OF T4. Distances separating genes have been computed using the same T4 mapping function as in Figure 4. The recombination data used in constructing this map were collected in the laboratories of Drs. R. S. Edgar and R. H. Epstein, to whom I am indebted for permission to use it.


r48
49
55
47
46
45
44
62
43
42
41
40
61
58
56
39
60
rIIA
rIIB
52
38
37
36
35
34
33
59
32
td
63
31
30
54
48
29
28
27
51
26
25
24
66
23
22
21
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
53
65
50
64
3
2
57
1

The most striking feature of the T2 map is its general similarity to that of T4. The order of the genes in the two maps is the same with one possible exception, and the similarity extends even to the spacings between genes, which are, on the whole, virtually identical in the two maps. There are three exceptions, two minor and one major, to this rule of identical spacings. In T2 the distance between genes 7 and 14 is expanded and the distance between genes 23 and 25 is contracted, relative to T4. These differences, however, are less notable than the major difference between the two phages with respect to the sizes of genes 34 and 35; this difference is explored in Section A.3.c of the Results.

The positions of five of the seven newly identified genes in T2 are indicated on the T2 map by arrows with mutant numbers in parentheses. Three of these genes (represented by mutants FS48, FS19, and 10) fall into the well-mapped late region, while two (represented by mutants 5a and 5b) fall into the relatively poorly mapped region containing a few genes whose mutants (in T4) show DNA-delayed phenotypes (21, 102). The phenotypes of mutants defective in the newly identified genes have not been determined. Mutants defective in two other newly identified genes have not been mapped.

T2 gt-1, a mutant defective in alpha-glucosyl transferase (43), was mapped as follows. An E. coli strain, 704, was selected for its ability to suppress most T2 and T4 amber mutants and for its ability to accept T-even phages without glucose attached to the HMC residues in their DNA. This strain was used as a host for crosses between T2 gt-1 and T2 early amber mutants. The progeny were plated on strain 704,

which should plate all the progeny of the cross, and on strain B/5, which, since it does not accept non-glucosylated phages and is restrictive for amber mutants, should plate only the wild-type recombinants formed in the cross. From the results, gt-1 appears to be closely linked to gene 47 and probably lies between genes 47 and 55. A more definite assignment is withheld because of the quantitative aspects of crosses between gt-1 and mutants defective in genes 43 through 46; recombination frequencies between these mutants and gt-1 were often lower than those between these mutants and gene 47 or gene 55 mutants. This effect is not due to an overall reduction of recombination frequencies in crosses involving gt-1, since maximal frequencies of recombination occurred in crosses between gt-1 and other, essentially unlinked mutants. It is possible that the non-glucosylated state of the gt-1 parent plays some role in this effect.

In summary, mapping experiments with T2 mutants have shown that the map of T2 bears a striking resemblance to that of T4, both in the order of the genes and in the map distances which separate them; this resemblance is strong evidence for the close relationship between T2 and T4. A few differences in spacing were noted, the most marked one occuring in the tail fiber region. The properties of recombination in T2 and T4 were shown to be very similar. The T2 gene for the alpha-glucosyl transferase has been mapped near one end of the T2 early region, and some newly identified genes have been given positions in the T2 map.

b. T6

1. Complementation Testing

Before this study, the genetics of T6 was virtually non-existent. T6 r mutants had been isolated (24, 82), but no reports had been published on any other types of mutants. Nonetheless, the strong resemblance between T6 and the other T-even phages in other respects suggested that a genetic description of T6 would probably resemble those of T2 and T4.

From a stock of T6, grown in the presence of 5-bromodeoxyuridine as described in Materials and Methods, 103 amber mutants were isolated. On the assumption that the genetic description of T6 would resemble those of T2 and T4, an attempt was made to characterize these T6 mutants by their behavior in complementation spot tests against T4 mutants. On the first attempt, these tests were not satisfactory; the positive tests were quite weak and often difficult to distinguish from the negative tests. It was later found, however, that the tests could be made satisfactory by reducing the bacterial concentration. This difference between the T4-T6 tests and the T2-T4 tests is almost undoubtedly due to the strong depressor effect which occurs in T4-T6 mixed infections and which is treated in Section B.3.a of the Results. The modified tests provided a successful classification of the T6 mutants, as judged by the same criteria used for the T2 mutant classification.

The stepwise procedure by which defects of the T6 mutants were assigned to genes was essentially the same as for the T2 mutants, with one added step; the fourteen T6 mutants whose defects could not be assigned to

homologs of T4 genes were tested against T2 mutants with defects in newly identified genes. These test worked without reducing the bacterial concentration, and demonstrated that the defects of some T6 mutants could be assigned to genes homologous with genes identified in T2 but not in T4. During the spot testing of the T6 mutants, occasionally exceptional results were observed. The results were of the same types as those observed in the spot testing of the T2 mutants and could be similarly accounted for. A summary of the spot test results is presented in Table 3. A total of 42 genes have been identified in T6; 34 are homologous to genes already identified in T4, 3 are homologous to genes identified in T2 but not in T4, and 5 are apparently new.

The table supports the notion of a reasonably close relationship between T6 and the other T-even phages in a number of ways. First, the possibility of performing T4-T6 and T2-T6 complementation spot tests argues that virtually all possible T4-T6 and T2-T6 hybrids are viable. The one exception to this rule involves genes 37 and 38, and is discussed in Section A.3.a of the Results. Second, a limited number of T6 mutants has sufficed to establish that a considerable fraction of the genes already identified in T2 and T4 have homologs in T6. Third, T6 must not contain very many genes which T2 and T4 lack, since over 90% of the T6 mutants occurred in genes already identified in T2 or T4.

As a guide for those who might wish to work with these mutants in the future, a composite list of amber mutants available in T2, T4, and T6 is presented in Table 4.

TABLE 3

GENE DEFECTS OF T6 AMBER MUTANTS

Gene Number	Mutants
1	67, 114a
2	92, 116
53	78, 97, 144
5	22, 106
6	58, 64
7	9, 27, 50, 59, 60, 82, 24a
8	80
10	28
12	14, 79, 117
13	18, 39, 137
14	63, 84, 73a
16	7
17	38, 71
26	57
51	73b
27	20, 34, 118
29	4, 8, 99, 104, 111, 143, 52b
30	44a
63	13, 88, 128, 134, 141
32	53, 75
34	15, 33, 83, 90, 107, 110, 146
35	26, 37, 62
37	3, 126, 24b
52	115a
39	122, 114b
56	25, 89, 95, 140a
41	44b
42	16, 31, 108
43	11, 41, 45, 46, 74, 100, 120, 129, 139
44	38, 70
45	133
46	76, 135, 52a
47	12, 61, 69
49	115b
(T2am10)	6

TABLE 3 (Continued)

Gene Number	Mutants
(T2am26) (T2am5b)	1, 36, 47, 56 65, 86, 96 103, 140b 113, 125 123 127 130

Mutants are listed with the genes in which their defects lie; single mutants are listed once and double mutants twice, once with the suffix "a" and once with the suffix "b". Each gene is given the number of its T4 homolog, and the numbered genes are presented in the order in which they occur in the T4 map. The three homologs of genes identified in T2 but not in T4 are indicated by parentheses containing the number of the representative T2 mutant in the homologous gene. Newly identified genes are not numbered.

TABLE 4. COMPOSITE LIST OF AMBER MUTANTS IN T2, T4, AND T6. Each number is the number of single amber mutants with defects in the indicated gene of the indicated phage. The numbers for T4 are minimum estimates and include only a part of all the amber mutants isolated in the laboratories of Drs. R. W. Edgar and R. H. Epstein. The T2 and T6 mutants are those described in this study.

TABLE 4

COMPOSITE LIST OF AMBER MUTANTS IN T2, T4, AND T6

Gene	T2	T4	T6
e	0	2	0
1	1	3	1
57	1	7	0
2	1	3	2
3	0	0	0
64	1	2	0
50	0	1	0
65	0	1	0
4	1	1	0
53	1	3	3
5	3	3	2
6	3	21	2
7	3	13	6
8	2	3	1
9	0	1	0
10	0	2	1
11	0	3	0
12	2	6	3
13	0	1	3
14	4	14	2
15	0	2	0
16	1	4	1
17	1	8	2
18	0	1	0
19	0	0	0
20	1	4	0
21	2	7	0
22	0	4	0
23	5	31	0
66	0	1	0
24	0	6	0
25	1	7	0
26	2	4	1
51	1	1	0
27	2	7	3
28	0	1	0
29	4	10	6
48	0	10	0
54	2	1	0
30	1	5	0

Gene	T2	T4	T6
31	0	4	0
63	3	1	5
32	3	3	2
59	0	2	0
33	3	3	0
34	0	20	7
35	1	2	3
36	4	1	0
37	3	8	2
38	4	7	0
52	1	8	0
60	0	9	0
39	5	29	1
56	9	9	3
58	1	1	0
61	0	4	0
50	0	0	0
41	4	5	0
42	5	4	3
43	8	12	9
62	0	2	0
44	1	3	2
45	1	1	1
46	6	13	2
47	5	2	3
55	3	1	0
(T2am10)	3	0	1
(T2am26)	3	0	4
(T2am89)	1	0	0
(T2amFS19)	1	0	0
(T2amFS48)	1	0	0
(T2amFS78)	1	0	0
(T2am5b)	1	0	3
(T6am103)	0	0	1
(T6am113)	0	0	2
(T6am123)	0	0	1
(T6am127)	0	0	1
(T6am130)	0	0	1

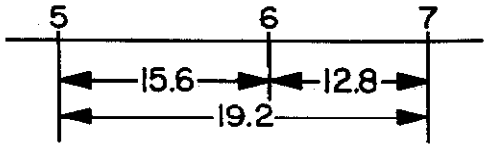
2. Mapping

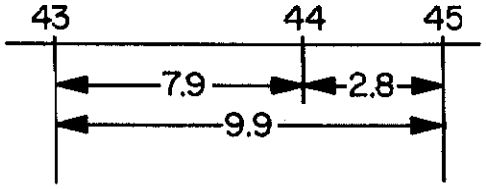
No attempt was made to construct a complete map of T6, but a number of crosses were performed in order to establish gene orders in regions of interest and to determine whether recombination in T6 resembles that in T2 and T4. The results of some of these crosses are presented in Figure 6; the orders of the genes in three regions of the T6 map are probably the same as in T2 and T4, and the frequencies of recombination observed in these T6 crosses are comparable to those observed in T2 and T4 crosses. Furthermore, these data demonstrate negative interference which resembles that seen in T2 and T4, and other crosses between more distantly linked T6 mutants have demonstrated that the maximal percentage of recombination in T6, about 43%, resembles that in T2 and T4. It is interesting to note that the percentages of recombination within the tail fiber region of T6 (genes 34-37) resemble the corresponding percentages of T2 much more closely than those of T4. This resemblance is treated at greater length in Section A.3.c of the Results.

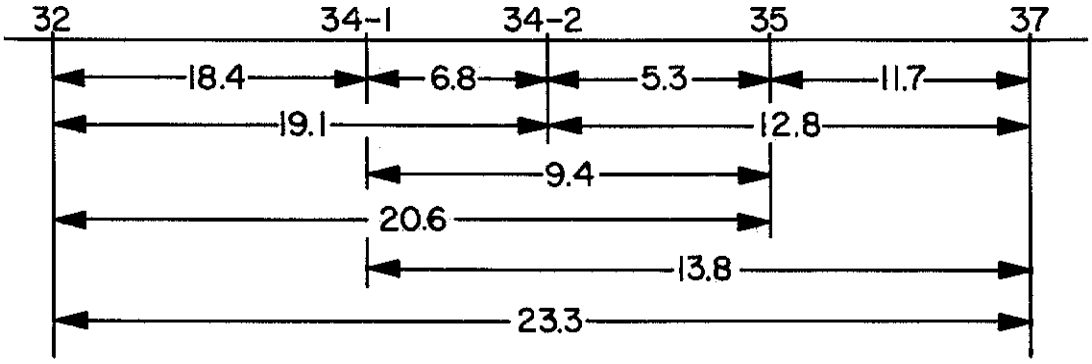
c. RB69

RB69 is one of several newly isolated bacteriophages whose origins are described in Materials and Methods. Shortly after its isolation it was recognized to be a relative of T2, T4, and T6 by virtue of its neutralization by antiserum prepared against T4. Of the sixty other phages isolated at the same time as RB69, thirty were judged to belong to the T-even species by this serological criterion. When each of these phages was tested in mixed infection with T4, all but RB69 were strongly

FIGURE 6. MAPS OF THREE REGIONS OF T6. Each number in the figure is the observed percentage of recombination in a cross between mutants in the genes at the ends of the corresponding arrow. For comparable values in T2 crosses, see Figure 1. The numbers 34-1 and 34-2 signify the most distantly linked mutants in gene 34 of T6.


5
6
7
15.6
12.8
19.2


43
44
45
7.9
2.8
9.9


32
34-1
34-2
35
37
18.4
6.8
5.3
11.7
19.1
12.8
9.4
20.6
13.8
23.3

excluded from the progeny, as described in section B.3.c of the Results. RB69, unlike the others, excluded T4, and because of its uniqueness in this respect, it was selected for genetic characterization.

1. Comparison with T2, T4, and T6

Before RB69 was characterized genetically, it was characterized by other criteria to determine its relationship to the other T-even phages. These criteria may conveniently be divided into those which deal with characteristics of the phage particle and those which deal with the features of the intracellular growth cycle.

a. Characteristics of the Phage Particle

Representative electron micrographs of RB69 are presented in Figure 7, with micrographs of T2, T4, and T6 at the same magnifications in Figures 8, 9, and 10 for comparison. From these micrographs it is clear that RB69 bears a strong resemblance to T2, T4, and T6. It has a polygonal head about 1120 Å long and about 810 Å wide, a striated sheath about 870 Å long and 170 Å wide, an endplate with spikes, tail fibers with the characteristic kink in the middle, and a neck region, devoid of sheath, at the joint between the head and the tail. Contracted RB69 particles have the empty head, contracted sheath, splayed-out configuration of endplate spikes, and exposed core typical of T2, T4, and T6. In addition, the contracted particles often reveal clearly the presence of a collar in the neck region.

FIGURE 7. ELECTRON MICROGRAPHS OF RB69. Phage particles were purified by differential centrifugation, as described in Materials and Methods, and stained with 1% uranyl acetate. The final magnification of the main portion of the figure is 82,960 X, and that of the insert is 340,000 X. Note the tail fibers, end plate spikes, sheath striations, and contracted phages, all of which are also found in preparations of the classical T-even phages.

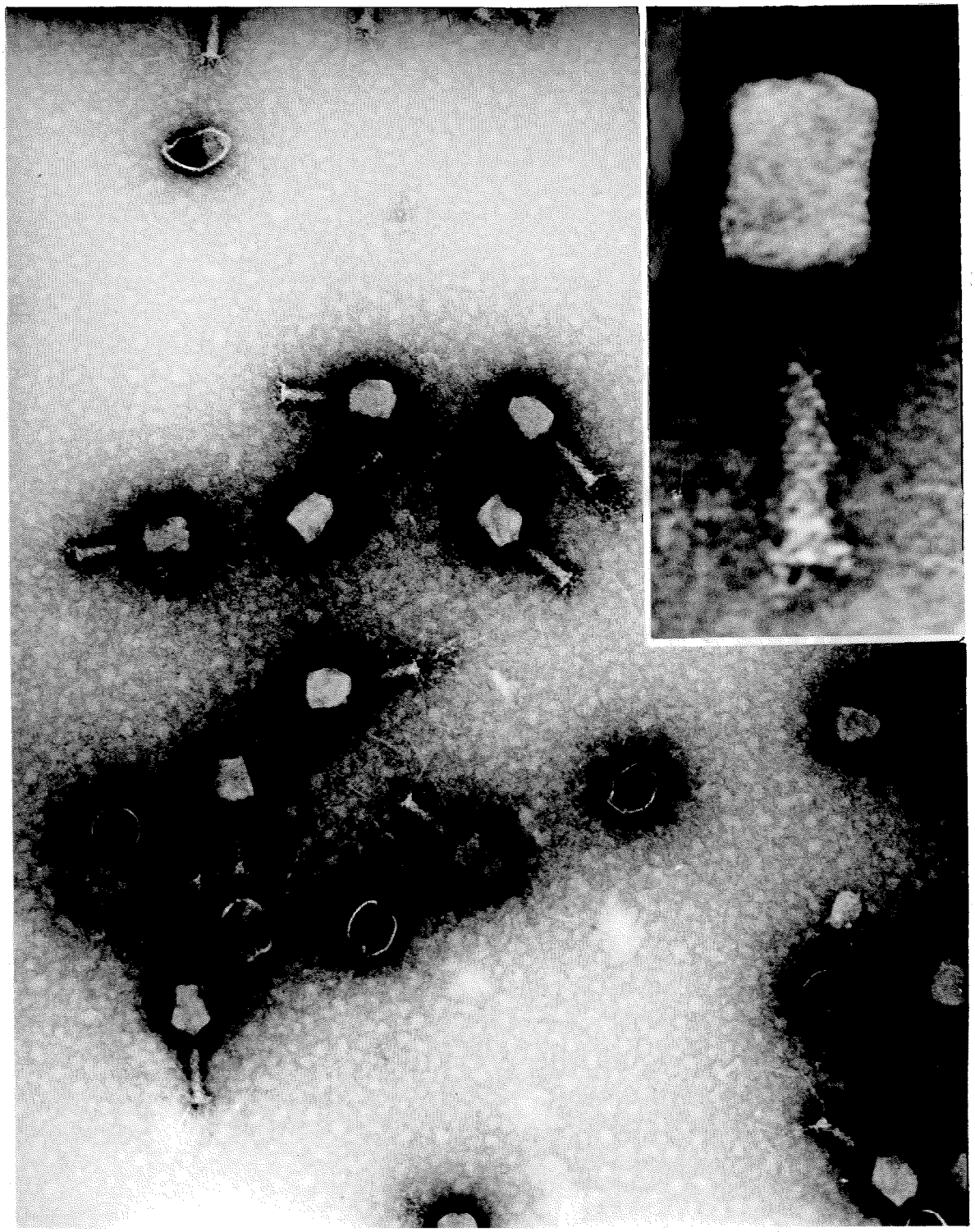


FIGURE 8. ELECTRON MICROGRAPHS OF T2. Phage particles were purified by differential centrifugation, as described in Materials and Methods, and stained with 1% uranyl acetate. The final magnification of the main portion of the figure is 82,960 X, and that of the insert is 340,000 X.

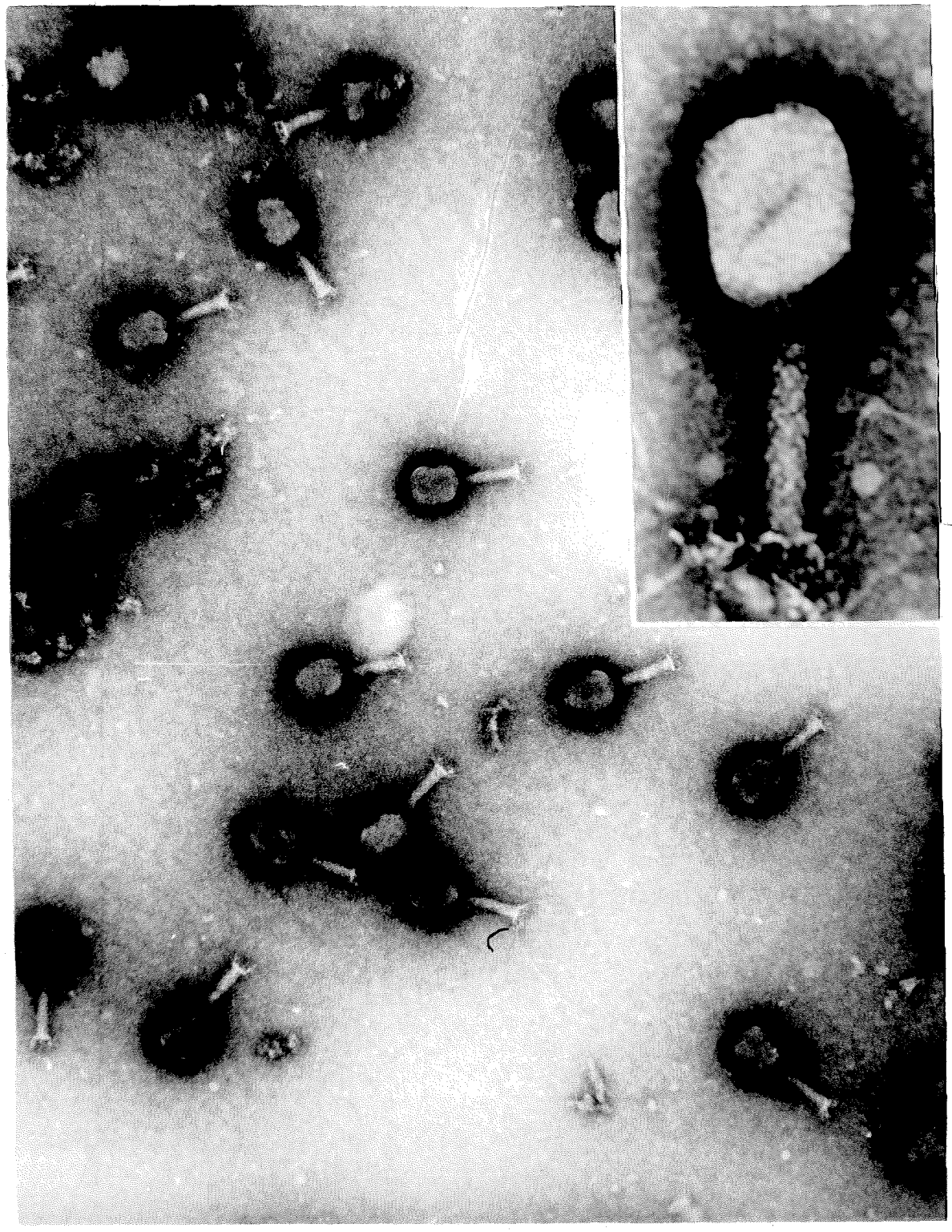


FIGURE 9. ELECTRON MICROGRAPHS OF T4. Phage particles were purified by differential centrifugation, as described in Materials and Methods, and stained with 1% uranyl acetate. The final magnification of the main portion of the figure is 82,960 X, and that of the insert is 340,000 X.

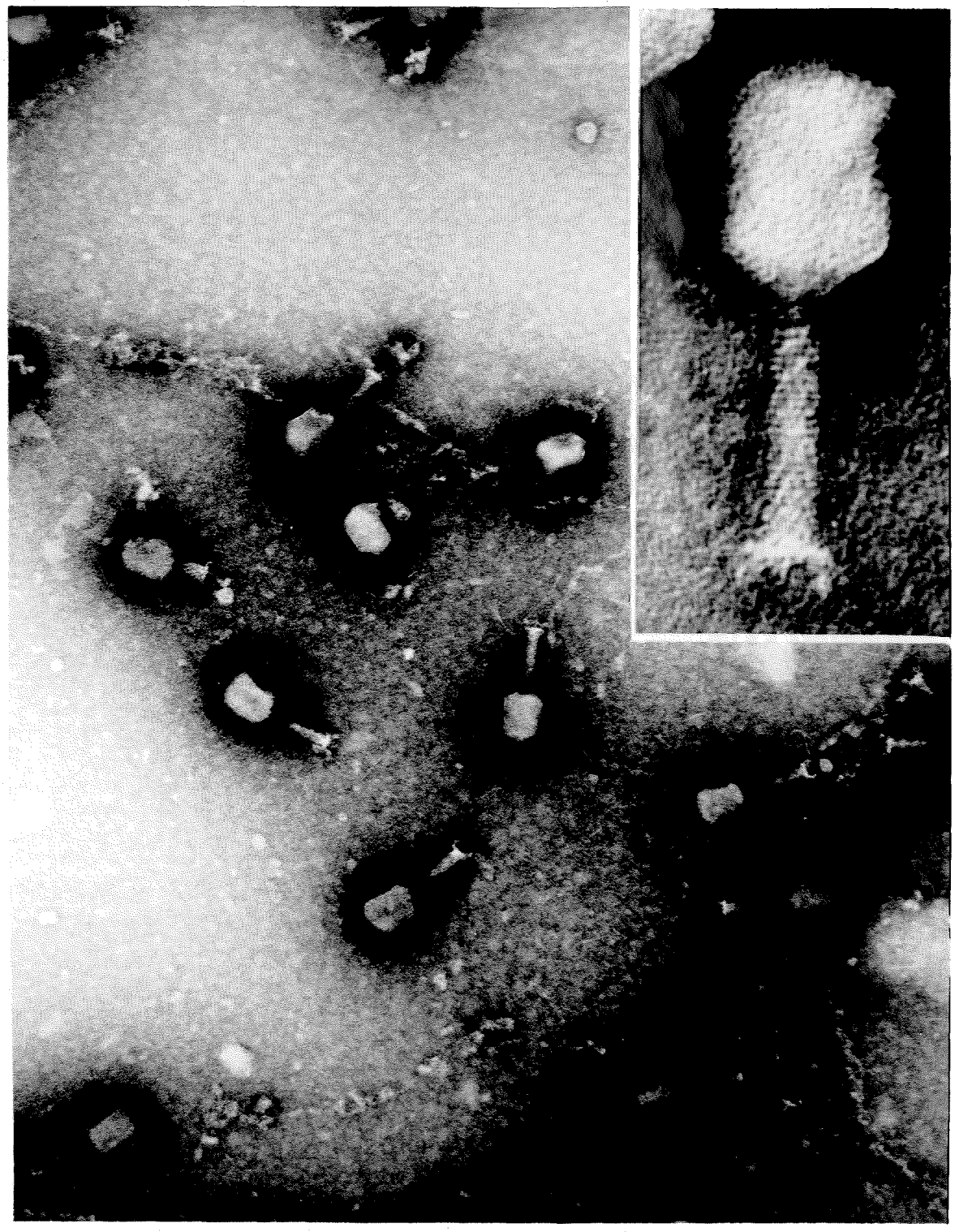
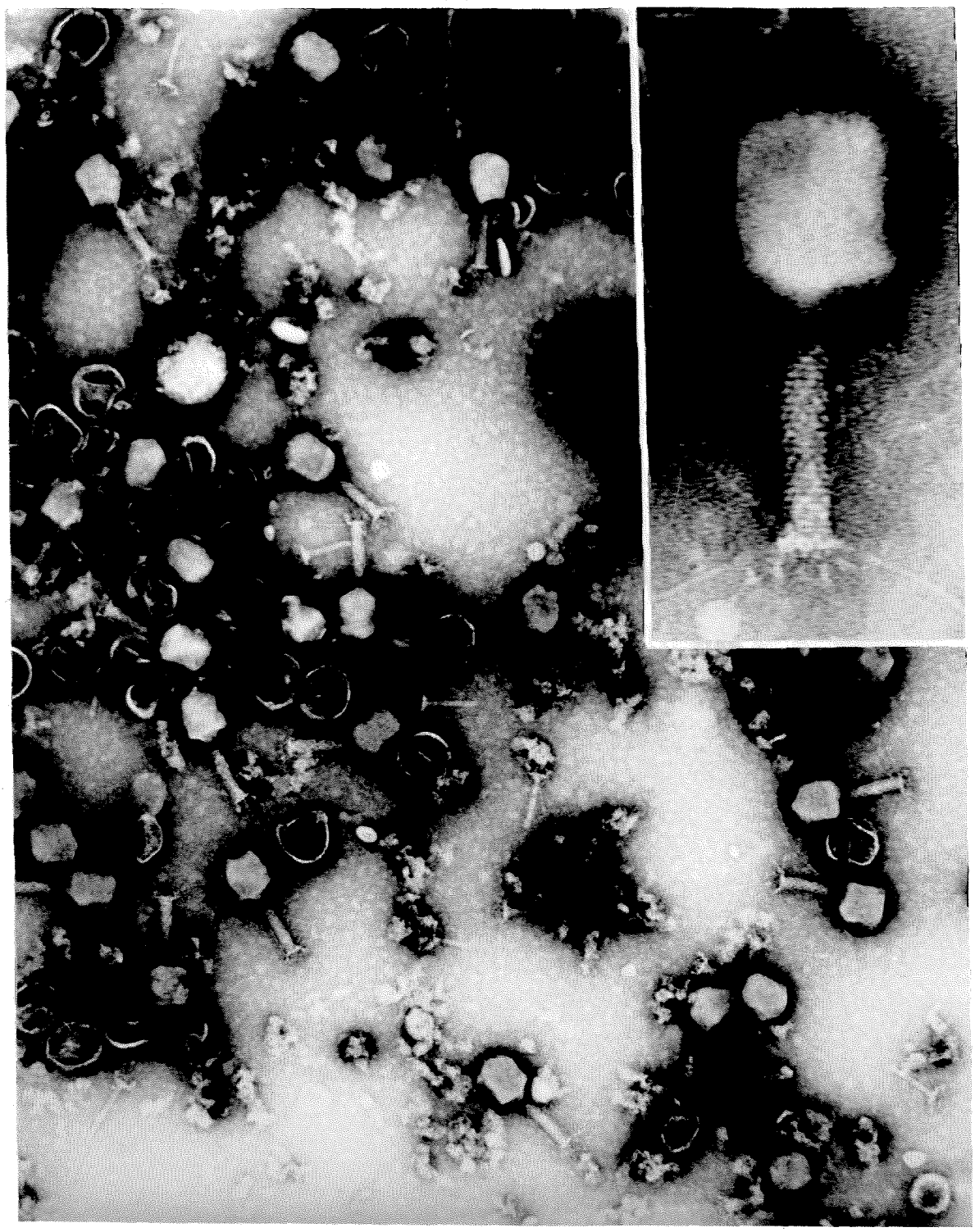


FIGURE 10. ELECTRON MICROGRAPHS OF T6. Phage particles were purified by differential centrifugation, as described in Materials and Methods, and stained with 1% uranyl acetate. The final magnification of the main portion of the figure is 82,960 X, and that of the insert is 340,000 X. This preparation was apparently not as purified as those of the preceding figures.



This close similarity between RB69 and the other T-even phages was confirmed by determinations of the density and sedimentation constant of the RB69 phage particle. The results of a density gradient equilibrium centrifugation of RB69 and T4 are presented in Figure 11, from which it appears that RB69 particles have a density of 1.515 g./ml. The results of a band velocity sedimentation of RB69 particles are presented in Figure 12; they demonstrate that RB69 particles sediment as a single species, and they are used to calculate a sedimentation constant of 879 s for RB69 in Figure 13.

