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DNA SYNTHESIS IN THE NONLETHAL RECOMBINATION REPAIR DEFICIENT X AND Y MUTANTS OF BACTERIOPHAGE T4

*City University of New York* PH.D. 1980

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# DNA SYNTHESIS IN THE NONLETHAL RECOMBINATION REPAIR DEFICIENT X AND Y MUTANTS OF BACTERIOPHAGE T4

by

ROBERT J. MELAMEDE

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1980

Robert Melamede

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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# LIST OF ABBREVIATIONS

DNA = deoxyribonucleic acid

Tdr = thymidine

dTMP = deoxythymidine monophosphate

dTTP = deoxythymidine triphosphate

BrdU = bromodeoxyuridine

## INTRODUCTION

The most basic of all homeostatic mechanisms is in all probability DNA repair. A critical balance must be maintained between the rate of mutation - the chemical foundation for evolution - and the need for stable genetic information to provide the base on which the change can occur. The essential nature of genetic information and its relative lack of redundancy make DNA particularly susceptible to the potentially damaging influences of the intracellular environment. Lethal and mutagenic hazards are many and varied. Heat, pH, chemicals and various forms of radiation are responsible for a continuous and unavoidable assault on the chromosomal material.

DNA damages may be divided into four basic categories: strand breakage, base alterations, sugar damages, and protein-DNA crosslinks (Cerutti, 1974). The effect that a given damage will have is largely determined by how fast and how efficiently the damage is repaired, if it is repaired. Historically, the first indications that a genetic repair process might exist are found in the early experiments of Hollaender and Curtis (1935). They found that ultraviolet light irradiation caused an extension of the lag phase in the growth of bacterial cultures. During this extended lag phase the irradiated cultures initially increased in viable counts, whereas the nonirradiated cultures did not. The significance of these findings is underscored by the earlier discovery that the action spectrum for ultra-

violet light killing of bacteria corresponded to the ultraviolet absorption spectrum of nucleic acids (Gates, 1928).

More concrete evidence for a heritable genetic repair mechanism was provided by the finding that the relative resistance to ultraviolet light demonstrated by bacteriophage T4 could be combined into the more sensitive T2 (Luria, 1947). Interestingly, the initial interpretation was that the wildtype state was one of hypersensitivity and therefore T4 was apparently lacking the u gene that accounted for this sensitivity (Streisinger, 1956). This erroneous interpretation was put to rest in 1963 when Harm (1963) showed that killed T4 could confer resistance to T2 during coinfection; yet when T2 was reexamined in the absence of the killed T4, it again demonstrated the sensitive phenotype.

As investigations into the u-repair process were proceeding, other workers were examining another apparent repair process known as photoreactivation (Kelner, 1949; Dulbecco, 1949). Kelner and Dulbecco's early studies showed that the damaging effects of ultraviolet light could be reversed by subsequent exposure to visible light.

Today the events of photoreactivation are known in great detail. Although this repair system is apparently limited to ultraviolet-induced pyrimidine dimers, it is simple and efficient. The photoreactivating enzyme binds to the pyrimidine dimer, and in the presence of visible light it absorbs a photon of the proper wavelength (510nm) resulting in the cleavage of the dimer and the release of

the enzyme (Ruppert 1962 a and b). This type of repair is very efficient and there is virtually no chance of error (Witkin, 1976). The widespread occurence of the photoreactivating enzyme throughout the spectrum of organisms demonstrates its evolutionary significance. Yet, its presence in extremely low concentrations, $10^{-5}$ of total protein extract (Ruppert, 1974), as well as its limited substrate specificity (Ruppert, 1960), clearly narrows the effects that this repair process can have on overall genetic maintenance.