Although it has not been rigorously established that the genetic material of RB69 is DNA, it can be said 1) that RB69 can form rare recombinants with T2, T4, and T6 whose genetic material is DNA, 2) that phenol extraction of RB69 particles results in a solution with the high viscosity typical of DNA solutions, and 3) that the density of the ultraviolet-absorbing material in this solution is typical of DNA. Consequently it was assumed that the material inside the head of RB69 was DNA, and the density and sedimentation constant of this material were determined. A tracing from a density gradient equilibrium centrifugation of RB69 DNA is presented in Figure 14; it is clear that this DNA has a monodisperse density distribution, with a mean density of 1.702 g./ml. Attempts to determine the sedimentation constant of RB69 DNA were fraught with the same difficulties encountered in sedimentation studies on T4 DNA. The sedimenting band often spread quite markedly, and in an asymmetric fashion which indicated a concentration dependence of the sedimentation constant. Nonetheless, a rough sedimentation constant

FIGURE 11. THE DENSITY OF RB69 PHAGE PARTICLES. Tracing of a photograph from a CsCl density gradient equilibrium centrifugation of a mixture of RB69 and T4 phage particles. Phage particles were purified as described in Materials and Methods, and the centrifugation was carried out for 13 hr. at 44,770 rpm in the Spinco Model E Analytical Ultracentrifuge. The input density of the cell contents was 1.501 g./ml. The broad lighter band seen in the tracing is apparently due to a form of T4 phage particle in which the penetration of CsCl into the phage head is incomplete. It disappeared upon prolonged centrifugation or when the phages were preheated in CsCl solution for 2 hr. at 48°C. Centrifugation of RB69 phage particles alone showed them to be monodisperse in density. The density of the T4 marker was determined by R. J. Huskey (103), relative to a $\lambda b_2 b_5$ marker of assumed density 1.4843 g./ml. The axis of rotation is to the left of the figure, and density increases from left to right.

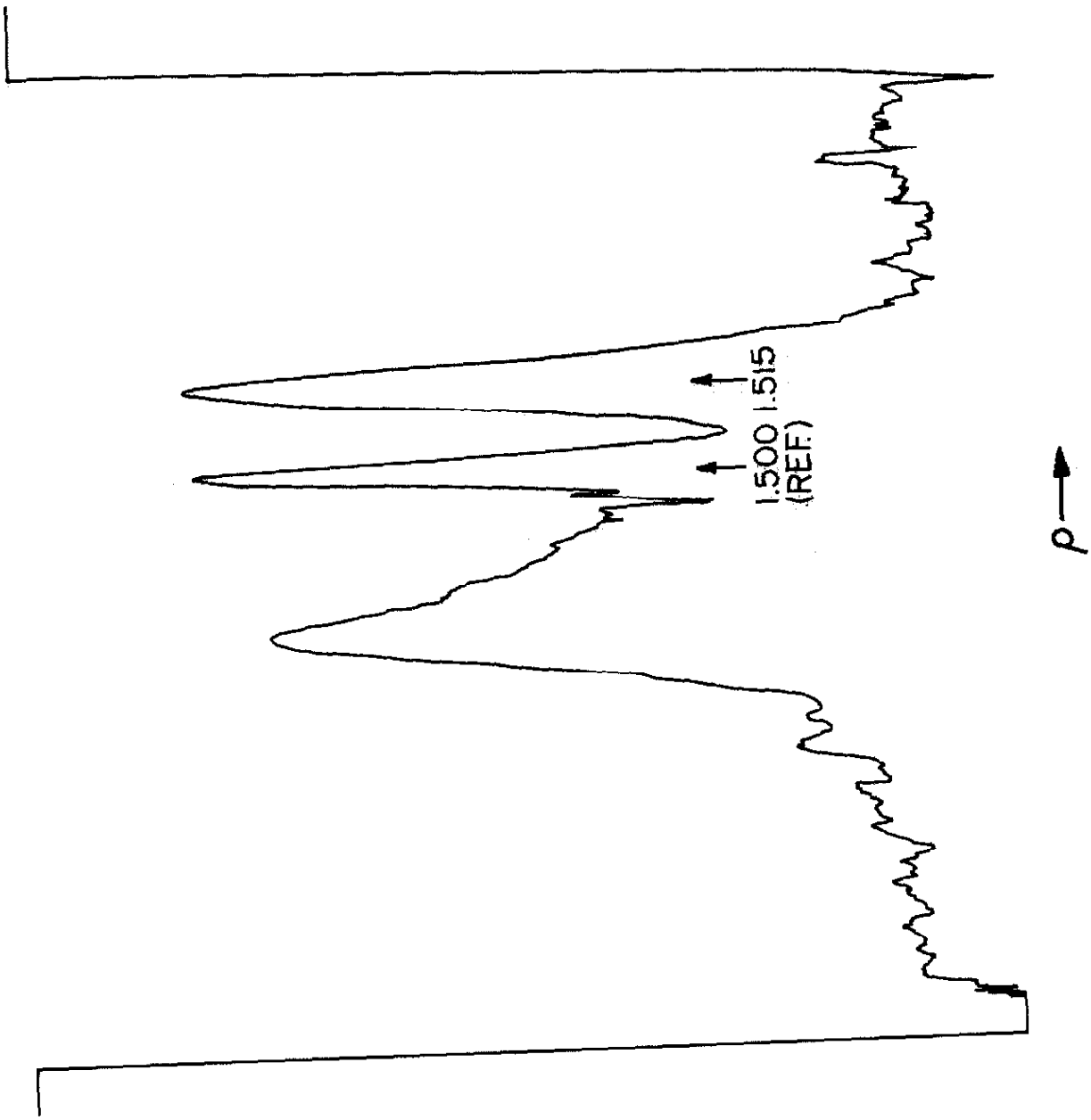

1.500
1.515
(REF.)
p

FIGURE 12. BAND VELOCITY SEDIMENTATION OF RB69 PHAGE PARTICLES. A composite tracing of several photographs taken during a band velocity sedimentation of RB69 phage particles. The lamella consisted of 0.020 ml. of a suspension of RB69 phage particles at a titer of about 2×10^{11}/ml. The supporting solution was a CsCl solution of density 1.336, and sedimentation was carried out at 12,590 rpm. Photographs were taken at 2-minute intervals, and the number above each peak is the number of the photograph from which that tracing was made. The axis of rotation is to the left of the figure.

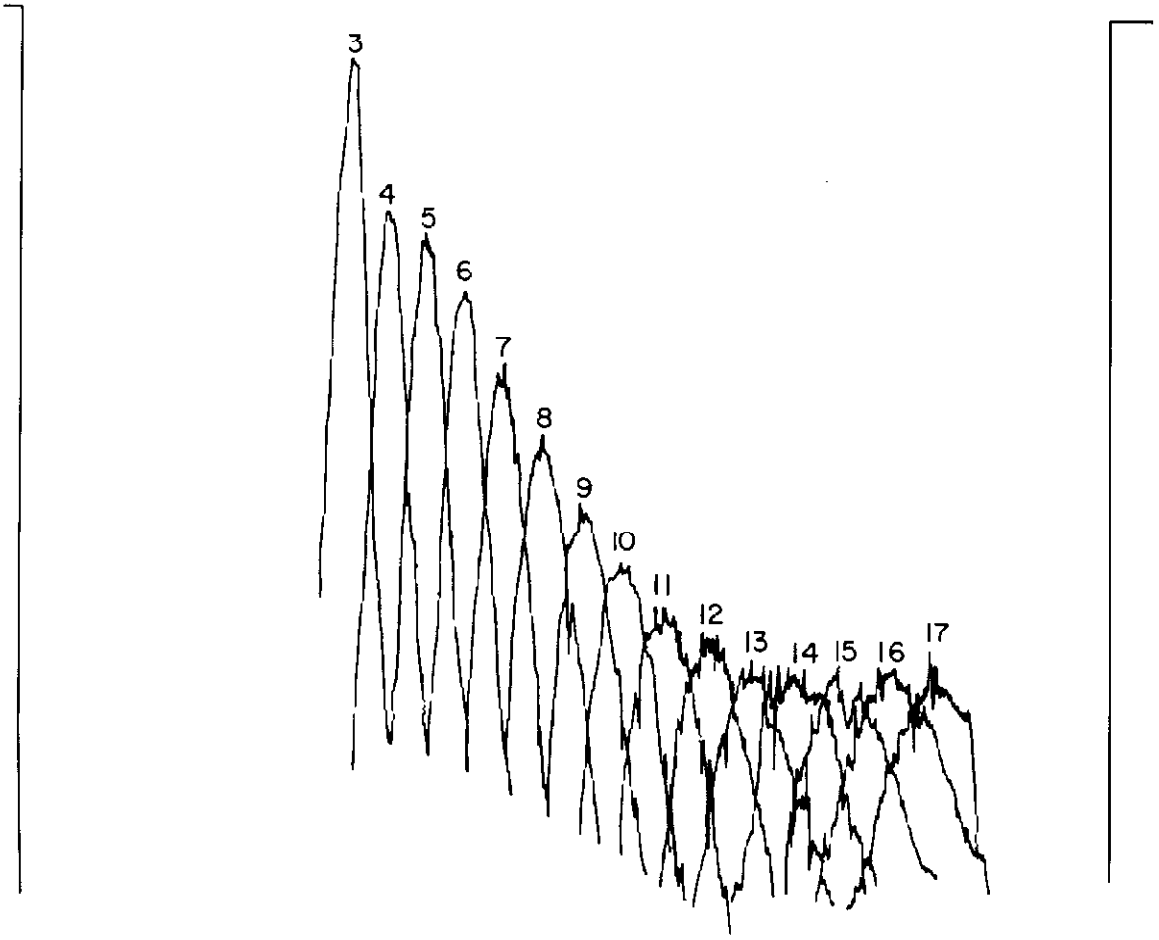

3
4
5
6
7
8
9
10
11
12
13
14
15
16
17

FIGURE 13. THE SEDIMENTATION CONSTANT OF RB69 PHAGE PARTICLES. Data from Figure 12 were used in the construction of this figure. The symbols are defined as follows: r is the distance of the band from the center of rotation in cm., s is one Svedberg (10^{-13} sec.), η_{solv} is the viscosity of the supporting CsCl solution, which was interpolated from the values reported by Bruner and Vinograd (104), σ is the density of the cesium form of the phage particle, and ρ is the density of the supporting CsCl solution, which was determined by a refractive index measurement. The correction of the cesium form sedimentation constant to the sodium form sedimentation constant is performed simply by calculating the difference in mass of these two forms, assuming that each phosphate in the phage DNA binds one cation, that RB69, like T4, contains 2×10^5 base pairs in one phage DNA complement, and that RB69 is 50% protein, 50% DNA. The correction of the sedimentation constant to standard conditions is by the formula of Svedberg and Pedersen (105).

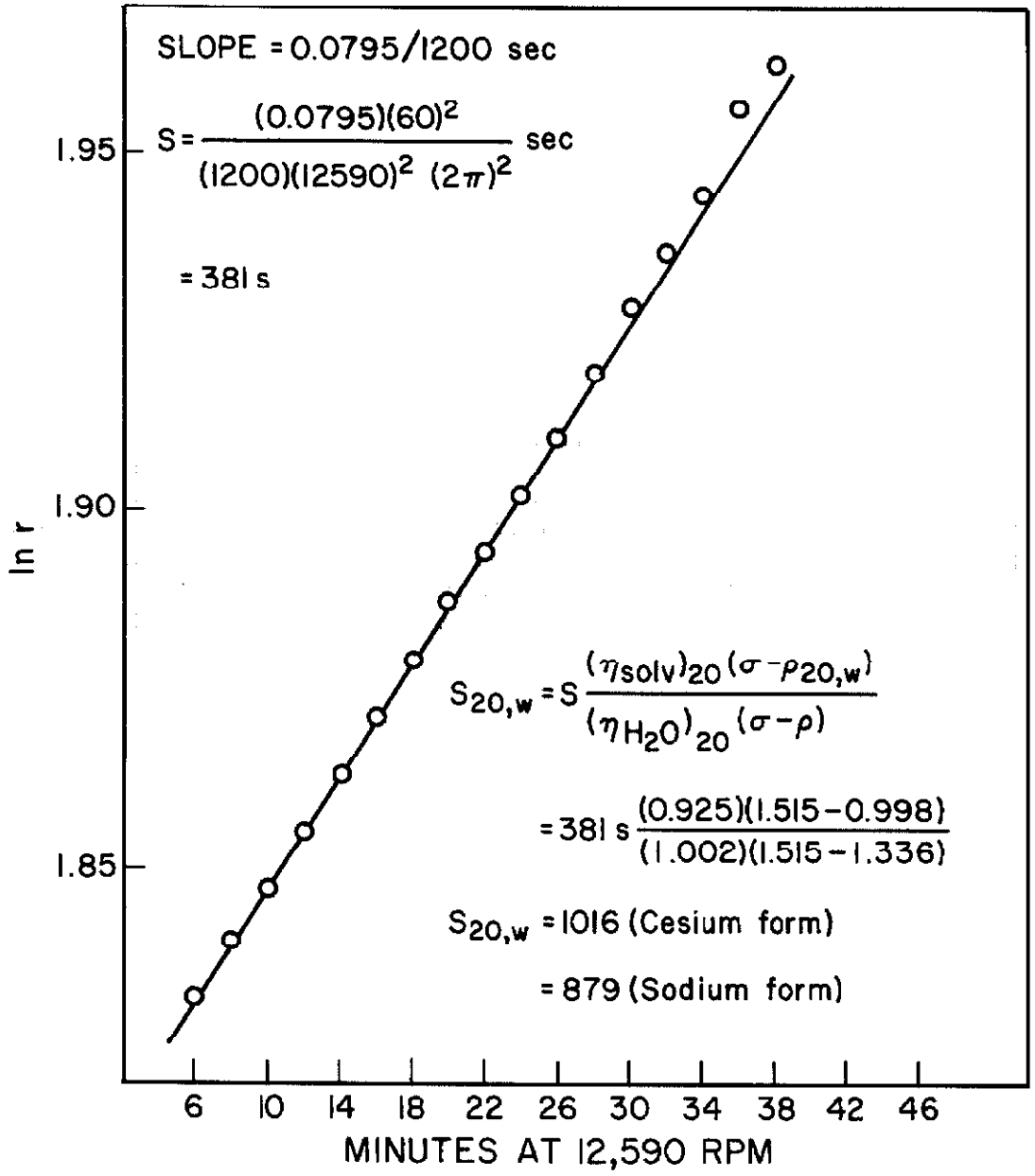
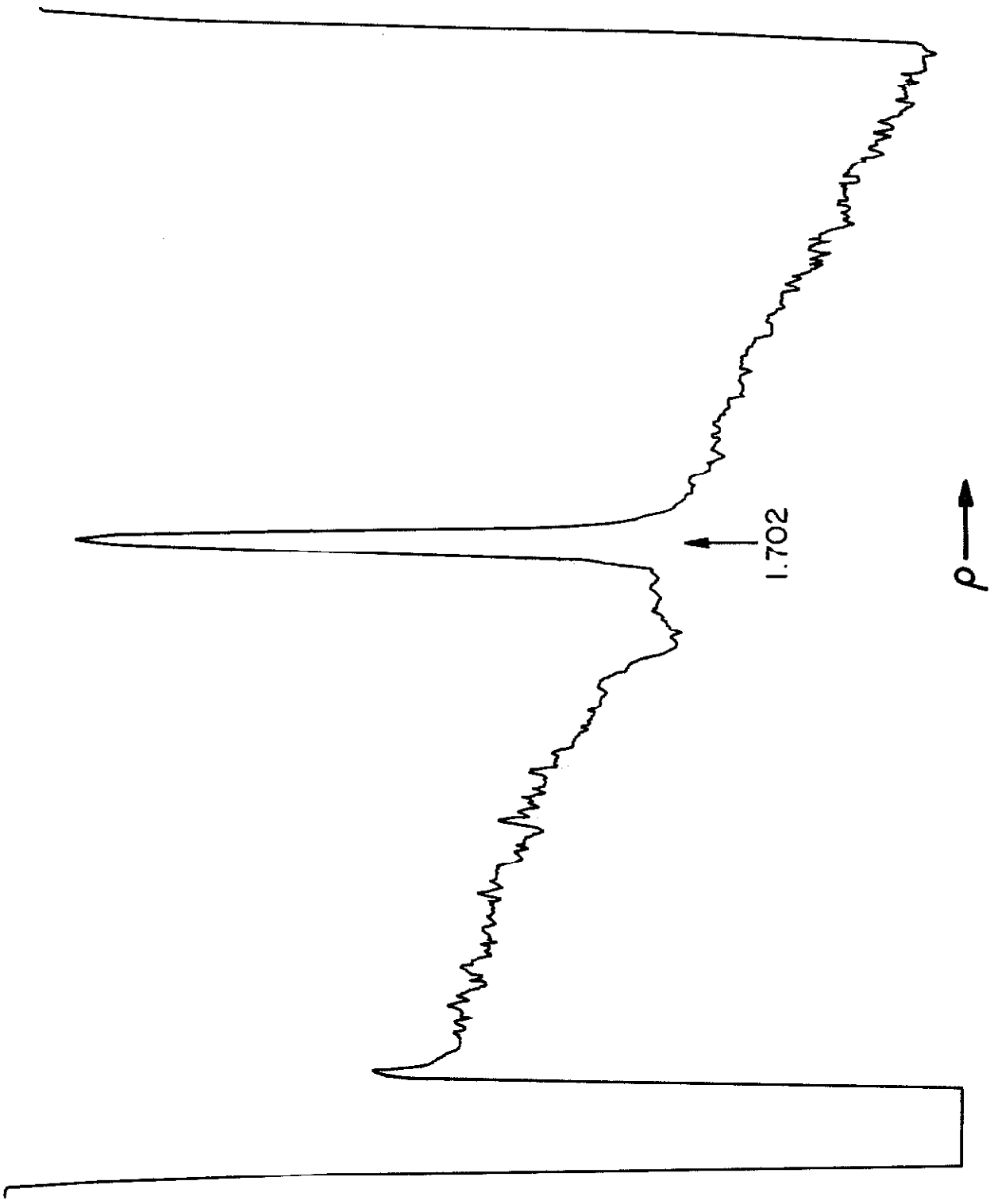

SLOPE = 0.0795/1200 sec
$S = \frac{(0.0795)(60)^2}{(1200)(12590)^2\ (2\pi)^2}$ sec
= 381 s
$S_{20,w} = S \frac{(\eta_{solv})_{20}(\sigma - \rho_{20,w})}{(\eta_{H_2O})_{20}(\sigma - \rho)}$
$= 381\ s \frac{(0.925)(1.515-0.998)}{(1.002)(1.515-1.336)}$
$S_{20,w}$ = 1016 (Cesium form)
= 879 (Sodium form)
ln r
1.95
1.90
1.85
6
10
14
18
22
26
30
34
38
42
46
MINUTES AT 12,590 RPM

FIGURE 14. THE DENSITY OF RB69 DNA. RB69 DNA was extracted from purified RB69 phages with phenol, as described in Materials and Methods. Its density was determined by measuring the position of the equilibrium band relative to the meniscus and the bottom of the centrifuge cell, measuring the density of the cell contents before and after the centrifugation, and using equations 15 and 18 of Vinograd and Hearst (106) to calculate the density.


1.702
d

could be determined for RB69 DNA, and its reliability was checked by performing a similar determination of the sedimentation constant of T2 DNA. Since this determination agreed well with the published values for the sedimentation constant of T2 DNA, the sedimentation constant of RB69 DNA was judged to be reasonably reliable.

A summary of the properties of the RB69 phage particle is presented in Table 5, with the corresponding properties of T2, T4, and T6 for comparison.

b. Characteristics of the Growth Cycle

The adsorption rate constant for RB69 was quite similar to those of T2, T4, and T6. Its host range, however, differed from those of T2, T4, and T6, indicating a somewhat different specificity of attachment to the bacterial surface. The results of a one-step growth experiment, presented in Figure 15, revealed close similarities between the intracellular growth parameters of RB69 and those of T4, and RB69-infected cells could be artificially opened by chloroform treatment before the end of the latent period, suggesting the existence of an RB69-induced lysozyme.

2. Complementation Testing

From a stock of RB69, grown in the presence of 5-bromodeoxyuridine as described in Materials and Methods, 82 amber mutants were isolated. Since RB69 appeared to be a close relative of

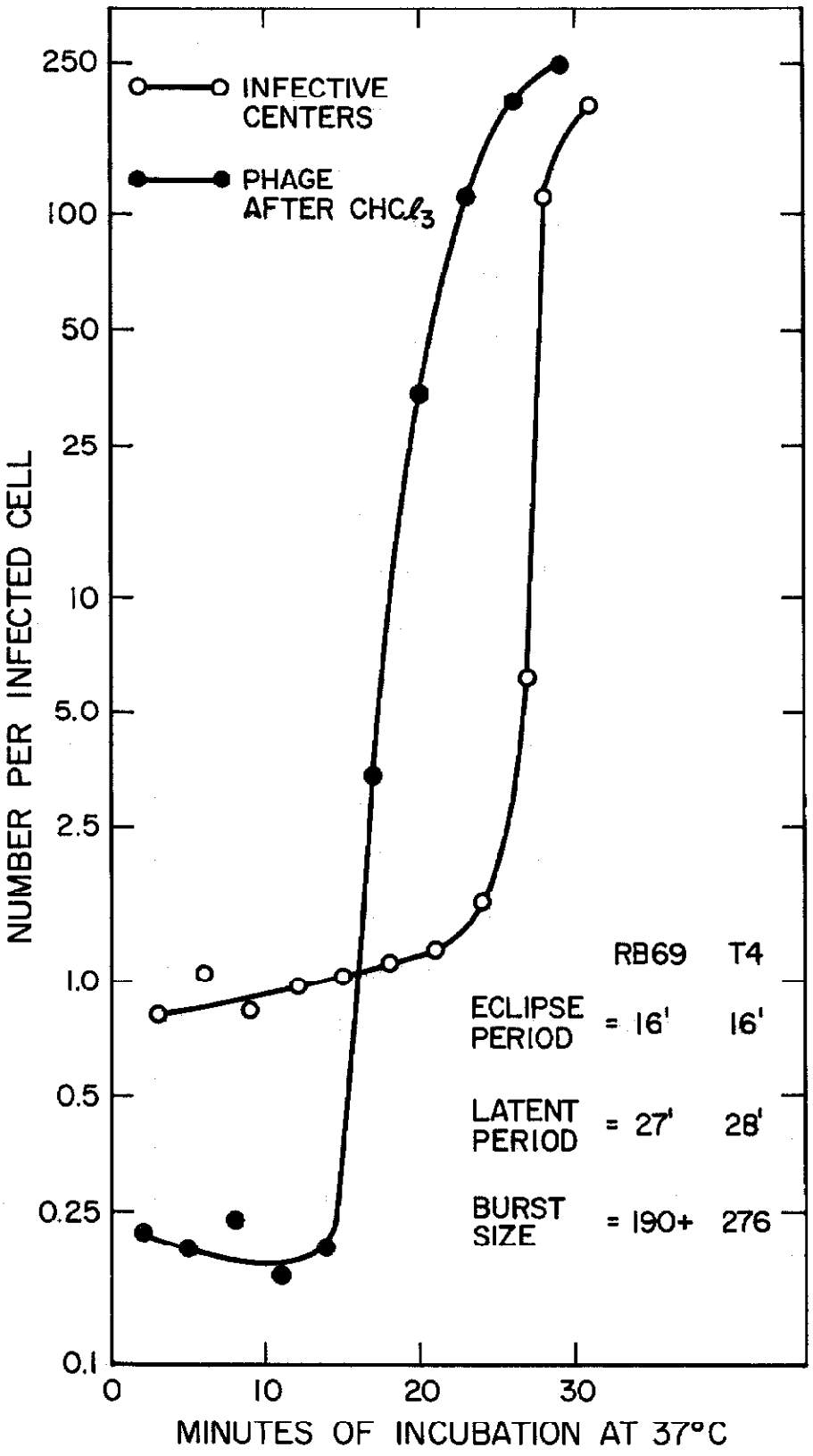
TABLE 5

PROPERTIES OF RB69 AND THE CLASSICAL T-EVEN PHAGES

	PHAGE PARTICLES			PHAGE DNA	
PHAGE	MORPHOLOGY	ρ (g./ml.)	S_{20}, w	ρ (g./ml.)	S_{20}, w
RB69	T-even, with collar	1.515	879s	1.702	(64)
T2	T-even, without collar	1.507 (103)	672s 1017s (107)	1.702 (108)	(64)
T4	T-even, with collar	1.500 (103)	758s 1110s (109)	1.701 (108)	65±1 (72)
T6	T-even, with collar	1.515 (103)	-	1.711 (108)	65±1 (72)

The densities of phage particles other than RB69 were determined by R. J. Huskey, relative to a $\lambda b_2 b_5$ marker of presumed density 1.4843 g./ml. (103). The parentheses surrounding the values for the sedimentation constants of RB69 DNA and T2 DNA are meant to indicate that difficulties were encountered in measuring these constants. All other values are from the indicated sources.

FIGURE 15. ONE STEP GROWTH CURVE OF RB69. Adsorption was for 10 minutes at 37°C. to B/5 which had been prepared for infection and to which KCN had been added to achieve a final concentration during adsorption of 0.002 M. The complexes were then diluted 40,000 fold into 37°C. broth, and samples were withdrawn at the indicated times thereafter for measurement of infective centers and intracellular phages. The end of the eclipse period is defined as the time when there is an average of one intracellular phage per cell, and the end of the latent period is defined here as the time when the number of extracellular phages reaches the square root of the final burst size (on a logarithmic scale, the half-rise point in the extracellular phage curve). The values for the growth parameters of T4 were determined by J. Eder during his tenure as a summer trainee under Dr. R. S. Edgar.


250
100
50
25
10
5.0
2.5
1.0
0.5
0.25
0.1
INFECTIVE CENTERS
PHAGE AFTER CHCl3
NUMBER PER INFECTED CELL
RB69 T4
ECLIPSE PERIOD = 16' 16'
LATENT PERIOD = 27' 28'
BURST SIZE = 190+ 276
0
10
20
30
MINUTES OF INCUBATION AT 37°C

T4, an attempt was made to characterize these mutants by their complementation properties in spot tests against T4 mutants. The number of plaques obtained in these spot tests was too small to permit a distinction between positive and negative results. Raising the phage concentrations ten-fold improved the tests somewhat, but the results were still quite unsuitable for a characterization of the RB69 mutants. As will be discussed at greater length in Section B of the Results, the failure of these spot tests can be attributed at least in part to the very strong exclusion which occurs in T4-RB69 mixed infections. However, the rare recombinants formed in such spot tests constitute evidence for a reasonably close relationship between T4 and RB69.

Since the intertype spot tests were unsatisfactory, the defects of the RB69 mutants were assigned to genes on the basis of their complementation patterns with one another. A stepwise testing procedure, similar in principle to the procedures for T2 and T6 mutants, was used, and the final assignments were checked in the same two ways that the T2 and T6 assignments had been checked.

A summary of these assignments is presented in Table 6; 37 genes have been identified in RB69, and the defects of a total of 77 single mutants and 5 double mutants have been assigned to these genes. The number of RB69 genes identified by these mutants compares favorably with the number of T2 and T6 genes identified by similar numbers of T2 and T6 mutants, indicating that RB69 probably has about as many genes as the classical T-even phages.

TABLE 6. GENE DEFECTS OF RB69 MUTANTS. Each mutant is listed with the other mutants which have defects in the same gene. Single mutants are listed once, and double mutants twice, once with the suffix "a" and once with the suffix"b". The double mutants 1, 12, 13 and 72 have been resolved into two single mutants each by crosses with RB69 wild type. The genes have not been numbered, in order to avoid confusion with the numbering for the classical T-even phages, and they are presented in their map order.

TABLE 6

GENE DEFECTS OF RB69 MUTANTS

10, 61 40, 106, 13b 17, 33, 43, 56, 63, 79, 90 89 8, 71, 94
26, 36, 57, 66, 70, 78, 1b, 14a 45, 62, 96 21a, 34, 59, 1a 99, 102 82, 14b
37 31, 48 105 4, 12b 11, 67
27, 47, 75 12a 29 24, 58, 93 6a, 53, 92, 104
42, 68 9, 35, 49 74 95, 101 25, 50
41 39 28 2, 16, 76 30, 38, 72b
65 6, 83, 91 85 51 55
18, 60, 97 100, 13a, 72a

3. Characterization of Mutant Phenotypes

Since the genes identified in RB69 could not be shown to be homologous to T4 genes, no a priori estimate of the effects of mutational defects in these genes could be made. Consequently, one mutant was selected to represent each gene and the results of infection of a restrictive host by these mutants were characterized in two ways; first by determining whether the infected cells lysed, and second by observing whether DNA was synthesized in the infected cells.

The experiments on the lysis of the infected cells were performed because it had been demonstrated in T4 that mutants defective in the synthesis of phage DNA are also defective in the formation of late phage proteins, including the phage-induced lysozyme; these mutants consequently fail to lyse the infected cell, and can thereby be identified. Some typical results from these experiments are presented in Figure 16, and the resulting classification of the RB69 mutants is presented in Table 7. It is clear that a significant fraction of these mutants fail to lyse the restrictive host.

Measurements of DNA synthesis in the infected cells were carried out in the simplified manner described in Materials and Methods. Some typical results are presented in Figure 17, and the resulting classification of the mutants is presented in Table 7. As in T4 (21), there is a good correlation between the ability of a mutant to lyse the restrictive host and its ability to initiate DNA synthesis. On the basis of this similarity to T4, those RB69 mutants which fail to initiate DNA synthesis and fail to lyse the restrictive host may tentatively be described as

FIGURE 16. LYSIS EXPERIMENTS WITH RB69 AMBER MUTANTS. The experiments were performed as described in Materials and Methods. Two mutants which lysed the cells well and two which failed to lyse the cells are presented for comparison. As can be seen by comparing with the curve for uninfected bacteria, mutants which do not lyse the cells nonetheless prevent further bacterial growth.