The need for a more versatile DNA repair process has been met by excision repair. This enzymatic sequence can remove and replace a wide variety of damaged DNA sections following the initial incision (Setlow and Carrier, 1966). Despite potentially damaging events inherent in this repair process (both endo and exonucleoclytic activities), the restoration of genetic integrity occurs with a high degree of efficiency and fidelity (Witkin, 1966; Witkin, 1969). Incision presumably occurs on the 5' phosphate side of the damaged base, or at the 5' phosphate neighboring the damaged base as has been demonstrated with enzymes isolated from T4 (Friedberg and King, 1971), Micrococcus luteus (Kaplan et al., 1969) and Escherichia coli (Braun and Grossman, 1974). The nick that is produced serves as a substrate for 5' to 3' exonuclease action that in turn removes the damaged DNA section. In Escherichia coli the 5' to 3' nuclease activity of pol I and an independent

exonuclease, exonuclease VII, have been found to be capable of this reaction (Kelly et al., 1969; Cooper and Hanawalt, 1969; Glickman, 1974; Chase and Richardson, 1974). Pol I, II or III can repolymerize the gap that has been thus created. Upon completion of repair synthesis the nick that remains may be sealed by DNA ligase (Howard-Flanders and Boyce, 1966). Interestingly, the initial incision products are also substrates for ligase action. In Escherichia coli, the potential competition between the repair nuclease activity, and the prevention of this activity by ligation, has been eliminated by the apparent inhibition of ligase action by the uvrC gene product under repair conditions (Seeberg and Rupp, 1975).

The successful purification of many proposed excision repair enzymes has provided a means for reproducing some of the repair processes in vitro. The uvrA gene product has recently been purified from Escherichia coli (Braun et al., 1974). It is an endonuclease activity that acts specifically on ultraviolet light-induced DNA damages. This finding was no surprise as the uvrA mutants were isolated by virtue of ultraviolet light sensitivity and were shown to be incapable of dimer excision (Howard-Flanders et al., 1966). More recently, however, Seeberg (1978) has shown that the dimer-specific endonuclease associated with the uvrA gene requires both the uvrB gene product and ATP in order to function. The T4v gene product (Harm, 1961), is produced early during infection (Friedberg and King, 1971),

and codes for an ultraviolet-specific endonuclease, endonuclease V, (Friedberg and King, 1971; Yasuda and Sekiguchi, 1970).

Purified repair enzymes have been used to successfully restore the biological activity of damaged DNA (Hamilton et al., 1974). Ultraviolet-irradiated Bacillus subtilis DNA was incised with the Micrococcus luteus ultraviolet endonuclease. Subsequently, the 5'→3' exonuclease activity of Pol I excised an oligonucleotide which included the pyrimidine dimer. The latter enzyme was also used for the repolymerization of the removed nucleotides. The final ligation step was carried out by Escherichia coli polynucleotide ligase, and successful repair was measured by transformation. When transforming activity had been reduced as much as sixty percent by ultraviolet light irradiation, repair was observed. Higher doses created damages that could not be repaired, presumably because incisions within a critical distance lead to the production of double stranded breaks. Clearly, despite the apparent risks involved with excision repair, genetic restoration occurs with a high degree of accuracy in vitro.

Recently, additional endonucleases have been discovered that are specific for damages other than those induced by ultraviolet light. Enzymes apparently specific for apurinic DNA damages (Verly and Paquette, 1972), for x-ray-induced lesions (Strniste and Wallace, 1975), and for gamma ray-induced lesions (Hariharan and Cerutti, 1975) have been

described. How many of these various activities represent distinct enzymes has not been determined.

The vital nature of excision repair has been demonstrated not only on a molecular level, but also at the human clinical level. Xeroderma pigmentosum is a rare autosomal recessive disease which results in a very high susceptibility to ultraviolet light-induced skin cancers. All of the complementation subgroups found among individuals suffering from this disease have been characterized by an inability to excise pyrimidine dimers (Cleaver, 1968). The defect appears to be at the incision step (Setlow et al., 1970). Other diseases that have been associated with repair deficiency include Fanconi's anemia (Sasaki and Tonomura, 1973; Finkelberg et al., 1974; Poon et al., 1974), Ataxia telangiectasia (Higurashi et al., 1973) and progeria (Epstein et al., 1973).