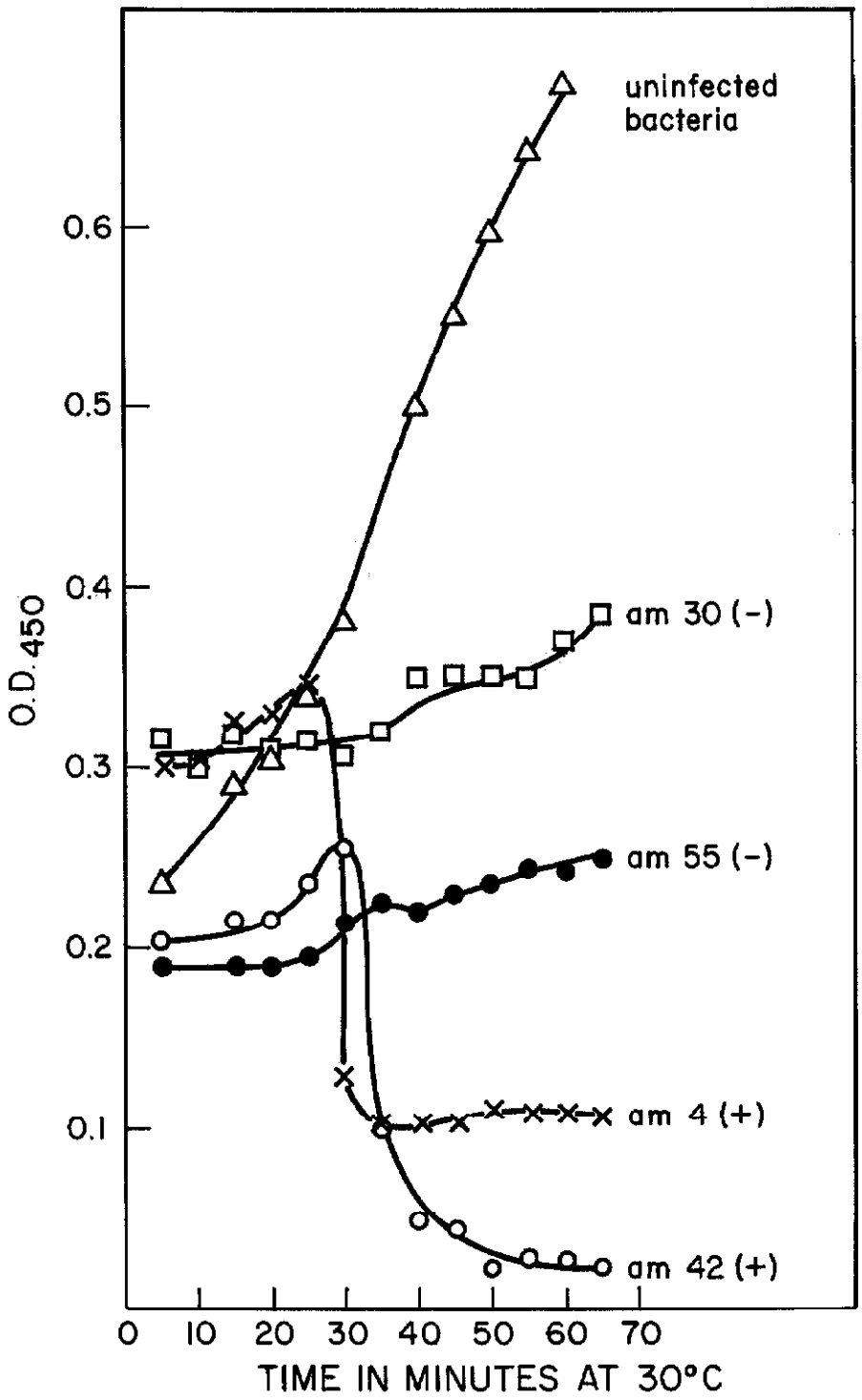

uninfected
bacteria
am 30 (-)
am 55 (-)
am 4 (+)
am 42 (+)
O.D.450
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
70
TIME IN MINUTES AT 30°C

FIGURE 17. DNA SYNTHESIS BY RB69 AMBER MUTANTS. The measurements of incorporation of tritiated thymidine were performed by the simplified method described in Materials and Methods. Two mutants showing normal kinetics of DNA synthesis, one showing arrested kinetics, and one showing no DNA synthesis have been selected for comparison.

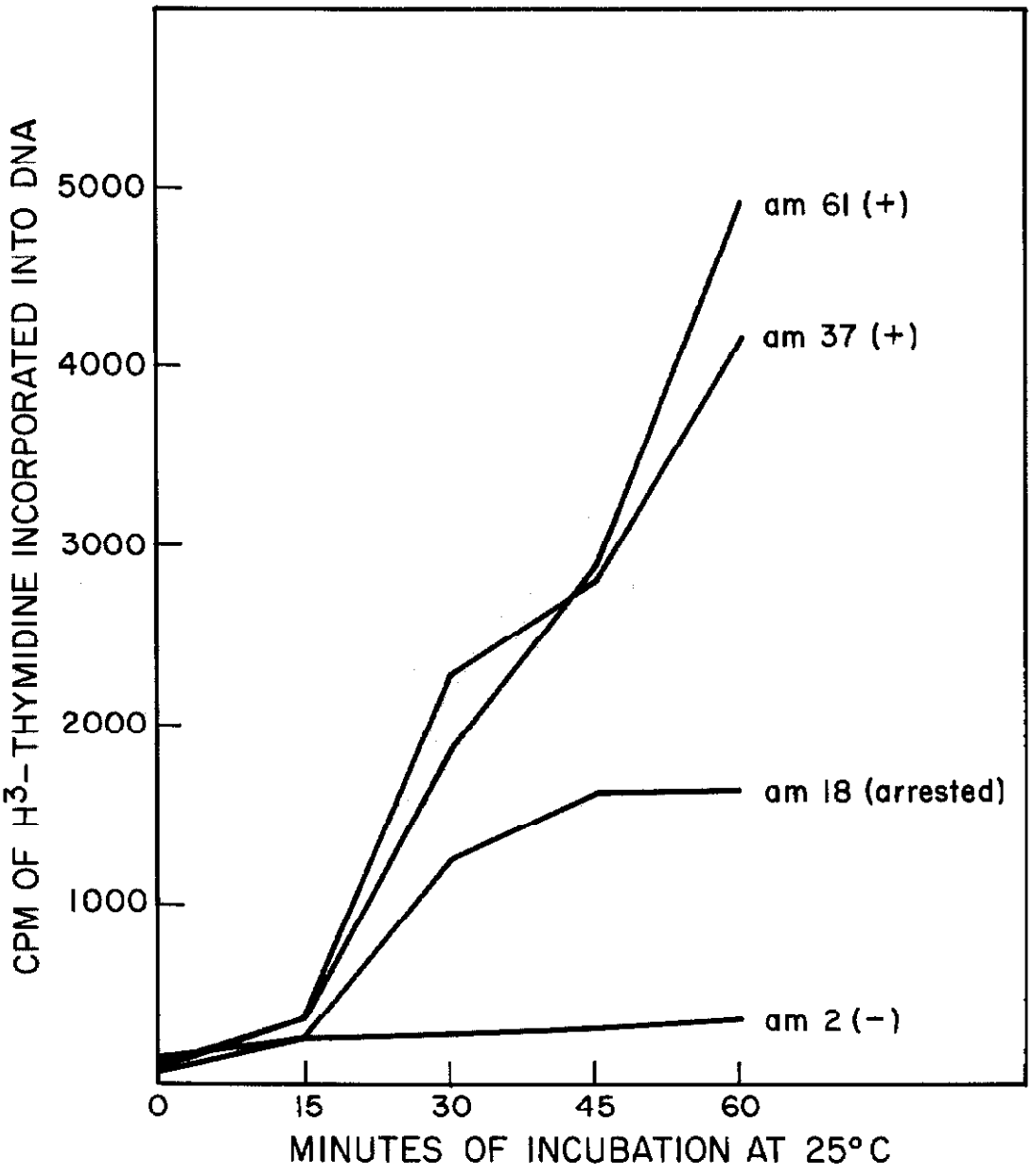

CPM OF H3-THYMIDINE INCORPORATED INTO DNA
5000
4000
3000
2000
1000
0
15
30
45
60
MINUTES OF INCUBATION AT 25°C
am 61 (+)
am 37 (+)
am 18 (arrested)
am 2 (-)

TABLE 7. CHARACTERIZATION OF RB69 MUTANT PHENOTYPES. Slow, late lysis occurred 60 minutes or later after infection, and consisted of a gradual reduction of the optical density of the culture over a period of twenty to thirty minutes. (It is not known whether RB69 shows lysis inhibition.) Low DNA synthesis began at the normal time but proceeded at a rate less than half that of normal. Delayed DNA synthesis began considerably later than normal and proceeded at a somewhat slower rate. Arrested DNA synthesis began at the normal time and proceeded initially at the normal rate; quite soon, however, it slowed down and reached a rate less than 20% of normal (see Figure 17).

TABLE 7

CHARACTERIZATION OF RB69 MUTANT PHENOTYPES

MUTANT	RESULTS OF INFECTION OF RESTRICTIVE HOST	
	CELL LYSIS	DNA SYNTHESIS
61	NORMAL	NORMAL
106	NORMAL	NORMAL
17	NORMAL	NORMAL
89	NORMAL	NORMAL
94	NORMAL	NORMAL
36	NORMAL	NORMAL
45	NORMAL	NORMAL
59	NORMAL	LOW
102	NORMAL	NORMAL
82	NORMAL	NORMAL
37	NORMAL	NORMAL
48	NORMAL	NORMAL
105	NORMAL	NORMAL
4	NORMAL	NORMAL
11	NORMAL	NORMAL
27	NORMAL	NORMAL
12a	----	NONE
29	SLOW, LATE	DELAYED
24	NONE	NONE
6a	NORMAL	NORMAL
42	NORMAL	NORMAL
9	NORMAL	NORMAL
74	NORMAL	NORMAL
95	SLOW, LATE	DELAYED
25	SLOW, LATE	DELAYED
41	----	NONE
39	SLOW, LATE	DELAYED
28	----	NONE
16	NONE	NONE
30	NONE	NONE
65	NONE	NONE
7	SLOW, LATE	NONE
85	----	NONE
51	----	NONE
55	NONE	NONE
18	NORMAL	ARRESTED
13a	NORMAL	ARRESTED

"early mutants", and those which initiate normal DNA synthesis and lyse the restrictive host may tentatively be described as "late mutants". Of the 37 mutants, each representing one gene, 20 appear to be late, 11 appear to be early, 4 have a DNA-delayed phenotype, and 2 have a DNA-arrested phenotype.

The characterization of the mutant phenotypes of the 20 late mutants was carried somewhat further by some preliminary electron microscope observations on defective lysates. Among the defective lysates examined, phage-like particles were seen only in those of mutants 6a, 9 and 10. Phage heads, unattached to tails, were seen in defective lysates of mutants 4, 11, 14, 21a, 27, 36 and 45, and phage tails, unattached to heads, were seen in defective lysates of mutants 31 and 37. These observations are summarized in Figure 21, below.

Although a slight modification of the procedure of Edgar and Wood (110) made it possible to develop an <u>in vitro</u> system for the assembly of RB69, its efficiency was only about 1% of that of the T4 system, and it was not clear which steps in the assembly of RB69 it could carry out. Consequently it could make only minor contributions to the characterization of the late mutant phenotypes, and these contributions are indicated in Figure 21 by dotted lines.

On the basis of the above observations, the following rough characterization of the functions of the RB69 late genes can be made. The genes represented by mutants 6a, 9, and 10 are apparently involved in the activation of particles, and it is argued below that the genes represented by mutants 6a and 9 are probably involved in attaching tail fibers to

these particles. The genes represented by mutants 31 and 37, and possibly those represented by mutants 82, 99, and 105 as well, are apparently involved in the synthesis of the head. The genes represented by mutants 4, 11, 17 and 27, and possibly that represented by mutant 89, are probably involved in the synthesis of the tail. The genes represented by mutants 21a, 36 and 45 are probably involved in converting the head into a form to which tails can be attached.

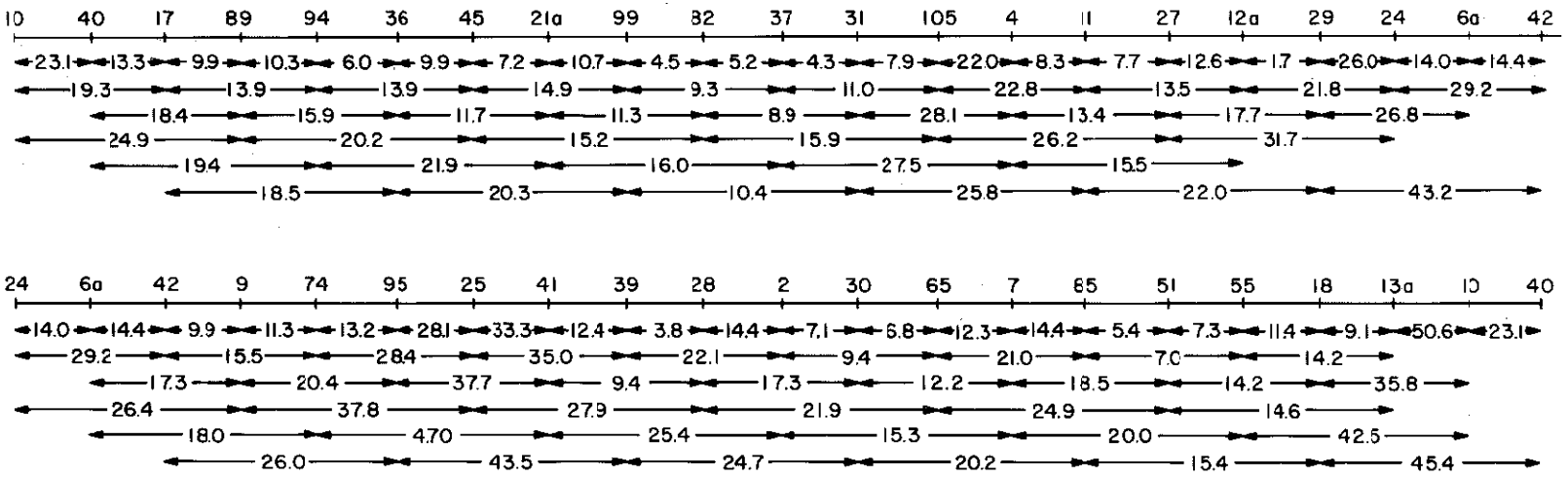
4. Mapping

a. Gene Order

In order to obtain a more complete genetic description of RB69, the newly isolated amber mutants were mapped. Since it was expected that the early mutants of RB69 might show functional clustering, as in T4, they were crossed first with one another, and later with the late mutants; the expected clustering was observed, and from these crosses, it was clear that the general features of recombination in RB69 were quite similar to those in T4. With these and subsequent crosses, the map of RB69 was gradually built up; when the final map order had been established, each mutant had been crossed with its five nearest neighbors on either side, and a total of 381 of the 666 possible crosses had been performed at least once each. The final map order and data from some of the crosses are presented in Figure 18.

Because of the high recombination frequencies which are observed between some pairs of neighboring mutants, a rigorous treatment of the data in Figure 18 only permits the mutants to be divided into four well-

FIGURE 18. RECOMBINATION DATA FROM RB69 CROSSES. Each number in the figure is the observed percentage of recombination between the mutants at the ends of the arrow. The calculation of percentages of recombination involves a correction for plating efficiency differences of the permissive and restrictive strains used, as described in Materials and Methods. The average number of determinations per number in the figure is 1.2.


10 40 17 89 94 36 45 21a 99 82 37 31 105 4 11 27 12a 29 24 6a 42
23.1 13.3 9.9 10.3 6.0 9.9 7.2 10.7 4.5 5.2 4.3 7.9 22.0 8.3 7.7 12.6 1.7 26.0 14.0 14.4
19.3 13.9 13.9 14.9 9.3 11.0 22.8 13.5 21.8 29.2
18.4 15.9 11.7 11.3 8.9 28.1 13.4 17.7 26.8
24.9 20.2 15.2 15.9 26.2 31.7
19.4 21.9 16.0 27.5 15.5
18.5 20.3 10.4 25.8 22.0 43.2
24 6a 42 9 74 95 25 41 39 28 2 30 65 7 85 51 55 18 13a 10 40
14.0 14.4 9.9 11.3 13.2 28.1 33.3 12.4 3.8 14.4 7.1 6.8 12.3 14.4 5.4 7.3 11.4 9.1 50.6 23.1
29.2 15.5 28.4 35.0 22.1 9.4 21.0 7.0 14.2
17.3 20.4 37.7 9.4 17.3 12.2 18.5 14.2 35.8
26.4 37.8 27.9 21.9 24.9 14.6
18.0 4.70 25.4 15.3 20.0 42.5
26.0 43.5 24.7 20.2 15.4 45.4

mapped groups, one consisting of mutants 40 through 105 on the map, the second of mutants 4 through 29, the third of mutants 24 through 95, and the fourth of mutants 41 through 13a. From other crosses not presented in the figure, the order of the first three groups with respect to one another is very probably correct as presented, but the relative orientation of the fourth group with respect to the first three is not rigorously established. The orientation presented is the one which gives the best agreement with all of the available data, and it happens that this orientation also gives the greatest similarity between the maps of RB69 and T4 with respect to the distribution of mutant phenotypes. There is no evidence for or against the circularity of the RB69 map.

b. Properties of Recombination

Negative interference was observed in RB69 crosses, and was quantitatively similar to that of T4 and T2 crosses; the extent of this effect is shown in Figure 19. In an attempt to circumvent the difficulty of negative interference, the T4 mapping function described above was applied to the RB69 recombination data, and the results are presented in Figure 20; the additivity demonstrated by the computed distances (61 points lie above the line, 53 below it) suggests that the T4 mapping function is an adequate solution to the problem of negative interference in RB69. A check on the adequacy of the T4 mapping function is possible if the RB69 map is assumed to be circular; the sum of all the shortest intervals in RB69 comes out to be 1.33 map lengths, closer to

FIGURE 19. ADDITIVITY OF RB69 RECOMBINATION PERCENTAGES. The ordinate of each point is the observed percentage of recombination between two non-adjacent mutants; the abscissa is the sum of the recombination percentages for the shortest intervals lying between the same two mutants. The line drawn is for the case in which the observed and summed percentages are equal. The distribution of lengths among the shortest intervals used is indicated by the histogram on the abscissa.

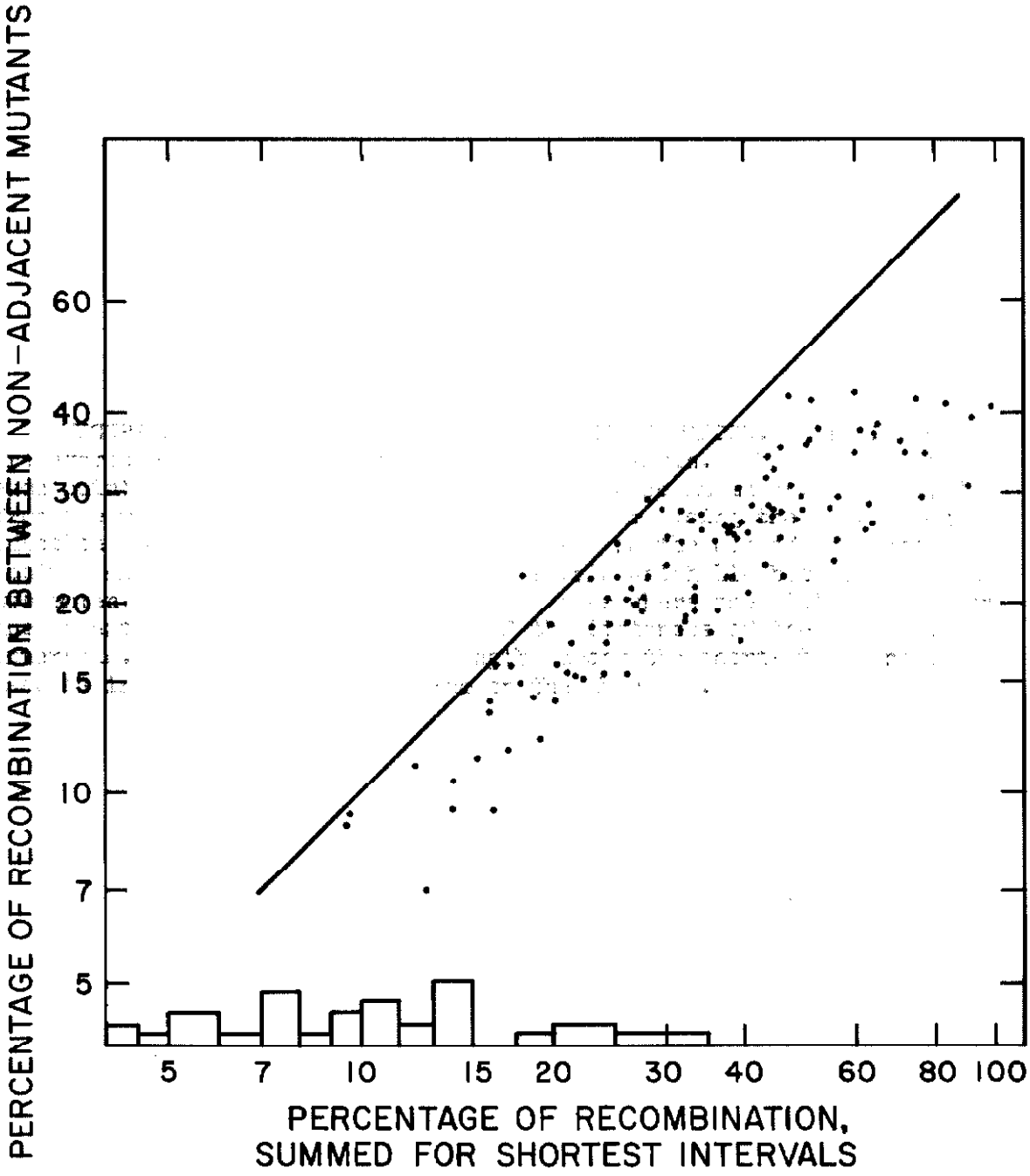
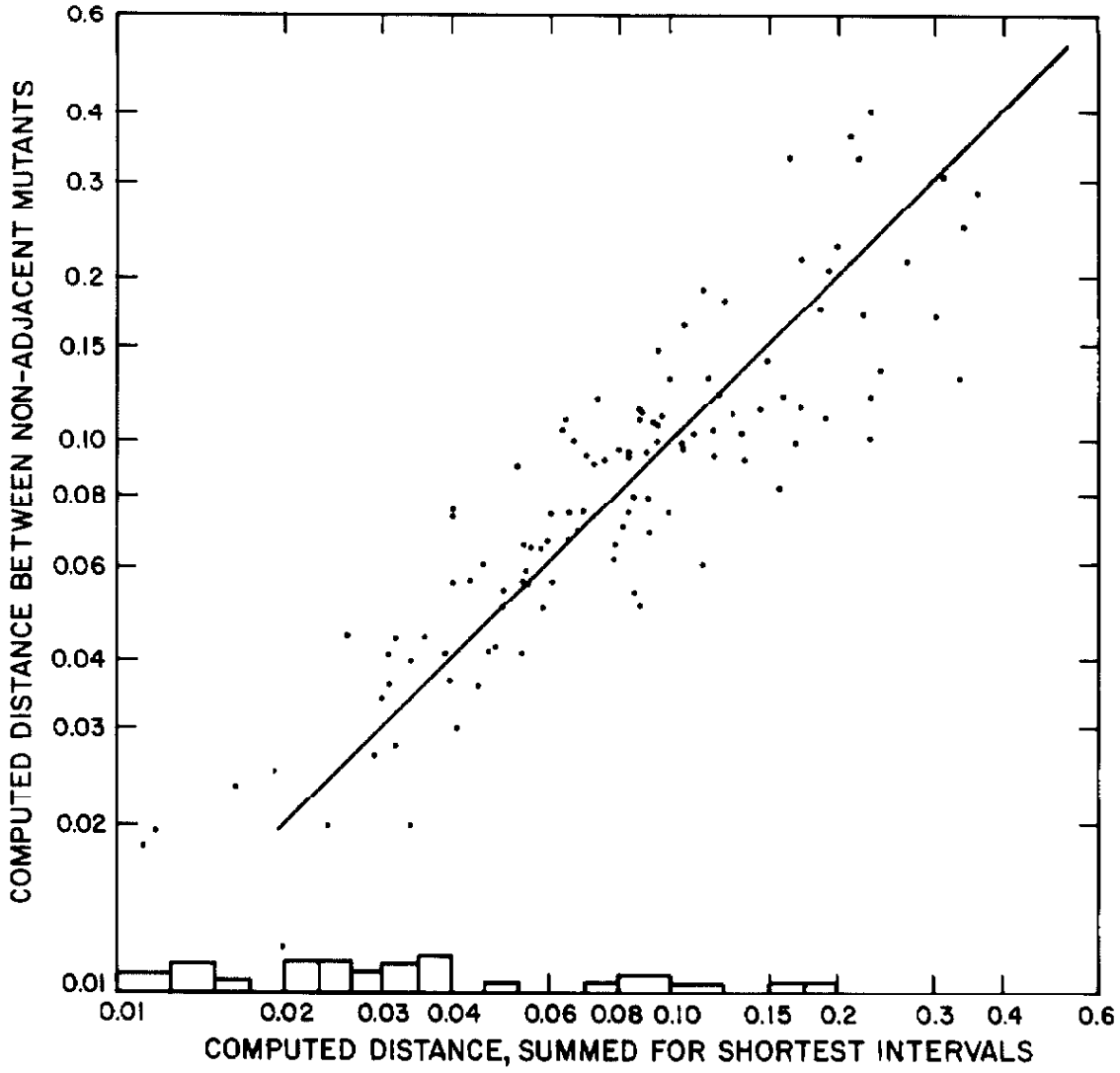

PERCENTAGE OF RECOMBINATION BETWEEN NON-ADJACENT MUTANTS
60
40
30
20
15
10
7
5
5
7
10
15
20
30
40
60
80
100
PERCENTAGE OF RECOMBINATION,
SUMMED FOR SHORTEST INTERVALS

FIGURE 20. ADDITIVITY OF RB69 COMPUTED DISTANCES. All distances are expressed as fractions of the total map length. The ordinate of each point is the distance between two non-adjacent mutants, computed from the recombination percentage between them, using the T4 mapping function described in the text; the abscissa is the sum of the corresponding distances for the shortest intervals which lie between the same two mutants. The line drawn is for the case where the distance between the mutants is the same as the sum of the distances of the shortest intervals. The distribution of lengths among the shortest intervals used is indicated by the histogram on the abscissa.


COMPUTED DISTANCE BETWEEN NON-ADJACENT MUTANTS
0.6
0.4
0.3
0.2
0.15
0.10
0.08
0.06
0.04
0.03
0.02
0.01
0.01
0.02
0.03
0.04
0.06
0.08
0.10
0.15
0.2
0.3
0.4
0.6
COMPUTED DISTANCE, SUMMED FOR SHORTEST INTERVALS

the ideal value of 1.00 map lengths than the 1.44 map lengths observed for the sum of shortest intervals in T4 data corrected in the same way.

c. The Map

A map for RB69, constructed using the distances computed with the T4 mapping function, is presented in Figure 21, with a comparable map for T4 in Figure 22 for comparison. For the purposes of constructing the RB69 map, it was assumed 1) that the map is circular, and 2) that the orientation of the well-mapped groups with respect to one another is as presented, even though the data do not rigorously establish either point. The most striking feature of the RB69 map is its general similarity to the map of T4, even though homologous genes cannot be identified in the two phages and the characterization of the RB69 mutant phenotypes is quite incomplete. Functional groupings of the RB69 mutants corresponding to the early and late regions of T4 are obvious, and even the distribution of mutant phenotypes within the late region of RB69 bears a considerable resemblance to that of T4. From the similarity of these distributions, it seems likely that the region of the RB69 map which includes mutants 74, 9, 42, and 6a is the tail fiber region of RB69. If so, then this region occupies a fraction of the total map comparable to that occupied by the tail fiber regions of T2 and T6, and consequently much smaller than that occupied by the tail fiber region of T4.

FIGURE 21. THE MAP OF RB69. Distances separating mutants have been computed using the T^4 mapping function, as described in the text. Mutant phenotypes are indicated in the boxes, and the symbols are according to Epstein, et al.(21); the dotted lines indicate somewhat speculative assignments, based primarily on data from the *in vitro* assembly system. As with T2, the sizes of the large gaps are necessarily somewhat uncertain.

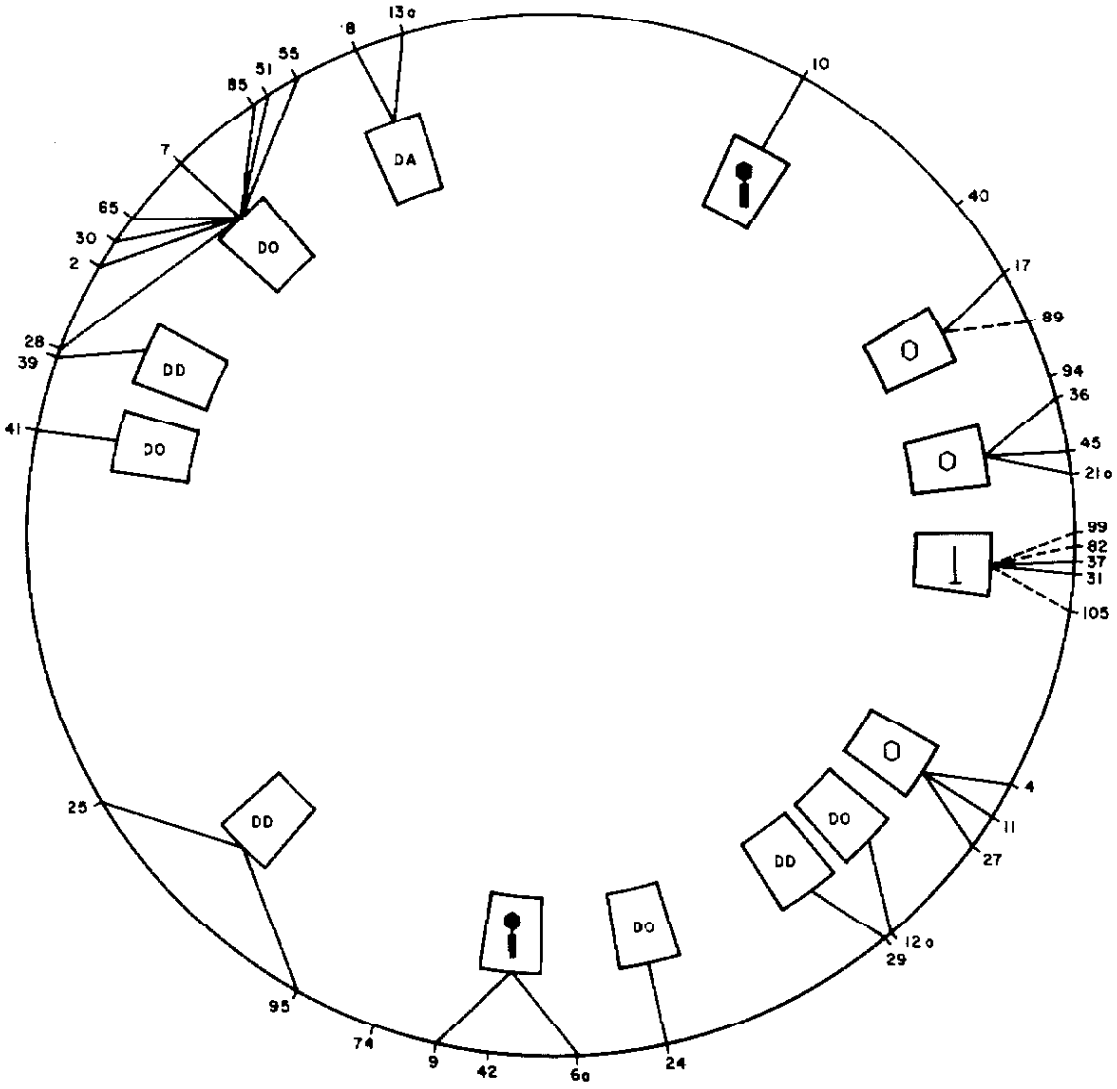
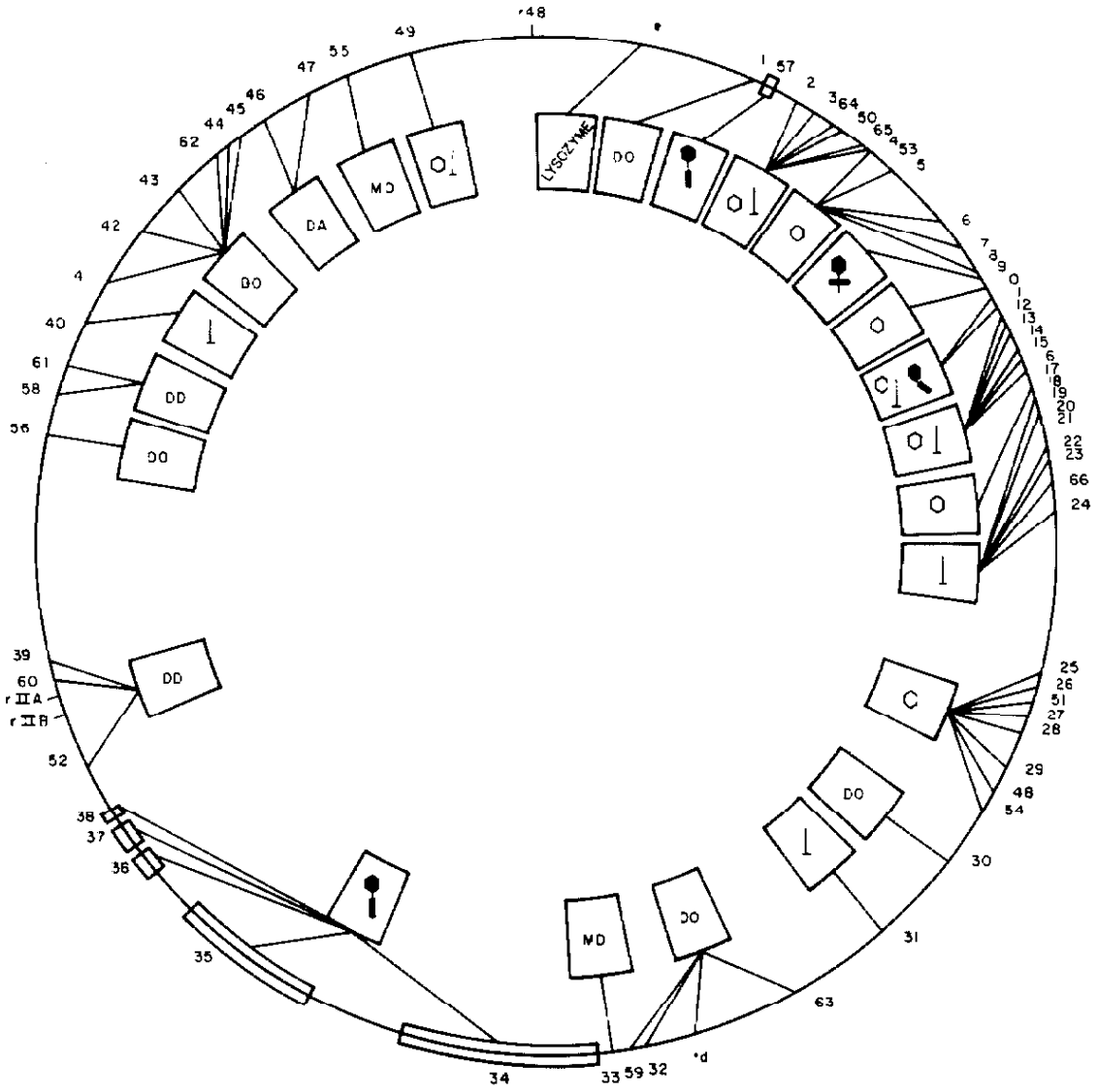

13a
8
55
51
85
7
65
30
2
28
39
41
25
95
74
9
42
6a
24
29
12a
27
11
4
105
99
82
37
31
21a
45
36
94
89
17
40
10
DA
DO
DD
DO
DD
DO
DD
DO
DO
DD
O
O
O
I

FIGURE 22. THE MAP OF T4. Distances separating genes have been computed using the same T4 mapping function as was used for the construction of the RB69 map in Figure 24. Mutant phenotypes are indicated in the boxes, and the symbols are as in Figure 21. All the recombination data and the data on the characterizations of mutant phenotypes have been collected in the laboratories of Drs. R. S. Edgar and R. H. Epstein, to whom I am indebted for permission to reproduce this map.


48
49
55
47
46
45
44
62
43
42
4
40
61
58
56
39
60
rIIA
rIIB
52
38
37
36
35
34
33
59
32
d
63
31
30
54
48
29
28
27
51
26
25
66
24
23
22
21
20
19
18
17
16
15
14
13
12
0
9
8
7
6
5
53
65
50
64
3
2
57
1
LYSOZYME
DO
MD
DA
DD
C

d. Summary

These genetic analyses of the T-even phages have confirmed the rather close relationship which was already known to exist between T2, T4, and T6, and they have also shown that the newly isolated phage RB69 bears a strong resemblance to these phages. The close relationship among the classical T-even phages has been substantiated by the success of intertype complementation spot tests, which shows that a large number of different T2-T4, T4-T6, and T2-T6 recombinant hybrids must be viable, by the identification in T2 and T6 of homologs for a considerable fraction of the genes already identified in T4, by the similarities in the properties of recombination in the three phages, and by the striking resemblance of the T2 map to that of T4. The resemblance between RB69 and the classical T-even phages has been shown by the appearance of the RB69 phage particle in the electron microscope, by various physical characteristics of the RB69 phage particle, by the parameters of the RB69 growth cycle, by the number of genes identified in RB69, by the similarity of RB69 mutant phenotypes to those of T4, by the properties of recombination in RB69, and by the T4-like distribution of mutant phenotypes around the RB69 map. Since intertype spot tests between RB69 mutants and those of T4 could not be performed, homologous relationships between RB69 genes and T4 genes could not be established.

3. Detailed Examination of the Tail Fiber Region

From the genetic characterizations of T2 and T6 presented above, it appears that these phages differ from T4 most markedly in the region of the genome which controls the synthesis of the phage tail fibers. Within this region, T2 and T6 differ from T4 in the sizes of genes 34 and 35, and in the compatibilities of the products of genes 37 and 38. Since differences between the phages in other regions were considerably less striking, the tail fiber region was selected for a closer study of the relationships among the phages.

a. Intertype Complementation Tests

The results of liquid culture complementation tests between mutants in the tail fiber genes of all three phages are presented in Table 8. In tests between mutants of the same phage, one striking feature can be noted; complementation between genes 34 and 35 and between genes 36 and 37 is consistently low. Stahl et al. have shown that complementation between genes 34 and 35 of T4 is low because of polarity effects on the expression of these genes (111), and they have discovered similar polarity effects for another pair of adjacent genes in T4 (genes 27 and 51). The lowered complementation seen here can also be explained by polarity effects, and the similarity of these effects in T2, T4 and T6 is further evidence for their close relationship.

In tests between different phages, several important features can be noted. From the tests between the wild types of different phages, it is

TABLE 8. INTERSPECIFIC COMPLEMENTATION AMONG TAIL FIBER MUTANTS. Each value in the table is the burst size of a mixed infection of strain B/5 with the two indicated phages. The burst sizes were determined by dividing the final yield by the number of input bacteria. Conditions were as described in Materials and Methods, with a multiplicity of at least five of each phage.

		T2						T4						T6			
		Wild Type	Amber In 34	Amber In 35	Amber In 36	Amber In 37	Amber In 38	Wild Type	Amber In 34	Amber In 35	Amber In 36	Amber In 37	Amber In 38	Wild Type	Amber In 34	Amber In 35	Amber In 37
T6	Amber In 37	11.4	9.8	10.6	7.3	0.0	8.4	9.5	0.5	0.9	0.2	0.0	0.1	91.8	47.0	68.5	0.0
	Amber In 35	26.2	2.6	0.1	28.8	30.0	45.6	1.0	0.1	0.0	0.1	0.1	0.1	56.6	15.9	0.0	
	Amber In 34	13.9	0.0	8.9	15.3	16.5	14.7	2.9	0.0	0.2	0.0	0.1	0.1	30.6	0.1		
	Wild Type	149	94	234	210	117	185	3.2	0.4	1.0	0.2	0.1	0.1	64.4			
T4	Amber In 38	19.2	19.8	17.1	4.6	0.4	0.2	183	141	129	105	118	0.1				
	Amber In 37	13.7	14.1	7.7	1.6	0.0	0.0	121	105	106	61.1	0.0					
	Amber In 36	13.7	11.9	10.6	0.0	11.7	5.8	175	121	124	0.0						
	Amber In 35	19.1	3.0	0.0	20.5	22.1	24.4	196	49.7	0.0							
	Amber In 34	8.1	0.1	6.1	3.4	7.5	7.1	135	0.0								
	Wild Type	100	74.0	142	122	121	132	146									
T2	Amber In 38	46.0	32.5	40.2	29.4	21.2	0.2										
	Amber In 37	13.6	14.3	21.5	4.3	0.0											
	Amber In 36	21.6	13.9	25.6	0.0												
	Amber In 35	43.0	3.5	0.0													
	Amber In 34	27.6	0.0														
	Wild Type	46.5															

clear that the T4-T6 mixed infection differs from the others in its extremely low burst size. This result was observed long ago in mixed infections between unrelated phages, by Delbrück (31), and has been called a "depressor effect". The T4-T6 depressor effect is discussed at greater length in Section B.3.a of the Results.

Tests between the wild type of one phage and mutants of another reveal evidence for the exclusion which is the subject of Section B of the Results. In T2-T4 infections, for example, mixed infections between T2 wild type and T4 mutants give much lower burst sizes than those between T4 wild type and T2 mutants. This asymmetry is very probably a result of the partial exclusion of T2 by T4. From similar asymmetries in T4-T6 and T2-T6 mixed infections, it would be predicted that T4 excludes T6 and that T6 excludes T2; both predictions are verified in Section B of the Results.

The complementation properties of gene 37 and gene 38 mutants provide an interesting example of specific gene product incompatibilities between T2, T4, and T6. The T2 and T6 mutants defective in these genes behaved exceptionally in spot tests; all of them failed to complement both gene 37 and gene 38 mutants of T4. The five T2 mutants, when tested against one another, fell into two complementation groups, and the three T6 mutants fell into a single group. Tests between T2 and T6 mutants revealed that the T6 group was homologous to one of the T2 groups. When T2 was mapped, mutants from the two groups mapped in adjacent positions between genes 36 and 52; on this basis one T2 group was given the status of T2 gene 37 and the other the status of T2 gene 38. From the T2-T6 comple-

mentation tests, the T6 group was then given the status of T6 gene 37. With these ascribed statuses, an interpretation can be made of the unusual complementation properties of these mutants, which are reflected both in the initial spot tests and in Table 8. (This interpretation was also made independently by Stahl and Murray (112).)

It is assumed that in T2, T4, and T6 the products of genes 37 and 38 normally combine to form a component of the tail fiber. In mixed infection of a restrictive host with a gene 37 amber mutant of one phage and a gene 38 amber mutant of another, the first phage produces its normal gene 38 product, but no gene 37 product, and the second phage produces its normal gene 37 product but no gene 38 product; thus the only gene 37 and gene 38 products produced are from different phages, and the ability of the cell to produce progeny phages with tail fibers depends on the compatibility of these two products. The results of Table 8 can be readily explained if the products of T2 and T6 are compatible but those of T2 and T4 are not. Whether the products of T4 and T6 are compatible cannot be decided from Table 8 because of the T4-T6 depressor effect; from the spot test behavior of the T6 mutants, however, it would appear that they are not.

This interpretation is supported by three separate pieces of evidence. First, Edgar and Lielausis (113) have shown that gene 37 controls an antigen which appears on the phage particle. Second, evidence presented below demonstrates that gene 38 determines the host range specificity of the phage particle, suggesting that the product of gene 38 is also incorporated into the phage particle. Third, in the pathway of tail

fiber assembly elucidated by the in vitro experiments of Edgar and Wood (110, 114), the interaction of the products of genes 37 and 38 is one of the initial steps. If the interpretation is correct, then in the evolutionary divergence of the T-even phages genes 37 and 38 have become a "co-adapted" pair of genes. (Other cases of co-adaptation have been found in the tail fiber region as well, but the degree of co-adaptation is generally less; in the complementation tests between tail fiber mutants of different phages, the progeny must have tail fibers of mixed origin, and the adsorption rates for these progeny are generally lower than for either parental phage.)

b. Localization of Host Range Determinants

It has been known for some time that T2, T4, and T6 can easily be distinguished by bacterial strains which are selectively resistant to each phage. The resistance of these strains resides in their inability to adsorb the resisted phage (89), and by implication the tail fibers, the organs of adsorption, must be sufficiently different in the three phages so that changes in the bacterial surface can prevent the attachment of one type of tail fiber while still allowing the attachment of the others. Streisinger (85) showed that these "host range" differences of T2 and T4 demonstrate phenotypic mixing, as would be expected of properties determined by the phage protein overcoat, and that particles of mixed host range phenotype occurred, as would be expected if the host range is determined by the tail fibers, of which each phage bears several. He failed to find any recombinants between the host range determinants

of T2 and T4, and on this basis he judged these determinants to be allelic.

Since genes 34-38 control the formation of the tail fibers, it was expected that the host range determinants would fall in this region of the map; in order to localize these determinants further, the distribution of host range genotypes among wild type progeny from crosses between T2 wild type and various T4 tail fiber mutants was determined. The wild type progeny from these crosses necessarily contain the T2 wild type allele of the T4 amber mutation, and the fraction of them which also have the T2 host range should reflect the linkage between this wild type allele and the host range determinants. The results are presented in Table 9; the host range determinants are clearly linked to each of the genes in the tail fiber region, most strongly to genes 37 and 38. Since these results suggested a position for the host range determinants near the gene 38 end of the region, wild type recombinant progeny from crosses between mutants of the three phages at this end of the region were characterized. These wild type progeny are necessarily recombinant within the tail fiber region, and their host ranges should reflect the relative linkage of the host range determinants to the two genes which carry mutations in the parental phages. The results are presented in Table 10; it is clear that the host range differences between the phages are governed by determinants which lie closer to genes 37 and 38 than to genes 35 and 36. In the one case which could be tested (T2-T4), the determinants appeared to lie closer to gene 38 than to gene 37.

It should be noted that these experiments only map the host range determinants; they cannot assign them to complementation groups. If the

TABLE 9

HOST RANGES OF T2-T4 PROGENY

CROSS	PERCENTAGE OF WILD TYPE PROGENY WITH T2 HOST RANGE GENOTYPE
T2 WILD TYPE x T4 WILD TYPE	10
T2 WILD TYPE x T4 AMBER IN 34	56
T2 WILD TYPE x T4 AMBER IN 35	84
T2 WILD TYPE x T4 AMBER IN 36	92
T2 WILD TYPE x T4 AMBER IN 37	97
T2 WILD TYPE x T4 AMBER IN 38	100

Progeny phages were plated on B/5 to select the wild types and host range genotypes were determined by stabs to plates seeded with B/2 and S/4. Occasional stabs growing well on both B/2 and S/4 were assumed to arise from heterozygotes, and were included in the table as having both T2 and T4 host ranges. 100 plaques were stabbed for each determination.

TABLE 10

HOST RANGES OF TAIL FIBER RECOMBINANTS

CROSS	PERCENTAGE OF WILD TYPE PROGENY WITH HOST RANGE GENOTYPE OF		
	T2	T4	T6
T2 AMBER IN 36 x T4 AMBER IN 37	95	5	
T2 AMBER IN 36 x T4 AMBER IN 38	99	1	
T2 AMBER IN 37 x T4 AMBER IN 36	2	98	
T2 AMBER IN 38 x T4 AMBER IN 36	0	100	
T2 AMBER IN 37 x T4 AMBER IN 38	100	0	
T2 AMBER IN 38 x T4 AMBER IN 37	0	100	
T2 AMBER IN 35 x T6 AMBER IN 37	100		0
T2 AMBER IN 36 x T6 AMBER IN 37	100		0
T2 AMBER IN 37 x T6 AMBER IN 35	2		98
T2 AMBER IN 38 x T6 AMBER IN 35	1		99
T4 AMBER IN 35 x T6 AMBER IN 37		100	0
T4 AMBER IN 36 x T6 AMBER IN 37		100	0
T4 AMBER IN 37 x T6 AMBER IN 35		0	100
T4 AMBER IN 38 x T6 AMBER IN 35		1	99

Progeny phages were plated on B/5 to select the wild type recombinants, and host range genotypes were determined by stabs to plates seeded with the appropriate indicator strains. Occasional stabs growing on both indicators were included in the table as having both host ranges. 100 plaques were stabbed for each determination. The wild type progeny of the crosses between T2 and T4 gene 37 and gene 38 mutants were quite rare, as might have been expected from the incompatibilities described above; consequently it is not certain that they represent the products of single exchange events between T2 and T4. Wild type progeny from crosses of gene 37 and gene 38 mutants of T2 and T6 or T4 and T6 grew so poorly that their host ranges could not be determined.

host range of the phage is determined by a known tail fiber gene, it is probably gene 38. However, if the host range is determined by an as yet unidentified gene, these results indicate only that this gene must lie quite close to gene 38. A distinction between these possibilities might be made by characterizing the host range phenotype of progeny phages resulting from complementation between tail fiber mutants of different phages. Only preliminary characterizations of this type have been made, but they indicate that the host range is probably determined by gene 38, and not some other, unidentified, gene.

In the light of the incompatibilities described above, it is interesting to note that the component of the tail fiber which is formed by the interactions of the products of genes 37 and 38 carries the host range specificity of the phage, and therefore probably interacts intimately with the bacterial surface.

c. The Sizes of Genes 34 and 35

Evidence has already been presented above for differences among the three classical T-even phages with respect to the genetic sizes of genes 34 and 35. These differences are examined more closely in Table 11; from the table it is clear that the maximum estimates of the lengths of these genes in T2 and T6 are considerably lower than the minimum estimates of their lengths in T4.

The phages might differ either in the physical lengths of these genes or in the frequencies of recombination per nucleotide pair within them. A difference in physical lengths would imply a difference in the

TABLE 11

THE LENGTHS OF GENES 34 AND 35 IN T2, T4 AND T6

GENE	ESTIMATE	PHAGE	PERCENTAGE OF TOTAL MAP LENGTH	T2/T4	T6/T4	T2 MAX / T4 MIN	T6 MAX / T4 MIN
34	MAXIMUM	T2	3.4	0.34	0.46	0.52	0.71
		T4	9.9				
		T6	4.6				
	MINIMUM	T2	3.0	0.46	0.12		
		T4	6.5				
		T6	0.8				
35	MAXIMUM	T2	1.9	0.21	0.24	0.40	0.47
		T4	9.2				
		T6	2.2				
	MINIMUM	T2	---*	---*	---**		
		T4	4.7				
		T6	---**				

*Only one amber mutant has been isolated in gene 35 of T2, and thus a minimum estimate is meaningless.

**Three amber mutants have been isolated in gene 35 of T6, but these have not been crossed with one another to determine a minimum size for gene 35 in T6.

Maximum estimates were obtained by taking the two outside markers closest to the two ends of a gene but lying outside the gene, measuring the frequency of recombination between them, and converting this frequency to a fraction of the total map length with the mapping functions described in the text, corrected so as to give circular additivity. Minimum estimates were obtained by taking the two most separated markers within a gene, measuring the recombination frequency between them, and converting to a fractional distance in similar fashion.

size of the gene products as well; the compatibility of the gene 34 and gene 35 products of one phage with the products of other tail fiber genes from other phages makes this interpretation unlikely, but does not rule it out. In an attempt to obtain more definitive evidence, the internal structures of these genes were examined in T2 and T4, where the most mutants were available. Crosses were performed between a series of five gene 34 mutants of T4, and six gene 34 mutants of T2, and the results are presented in Table 12. It is clear that gene 34 intragenic recombinants are formed in crosses between the phages almost as frequently as in crosses between mutants of the same phage, suggesting a considerable similarity between the two phages with respect to the internal structure of gene 34. Furthermore, the T2 mutants can be roughly ordered in the T4 map (T2 amber 74 appears to be closest to T4 amber B258, T2 amber 75 to T4 amber A455, etc.), and this ordering is consistent with their independently determined order. Since the T2-T4 host range difference had been localized to gene 38, this ordering could be checked by treating each of the T2-T4 crosses in Table 12 as a three factor cross, with the host range difference as a third factor. The results, presented in Table 13, confirm the rough ordering and thereby provide further evidence for similarities between the two phages with respect to the internal structure of gene 34. Dramatic evidence of the difference in recombination frequencies between T2 and T4 is furnished by the T4 mutants amber B258 and amber N58, and the T2 mutants amber 75 and amber 73; the T4 mutants give 23.9% recombination, but they fall within the interval bounded by the T2 mutants, which give 5.4% recombination.

TABLE 12

T2-T4 CROSSES WITHIN GENE 34

		T2						T4				
		AMBER 12	AMBER 73	AMBER 135	AMBER 79	AMBER 74	AMBER 75	AMBER B25	AMBER B265	AMBER N58	AMBER B258	AMBER A455
T4	AMBER A455	11.7	5.3	4.5	3.2	5.7	1.3	30.9	29.5	28.4	17.4	
	AMBER B258	9.7	2.2	3.9	1.3	1.1	2.9	32.3	26.3	23.9		
	AMBER N58	8.7	0.4	0.2	0.9	3.1	8.4	18.5	13.4			
	AMBER B265	8.3	5.8	5.2	4.0	6.2	12.7	10.4				
	AMBER B25	4.4	5.3	5.0	3.9	6.4	13.0					
T2	AMBER 75	24.8	5.4	6.6	2.1	1.3						
	AMBER 74	21.0	5.5	6.2	2.9							
	AMBER 79	15.1	1.4	1.3								
	AMBER 135	20.7	0.8									
	AMBER 73	16.8										
	AMBER 12											

Each value in the table is a recombination percentage calculated by doubling the percentage of wild types among the progeny of a cross between the indicated mutants. A correction for differences in plating efficiency is applied as described in Materials and Methods.

TABLE 13

ORDERING OF T2 AND T4 MUTANTS IN GENE 34

		T2					
		AMBER 12	AMBER 73	AMBER 135	AMBER 79	AMBER 74	AMBER 75
T4	AMBER A455	47	53	40	43	37	43
	AMBER B258	37	37	30	32	33	9
	AMBER N58	28	23	10	9	6	3
	AMBER B265	23	16	17	11	3	11
	AMBER B25	26	9	5	4	10	9

Each value in the table is the percentage of wild type recombinants having the T2 host range genotype in a cross between the two indicated mutants. Since progeny of a T2-T4 cross selected to contain the gene 34 of T2 are only 56% T2 in host range, this is the maximum figure expected in the table. Somewhat arbitrarily, values larger than 20% were taken to indicate one ordering of the mutants, while values smaller than 20% were taken to indicate the other. This method gave no inconsistencies in ordering, and the position of each T2 mutant in the series of T4 mutants is indicated by a dot.

All of the above data argue for a considerable similarity between T2 and T4 in the internal structure of gene 34. Consequently, it seems likely that the two phages differ in factors which affect the frequency of recombination per nucleotide pair in this region. Whether the sizes of genes 34 and 35 are genetically overestimated in T4 or genetically underestimated in T2 and T6 cannot be decided directly from these data, but two recent pieces of evidence point toward the former possibility.

First, Mosig has recently undertaken to construct a physical map for T4, taking advantage of the discovery (115, 116) of a class of particles of T4 which contain only two-thirds the normal amount of DNA. Since this DNA is apparently a circularly permuted fraction of the total genome, the probability of the joint occurrence of two genes in the same two-thirds phage can be used as a measure of the physical distance between them. In the physical map which Mosig has constructed by this method, genes 34 and 35 are much smaller than in the genetic map, whereas other intervals retain the size which they have in the genetic map (117).

Second, Womack has recently measured the probability of rescuing different markers from the same ultraviolet-inactivated stock of T4 (118). Since markers are almost centainly rescued from such a stock by recombination away from lethal ultraviolet-induced damages, and since such damages are probably distributed randomly over the genome, a high probability of rescue for a given marker might be interpreted as signifying an intrinsically higher probability of recombination per nucleotide pair in the area of that marker. Womack found that markers in genes 34 and 35 showed such high probabilities of rescue, whereas markers in most other regions

of the map did not (118).

Both pieces of evidence suggest that the sizes of genes 34 and 35 are genetically overestimated in T4, and that their relative genetic sizes in T2 and T6 more closely approximate the true physical sizes. It seems that the characterization of the basis for this difference between the phages might prove of value for an understanding of recombination mechanisms in phage.

d. Intertype Crosses

The crosses between gene 34 mutants of T2 and T4 demonstrated considerable homology between the two phages in this region; an attempt was made to survey the degree of homology between them in the rest of the tail fiber region by performing crosses between mutants defective in other tail fiber genes. The results from these crosses are presented in Table 14; in the crosses involving T2 and T4 mutants, the recombination percentages decrease as the mutants approach the gene 38 end of the region, suggesting that homology decreases in this direction as well. A similar decrease, although less pronounced, occurs in the recombination percentages for crosses between T4 mutants; the normalizations presented in Table 15 show that it does not account for the decrease in T2-T4 recombination percentages.

The apparent progressive loss of homology can be taken at face value, or it can be interpreted as the result of a spreading effect generated by one major inhomology in genes 37 and 38. What little evidence there is for evaluating the second interpretation suggests that it requires a

TABLE 14

T2-T4 CROSSES

		T2					T4				
		AMBER IN 34	AMBER IN 35	AMBER IN 36	AMBER IN 37	AMBER IN 38	AMBER IN 34	AMBER IN 35	AMBER IN 36	AMBER IN 37	AMBER IN 38
T4	AMBER IN 38	4.6	1.8	0.5	0.1	0.0	35.1	20.7	14.8	10.0	
	AMBER IN 37	5.6	1.1	0.3	0.0	0.3	29.1	17.9	10.4		
	AMBER IN 36	4.7	1.0	0.0	0.3	0.8	34.8	14.3			
	AMBER IN 35	5.8	0.0	3.1	2.8	3.6	33.8				
	AMBER IN 34	3.1	7.0	8.9	6.6	10.9					
T2	AMBER IN 38	39.0	20.7	25.6	17.8						
	AMBER IN 37	22.2	11.0	9.6							
	AMBER IN 36	13.7	7.5								
	AMBER IN 35	12.1									
	AMBER IN 34										

Each value in the table is the recombination percentage observed in a cross between the two indicated mutants. Corrections for plating efficiency differences were applied as described in Materials and Methods.

TABLE 15

NORMALIZATIONS OF T2-T4 RECOMBINATION PERCENTAGES

		NORMALIZED RELATIVE TO T2-T2 DATA					NORMALIZED RELATIVE TO T4-T4 DATA				
		T2					T4				
		AMBER IN 34	AMBER IN 35	AMBER IN 36	AMBER IN 37	AMBER IN 38	AMBER IN 34	AMBER IN 35	AMBER IN 36	AMBER IN 37	AMBER IN 38
T4	AMBER IN 38	.118	.087	.020	.006		.131	.087	.034	.010	
	AMBER IN 37	.252	.100	.031		.017	.192	.061	.029		.030
	AMBER IN 36	.343	.133		.031	.031	.135	.070		.021	.054
	AMBER IN 35	.480		.413	.255	.174	.172		.217	.156	.174
	AMBER IN 34		.579	.650	.297	.280		.207	.256	.227	.311

Each number in the table is the quotient obtained when the appropriate T2-T4 recombination percentage is divided by the corresponding T2-T2 or T4-T4 recombination percentage. All the recombination percentages used have been presented in Table 14.

stronger spreading effect than is normally seen in T4 crosses (119).

e. Summary

The detailed examination of the tail fiber regions of the T-even phages has led to the following conclusions; 1) the similar polarity effects within this region are further evidence for the close relationship of these phages to one another, 2) genes 37 and 38 provide a unique example of a pair of co-adapted genes in the T-even phages, 3) the host range differences between the phages are determined by gene 38, and hence its product interacts most intimately with the bacterial surface, 4) the phages differ with respect to factors which influence the probability of recombination per nucleotide pair in the region of genes 34 and 35, and 5) T2 and T4 show a progressive loss of homology proceeding from gene 34 to gene 38.

B. Studies of Mixed Infections Between Some T-even Phages

1. Introduction

The first systematic studies of mixed infections between the T-even phages were reported in 1946 by Delbrück and Bailey(24), who noted that a characteristic fraction of the mixedly infected cells yielded both types of phages, depending on the pair of infecting phages; for the pairs T2-T4 and T2-T6, the fraction of mixed yielders was 80-90%, whereas for the pair T4-T6, it was only 10-20%. In the same study, Delbrück and Bailey provided the first evidence for phenotypic mixing and genetic exchange between different members of the T-even species.

In 1956, Streisinger (32, 85, 120) found that mixed infections between T2 and T4 were characterized by an "unequal yield effect"; the markers of progeny phages were derived more often from the T4 parent than from the T2 parent. This observation was followed up by Streisinger and Weigle (33), who revealed that ~~different properties~~ of T2 were transmitted with different efficiencies to the progeny of the mixed infection; while host range and rII markers from the T2 parent appeared in about 13% of the progeny, other T2 properties, such as the inability to plate on certain K strains of E. coli, the susceptibility to exclusion by T4, and the glucosylation pattern of the HMC residues in the DNA, appeared in less than 1%. In 1966, de Groot used special methods to isolate a T2-T4 hybrid which was not excluded by T4 and did not exclude T2 (121). This hybrid he described as having the "exclusion resistance" of T4 but the "excluding ability" of T2, and by crosses between it and T4 he showed linkage between the excluding ability of T4 and its rII and host range markers (122, 123).