Recombination repair was the third repair process to be elucidated. The precise molecular events that are responsible for this mode of repair have not yet been defined. Clearly this problem is a reflection of the difficulty that has been experienced in unraveling recombination itself. The ultraviolet light sensitivity of recombination deficient *Escherichia coli* and bacteriophage T4 mutants gave the first hints of a conservational aspect associated with genetic exchanges. Howard-Flanders first suggested that intact chromosomes could be constructed from ultraviolet light-damaged DNA in the absence of excision repair or

photoreactivation via the exchange of sister chromatids (Howard-Flanders, 1968). It was postulated (Howard-Flanders, 1968) that replication could proceed past a dimer but that when this occurred a gap was left opposite the dimer. In prokaryotes these gaps have been subsequently found to be between 500 and 1000 nucleotides in length (Iyer and Rupp, 1971). The correlation between the size of ultraviolet light-induced replication gaps and the size of Okazaki pieces resulting from the discontinuous mode by which DNA is synthesized (Sugimo and Okazaki, 1972), suggests that in fact the gap length may be determined by the distance from the dimer to the next initiation site for the subsequent Okazaki piece (Hanawalt, 1975). The gap with its associated free ends and single-stranded region has been implicated in the initiation of recombination (Rupp et al., 1971). The efficiency with which recombination is induced by this physical condition is remarkably high. Rupp et al., (1971) have shown, using a density label as a DNA marker, that one recombinant molecule can be detected for every dimer beyond which replication has proceeded. Along similar lines, Shahn (1968) has shown that ultraviolet irradiation increases the number of fragments from irradiated parental phage that could be recovered in progeny from unirradiated phage during mixed infection.

Further confirmation of the recombinational repair model is seen when the effects of bifunctional alkylating agents such as mitomycin and psoralen are considered.

Recombinational repair seems to be initiated after exposure to these drugs by a double cleavage on one strand of DNA such that the damaged base on that strand is excised. The damaged nucleotide, however, remains attached to its complement by the bifunctional agent. Once the recombinogenic gap has led to genetic exchange, the other damaged strand may be repaired by conventional excision repair (Cole, 1973).

The complementary action of excision and recombination in repairing DNA damages caused by bifunctional alkylating agents has been nicely demonstrated in bacteriophage lambda (Howard-Flanders *et al.*, 1974). Free phage were treated with psoralen plus light and then the damaged phage were used to infect bacteria that were carrying an untreated homoimmune phage. Recombination frequencies were found to be dependent on the presence of the *uvrA*/*uvrB* gene products.

Additional complexities of the recombinational repair process are revealed upon examining the mutational subgroups that affect recombination and repair in *Escherichia coli*. The *recA* function appears to play a crucial role in recombinational repair in *Escherichia coli* (Howard-Flanders and Theriot, 1966; Clark and Margulies, 1965). A number of phenotypes have been associated with *recA* mutants. Some of the obvious ones include sensitivity to ultraviolet light and to x-irradiation (Clark and Margulies, 1965; Howard-Flanders and Theriot, 1966), the "rec less" degradation of DNA after ultraviolet irradiation, (Clark

and Chamberlin, 1966) and the inviability of mutants that lack both the recA and DNA polymerase I activities (Monk and Kinross, 1972).

The recBC mutations affect the same pathway as those of recA. The recBC mutants, however, exhibit less of an effect on recombination and repair than do the recA mutants (Howard-Flanders and Boyce, 1966). The recBC mutants appear to lack a multifunctional nuclease, exonuclease V (Tomizawa and Ogawa, 1972). The complexity of this enzyme has tended to compound rather than simplify the formulation of a working model for recombinational repair. An ATP-stimulated exonuclease activity that acts on both single and double stranded DNA, an ATP-stimulated endonuclease activity that acts on closed single stranded circles, and a DNA-dependent ATPase activity have been attributed to this enzyme (Oishi, 1969; Goldmark and Linn, 1970). The recBC pathway is dependent on the recA gene product (Hori and Clark, 1973), as well as exonuclease I (Kushner et al., 1971). The latter enzyme is coded for by the sbcB locus (Kushner et al., 1971, 1974; Yajko, 1974). When exonuclease I is missing and the recBC pathway is not functioning, another recA dependent pathway, that of recF, can be detected (Hori and Clark, 1973). Supportive evidence for this recently discovered pathway is found by analyzing the ultraviolet light survival curves of these mutants. The recBCrecF double mutants exhibit additive sensitivities when compared to the single mutants (Brendel and Hayes, 1973).