In this section of the Results, conditional lethal mutants are used to extend and clarify these observations. The T2-T4 mixed infection is examined first in detail, in the hope of understanding the mechanism of T2-T4 partial exclusion. Other mixed infections are then examined in the hope of revealing other stages in the evolution of exclusion.

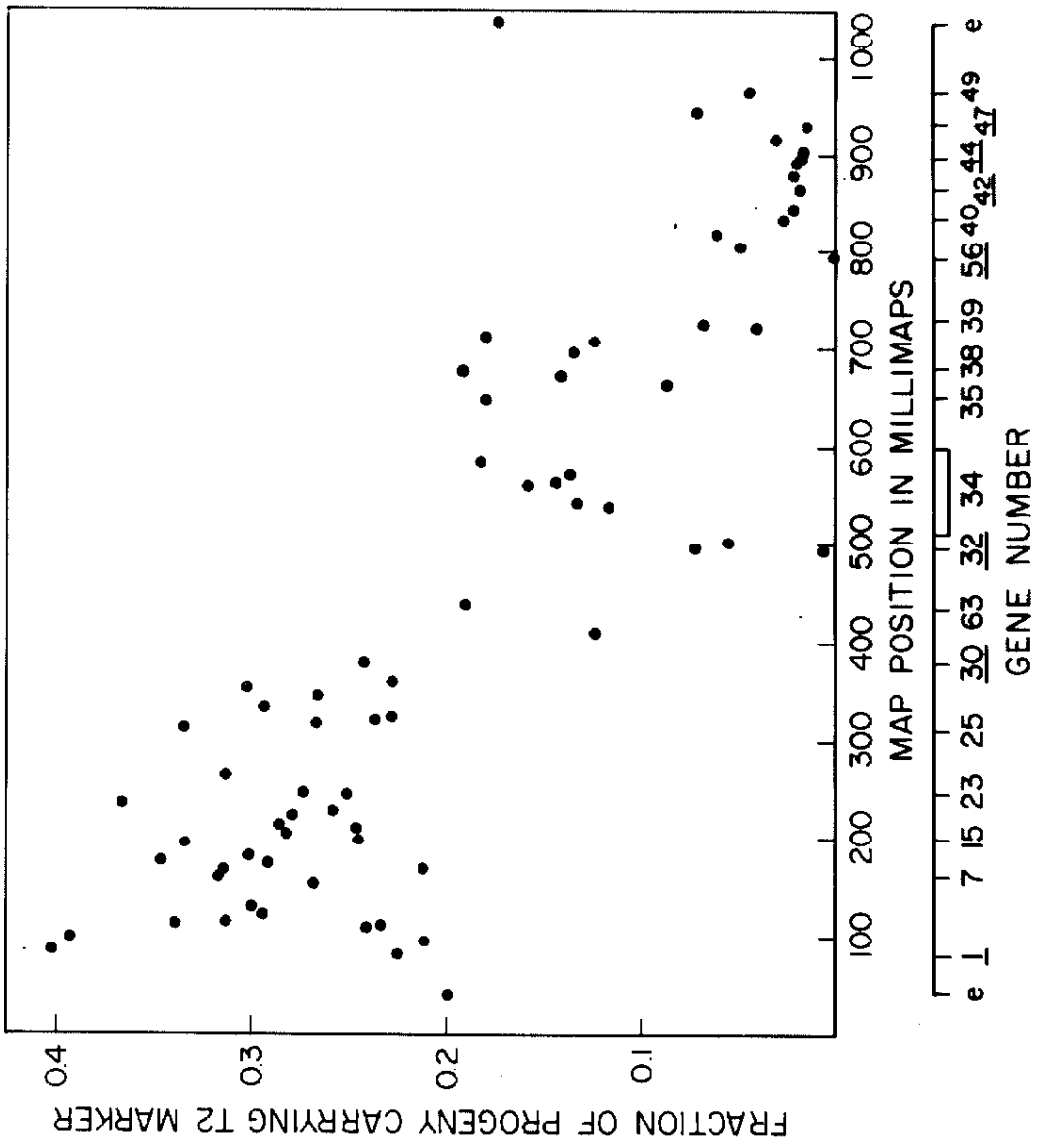
2. Mixed Infections Between T2 and T4

a. Preferential Exclusion of Two Regions of the T2 Genome

The efficiencies with which a large number of different T2 markers are transmitted to the progeny of T2-T4 mixed infections were determined by "crosses" between various T4 amber mutants and T2 wild type. The fraction of wild types (non-amber mutants) was measured before infection, to ensure that approximately equal input of the two parents had been achieved, and among the progeny, to determine the efficiency with which the T2 wild type allele of the T4 amber mutation was transmitted to the progeny. As expected from the observations described above, the fraction of wild types among the progeny was always less than among the parents and varied considerably from cross to cross.

As Figure 23 shows, the data exhibit quite a bit of scatter; nevertheless T2 markers from two regions of the map (one around gene 32, the other from gene 39 to gene 49) appear much less frequently among the progeny than those from other regions. These "preferentially excluded" regions contain between them most of the early genes and very few late genes, but their preferential exclusion cannot be explained by this fact

FIGURE 23. EFFICIENCY OF TRANSMISSION OF VARIOUS T2 MARKERS TO THE PROGENY OF T2-T4 MIXED INFECTIONS. The conditions used are those employed for crosses, as described in Materials and Methods, with CR63 as the host. A mixture of anti-T2 and anti-T4 antisera was used to eliminate unadsorbed phages. Crosses in which the parental input ratio was greater than 3:2 or less than 2:3, and crosses with a T4 multiplicity of less than 4 were not used in constructing the figure. At least one mutant defective in each identified gene of T4 was crossed with T2 wild type, and each mutant is represented by a point in the figure. Each point is the average of from 1 to 13 determinations; the average number of determinations per point is 3.1. The abscissa is constructed by cutting the T4 map at the top (at mutant r 48) and opening it out into a line so that the clockwise direction is from left to right. The map position is reckoned in thousandths of the total map (millimaps), proceeding clockwise from r 48. The numbers of early genes are underlined. The values for the ordinate of each point are corrected for slight inequalities in the input of the relevant crosses.


FRACTION OF PROGENY CARRYING T2 MARKER
0.4
0.3
0.2
0.1
100 200 300 400 500 600 700 800 900 1000
MAP POSITION IN MILLIMAPS
e 1 7 15 23 25 30 63 32 34 35 38 39 56 40 42 44 47 49 e
GENE NUMBER

alone; the early genes 1 and 30 which lie outside these regions are not preferentially excluded, and the late gene 40 which lies within one of the regions is.

b. The Recombinant Nature of Progeny Carrying T2 Markers

Since most T2-T4 progeny carry the T4 alleles of T2 markers from the preferentially excluded regions, those which carry T2 markers from other regions should be mostly T2-T4 recombinants. This implication was checked by measuring the fraction of such progeny at various times in the latent period; since the frequency of recombinants increases during the latent period (124), this fraction should also rise. The results presented in Figure 24 show that this is the case.

Further experiments indicated that even those progeny carrying T2 markers from the preferentially excluded regions are mostly T2-T4 recombinants. Another study (125) had indicated that the frequency of recombination between closely linked mutants in T4 could be greatly increased by a judiciously applied period of interruption in DNA synthesis. A T2-T4 cross was performed in which the T4 parent carried an amber mutation in an early gene and both parents carried temperature sensitive mutations in gene 42; since gene 42 controls the dCMP hydroxymethylase, DNA synthesis in this cross could be interrupted at will by raising the temperature, and the fraction of progeny carrying a T2 marker from a preferentially excluded region could be measured as in Figure 23. The effects of a series of high temperature pulses are shown in Figure 25, together with the effects of a similar series of pulses on recombination

FIGURE 24. THE FRACTION OF PROGENY CARRYING GIVEN T2 MARKERS AS A FUNCTION OF TIME IN THE LATENT PERIOD. Cross conditions were used as described in Materials and Methods, and each experiment was a mixed infection between T2 wild type and the indicated mutant of T4. After dilution, samples were withdrawn at the indicated times and chloroformed; the fraction of wild types in each of these samples was determined. The results of the cross of am 269 with T2 wild type may not be representative of the kinetics of the mixedly infected cells, since some of the wild type progeny in this case come from cells infected with T2 alone.

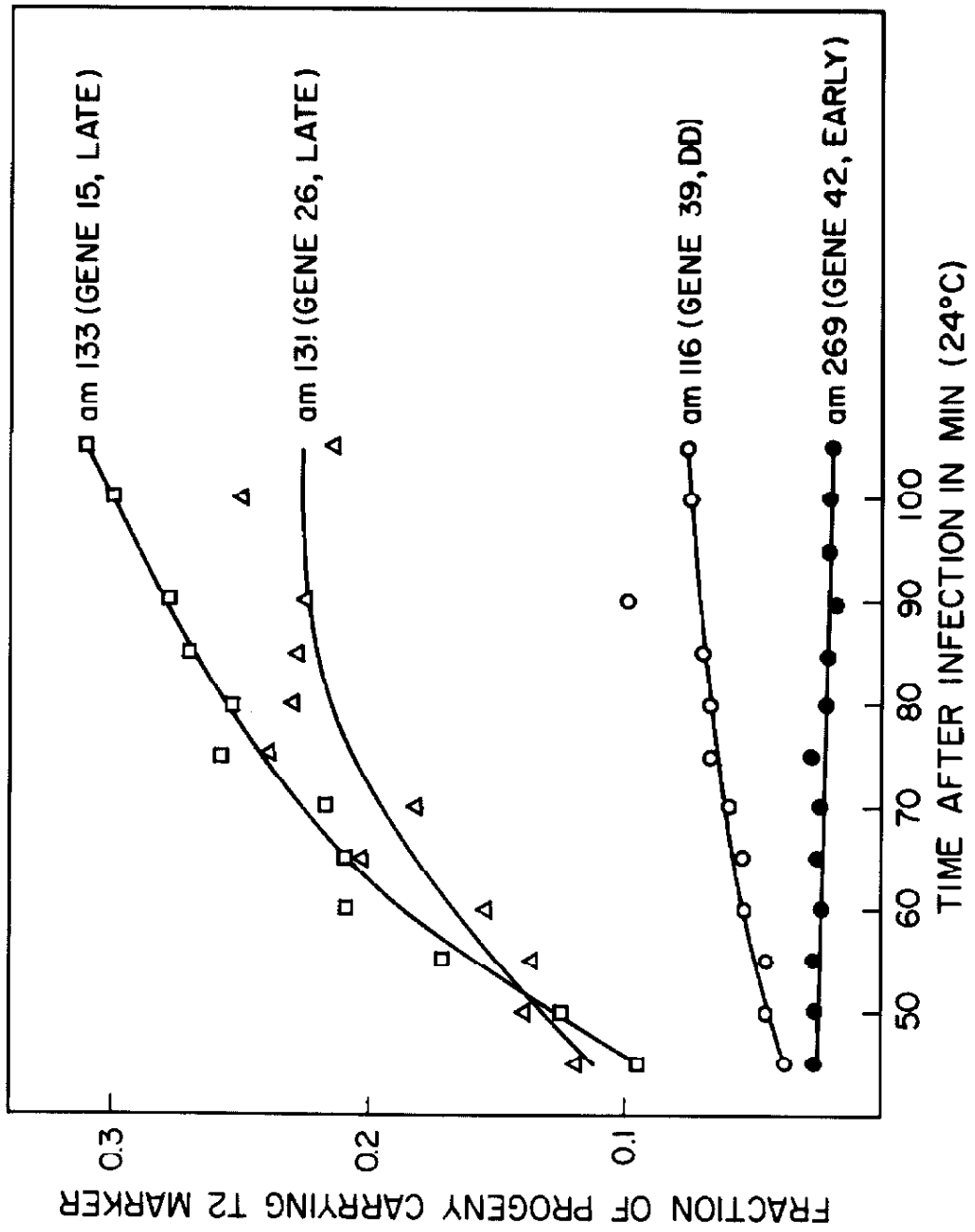
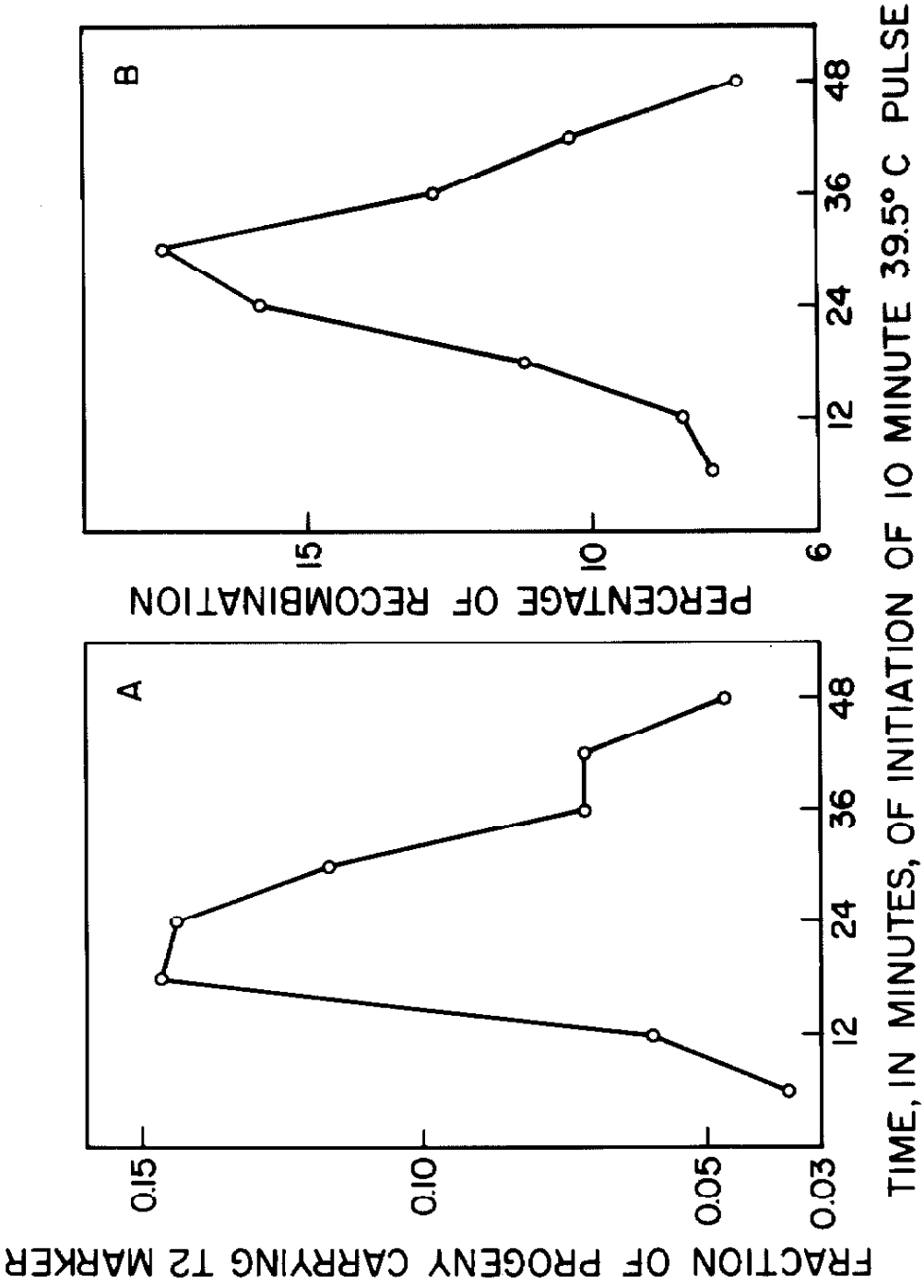

am 133 (GENE 15, LATE)
am 131 (GENE 26, LATE)
am 116 (GENE 39, DD)
am 269 (GENE 42, EARLY)
0.3
0.2
0.1
50
60
70
80
90
100
TIME AFTER INFECTION IN MIN (24°C)
FRACTION OF PROGENY CARRYING T2 MARKER

FIGURE 25. THE EFFECTS OF INTERRUPTING DNA SYNTHESIS ON THE FRACTION OF PROGENY CARRYING A T2 EARLY MARKER AND ON T4 RECOMBINATION. A. The experiment was a mixed infection between the T4 double mutant am 82 ts LB-1 and T2 ts 62; am 82 is defective in gene 44, an early gene, and T4 ts LB-1 and T2 ts 62 are both defective in gene 42, an early gene, which controls the production of the dCMP hydroxymethylase. The mixedly infected cells were incubated at 25°C. and at 6 minute intervals samples were shifted to 39.5°C. for 10 minutes and then returned to 25°C. When the cells had lysed, the fraction of progeny carrying the T2 am^+ marker was determined. B. The experiment was a cross between two T4 double mutants, T4 ts LB-3 rIIa41 and T4 ts LB-3 rIIb45. The mutant tsLB-3 is defective in gene 42; the two rII mutants are defective in the A and B cistrons, respectively, and normally give about 6.3% recombination. The mixedly infected cells were treated exactly as in part A, and the frequency of recombination between the rII mutants was measured in each sample. The high temperature pulses are intended to interrupt DNA synthesis by preventing the temperature-sensitive dCMP hydroxymethylases from acting; how effectively they do so has not been determined.


A
B
0.15
0.10
0.05
0.03
15
10
6
12
24
36
48
FRACTION OF PROGENY CARRYING T2 MARKER
PERCENTAGE OF RECOMBINATION
TIME, IN MINUTES, OF INITIATION OF 10 MINUTE 39.5°C PULSE

frequencies in T4, for comparison. The similarity indicates that progeny carrying a T2 marker from the preferentially excluded region are mostly recombinants.

c. The Absence of Progeny with T2 Exclusion Sensitivity

The recombinant nature of progeny carrying T2 markers can be accounted for by assuming localized exclusion sensitivity determinants in the T2 genome which, because of some action exerted against them by T4, are transmitted very infrequently to the progeny. Progeny phages carrying a given T2 marker would then necessarily arise from recombination events between that marker and the determinants, and determinants at several locations between genes 39 and 49 and one or more locations in the vicinity of gene 32 could account for the preferential exclusion of T2 markers in these regions.

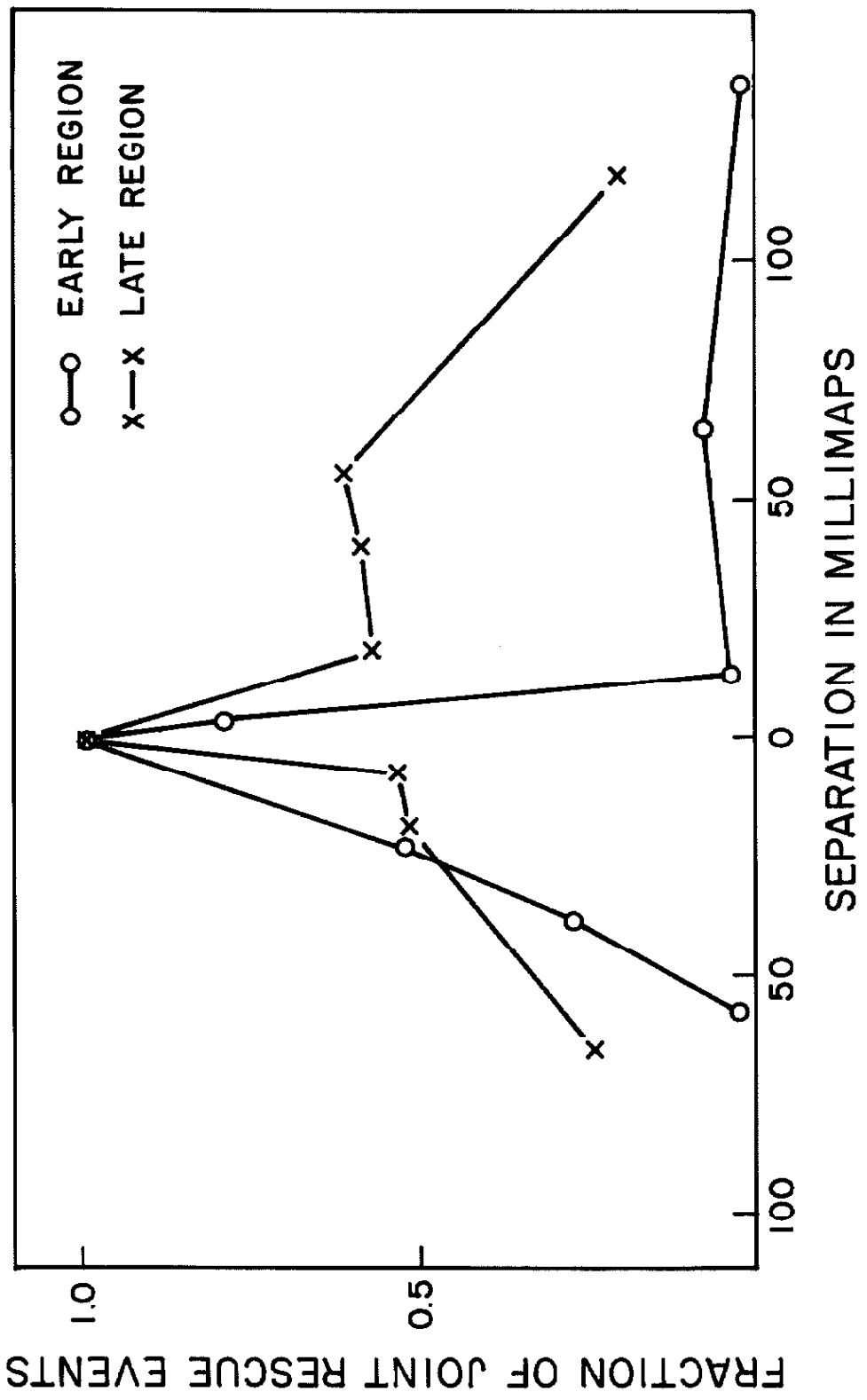
Any rare progeny phages carrying the proposed determinants should be sensitive to exclusion by T4, and a search for them was conducted among progeny selected to carry T2 markers from the preferentially excluded regions. 48 T2-T4 progeny, 24 containing T2 markers from the region between genes 39 and 49 and 24 containing T2 markers from the region near gene 32, were tested in mixed infections with T4 mutants. Each progeny phage was tested with 5 different T4 mutants, and in no case was there any evidence that the progeny phages were excluded by T4; if progeny phages carrying the determinants exist, they must be quite infrequent.

A more intensive search for these progeny was conducted using a spot test developed for the purpose. This spot test, which depends on

the preferential exclusion of certain regions of the T2 genome, was designed to differentiate between phages with T2 and T4 exclusion properties, and clearly differentiates between T2 and T4 themselves. From a T2-T4 cross 300 progeny phages, selected to contain T2 markers from a preferentially excluded region, were spot tested; all of them had T4 exclusion properties. This result suggests that virtually none of the progeny carries the proposed determinants.

Since the determinants do not appear among the progeny, linkage between T2 markers on either side of them should disappear, and this affords a way of determining their location more precisely. Crosses were performed between T2 wild type and a number of T4 double mutants, each carrying an amber mutation in gene 44 and a temperature sensitive mutation in a nearby gene. For each cross, the fraction of the progeny carrying the T2 wild type allele of the T4 amber mutation and the fraction carrying the T2 wild type allele of the T4 temperature sensitive mutation were determined, and the fraction expected to carry both T2 alleles by chance was calculated. The fraction actually carrying both T2 alleles was compared to this calculated fraction, and any excess was taken as evidence of linkage between the two alleles. A similar series of crosses was performed with T4 double mutants of the late region, for comparison. The results are presented in Figure 26; the loss of linkage which occurs between genes 45 and 46 in the early region suggests the presence there of a determinant of the proposed type, and a similar loss of linkage between genes 41 and 42, although less pronounced, suggests the location of another determinant. No determinants were detected in the late region.

FIGURE 26. LINKAGE OF T2 EARLY AND LATE MARKERS IN T2-T4 MIXED INFECTIONS. Crosses were performed as described in the text between T2 wild type and two series of T4 double mutants, the members of one series carrying am 82 (gene 44) and ts mutations in neighboring genes, the members of the other series carrying am B20 (gene 14) and ts mutations in neighboring genes. The ordinate of each point is the fraction of am^+ progeny which are also ts^+, corrected for the fraction which would be expected to be ts^+ by chance alone. It is thus a measure of the linkage between the am^+ and ts^+ markers of the T2 parent. The abscissa of each point is the map position of the ts mutant used, relative to the am mutant used. The results from crosses involving T4 double mutants in the early and late regions plotted separately. For the late region, the ts mutations used were (from left to right) in genes 5, 10, 12, 19, 23, 24, and 51. For the early region, the ts mutants used were (from left to right) in genes 41, 42, 43, 45, 46, 49 and e. The loss of linkage which occurs between genes 45 and 46 in the early region is indicative of the existence there of a determinant of the postulated type. A possible second determinant is located between genes 41 and 42. No determinants are detected in the late region.


EARLY REGION
LATE REGION
FRACTION OF JOINT RESCUE EVENTS
1.0
0.5
100
50
0
50
100
SEPARATION IN MILLIMAPS

The picture which emerges from these studies may be briefly summarized as follows. In mixed infections between T2 and T4, T4 acts on determinants in the T2 genome, and as a result these determinants appear in very few, if any, progeny. These determinants are inferred to be at several positions in the early region of T2, including one between genes 45 and 46 and another between genes 41 and 42, and at one or more positions in the vicinity of gene 32. Markers from the T2 parent appear among the progeny by virtue of recombination events which separate them from these determinants.

d. The Limitation of T2 DNA Replication by T4

At least four possible mechanisms for the exclusion of the T2 determinants from the progeny can be suggested; DNA containing these determinants might be degraded, it might be prevented from replicating, it might not be wrapped up in progeny heads, or it might render progeny phage which contain it inviable. If it is degraded, the degradation must be localized to the vicinity of the determinants; extensive degradation of this type might be expected to destroy the functional capacity of T2 genes near the determinants, but not that of genes far away. The results of experiments to determine these functional capacities are presented in Tables 16 and 17, and they suggest that T2 DNA in the vicinity of the determinants is not rapidly and extensively degraded.

If T2 DNA is prevented from replicating, experiments with density-labelled T2 phages might reveal this block. Because of the high frequencies of recombination between T2 and T4 and because the details of T-even

TABLE 16

FUNCTIONAL CAPACITY OF T2 GENES NEAR DETERMINANTS

GENE CONTAINING T4 AMBER MUTATION	BURST SIZE AS PERCENTAGE OF T2++ x T4++ CONTROL
63	125
32	162
59	149
33	105
52	70
rIIA	70
60	113
39	99
56	192
58	140
61	91
40	72
41	51
42	80
43	165
62	106
44	150
45	97
46	25
47	23
Average	104

Crosses were performed between T2 wild type and the indicated T4 amber mutants in the restrictive host B/5. The burst sizes were computed and compared to those of simultaneously performed T2 wild type by T4 wild type controls. These burst sizes are measures of the extent to which the excluded T2 can provide the functions which the T4 mutants lack, and thus they measure the functional capacities of the corresponding T2 gene.

TABLE 17

FUNCTIONAL CAPACITY OF T2 GENES FAR FROM DETERMINANTS

GENE CONTAINING T4 AMBER MUTATION	BURST SIZE AS PERCENTAGE OF T2++ x T4++ CONTROL
e	71
1	46
57	133
2	20
3	159
64	116
50	38
65	186
4	49
53	15
5	36
6	31
7	6
8	72
9	96
10	53
11	41
12	32
13	66
14	116
15	82
16	42
17	41
18	77
19	70
20	17
21	20
22	24
23	18
24	20
25	81
26	34
51	119
27	61
28	123
29	39
30	75
48	44
54	40
31	35

TABLE 17 (Continued)

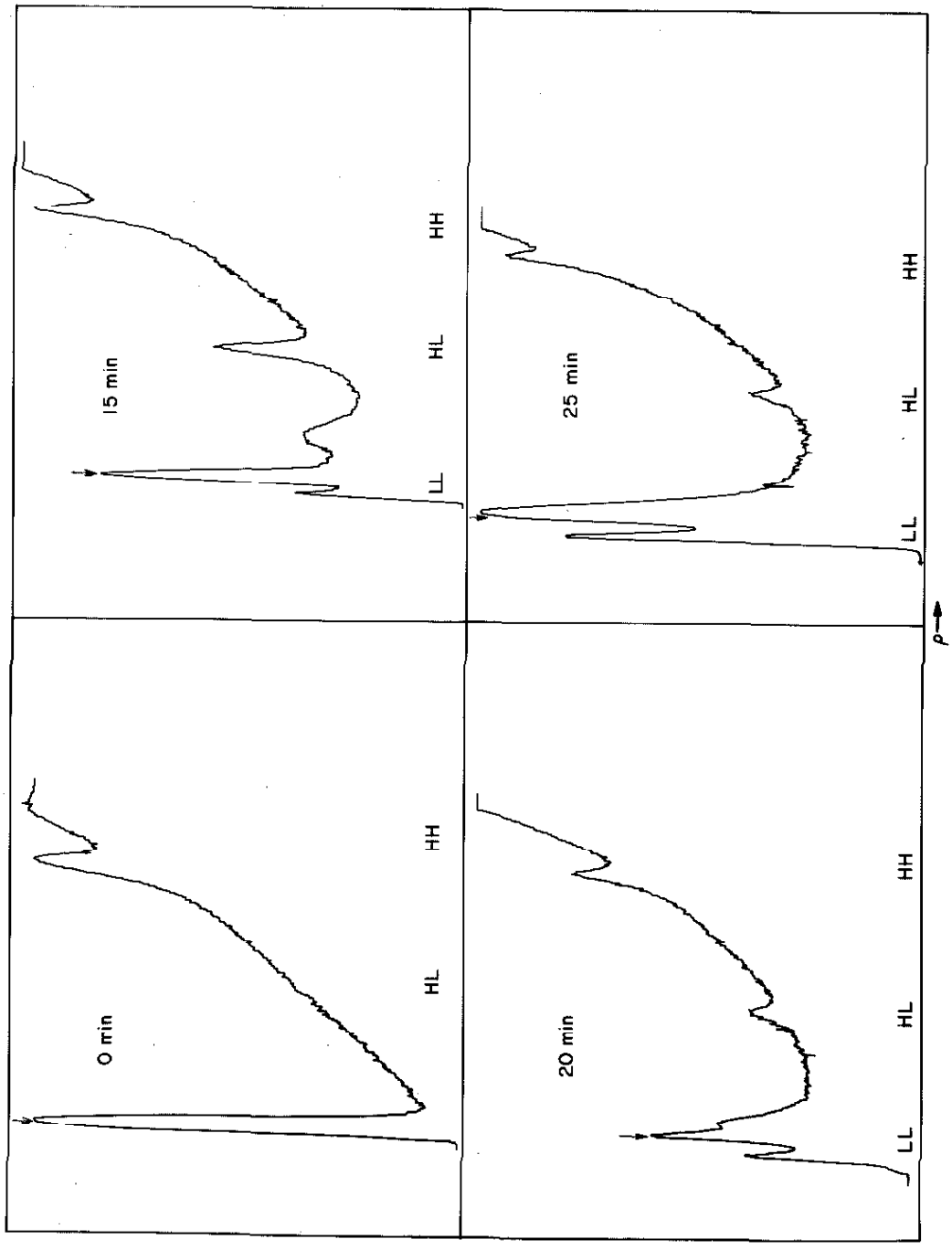
GENE CONTAINING T_4 AMBER MUTATION	BURST SIZE AS PERCENTAGE OF T2++ x T_4++ CONTROL
34	17
35	6
36	16
37	7
38	25
Average	56

Crosses were performed between T2 wild type and the indicated T_4 amber mutants in the restrictive host B/5. The burst sizes were computed and compared to those of simultaneously performed T2 wild type by T_4 wild type controls. These burst sizes are measures of the extent to which the excluded T2 can provide the functions which the T_4 mutants lack, and thus they measure the functional capacities of the corresponding T2 gene.

phage DNA replication are not known, however, it is difficult to make detailed predictions about the results of such experiments. Accordingly, a preliminary experiment was done to characterize the replication of density-labelled T2 DNA. 5-BU-labelled T2 was prepared as described in Materials and Methods and used to infect strain BB; the intracellular DNA was extracted at various times after infection and examined by density gradient equilibrium centrifugation. The results are presented in Figure 27; the large relatively light peak is assumed to be undegraded host DNA, and four phage DNA species can be detected. The parental (HH) DNA is present from the beginning of the infection and, interestingly, some of it still remains as late as 25 minutes after infection. Material of hybrid density (HL) appears as early as 10 minutes after infection (not shown) and some of it also persists late in infection. Light material (LL) is first detected at 15 minutes after infection and accumulates at later times. In addition, a fourth species can easily be seen in the 15 and 20 minute tracings and can be inferred in the 25 minute tracing; this material has a density intermediate between hybrid and light, and it becomes lighter with time. Its properties are those expected of the replicating form of T-even DNA proposed by Frankel (125), providing only that this replicating form contains parental DNA. The other two non-parental species have the densities expected of the products of semi-conservative replication.