Recently a novel form of repair that is dependent on the *recA* gene product has been reported. It appears that single strand gaps that are not repaired by the other repair processes lead to the induction of what has come to be known as the SOS repair pathway (DeFais *et al.*, 1971; Radman, 1975, and Witkin and George, 1973). Although of minor importance in terms of the amount of repair attributed to this pathway, all ultraviolet light-induced mutagenesis in *Escherichia coli* and bacteriophage lambda appears to be due to the error generating events inherent in SOS repair (Witkin, 1976). The nature of the biochemical processes that are responsible for this repair pathway are unknown, however, a "long patch" excision repair and a chloramphenicol sensitive postreplicative repair seems to be involved (See Witkin 1976 for review).

The precise biochemical function(s) of the *recA* gene product remain unclear. The universal involvement of *recA* in all forms of recombination found in *Escherichia coli* has pointed up the need to understand what the *recA* gene product does. Recently there has been some success along these lines. McEntee *et al.* (1976) have been able to purify the *recA* protein using a radiochemical assay and polyacrylamide gels. Little and Kleid (1977) have identified the *recA* protein as being the X protein that is induced by various factors that damage DNA. Additional evidence has recently demonstrated that the *tif* mutation, which yields constitutive synthesis of the SOS functions,

is the result of an altered recA gene product (Gudas and Mount, 1977). Perhaps most interesting is the finding that the recA protein inactivates phage λ repressor via proteolytic cleavage (Roberts et al., 1978) thus explaining the role of the recA protein in prophage induction and providing a model for its other functions.

The aim of this investigation is to help define recombinational repair in bacteriophage T4. The obligatory intermeshing of replication and recombination in T4 is evidenced by the many mutants that affect both processes (Epstein et al., 1963). The details that are known with respect to DNA replication in T4 and the link to recombination suggests that recombinational repair in T4 may in fact be more complicated than that found in *Escherichia coli*.

T4 DNA replication begins soon after infection. Kozinski has detected DNA synthesis as early as 2 minutes after infection using equilibrium density gradient centrifugation analysis (Kozinski and Kozinski, 1967). A protein-directed attachment of DNA to membrane has been convincingly demonstrated with the use of specific isotopic labels for DNA or membrane by 3-4 minutes after infection (Miller, 1972). By 5-6 minutes after infection the additional of BrdU creates a shift in density of the parental DNA (Carlson, 1974). At this stage of development, electron microscopic experiments have demonstrated that DNA initiation occurs at internal sites on the parental

molecule and proceeds bidirectionally from several initiation points to produce eye forms with displaced 3' ends extending as whiskers (Delius _et al._, 1971). At 7-8 minutes after infection in the presence of a density label true hybrids are found, that is, there are no conserved parental molecules and no complete progeny (Emanuel, 1972). DNA examined on neutral sucrose gradients after 7-8 minutes of infection is found to be approximately 1/5 the size of the parental molecules (Miller, 1975). Interestingly, this is the size that corresponds to the distance between initiation points (Miller, 1975).

After 8 minutes of infection parental DNA acquires single-stranded nicks (Kozinski and Kozinski, 1967) which is apparently the first step in the T4 recombination process. (This nicking also coincides with the transition to late gene transcription). Nicking is followed by the formation of gaps created by the nuclease activity that is directed by genes 46 and 47 (Prashad and Hosoda, 1972). In the presence of DNA binding protein, the gaps allow for the formation of noncovalently linked recombinant molecules (Tomizawa _et al._, 1966). The noncovalent "joint" molecules are converted to true recombinants by T4-induced DNA polymerase and polynucleotide ligase (Anraku and Lehman, 1969). It is the latter step that is presumably responsible for the formation of concatemeric lengths of single stranded DNA.