It might have been predicted that if T4 prevents replication of T2 DNA, mixed infection between T4 and 5-BU-labelled T2 would lead to a persistent peak of unreplicated (HH) T2 parental DNA; this experiment

FIGURE 27. REPLICATION OF 5-BU-LABELLED T2 DNA. Each tracing is of a photograph from a density gradient equilibrium centrifugation of DNA extracted at the indicated time from cells infected with 5-BU-labelled T2 phage. The infection was carried out at 30°C., with strain BB as host. DNA was extracted by the gentle phenol procedure described in Materials and Methods, and presumably is of quite high molecular weight. The axis of rotation is to the left in these and subsequent tracings, and thus density increases from left to right. The large light peak which is indicated by an arrow in these and subsequent tracings is presumed to be the host DNA.


0 min
15 min
20 min
25 min
LL
HL
HH
p

shows that such a peak would have to be detected against a rather high background of T2 parental DNA which does not replicate for unknown reasons, and thus makes its presence or absence a poor criterion for deciding whether T4 prevents replication of T2 DNA. A better criterion might be the participation of the T2 parental DNA in the formation of the replicating form, and consequently experiments were conducted to determine this participation. 5-BU-labelled T2 was used in mixed infection with T4 and in mixed infection with T2 as a control, and intracellular DNA was extracted and analyzed as above. The results are presented in Figures 28 and 29. The same species are detected as above, with the exception of the replicating form, and its apparent absence can probably be explained by the mechanics of the experiments. The parental DNA in these experiments is partly heavy (HH) and partly light (LL), whereas in the above experiment it was all heavy; if the replicating form assimilates most of the parental DNA inside the infected cell, it would be lighter in these experiments and would thus fall in the region of the gradient obscured by the host DNA. Since the replicating form could not be observed directly, shearing was tried as an indirect method; shearing the replicating form should release material of hybrid density in proportion to the extent to which the labelled T2 parent participated in its formation. The results of the shearing experiments are presented in Figure 30, and it is clear that the 5-BU-labelled T2 parental DNA participates in forming the replicating form much more fully in mixed infection with T2 than in mixed infection with T4.

FIGURE 28. REPLICATION OF 5-BU-LABELLED T2 DNA IN MIXED INFECTION WITH NORMAL T2. Each tracing is of a photograph from a density gradient equilibrium centrifugation of DNA extracted at the indicated time from cells mixedly infected with 5-BU-labelled T2 and normal T2. The infection was carried out at 30°C., with strain BB as host. DNA was extracted by the gentle phenol procedure described in Materials and Methods, and presumably is of quite high molecular weight. The large light peak which is indicated by an arrow in all tracings is presumed to be the host DNA.

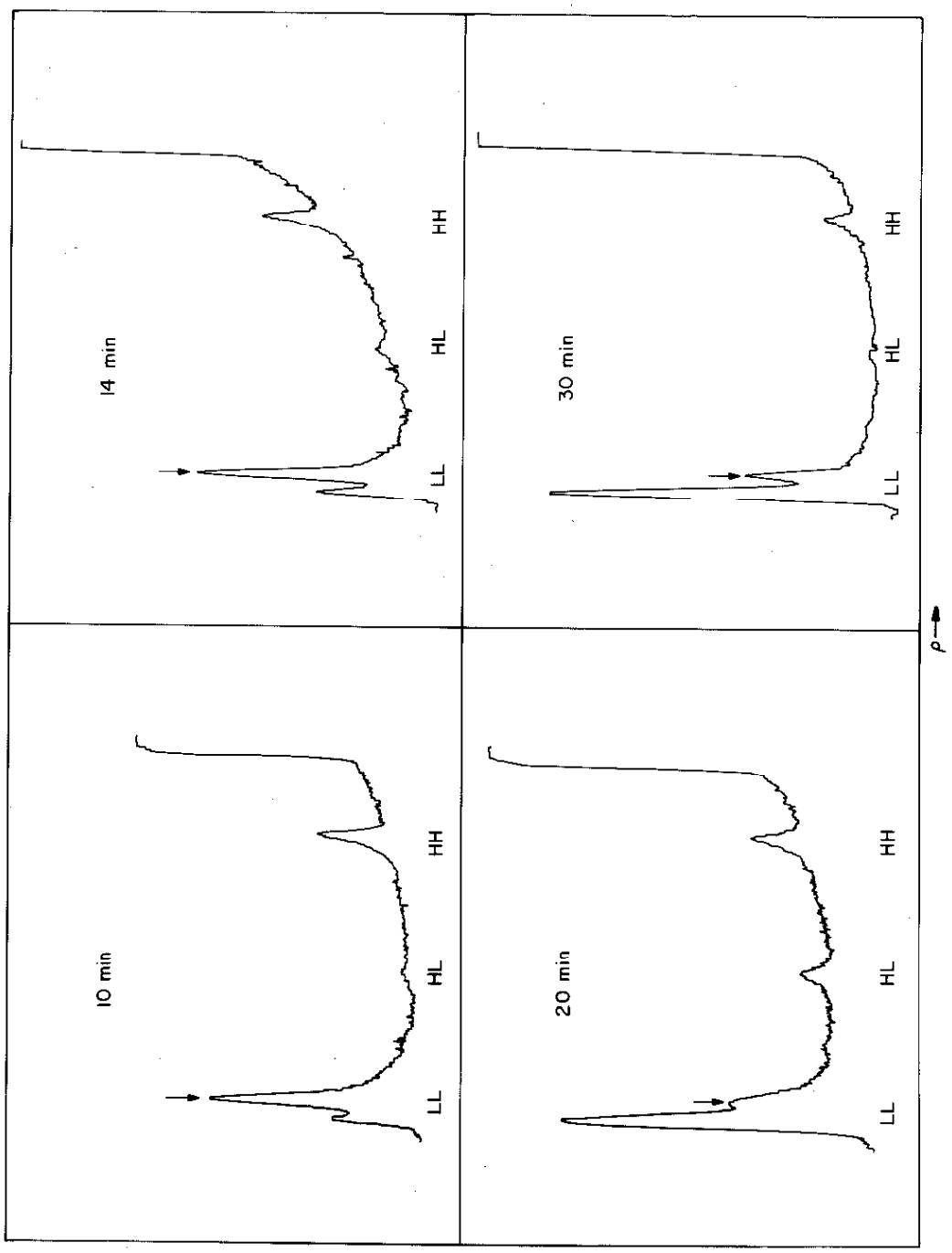

14 min
LL
HL
HH
30 min
LL
HL
HH
10 min
LL
HL
HH
20 min
LL
HL
HH
ρ

FIGURE 29. REPLICATION OF 5-BU-LABELLED T2 DNA IN MIXED INFECTION WITH NORMAL T4. Each tracing is of a photograph from a density gradient equilibrium centrifugation of DNA extracted at the indicated time from cells mixedly infected with 5-BU-labelled T2 and normal T4. The infection was carried out at 30°C., with strain BB as host. DNA was extracted by the gentle phenol procedure described in Materials and Methods, and presumably is of quite high molecular weight. The large light peak which is indicated by an arrow in all tracings is presumed to be the host DNA.

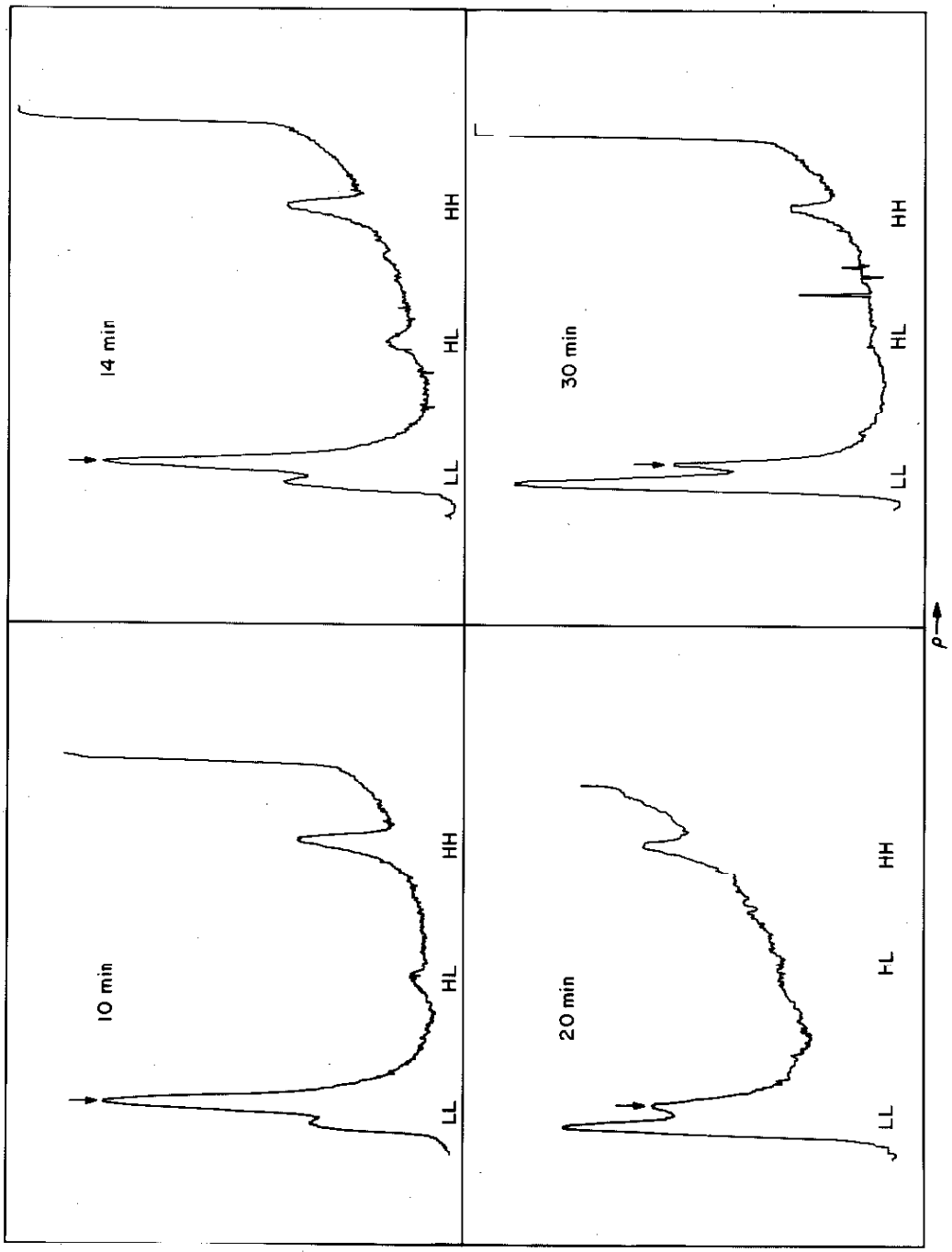
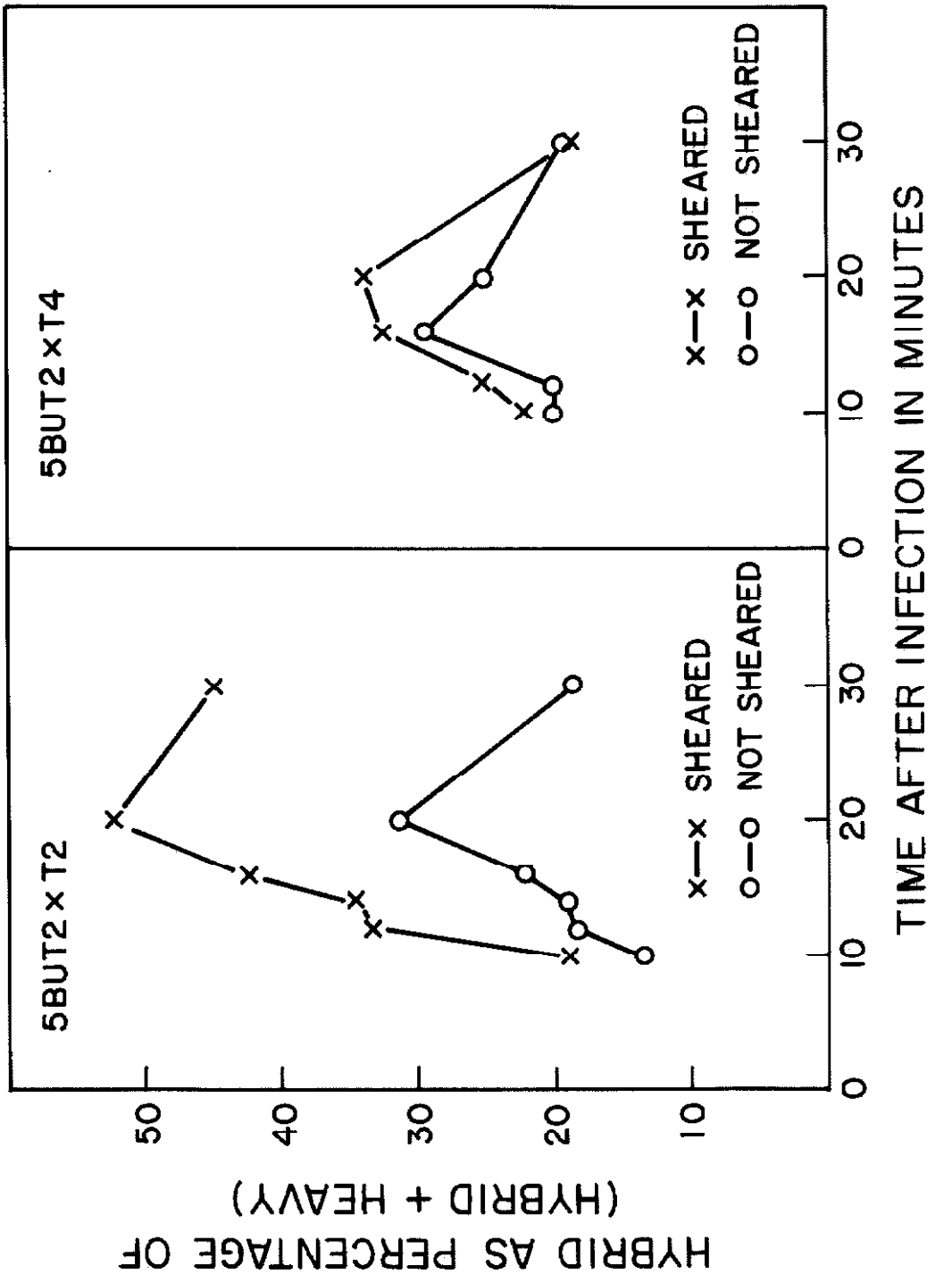

14 min
LL
HL
HH
10 min
LL
HL
HH
30 min
LL
HL
HH
20 min
LL
HL
HH
ρ

FIGURE 30. EFFECT OF SHEARING ON INTRACELLULAR DNA FROM T2-T2 AND T2-T4 MIXED INFECTIONS. Each sample was examined first by density gradient equilibrium centrifugation, and then an aliquot was sheared and examined in the same way. Shearing was accomplished by three passages at moderate speed through a no. 25 serological needle; although it is not easy to determine the shear in such passages, it is likely that molecules of the order of half-T4-length molecules or less predominated among the shear products. The equilibrium bands were noticeably broadened by the shearing. The amounts of hybrid and heavy material in each sample were determined by tracing photographs from the density gradient equilibrium centrifugation, cutting out the peaks of heavy and hybrid density, and weighing the graph paper contained under these peaks. Two determinations, on separate photographs from the same centrifugation, were performed for each point in the figure, and these two determinations were usually in good agreement. The amount of hybrid material is expressed as a fraction of the sum of heavy and hybrid materials, primarily bacause there was no other way to standardize the samples with respect to differences in recovery; both the amount of host DNA and the amount of light phage DNA change during the infection. It is clear that shearing releases hybrid material in the T2-T2 infection but not in the T2-T4.


5BUT2 x T2
5BUT2 x T4
x—x SHEARED
o—o NOT SHEARED
HYBRID AS PERCENTAGE OF
(HYBRID + HEAVY)
10
20
30
40
50
0
10
20
30
0
10
20
30
TIME AFTER INFECTION IN MINUTES

This result is consistent with the notion that T4 acts on the T2 exclusion sensitivity determinants to prevent DNA carrying them from participating in the replicating form. The absence of these determinants from the progeny suggests that only DNA which is part of the replicating form is incorporated into progeny particles.

e. The Nature of the Replication Limitation

An attempt was made to determine the mechanism of the T4 action against the T2 determinants. The genetic control of the action was investigated first by asking whether any known T4 genes determined the specificity of the action. The crosses of Figure 23 were repeated in the restrictive host B/5, and the genetic composition of the progeny was compared to that of progeny from the permissive host. If any of the T4 amber mutants had been defective in the gene controlling the specificity of the action, the results in the two hosts should have differed markedly; as Figures 31 and 32 show, none did. An attempt to isolate a T4 mutant defective in excluding ability was unsuccessful; 1800 plaques from a highly mutagenized stock were examined by the spot test described above, and none had lost the ability to exclude T2.

Since the genetic control of the action could not be revealed, an attempt was made to determine the nature of the T2 exclusion sensitivity determinants. To investigate whether the differences in glucosylation of T2 and T4 DNA's were related to the determinants, exclusion experiments were performed between T2 and T4 phages whose degree of glucosylation had been altered by growth in hosts defective in the synthesis of UDP-

FIGURE 31. EFFICIENCY OF TRANSMISSION OF VARIOUS T2 MARKERS TO THE PROGENY OF T2-T4 MIXED INFECTIONS. The conditions are the same as in Figure 23, except that the crosses were performed in the restrictive host, B/5, so that each cross is performed in the absence of one particular T4 gene function. Each point is the average of from one to six determinations; the average number of determinations per point in the figure is 2.1. There is a striking similarity to Figure 23, as is demonstrated in Figure 32.

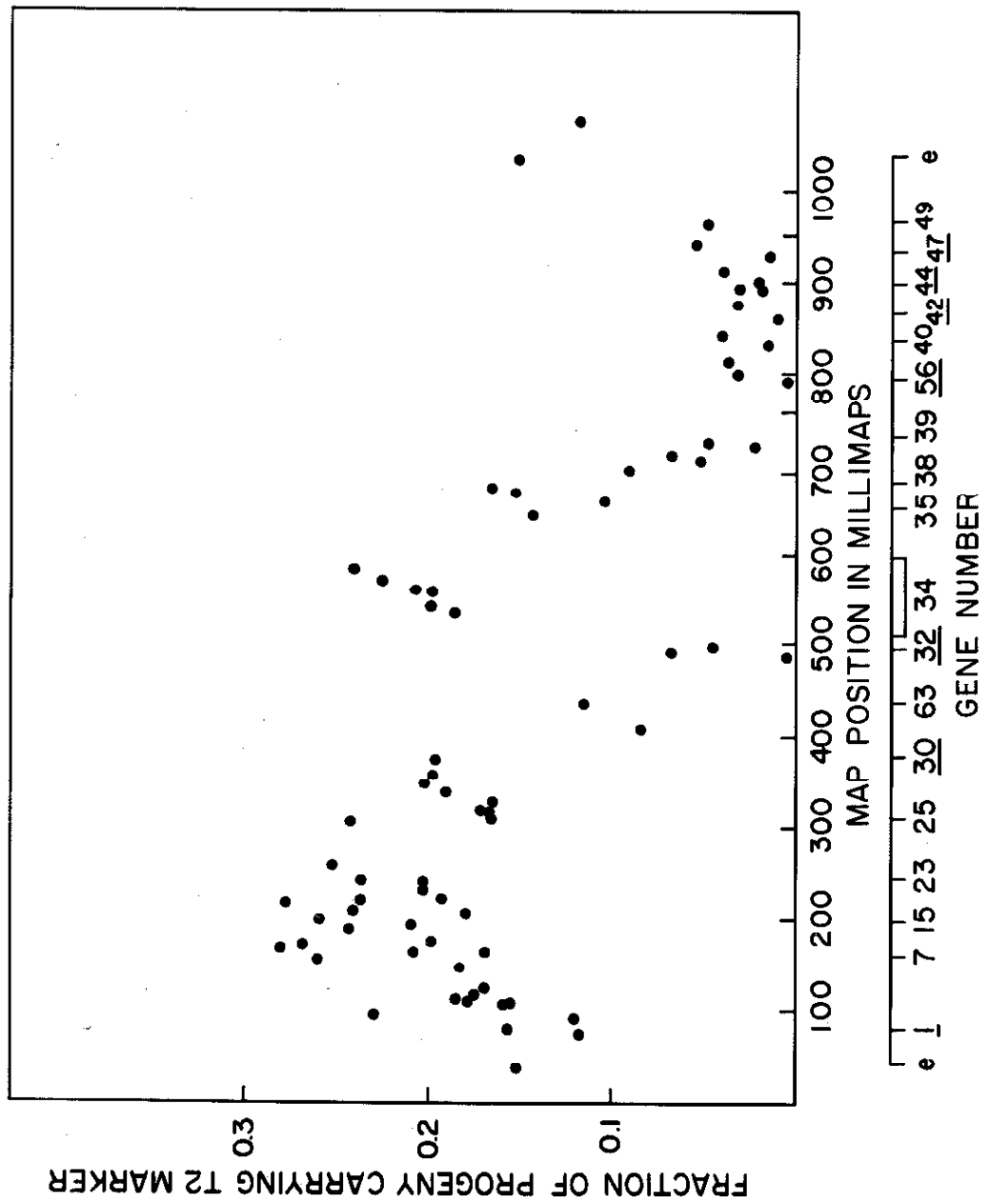
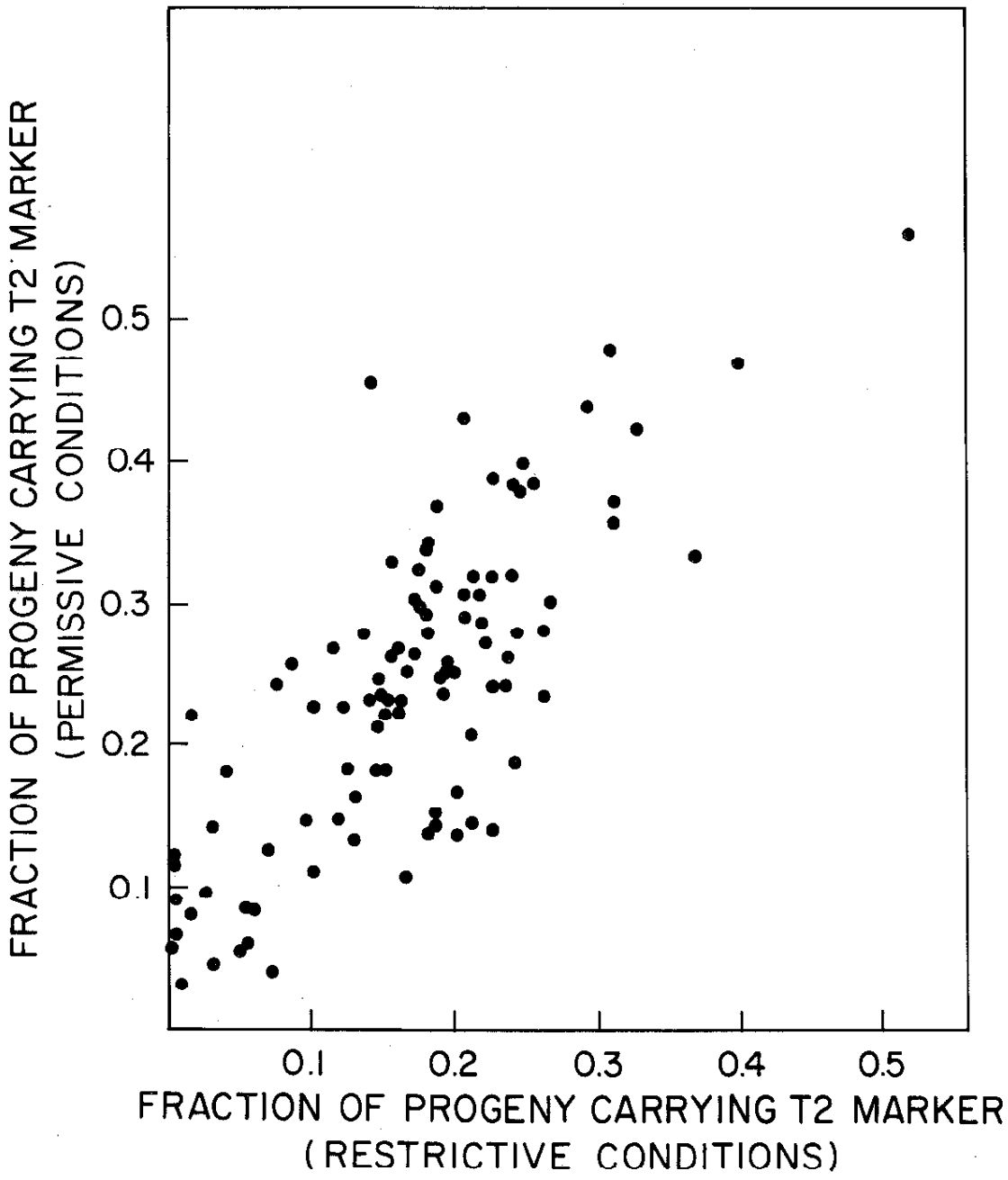

FRACTION OF PROGENY CARRYING T2 MARKER
0.3
0.2
0.1
100 200 300 400 500 600 700 800 900 1000
MAP POSITION IN MILLIMAPS
e 1 7 15 23 25 30 63 32 34 35 38 39 56 40 42 44 47 49 e
GENE NUMBER

FIGURE 32. COMPARISON OF THE EXCLUSION OF T2 BY T^4 IN THE PRESENCE AND ABSENCE OF VARIOUS GENE FUNCTIONS OF T^4. The points in this figure are drawn from Figures 23 and 31, and from some other crosses not included in those figures. The fraction of the progeny carrying a given T2 marker is compared for the case in which the corresponding gene function of T^4 is performed (permissive conditions, Figure 23) and the case in which it is not performed (restrictive conditions, Figure 31). The similarity of the results in the two cases is indicated by the fact that the results scatter about a line with a slope of nearly 1. These results have been confirmed in more detail by studies with temperature sensitive mutants in the early region.


FRACTION OF PROGENY CARRYING T2 MARKER
(PERMISSIVE CONDITIONS)
0.1
0.2
0.3
0.4
0.5
0.1
0.2
0.3
0.4
0.5
FRACTION OF PROGENY CARRYING T2 MARKER
(RESTRICTIVE CONDITIONS)

glucose (126, 127, 128). The results are presented in Table 18; it appears that alterations in the glucosylation of either parent or of the progeny do not affect the extent of exclusion. Since the "unglucosylated" phages used probably still contained small amounts of glucose in their DNA (126), however, these experiments cannot rule out the possibility that glucosylation plays some role in specifying the T2 determinants.

f. Summary

In T2-T4 mixed infections, T4 acts against exclusion sensitivity determinants located in two regions of the T2 genome, one between genes 39 and 49 and the other near gene 32; this action prevents these determinants from appearing among the progeny and it restricts DNA bearing them from participating in the formation of the replicating structure. It does not prevent T2 genes in the immediate vicinity of the determinants from functioning. Markers from the T2 parent appear among the progeny by virtue of recombination events which separate them from the determinants. The specificity of the T4 action is not controlled by any identified T4 gene, and glucosylation probably does not play a role either in the action itself or in specifying the determinants.

TABLE 18

THE EFFECTS OF VARIATIONS IN GLUCOSYLATION ON THE T2-T4 EXCLUSION

		HOST			
		B/5	$B/4_o$	Sh15	$SH/4_o$
ACCEPTANCE OF NON-GLUCOSYLATED PHAGES		-	-	+	+
ABILITY TO GLUCOSYLATE		+	-	+	-
T4 PARENT*	T2 PARENT				
GLUCOSYLATED	GLUCOSYLATED	.063	.021	.049	.001
GLUCOSYLATED	UNGLUCOSYLATED	--	--	.030	.001
UNGLUCOSYLATED	GLUCOSYLATED	--	--	.065	.0001
UNGLUCOSYLATED	UNGLUCOSYLATED	--	--	.042	.0001

*Since some of the hosts used were resistant to T4, it was necessary to use as the T4 parent a T2-T4 hybrid with the host range of T2 but the exclusion properties of T4. Such a hybrid was obtained from a cross of T4 tsL13 (gene 42) by T2 wild type, by selecting progeny with the host range of T2 and the temperature sensitive marker from the T4 parent. One such progeny phage was selected, and its exclusion properties were tested in mixed infections with T2 and T4 wild type; since it excluded T2 and was not excluded by T4, it was judged to have the exclusion properties of T4.

Each number in the table is the fraction of progeny carrying a T2 marker from a preferentially excluded region, in a cross between the indicated parents in the indicated host. Unglucosylated phages were prepared by one cycle of growth in UDPG-defective hosts, as described in Materials and Methods. A slight correction has been applied to each number in the table for the difference in burst sizes of the two parents on the indicated host. The average burst sizes obtained from the different hosts were: B/5, 60; $B/4_o$, 5.8; Sh15, 327; and $Sh/4_o$, 22. The reason for the very low values for crosses performed in $Sh/4_o$ is not known.

3. Mixed Infections Between Other T-even Phages

a. Mixed Infections Between T4 and T6

An attempt was made to discover the basis for the depressor effect which characterizes T4-T6 mixed infections (see Section A.3.a of the Results). Measurements of the rate of DNA synthesis in the T4-T6 mixed infection and in infections with each phage separately are presented in Figure 31; in the mixed infection the rate is more than four-fold lower. This reduction alone would probably be sufficient to reduce the burst size by about a factor of four, but other effects must be invoked to account for the observed thirty-fold reduction. The nature of these additional effects was not investigated, but it may be of importance that the depressor effect is less marked in CR63 than in B/5 (T4-T6 mixed infections give burst sizes of about 15 in CR63, as opposed to about 3 in B/5). It is interesting to note that if the depressor effect is viewed as the results of a competition in which T4 attempts to exclude T6 and T6 attempts to exclude T4, the low rate of DNA synthesis is further evidence that exclusion operates by limiting DNA replication.

If CR63 is used as host, the genetic composition of the progeny of T4-T6 mixed infections can be determined. Since the results of T4-T6 complementation tests (see Table 8) had suggested that T4 excludes T6, crosses were performed between T6 wild type and a number of T4 mutants and the progeny were analyzed as in Figures 23 and 31. The results are presented in Table 19, along with the results of equivalent T2-T4 crosses for comparison; the similarity between the two argues that T4 also excludes T6 by acting against localized exclusion sensitivity determinants in the

FIGURE 31. DNA SYNTHESIS IN T4 AND T6 INFECTIONS. The rates of DNA synthesis were measured by the more precise method described in Materials and Methods. The host for all infections was B/5, and the total multiplicity for all infections was 10 (in the mixed infection, a multiplicity of 5 of each parent was used).

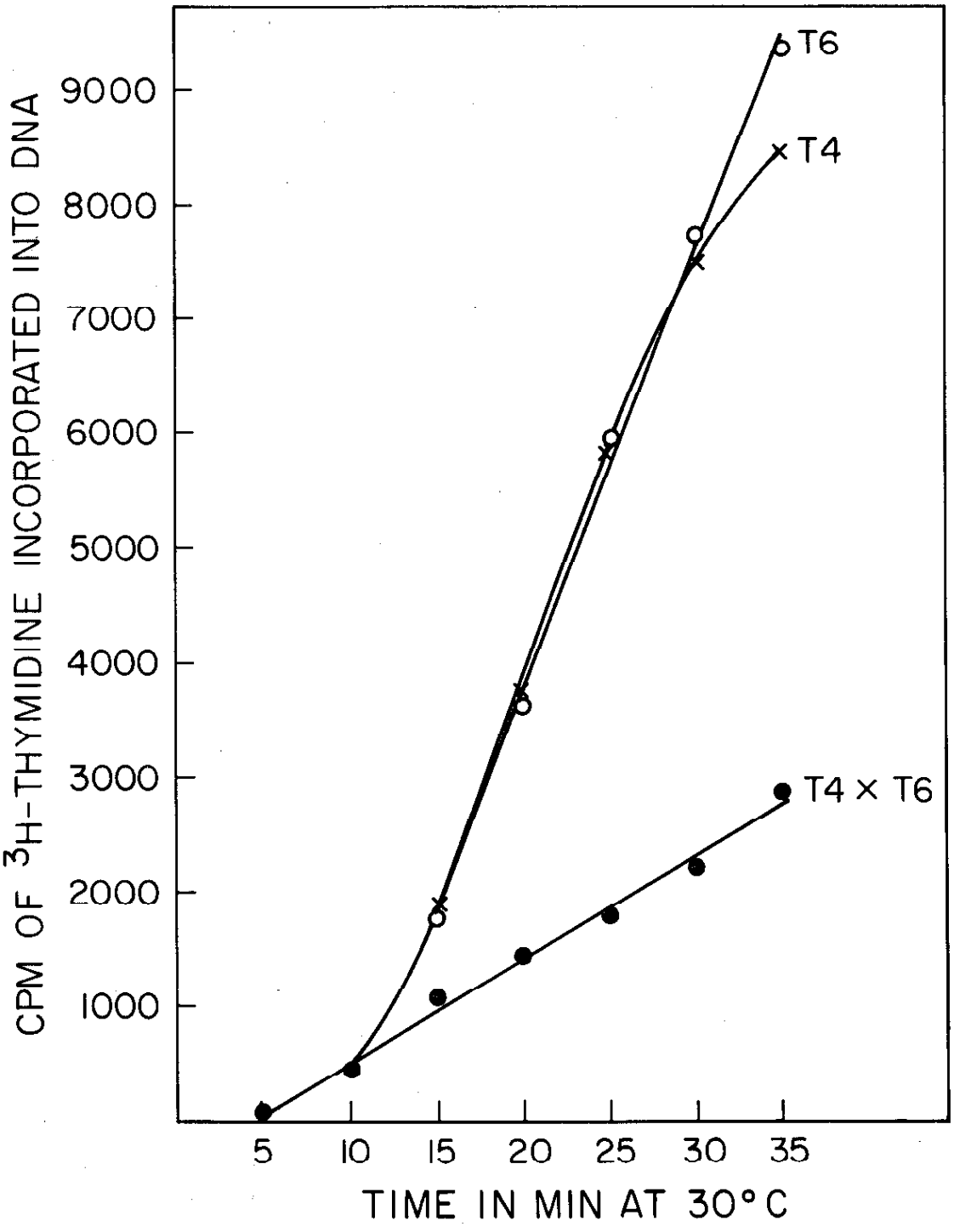

CPM OF ^{3}H-THYMIDINE INCORPORATED INTO DNA
9000
8000
7000
6000
5000
4000
3000
2000
1000
T6
T4
T4 × T6
5
10
15
20
25
30
35
TIME IN MIN AT 30° C

TABLE 19

COMPARATIVE EXCLUSION OF T2 AND T6 BY T4

GENE CONTAINING T4 AMBER MUTATION	PERCENTAGE OF PROGENY CARRYING T2 MARKER IN T4am x T2++ CROSS	PERCENTAGE OF PROGENY CARRYING T6 MARKER IN T4am x T6++ CROSS
1	22	33
9	32	50
14	24	44
20	26	16
23	27	26
31	12	15
33	5.5	12
35	18	19
36	8.7	16
39	6.8	7.1
41	2.2	9.7
42	1.9	3.5
43	2.1	1.7
44	1.8	2.8
46	1.8	3.8
47	1.5	5.0

All crosses were performed in CR63, a permissive host. The values for the T2-T4 crosses are drawn from Figure 28.

T6 genome. If the T6 map is assumed to resemble those of T2 and T4, these determinants fall in the same positions as in T2.

The depressor effect makes it difficult to determine whether the excluded T6 can function, as the excluded T2 can in T2-T4 mixed infections, and consequently no attempt was made to determine the functional capacity of T6 genes near the determinants. The success of T4-T6 spot tests, however, suggests that these genes can function to at least some extent.

In summary, mixed infections between T4 and T6 resemble those between T4 and T2 in two important respects; 1) there is an unequal yield effect, in which T4 predominates, and 2) T4 acts against localized determinants in the same regions of the excluded genome. They differ in that T6 can exert some counter-action which slows down the synthesis of T4 DNA, whereas T2 cannot.

b. Mixed Infections Between T2 and T6

Since intertype complementation tests (see Table 8) had suggested that T6 excludes T2, crosses were performed between T2 wild type and a number of T6 amber mutants. The results presented in Table 20 show that T6 does indeed exclude T2, although less strongly than T4 excludes either T2 or T6. There is rather weak evidence that T6 acts against localized determinants in the T2 genome, but if so, then the action must be less restricting than in either case examined above; since markers from the "preferentially excluded" regions appear in 10% or more of the progeny. These results can be accomodated by a variation of the

TABLE 20

COMPARATIVE EXCLUSION OF T2 BY T4 AND T6

GENE CONTAINING AMBER MUTANT	PERCENTAGE OF PROGENY CARRYING T2 MARKER IN T4am x T2++ CROSS	PERCENTAGE OF PROGENY CARRYING T2 MARKER IN T6am x T2++ CROSS
5	30	14
6	27	20
7	32	29
27	23	40
32	0.9	12
34	13	21
35	18	20
37	14	20
42	1.9	10
43	2.1	16
44	1.8	12
45	1.6	11
46	1.8	11

All crosses were performed in CR63, a permissive host. The values for the T2-T4 crosses are drawn from Figure 26.

model used above in which the T6 action only partially prevents the T2 determinants from appearing among the progeny. The implication that some T2-T6 progeny should retain the T2 determinants has not been checked.

The complementation tests of Table 8 had demonstrated that T2 can provide a limited amount of tail fiber functions in mixed infection with T6; the results presented in Table 21 show that T2 can perform some other functions too, although apparently not as efficiently as in mixed infection with T4. As in mixed infection with T4, T2 genes near the determinants function at least as well as genes far away.

In summary, T2-T6 mixed infections resemble T2-T4 and T4-T6 mixed infections in three ways; 1) there is an unequal yield effect, T6 predominating over T2, 2) T6 probably acts against localized determinants in the same regions of the excluded genome, and 3) the excluded T2 can nonetheless perform some functions. They differ from the other mixed infections in the strength of the exclusion, and in the extent to which the excluded T2 can perform various functions.

c. Mixed Infections Between T4 and Some New T-even Phages

To see whether the interactions observed between the classical T-even phages might occur among T-even phages in nature, mixed infections were performed between T4 and a group of newly isolated T-even-like phages. These phages have been described briefly in Materials and Methods; they were isolated from six different Long Island sewage treatment plants, and were selected for their ability to plate on E.coli strain B/5 and for their sensitivity to inactivation by anti-T4 and/or

TABLE 21

FUNCTIONAL CAPACITY OF T2 GENES IN MIXED INFECTIONS WITH T6

GENE CONTAINING T6 AMBER MUTATION	BURST SIZE AS PERCENTAGE OF T2++ x T6++ CONTROL
5	9.6
6	2.8
7	3.9
27	2.5
32	43
34	13
35	20
37	8.6
42	27
43	33
44	5.3
46	3.2
Average	13.2

Crosses were performed between T2 wild type and the indicated T6 amber mutant in the restrictive host B/5. The burst sizes were computed and compared to those of simultaneously performed T2 wild type by T6 wild type controls. These burst sizes are measures of the extent to which the excluded T2 can provide the functions which the T6 mutants lack, and thus they measure the functional capacity of the corresponding T2 genes.

anti-T6 antisera. Further characterizations presented in Tables 22 and 23 suggest that they represent a set of reasonably close relatives which share certain properties in common but can be distinguished by others. Their burst sizes cover the range expected of T-even phages, and they adsorb well to B/5 under standard conditions. Their ability to plate efficiently at 42°C. allowed them to be treated as non-temperature-sensitive in crosses with T4, and their ability to plate efficiently on S/4 permitted their host ranges to be distinguished from that of T4 on mixed indicators composed of B/5 and S/4 (on such an indicator mixture, the RB phages form clear plaques, while those of T4 are turbid).

Crosses were performed between a T4 temperature sensitive mutant defective in gene 42 (an early gene controlling the enzyme dCMP hydroxymethylase) and each of the new RB phages. Immediately after adsorption, the infected complexes were divided into two aliquots and one was incubated at 25°C., a permissive temperature for the temperature sensitive mutant, the other at 39.5°C., a restrictive temperature. The infective centers from the 25°C. aliquot were plated on the mixed indicator to determine how many of them yielded phages with the host range of the RB parent. After lysis, the burst sizes for each high temperature and low temperature infection were determined, and the progeny of the low temperature infection were analyzed to determine how many contained the host range marker of the RB phage parent and how many contained the RB phage wild type allele of the T4 temperature sensitive mutation. The low temperature progeny were also plated on the mixed indicator at 42°C., and the presence of turbid plaques on these plates, when it occurred, was

TABLE 22. PROPERTIES OF RB PHAGES. Plaque sizes were determined on strain B/5 at 30°C.; T2, T4, and T6 have medium plaque sizes on this scale. The extents of inactivation by anti-T4 and anti-T6 antisera were determined by R. O. Berry, and T4 and T6 give +++ degrees of inactivation with their respective antisera. Plating groups were determined by the ability to plate on some standard laboratory strains of E. coli and on various resistant derivatives of B/5; phages in the same group could not be distinguished from one another by this test, and the ten groups thus identified could be divided into two sets of five each on the basis of rather broad similarities in their plating patterns (sets I and II). The sensitivity to citrate was determined by plating on EHA plates, which contain citrate; presumably such sensitivity would reflect a requirement for divalent cations, which are chelated by citrate. Phages indicated by asterisks (*) were examined in the electron microscope, and were T-even-like.

TABLE 22

PROPERTIES OF RB PHAGES

PHAGE	SOURCE	PLAQUE SIZE	INACTIVATION BY		PLATING GROUP	SENSITIVITY TO CITRATE
			ANTI-T4	ANTI-T6		
T16	HERSHEY	MEDIUM	?	?	I D	-
RB1	HUNTINGTON	MEDIUM	++	++	I A	-
RB2	HUNTINGTON	MEDIUM	++	++	I B	-
RB3	HUNTINGTON	MEDIUM	+	++	I A	-
RB5	HUNTINGTON	MEDIUM	+	++	I A	-
RB6	HUNTINGTON	MEDIUM	+	++	I A	-
RB7	HUNTINGTON	MEDIUM	+	++	I A	-
RB8	HUNTINGTON	MEDIUM	+	++	I A	-
RB9	HUNTINGTON	MEDIUM	++	+++	I A	-
RB10	HUNTINGTON	MEDIUM	++	+++	I A	-
RB14*	OYSTER BAY	MEDIUM	+++	+++	I A	-
RB15	OYSTER BAY	MEDIUM	+++	+++	I A	-
RB17	OYSTER BAY	MEDIUM	++	++	I A	-
RB18	OYSTER BAY	MEDIUM	+++	+++	I C	-
RB19*	OYSTER BAY	MEDIUM	++	+	II B	-
RB23	FLUSHING	MEDIUM	++	++	I D	-
RB26	FLUSHING	SMALL	+++	+++	I A	-
RB27	FLUSHING	MEDIUM	+++	++	I A	-
RB30	FLUSHING	MEDIUM	+++	++	I A	-
RB46	GLEN COVE	MEDIUM	++	++	II A	-
RB51*	GREAT NECK	MEDIUM	+	+	II A	-
RB52	GREAT NECK	MEDIUM	++	++	II A	-
RB53	GREAT NECK	MEDIUM	++	+	II C	-
RB57	GREAT NECK	MEDIUM	++	+	II D	-
RB58	GREAT NECK	MEDIUM	+++	++	I A	-
RB60	GREAT NECK	SMALL	+++	+++	I E	-
RB61*	LONG BEACH	MEDIUM	?	?	II A	-
RB62	LONG BEACH	LARGE	+	+	II A	-
RB68	LONG BEACH	MEDIUM	++	++	II A	-
RB69*	LONG BEACH	MEDIUM	+	0	II E	-
RB70	LONG BEACH	MEDIUM	+	0	II A	-

TABLE 23. GROWTH PARAMETERS OF RB PHAGES. Adsorption was carried out for 10 minutes to strain B/5 at a final concentration of 2 x 10^8/ml. at 30°C.; under these conditions the classical T-even phages adsorb as follows -- T2, 96%; T4, 93%; and T6, 75%. Burst sizes were determined on B/5 at the indicated temperatures; it is interesting to note that even the most severe reductions in burst size at 39.5°C. have remarkably small effects on the ability of the phages to form plaques at 42°C. All efficiencies of plating (E.O.P.'s) are relative to strain B/5 at 30°C.

TABLE 23

GROWTH PARAMETERS OF RB PHAGES

PHAGE	PERCENT ADSORPTION	BURST SIZE		E.O.P. ON CR63	E.O.P. AT 42°C. ON B/5	E.O.P. ON S/4
		25°C.	39.5°C.			
T16	82	434	349	0.33	0.97	1.08
RB1	75	84	1.3	0.30	0.81	0.68
RB2	82	105	166	0.51	0.92	1.26
RB3	78	88	1.4	0.40	0.66	1.01
RB5	87	125	1.4	0.50	0.26	1.09
RB6	71	93	8.5	0.43	1.40	0.78
RB7	65	90	7.6	0.32	1.35	0.82
RB8	78	32	6.3	0.43	1.30	0.97
RB9	76	76	4.3	0.25	0.86	1.14
RB10	72	78	4.5	0.35	1.59	1.15
RB14	51	303	330	0.04	1.18	1.07
RB15	82	325	212	0.81	1.09	1.33
RB17	86	132	34	0.26	0.89	0.94
RB18	91	427	386	0.65	1.15	0.93
RB19	97	293	336	0.52	1.19	0.99
RB23	85	234	51	0.49	0.94	1.15
RB26	85	54	8.9	1.49	0.96	1.13
RB27	77	255	86	0.61	1.03	1.09
RB30	82	223	101	0.56	1.22	1.12
RB46	97	314	95	0.34	1.30	0.87
RB51	99	443	119	0.28	1.12	0.96
RB52	97	360	122	0.29	0.94	0.96
RB53	98	324	94	0.22	1.02	0.84
RB57	93	307	99	0.22	0.88	1.12
RB58	91	202	51	0.49	0.97	1.00
RB60	88	476	141	0.34	0.95	1.26
RB61	99	340	124	0.28	0.96	0.92
RB62	88	339	92	0.26	0.96	0.75
RB68	96	277	66	0.21	0.97	0.90
RB69	99	181	30	1.54	0.82	1.67
RB70	99	284	94	0.23	1.04	0.73

taken as evidence for the formation of recombinants. The results of these crosses are summarized in Table 24.

In almost all of the mixed infections, a significant fraction of the mixedly infected cells yielded phages with the host range of the RB phage parent and in about two-thirds of the cases examined, T4-RB phage recombinants could be detected; both observations suggest that most of the RB phages can, to some extent, grow together with T4 in the same cell. The genetic composition of the progeny shows, however, that all but one of these phages (RB69) are strongly excluded by T4. This exclusion differs from the cases examined above in that the host range markers of the excluded phages are transmitted to less than 10% of the progeny; attempts to explain it by modifications of the model used above must therefore account for the absence of frequent recombinants separating the host range marker and the exclusion sensitivity determinants.

The limited experiments performed do not permit a distinction between two equally satisfactory modifications of the model. The first of these assumes an additional exclusion sensitivity determinant near the host range marker of the RB phage parent, but normal potential for genetic exchange between T4 and the RB phages. The second assumes limited potential for genetic exchange between the phages, but exclusion sensitivity determinants only at the same places as in the above cases. Since more progeny generally carry the host range marker than carry the early marker from the RB phage parent, the second interpretation is tentatively favored. Whichever interpretation is true, the reasonably high burst sizes in the 39.5°C. aliquots show that the excluded RB phages can nonetheless function.

TABLE 24. MIXED INFECTIONS BETWEEN T4 AND THE RB PHAGES. All infections were between a T4 temperature sensitive mutant defective in gene 42 and the indicated RB phage. The RB yielder frequency is the fraction of the infected cells which yield phages with the RB host range. The reasonably high burst sizes of the 39.5°C. infections suggest that the RB phages can supply the dCMP hydroxymethylase which the T4 mutant lacks, even though they are strongly excluded in all but one case. Where recombinants between T4 and the RB phage parent were looked for, they were found in about two-thirds of the cases.

TABLE 24

MIXED INFECTIONS BETWEEN T4 AND THE RB PHAGES

RB PHAGE PARENT	RB YIELDER FREQUENCY	BURST SIZE		FRACTION OF 25°C. PROGENY CARRYING		RECOMBINANTS DETECTED
		25°C.	39.5°C.	RB PHAGE HOST RANGE	RB PHAGE ts+ ALLELE	
T16	0.14	78	77	0.047	0.014	yes
RB1	0.30	119	107	0.009	0.000	no
RB2	0.05	222	286	0.015	0.003	yes
RB3	0.30	70	85	0.011	0.000	yes
RB5	0.21	90	93	0.010	0.000	yes
RB6	0.32	67	77	0.011	0.001	no
RB7	0.42	45	73	0.034	0.000	yes
RB8	0.57	34	67	0.039	0.016	yes
RB9	0.43	66	91	0.029	0.000	yes
RB10	0.46	32	75	0.037	0.000	no
RB14	0.03	283	68	0.003	0.002	yes
RB15	0.11	81	28	0.046	0.000	yes
RB17	0.41	28	40	0.014	0.043	yes
RB18	0.10	32	169	0.072	0.061	no
RB19	0.36	24	30	0.157	0.069	no
RB23	0.44	180	?	0.033	0.013	-
RB26	0.93	89	?	0.055	0.005	-
RB27	0.68	206	43	0.038	0.011	-
RB30	0.66	196	56	0.041	0.021	-
RB46	0.58	107	30	0.041	0.041	-
RB51	0.51	91	34	0.040	0.033	-
RB52	0.43	125	29	0.043	0.022	-
RB53	0.51	98	31	0.059	0.032	-
RB57	0.51	133	37	0.044	0.034	-
RB58	0.45	224	54	0.025	0.004	-
RB60	0.68	145	76	0.054	0.043	-
RB61	0.62	73	33	0.075	0.051	-
RB62	0.50	82	29	0.075	0.044	-
RB68	0.56	80	25	0.059	0.035	-
RB69	0.96	172	23	0.988	0.990	-
RB70	0.56	113	35	0.059	0.040	-

In summary, mixed infections between T4 and the RB phages resemble those between the classical T-even phages in that there are unequal yield effects in which T4 predominates over other phages, and in that the excluded phages can still function. They differ from them in that the excluded phages make a smaller genetic contribution to the progeny.

d. Mixed Infections Between RB69 and T2, T4, and T6

RB69, in contrast to all the other RB phages, strongly excluded T4; these unusual exclusion properties led to a further study of its interactions with the classical T-even phages. Crosses were performed between RB69 amber mutants and T2 wild type, T4 wild type, and T6 wild type, and the results are presented in Table 25. RB69 excludes T2, T4, and T6 so strongly that only 1-2% of the cells yield phages with markers derived from them. Since some such cells are those which, by chance, escaped infection by RB69, the fraction of T-even phage yielders among truly mixedly infected cells may be even lower. These results are analogous to those described above for the interactions between T4 and the other RB phages, only a smaller fraction of the cells yield the excluded phage; they can be accommodated by the second modification of the model described above, and the very low frequency of recombinants in T4-RB69 spot tests supports the notion that genetic exchange between RB69 and the classical T-even phages is severely limited.

To determine whether the T-even phages can function in mixed infections with RB69, the crosses were repeated in the restrictive host. The results presented in Table 26, show that the T-even phages can provide

TABLE 25

EXCLUSION OF THE CLASSICAL T-EVEN PHAGES BY RB69

RB69 AMBER MUTANT	MUTANT PHENOTYPE	PERCENTAGE OF WILD TYPE PROGENY IN CROSS WITH		
		T2 WILD TYPE	T4 WILD TYPE	T6 WILD TYPE
am18	DNA ARRESTED	0.9	2.5	1.9
am65	DNA NEGATIVE	0.7	2.1	1.8
am9	(TAIL FIBERLESS)	0.5	1.6	1.9
am24	DNA NEGATIVE	0.8	2.6	1.8
am36	LATE	0.9	3.0	2.1
am94	LATE	0.6	1.6	2.0

All crosses were performed in CR63, a permissive host. Since the fraction of progeny bearing markers from the classical T-even phages is so low, all these progeny may be coming from cells which were not infected with RB69. In fact, only about 1-2% of the infected bacteria yielded wild type progeny in these crosses, indicating that the fraction of wild types among the progeny of truly mixedly infected cells may be considerably lower than the values shown.

TABLE 26

FUNCTIONAL CAPACITY OF THE CLASSICAL T-EVEN PHAGES
IN MIXED INFECTION WITH RB69

RB69 AMBER MUTANT	MUTANT PHENOTYPE	BURST SIZE AS PERCENTAGE OF CONTROL IN MIXED INFECTION WITH		
		T2 WILD TYPE	T4 WILD TYPE	T6 WILD TYPE
am18	DNA ARRESTED	6.8	11.3	5.8
am65	DNA NEGATIVE	2.6	8.4	11.9
am9	(TAIL FIBERLESS)	1.0	3.4	2.5
am24	DNA NEGATIVE	12.1	19.0	12.7
am36	LATE	2.1	6.8	5.1
am94	LATE	5.7	38.4	28.9

All crosses were performed in B/5, a restrictive host. Controls were mixed infections between RB69 wild type and T2, T4, or T6 wild type. The control burst sizes were all in the range of 100-150 phage per cell. Control infections of the restrictive host with the RB69 mutants alone yield about 1 phage per cell.

limited amounts of several functions lacking in different mutants of RB69. Their inability to provide other functions well can probably be attributed to incompatibilities between them and RB69.

In summary, mixed infections between RB69 and the classical T-even phages resemble those described above in that there is an unequal yield effect and in that the excluded phages can perform some functions. They differ from them in that very few of the mixedly infected cells yield phages with markers derived from the excluded parent, and in that the frequency of recombinants is very low.

e. Summary

These studies of mixed infections between other T-even phages have shown that the features of the T2-T4 mixed infection are seen to various extents in other mixed infections. The unequal yield effect was seen in all the other mixed infections studied, and in most cases a significant fraction of the cells were mixed yielders. In all cases the excluded phage could provide some functions, but the extent to which a function could be provided varied, depending both on the function and on the mixed infection. Evidence for the location of exclusion sensitivity determinants was obtained only in the T4-T6 and T2-T6 mixed infections, where the locations were the same as in the T2-T4 mixed infection. The general model developed to account for the features of the T2-T4 mixed infection could account for those of the other mixed infections as well, but in order to do so it was necessary 1) to allow variations in the stringency with which the excluding phage could

limit the replication of the excluded phage, and 2) to assume differences between the phages affecting the frequencies of intertype recombination and the ability of excluded phages to provide certain functions.

IV. Discussion

A. Relationships Among the T-even Phages

The close relationships among the classical T-even phages have been known for a long time, and the results presented above provide additional evidence about these relationships. The classification of T2 and T6 mutants by their behavior in complementation spot tests against T4 mutants depended on the production of viable T2-T4 and T6-T4 recombinants; its success implies that very many different recombinants, with different hybrid combinations of genes, must be viable.

The identification of T2 and T6 genes showed that most are homologous with genes already identified in T4, and conversely, that a large fraction of the T4 genes have homologs in T2 and T6. The map of T2 is nearly identical with that of T4, with respect to both gene order and the relative genetic distances between mutants; the limited number of crosses performed between T6 mutants showed gene orders identical to those in T2 and T4. Both T2 and T6 crosses were characterized by negative interference and maximum frequencies of recombination resembling those of T4. Similar polarity effects were also seen in T2, T4 and T6. Each of these findings confirms the close relationships among the classical T-even phages.

Although RB69 mutants could not be classified by their behavior in complementation spot tests against T4 mutants, RB69 nonetheless bears a fairly strong genetic resemblance to the classical T-even phages. RB69 amber mutants were easy to isolate, and they identified almost as many genes as in T2 and T6. The RB69 mutant phenotypes were like those

of T4, and their distribution around the map bore a strong resemblance to that of T4. RB69 crosses were like classical T-even phage crosses in their negative interference and in their maximum frequencies of recombination. The genetic resemblance of RB69 to the classical T-even phages was confirmed by its serological cross reaction with T4, by its particle morphology in the electron microscope, by various physical characteristics of the RB69 phage particle, and by the parameters of its intracellular growth cycle.

The strong similarity of all these members of the T-even species focusses attention on the limited number of differences between them. Among the classical T-even phages, the tail fiber region proved to be the site of the most important differences, and this fact in itself bears some comment. Because of the T-even phages' absolute parasitic dependence on their bacterial hosts and because of the apparent ease with which the bacterial surface can be altered by mutation so as to resist these phages, the adsorption phase of the phage life cycle is subject to considerable selective pressures. The various bacterial derivatives resistant to one or another of the T-even phages can be viewed as different ecological niches which are potentially exploitable by the T-even species as a whole. In order to exploit these niches, however, the T-even species must exhibit sufficient diversity with respect to adsorption specificities so that it is unlikely that a bacterial mutant will ever arise which is resistant to all of the T-even phages. The differences between the classical T-even phages in the tail fiber region can be viewed as the result of this selection for adsorption diversity. In support of

this view it can be noted that among the thirty-one RB phages, all members of the T-even species, ten different adsorption specificities could be detected. Since the classical T-even phages do not show similar diversity elsewhere in the map, there is probably less intensive selection for diversity in other aspects of the phage life cycle.

From the evolutionary point of view, there is some interest in the nature of the observed tail fiber differences, particularly those involving the products of genes 37 and 38. T2-T6 combinations of these gene products are compatible, but T2-T4 and T4-T6 combinations are not. Since the host range differences between the phages are determined by gene 38, the tail fiber component formed by the gene 37 and gene 38 products probably interacts most intimately with the bacterial surface, and the observed compatibility relationships suggest a basic difference between the bacterial receptors for T4 and those for T2 and T6. This basic difference probably involves the layers of the cell wall where these receptors reside; those for T4 reside in the lipopolysaccharide layer whereas those for T2 and T6 reside in the lipoprotein layer (80). Thus the ecological diversity which results from the existence in the bacterial cell wall of two independently modifiable layers is reflected in the polymorphic diversity of the T-even phages with respect to genes 37 and 38.

The evolutionary significance of the enhanced recombination frequencies in genes 34 and 35 of T4 is less clear. Some co-factor requiring mutations of T4 map in gene 34 (42, 129) and from the adsorption properties of some T2-T6 hybrids, it is clear that the gene 34 product of the tail fiber can influence the rate of adsorption strongly (130); it may be that

gene 34 determines the ability of the phage to adsorb under various environmental conditions, including variations in the concentrations of inorganic ions or required biochemical adsorption factors. If so, the variety of environments potentially open to phages would constitute a selective force for the development of diversity in gene 34, and such diversity would clearly be enhanced by higher gene 34 recombination frequencies.

The differences between the phages in the tail fiber region suggest that, in some respects, T2 and T6 are more closely related to each other than to T4: the gene 37 and gene 38 products of T2 and T6 are compatible, whereas those of both phages are incompatible with those of T4; T2 and T6 both adsorb to the lipoprotein layer of the cell wall, whereas T4 adsorbs to the lipopolysaccharide layer; and T4 has enhanced recombination frequencies in genes 34 and 35, whereas T2 and T6 do not. This conclusion, is substantiated by other observations: T2 and T6 lack the β-glucosyl-transferase possessed by T4 (67, 68), but they both have a "di-glucosylating" enzyme which T4 lacks (67, 68); they are both about twice as sensitive to ultraviolet light as T4 (83); and both are strongly excluded by T4, whereas the T2-T6 mixed infection is characterized by only a weak exclusion (see Sections B.2 and B.3 of the Results).

B. Interactions Between the T-even Phages

In the above view of the relationships among the classical T-even phages, the degree of partial exclusion in a mixed infection between two phages is correlated with their degree of evolutionary divergence. This correlation supports the contention that partial exclusion is a stage in the evolution of absolute exclusion; other cases of partial exclusion, such as the T5-PB case (26, 34) and the T7-Cro case (35), show that this stage occurs in other species as well, and argues that the results of this study on the interactions of the T-even phages may have general implications.

From the study of T2-T4 mixed infections, there emerged a relatively clear picture of the partial exclusion of T2 by T4. T4 acts against exclusion sensitivity determinants located in two regions of the T2 genome; this action prevents these determinants from appearing among the progeny, and markers from the T2 parent appear by being recombined away from them. Most early genes are located close to determinants, while most late genes are not. The action of T4 limits the replications of the parental T2 DNA by preventing it from participating in the formation of the replicating structure. The specificity of the action does not depend either on the product of any known T4 gene or on differences between the T2 and T4 parents with respect to DNA glucosylation.

The virtue of this picture is that, with a limited number of modifications based on reasonable hypotheses about the relationships between the phages involved, it applies to all the other mixed infections studied. In the T4-T6 mixed infection, the exclusion of T6 by T4 followed very much the same pattern as the exclusion of T2 by T4; exclusion sensitivity

determinants in the same regions of the T6 genome were detected, and T6 markers not close to such determinants were rescued with good efficiency. Although this mixed infection was characterized by a depressor effect not seen in the T2-T4 mixed infections, this effect can be accounted for as a case of competitive exclusion. In the T2-T6 mixed infection, the exclusion was considerably weaker; although there was evidence for preferential exclusion of the same regions as in the T2-T4 and T4-T6 cases, even the most strongly excluded T2 marker appeared in more than 10% of the progeny. This result can be accommodated by the ad hoc assumption that T6 imposes a considerably less stringent limitation on the replication of T2 DNA than in the T2-T4 and T4-T6 cases. T2 could provide some functions well in the T2-T6 mixed infection; its inability to provide other functions can be explained by assuming partial incompatibilities between T2 and T6.

In mixed infections between T4 and the newly isolated RB phages, early and late markers from the RB phage parent appeared with roughly similar and low frequencies among the progeny, although the late markers were usually transmitted with slightly higher efficiency. This result can be accounted for on the ad hoc assumption that recombination between T4 and the RB phages is reduced because of inhomologies. Despite the strong exclusion of the RB phages, they all provided dCMP hydroxymethylase quite well in the mixed infections. In the mixed infections between RB69 and the classical T-even phages, extremely strong exclusion was observed. This result can be accommodated if recombination between RB69 and the classical T-even phages is assumed to be extremely limited. Despite the

very strong exclusion, the classical T-even phages could nonetheless provide some functions in the mixed infection; their inability to provide other functions can again be attributed to incompatibilities.

The modifications of the T2-T4 picture required to explain the above results define a general model which applies to all the cases studied. In this model, one phage acts against determinants located in the genome of the other, limiting the replication of the other's DNA without affecting its ability to function. The stringency of the replication limitation, the efficiency with which markers are rescued from the excluded phage, and the ability of the excluded phage to provide specific functions all depend on the degree of evolutionary divergence of the two phages. If all the cases to which this model applies are considered to belong to the same evolutionary sequence, at least three stages in this sequence can be distinguished. In the first stage, typified by the T2-T6 mixed infection, replication of the excluded phage DNA is only weakly limited, markers can be rescued easily from it, and it can provide most functions well. In the second stage, typified by the T2-T4 and T4-T6 mixed infections, replication of the excluded phage DNA is severely limited, but markers can still be rescued easily from it, and it can still provide most functions well. In the third stage, typified by T4-RB phage mixed infections, replication of the excluded phage DNA is still severely limited, and markers are rescued from it only with difficulty, but it still provides functions well. A fourth stage, in which the excluded phage fails to provide functions well, is not difficult to imagine, and mixed infections

between RB69 and the classical T-even phages approach this stage.

In this sequence, the excluded phage loses first the ability to replicate, then the ability to recombine with the excluding phage, and finally the ability to provide functions. A rationale for this order can be provided as follows.

The T-even phage species, like other species, demonstrates polymorphic diversity. This diversity generates advantages, for it allows the species to exploit a range of different environments, and disadvantages, for it leads to the production of poorly adapted variants which can constitute a serious genetic load; the extent of diversity within the species is undoubtedly determined by a balance between the two.

A new environment which is potentially exploitable by the species tends to shift this balance in favor of further diversity, but in order for this diversity to become established, the resulting genetic load must be reduced. This reduction can be accomplished by partial isolating mechanisms which permit the total species diversity to be redistributed among subspecies in such a way that poorly adapted variants are produced less frequently. According to this view, mutations within the species which tend to limit genetic exchange between the mutant individual and the rest of the species will be maintained as partial isolating mechanisms only if they permit an advantageous redistribution of the species diversity. A mutation which leads to a limitation on the replication of the excluded phage DNA fulfills the condition and therefore constitutes a workable first step in the evolution of exclusion.

After the redistribution has occurred, the extent to which the resulting subspecies recombine will reflect a balance between the advantages and disadvantages of the recombinants produced. Because of their partial isolation, the subspecies will tend to diverge; as they do so, the disadvantages will come to outweigh the advantages, and the capacity for recombination will gradually be lost. Finally, after sufficient divergence, the functions of the subspecies will be sufficiently different so that the excluded phage fails to perform functions for the excluding one.

This interpretation also provides a rationale for the locations of the exclusion sensitivity determinants. The limitation on DNA replication is imposed through localized determinants, and genetic exchange between the phages is most severely limited in the regions surrounding them; consequently redistribution of the diversity governed by genes in these regions will be relatively slow. Since the redistribution constitutes a selective advantage, systems will be favored in which the determinants fall in regions where redistribution is least important. Regions in which the initial degree of diversity is low are consequently likely locations for the determinants. The early regions probably fit this description. The products of the early genes operate in the relatively constant intracellular environment and thus are not subject to the selection for diversity which applies to genes determining the phage coat.

C. Exclusion in Other Bacteriophage Species

Whether the general model developed above applies to partial exclusion in other bacteriophage species is not immediately clear, since only two such cases have been studied in any detail. PB excludes its relative T5 rather strongly; a considerable fraction of the mixedly infected bacteria are mixed yielders, but only about 1-2% of the progeny of mixed yielders have the host range of T5 (26, 34). In these respects, this mixed infection resembles those between T4 and the newly isolated RB phages, and it can be accounted for by similar specific elaborations on the general model. Since progeny phages with the host range of T5 retain the exclusion sensitivity of T5 as well, even though they contain other PB markers (26), it is necessary to postulate an exclusion sensitivity determinant quite close to the host range marker in T5.

T7 excludes its relative Cro moderately strongly; 93% of the mixedly infected cells are mixed yielders, but the progeny derive more than 70% of their markers from the T7 parent (35). As in the classical T-even phage mixed infections, different markers from the excluded Cro parent are transmitted to the progeny with different efficiencies, in a way which suggests localized exclusion sensitivity determinants in the Cro genome. The Cro host range marker is transmitted with low efficiency, again suggesting linkage between the host range marker and an exclusion sensitivity determinant. The marker transmitted with the lowest efficiency from the Cro parent still appears among about 10% of the progeny, and most of the progeny bearing Cro markers are recombinants, showing that genetic exchange between T7 and Cro is extensive. In all these respects this

mixed infection resembles the T2-T6 case, and it can be similarly accounted for.

These cases of exclusion in other species can be interpreted in terms of the general model which emerged out of the study of T-even phages, but the critical experiments to decide whether this interpretation is the correct one remain to be done.

D. Analogies with Host Controlled Modification

Exclusion as an isolating mechanism can be compared with the host controlled modification of DNA which appears to operate as an isolating mechanism among the bacteria (131). Two studies on host controlled modification suggest a similarity between the restricted phage in a host modification system and the excluded phage in the T-even exclusion system. Drexler and Christensen (132) studied the rescue of markers from restricted T1 in mixed infection with nonrestricted T1; markers from the restricted phage appeared in only a small minority of the progeny, and different markers were transmitted with different efficiencies to the progeny. From the pattern of marker transfer, the restricting system appeared to operate on one or more determinants in the restricted genome, and the position of these determinants could be roughly inferred. Interestingly, one of the determinants fell relatively close to a host range marker.

Analogous experiments with restricted and unrestricted lambda phages were performed by Dussoix and Arber (133) who observed a polarity in the efficiencies with which different markers were transmitted from the restricted phage. The marker m_6, at the left end of the lambda map, was transmitted much less efficiently than were markers at the middle and on the right half.

These cases probably should not be taken as true analogs of the exclusion seen in T-even mixed infections, since the frequency of progeny carrying markers from the restricted phage is considerably lower than in

the exclusion case and there is evidence for the breakdown of the restricted DNA (134). However, they do demonstrate that the restricting system in host controlled modification recognizes and acts against localized determinants in restricted phage DNA, as does the T-even excluding system.

This similarity between host controlled modification and exclusion suggests that DNA methylation may play a role in specifying the exclusion sensitivity determinants. Although T2, T4, and T6 induce different levels of DNA-methylating enzymes (135), no experiments were done to test this possibility.

E. Summary

Tentative evolutionary relationships have been assigned to the T-even phages T2, T4, T6, and RB69. T2, T4, and T6 probably constitute one subspecies, within which T2 and T6 are somewhat more closely related to each other than to T4, and RB69 constitutes a distantly related subspecies. The tail fiber region differences between T2, T4, and T6 can be accounted for by selection for adsorption diversity.

A general model has been developed to account for the interactions of these phages in mixed infections; in this model, one phage acts against localized exclusion sensitivity determinants in the genome of the other, limiting its replication without affecting its ability to function. If the various interactions studied are treated as different stages in the evolution of exclusion as an isolating mechanism, then in this evolution the excluded phage loses first the ability to replicate, then the ability to recombine with the excluding phage, and finally the ability to provide functions. This sequence and the location of the exclusion sensitivity determinants can be rationalized by treating exclusion in terms of its effects on polymorphism within the T-even species as a whole.

The general model can account for cases of partial exclusion in the T5 and T3-T7 species. Analogies between exclusion and host controlled modification suggest that these two isolating mechanisms may operate in similar ways.

BIBLIOGRAPHY

1. Dobzhansky, T., "Genetics and the Origin of Species," 3rd edition, pp. 258 - 259, Columbia University Press, New York, 1951.

2. Dobzhansky, T., ibid., pp. 254 - 275.

3. Dobzhansky, T., ibid., pp. 179 - 180.

4. Dobzhansky, T., ibid., pp. 135 - 178.

5. Dobzhansky, T., ibid., pp. 180 - 211.

6. Dobzhansky, T., ibid., p. 187.

7. Dobzhansky, T., ibid., p. 183.

8. Meselson, M., and J.J. Weigle, "Chromosome breakage accompanying genetic recombination in bacteriophage," P.N.A.S. 47, 857 (1961).

9. Adams, M.H., "Criteria for a biological classification of bacterial viruses," Ann. N.Y. Acad. Sci. 56, 442 (1953).

10. Burnet, F.M., "The classification of dysentery-coli bacteriophages. II. The serological classification of coli-dysentery phages," J. Path. Bact. 36, 307 (1933).

11. Burnet, F.M., E.V. Keogh, and D. Lush, "Immunological reactions of filterable viruses," Australian J. Exptl. Biol. Med. Sci. 15, 227 (1937).

12. Adams, M.H., "Classification of bacterial viruses: characteristics of the T5 species and of the T2, C16 species," J. Bact. 64, 387 (1952).

13. Adams, M.H., and E. Wade, "Classification of Bacterial Viruses: the relationship of two Serratia phages to coli-dysentery phages T3, T7 and D44," J. Bact. 68, 320 (1954).

14. Adams, M.H., and E. Wade, "Classification of bacterial viruses: characteristics of the T1, D20 species of coli-dysentery phages," J. Bact. 70, 253 (1955).

15. Brodetsky, A.M., and W.R. Romig, "Characterization of Bacillus Subtilis bacteriophages," J. Bact. 90, 1655 (1965).

16. Ruska, H., "Uber ein neues bei der bakteriophagen Lyse auftretendes Formelement," Naturwiss. 29, 367 (1941).

17. Ruska, H., "Morphologische Befunde bei der bakteriophagen Lyse," Arch. ges. Virusforsch. 2, 345 (1942).

18. Luria, S.E., and T.F. Anderson, "The identification and characterization of bacteriophages with the electron microscope," P.N.A.S. 28, 127 (1942).

19. Luria, S.E., M. Delbrück, and T.F. Anderson, "Electron microscope studies of bacterial viruses," J. Bact. 46, 57 (1943).

20. Brenner, S., G. Streisinger, R.W. Horne, S.P. Champe, L. Barnett, S. Benzer, and M.W. Rees, "Structural components of bacteriophage," J. Mol. Biol. 1, 281 (1959).

21. Epstein, R.H., A. Bolle, C.M. Steinberg, E. Kellenberger, E. Boy de la Tour, R. Chevalley, R.S. Edgar, M. Susman, G.H. Denhardt, and A. Lielausis, "Physiological studies of conditional lethal mutants of bacteriophage T4D," Cold Spring Harbor Symp. Quant. Biol. 28, 375 (1963).

22. Kellenberger, E., "Morphogenesis of Phage and its Genetic Determinants," New Prespectives in Biology 4, 234 (1964).

23. Eiserling, R.A., and E. Boy de la Tour, "Capsomeres and other structures observed on some bacteriophages," Path. Microbio. 28, 175 (1965).

24. Delbrück, M., and W.T. Bailey, Jr., "Induced mutations in bacterial viruses," Cold Spring Harbor Symp. Quant. Biol. 11, 33 (1946).

25. Luria, S.E., Rec. Gen. Soc. Am. 18, 102 (1949).

26. Wasserman, R.D., "The inheritance of host range differences between the related phages T5 and PB," Virology 9, 425 (1959).

27. Hausmann, R.L., E.P. Almeida Magalhaes, and C. Araujo, "Isolation and characterization of hybrids between bacteriophages T3 and T7," Amais de Microbiologia 9, 511 (1961).

28. Schildkraut, C.L., K.L. Wierzchowski , J. Marmur, D.M. Green, and P. Doty, "A study of the base sequence homology among the T-series of bacteriophages," Virology 18, 43 (1962).

29. Delbrück, M., "Bacterial viruses or bacteriophages," Biol. Revs. Cambr. Phil. Soc. 21, 30 (1946).

30. Delbrück, M., and S.E. Luria, "Interference between bacterial viruses. I. Interference between two bacterial viruses acting upon the same host, and the mechanism of virus growth," Arch. Biochem. 1, 111 (1942).

31. Delbrück, M., "Interference between bacterial viruses. III. The mutual exclusion effect and the depressor effect," J. Bact. 50,151 (1945).

32. Streisinger, G., "The genetic control of ultraviolet sensitivity levels in bacteriophages T2 and T4," Virology 2, 1 (1956).

33. Streisinger, G., and J. Weigle, "Properties of bacteriophages T2 and T4 with unusual inheritance," P.N.A.S. 42, 504 (1956).

34. Fodor, A.R., and M.H. Adams, "Genetic control of serological specificity in bacteriophage," J. Immunol. 74, 228 (1955).

35. Hausmann, R.L., E.P. Almeida Magalhaes, and C. Araujo, "Intracellular compatibility of coliphages T3, T7, and Cro," Anais de Microbiologia 10, 43 (1962).

36. Hausmann, R.L., E.P. Alemida Magalhaes, and C. Araujo, "Serological properties of two related coliphages, T7 and Cro," Anais de Microbiologia 10, 35 (1962).

37. Burnet, F.M., "The classification of dysentery-coli phages III. Correlation of serological classification with certain biochemical tests," J. Path. 37, 179-184. (1933). (Serological group 11 contains the T-even phages.)

38. See section B.3. c of the Results.

39. See Adams, M.H."Bacteriophages," Interscience Publishers, New York 1959.

40. Epstein, R.H., and C.M. Steinberg, in preparation.

41. Edgar, R.S., and I. Lielausis, "Temperature-sensitive mutants of bacteriophage T4D: their isolation and genetic characterization," Genetics 49, 649 (1964).

42. Edgar, R.S., G.H. Denhardt, and R.H. Epstein, "A comparative genetic study of conditional lethal mutations of bacteriophage T4D," Genetics 49, 635 (1964).

43. Revel, H.R., S. Hattman, and S.E. Luria, "Mutants of bacteriophages T2 and T6 defective in α-glucosyl transferase," Biochem. Biophys. Res. Comm. 18, 545 (1965).

44. Russell, R.L., unpublished results.

45. Hill, R., personal communication.

46. Sagik, B.P., "A specific reversible inhibition of bacteriophage T2," J. Bact. 68, 430 (1954).

47. Stahl, F.W., J.M. Crasemann, L. Okun, E. Fox, and C. Laird, "Radiation sensitivity of bacteriophage containing 5-bromodeoxyuridine," Virology 13, 98 (1961).

48. Adams, M.H., "Bacteriophages," Appendix, Interscience Publishers, New York, 1959.

49. R.S. Edgar, personal communication.

50. Tomizawa, J., and N. Anraku, "Molecular mechanisms of genetic recombination in bacteriophage II. Joining of parental DNA molecules of phage T4," J. Mol. Biol. 8, 516 (1964).

51. Vinograd, J., R. Radloff, and R. Bruner, "Band-forming centerpieces for the analytical ultracentrifuge," Biopolymers 3, 481 (1965).

52. I am indebted to J. Vinograd for the use of this densitometer, and to Rebecca Kent and Robert Watson for instruction in its use.

53. I am indebted to A.J. Hodge for the use of this instrument. Several people, including Don Robberson, Alex Lielausis, Dorothy Hayes and S.K. Malhotra, helped me with the electron microscopy.

54. Anderson, T.F., "Morphological and chemical relations in viruses and bacteriophages," Cold Spring Harbor Symp. Quant. Biol. 11, 1 (1946).

55. Williams, R.C., and D. Fraser, "Morphology of the seven T-bacteriophages," J. Bact. 66, 458 (1953).

56. Moody, M.F., "The shape of the T-even bacteriophage head," Virology 26, 567 (1965).

57. Moody, M.F., personal communication.

58. Edgar, R.S., personal communication.

59. Russell, R.L., unpublished results.

60. Hershey, A.D., J. Dixon, and M. Chase, "Nucleic acid economy in bacteria infected with bacteriophage T2. I. Purine and pyrimidine composition," J. Gen Physiol. 36, 777 (1953).

61. Sinsheimer, R.L., "The nucleic acids of the bacterial viruses," in The Nucleic Acids, (E. Chargaff and J.N. Davidson, eds.), Vol. III, Academic Press, New York, 1960. p. 187.

62. Wyatt, G.R., and S.S. Cohen, "The bases of the nucleic acids of some bacterial and animal viruses: the occurrence of 5-hydroxymethyl-cytosine," Biochem. J. 55, 774 (1953).

63. Jesaitis, M.A., "Differences in the chemical composition of the phage nucleic acids," Nature 178, 637 (1956).

64. Sinsheimer, R.L., "Nucleotides from T2r+ bacteriophage," Science 120, 551 (1954).

65. Jesaitis, M.A., "Nucleic acids of T2, T4, and T6 bacteriophages," J. Exp. Med. 106, 233. (1957).

66. Lehmann, I.R., and E.A. Pratt, "On the structure of glucosylated hydroxymethylcytosine nucleotides of coliphages T2, T4, and T6," J. Biol. Chem. 235, 3254 (1960).

67. Kornberg, S.R., S.B. Zimmerman, and A. Kornberg, "Glucosylation of deoxyribonucleic acid by enzymes from bacteriophage-infected Escherichia coli," J. Biol. Chem. 236, 1487 (1961).

68. Zimmerman, S.B., S.R. Kornberg, and A. Kornberg, "Glucosylation of deoxyribonucleic acid. II. α-glucosyl transferases from T2- and T6-infected Escherica coli," J. Biol. Chem. 237, 512 (1962).

69. Burton, K., M.R. Lunt, G.B. Petersen, and J.C. Siebke, "Studies of nucleotide sequences in DNA," Cold Spring Harbor Symp. Quant. Biol. 28, 27 (1963).

70. Lunt, M.R., J.C. Siebke, and K. Burton, "Glucosylated nucleotide sequences from T2-bacteriophage deoxyribonucleic acid," Biochem. J. 92, 27. (1964).

71. Lunt, M.R., and E.A. Newton, "Glucosylated nucleotide sequences from T-even bacteriophage deoxyribonucleic acid," Biochem. J. 92, 59 (1964).

72. Wetmur, J. personal communication.

73. Puck, T.T., A. Garen, and J. Cline, "The mechanism of virus attachment to host cells: I. The role of ions in the primary reaction," J. Exptl. Med. 93, 65 (1951).

74. Wollman, E.L., and G.S. Stent, "Studies on actimation of T4 bacteriophage by cofactor: I. The degree of activity," Biochim. et Biophys. Acta 6, 292 (1950).

75. Stent, G.S., and E.L. Wollman, "Studies on activation of T4 bacteriophage by cofactor: II. Mechanism of activation," Biochim.et Biophys. Acta 6, 307 (1950).

76. Stent, G.S., and E.L. Wollman, "Studies on activation of T4 bacteriophage by cofactor: III. Conditions affecting the activation process," Biochim. et Biophys. Acta 6, 374 (1951).

77. Delbrück, M.,"Biochemical mutants of bacterial viruses," J. Bact. 56, 1 (1948).

78. Anderson, T.F., "The inheritance of requirements for adsorption cofactors in the bacterial virus T4," J. Bact. 55, 651 (1948).

79. Demerec, M., and U. Fano, "Bacteriophage resistant mutants in Escherichia coli," Genetics 30, 119 (1945).

80. Weidel, W., H. Frank, and H.H. Martin, "The rigid layer of the cell wall of Escherichia coli strain B," J. Gen. Microbiol. 22, 158 (1960).

81. Russell, R.L., unpublished results.

82. Doermann, A.H., "Lysis and lysis inhibition with E. coli bacteriophage," J. Bact. 55, 257 (1948).

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

84. Novick, A., and L. Szilard, "Virus strains of identical phenotype but different genotype," Science 113, 34 (1951).

85. Streisinger, G., "Phenotypic mixing of host range and serological specificities in bacteriophages T2 and T4," Virology 2, 388 (1956).

86. Weigert, M., and A. Garen, "Base composition of nonsense codons in E. coli," Nature 206, 992 (1965).

87. Brenner, S., personal communication.

88. Capecchi, M.R., and G.N. Gussin, "Suppression in vitro: identification of a serine-sRNA as a 'nonsense' suppressor," Science 149, 417 (1965).

89. Luria, S.E., "Mutations of bacterial viruses affecting their host range," Genetics 30, 84 (1945).

90. Luria, S.E., "Genetics of bacterium-bacterial virus relationship," Ann. Missouri Botan. Gard. 32, 235 (1945).

91. Hershey, A.D., "Spontaneous mutations in bacterial viruses," Cold Spring Harbor Symp. Quant. Biol. 11, 67 (1946).

92. Hershey, A.D., "Mutation of bacteriophage with respect to type of plaque," Genetics 31, 620 (1946).

93. Sekely, L.I., "Mapping experiments with bacteriophage T2L," Virology 12, 118 (1960).

94. Baylor, M.B., A.Y. Hessler, and J.P. Baird, "The circular linkage map of bacteriophage T2H," Genetics 51, 351 (1965).

95. Hershey, A.D., and R. Rotman, "Linkage among genes controlling inhibition of lysis in a bacterial virus," P.N.A.S. 34, 89 (1948).

96. Hershey, A.D., and R. Rotman, "Genetic recombination between host range and plaque-type mutants of bacteriophage in single bacterial cells," Genetics 34, 44 (1949).

97. Hershey, A.D., and H. Davidson, "Allelic and non-allelic genes controlling host range specificity in a bacteriophage," Genetics 36, 667 (1951).

98. Baylor, M.B., D.D. Hurst, S.L. Allen, and E.T. Bertani, "The frequency and distribution of loci affecting host range in the coliphage T2H," Genetics 42, 104 (1957).

99. Dobzhansky, T., op. cit. p. 110 - 114.

100. Patterson, J.T., and W.S. Stone, "Evolution in the genus Drosophila," pp. 183-230, The MacMillan Company, New York, 1952.

101. Stahl, F.W., R.S. Edgar, and J. Steinberg, "The linkage map of bacteriophage T4," Genetics 50, 539 (1964).

102. Edgar, R.S.and R.H. Epstein, personal communication.

103. Huskey, R.J., personal communication.

104. Bruner, R., and J. Vinograd, "The evaluation of standard sedimentation coefficients of sodium RNA and sodium DNA from sedimentation velocity data in concentrated NaCl and CsCl solutions," Biochem. Biophys. Acta 108, 18 (1965). (Table 1.)

105. Svedberg, T., and K.O. Pedersen, "The Ultracentrifuge," Clarendon Press, Oxford. 1940.

106. Vinograd, J., and J.E. Hearst, "Equilibrium sedimentation of macromolecules and viruses in a density gradient," Prog. Chem. Org. Nat. Prod. 20, 372 (1962).

107. Bendet, I.J., J.L. Allison, and M.A. Lauffer, "Structural basis for dual sedimentation of bacteriophage: Electron microscopy," Virology 6, 571 (1958).

108. Erikson, R.L., and W. Szybalski, "The Cs_2SO_4 Density Gradient and its application for the study of T-even phage DNA: Glucosylation and replication," Virology 22, 111 (1964).

109. Kellenberger, E., A. Bolle, E. Boy de la Tour, R.H. Epstein, N.C. Franklin, N.K. Jerne, A. Reale-Scafati, J. Sechaud, I. Bendet, D. Goldstein, and M.A. Lauffer," Functions and properties related to the tail fibers of bacteriophage T4," Virology 26, 419 (1965).

110. Edgar, R.S. and W.B. Wood, "Morphogenesis of bacteriophage T4 in extracts of mutant-infected cells," P.N.A.S. 55, 498 (1966).

111. Stahl, F.W., N.E. Murray, A. Nakata, and J.M. Crasemann, "Intergenic cis-trans position effects in bacteriophage T4," Genetics 54, 223 (1966).

112. Stahl, F.W., and N.E. Murray, "The evolution of gene clusters and genetic circularity in microorganisms," Genetics 53, 569 (1966).

113. Edgar, R.S., and I. Lielausis, "Serological studies with mutants of phage T4D defective in genes determining tail fiber structure," Genetics 52, 1187 (1965).

114. Wood, W.B., personal communication, and King, J.A., personal commun.

115. Mosig, G., "Coordinate variation in density and recombination potential in T4 phage particles produced at different times after infection," Genetics 48, 1195 (1963).

116. Mosig, G., "Genetic recombination in bacteriophage T4 during replication of DNA fragments," Cold Spring Harbor Symp. Quant. Biol. 28, 35 (1963).

117. Mosig, G. personal communication.

118. Womack, F.C., "Cross-reactivation differences in Bacteriophage T4D," Virology 26, 758 (1965).

119. Berger, H., "Genetic analysis of T4D phage heterozygotes produced in the presence of 5-fluorodeoxyuridine," Genetics 52, 729 (1965).

120. Streisinger, G., "The genetic control of host range and serological specificity in bacteriophages T2 and T4." Virology 2, 377 (1956).

121. de Groot, B., "Marker rescue of the adsorption properties of bacteriophage T4 by bacteriophage T2," Antonie van Leeuwenhoek J. Microbiol. Serol. 32, 17-24 (1966).

122. de Groot, B., "The genetic nature of the exclusion of bacteriophage T2 by T4 with mixed infection on E. coli B." Proc. XI Int. Congr. Genetics, The Hague, I: abstract 3.22.

123. de Groot, B., "Partial exclusion of bacteriophage T2 by T4: an early function of the second linkage group," Genetica 37, 37 (1966).

124. Levinthal, C., and N. Visconti, "Growth and recombination in bacterial viruses," Genetics 38, 500 (1953).

125. Frankel, F.R., "Studies on the nature of replicating DNA in T4-infected Escherichia coli," J. Mol. Biol. 18, 127(1966).

126. Hattman, S., and T. Fukasawa, "Host-induced modification of T-even phages due to defective glucosylation of their DNA," P.N.A.S. 50, 297 (1963).

127. Shedlovsky, A., and S. Brenner, "A Chemical basis for the host-induced modification of T-even bacteriophages," P.N.A.S. 50, 300 (1963).

128. Fukasawa, T., and Saito, S., "The course of infection with T-even phages on mutants of E. coli K-12 defective in the synthesis of uridine diphosphoglucose," J. Mol. Biol. 8, 175 (1964).

129. Herskowitz, I. and J.A. King, personal communication.

130. Russell, R.L., unpublished results.

131. Wood, W.B., "Host specificity of DNA produced by Escherichia coli: Bacterial mutations affecting the restriction and modification of DNA," J. Mol. Biol. 16, 118 (1966).

132. Drexler, H., and J.R. Christensen, "Genetic crosses between restricted and unrestricted phage T1 in lysogenic and non-lysogenic hosts," Virology 13, 31 (1961).

133. Dussoix, D., and W. Arber, "Host specificity of DNA produced by Eschericia coli, II. Control over acceptance of DNA from infecting phage lambda," J. Mol. Biol. 5, 37 (1962).

134. Lederberg, S., "Suppresssion of the multiplication of heterologous bacteriophages in lysogenic bacteria," Virology 3, 496 (1957).

135. Gold, M., R. Hausmann, U. Maitra, and J. Hurwitz, "The enzymatic methylation of RNA and DNA, VIII. Effects of bacteriophage infection on the activity of the methylating enzymes," P.N.A.S. 52, 292 (1964).