For normal phage packaging to occur, the concatemers

must be chopped into phage lengths. Since the amount of DNA in T4 bacteriophage is longer than necessary to include a single copy of each T4 gene, the cutting into headful lengths generates a terminally redundant, circularly permuted genome (Streisinger, 1956). Mutations in gene 49 lead to an abnormally large accumulation of fast sedimenting DNA molecules (Frankel et al., 1971) suggesting that the gene 49 protein is responsible for cutting the DNA into headful lengths. Extracts from these mutants are found to be lacking an endonuclease activity.

There are two classes of mutants in T4 that affect recombination. Mutants that appear to involve the replication process directly are lethal and result in either no DNA synthesis, as is the case with mutants in genes 30, 32, 41, 43, 44, 45 and 62 (Epstein et al., 1963). Mutants that result in an abrupt cessation of synthesis early in the infective cycle are known as DNA arrest mutants (Epstein et al., 1963), and include genes 46 and 47 (Epstein et al., 1963). The nonessential genes (not lethal) that affect recombination include genes x (Harm, 1964), y (Boyle and Symonds, 1969), 1206 (Maynard-Smith and Symonds, 1973), w and 58 (Hamlett and Berger, 1975).

The accumulation of biochemical and genetic evidence has revealed aspects of the roles played by the essential gene products in T4 development. For example, the gene 32 protein binds in stoichiometric amounts to single-stranded DNA and appears to aid in denaturing the DNA ahead of the

replicating fork (Alberts and Fry, 1971). The gene 32 protein is also apparently capable of a direct interaction with T4 DNA polymerase (Huberman et al., 1971). Additional insights into the nature of the gene 32 activity has been elegantly demonstrated by Mosig and Bock (1976). They found that the C-terminal domain of the protein protects T4 DNA from degradation by the nuclease activity that is controlled by genes 46 and 47. The protective ability of the binding protein was clearly demonstrated by the relative lack of DNA degradation in double mutants of genes 32 and 46. Interestingly, in the absence of genes 32 and 46, as well as the host recBC genes, no DNA degradation was detectable.

The above findings also help to clarify to some extent the vital role played by the geres 46 and 47 nuclease activity. The enzyme activity controlled by genes 46 and 47 is multifunctional. It consists of a nuclease activity that is responsible for the breakdown of host DNA and thus provides DNA precursors for the developing phage via salvage pathways(Wiberg, 1966). The nuclease activity that is controlled by the 46-47 gene products also is necessary for the normal developmental processing of T4 DNA (Shalitin and Kaban, 1970). The precise molecular events involved in development are unknown. In the absence of the gene 46 and 47 product, phage DNA synthesis abruptly ceases about 12 minutes after infection (Epstein et al., 1953; Shah and Berger, 1971).

Sucrose density gradient analysis of the DNA in mutant-infected cells indicate that they do not form normal concatemeric DNA (Shalitin and Naot, 1971). In addition, the DNA from these mutant-infected cells seems to separate prematurely from membrane complexes (Shah and Berger, 1971; Hosoda et al., 1971). This latter finding, however, may be inaccurate, since the technique (M-band isolation) from which the information was derived, has since been found to be less specific than originally supposed (Kemper and Janz, 1976). Interestingly, the addition of chloramphenicol to cells infected with gene 46 and 47 mutants early infection can reverse the DNA arrest phenotype. Most other characteristics that are associated with these mutants, are however, not reversed (Hosoda et al., 1971; Shah and Berger, 1971; Shalitin and Naot, 1971; Wu et al., 1972).

Mutants of gene 59 are also of the DNA arrest type (Wu et al., 1972). They exhibit the same phenotypes with respect to phage DNA synthesis as mutants of genes 46 and 47, but they map at a different location (Epstein et al., 1963). Despite many similarities between gene 46 and 47 mutants and the gene 59 mutants, these DNA arrest mutants apparently act along different pathways. The multiple mutants exhibit cumulative effects with respect to recombination. As was the case with mutants of genes 46 and 47, the addition of chloramphenicol early in infection can prevent the DNA arrest phenotype. Rescue of DNA synthesis by this technique does not increase recombination or repair

of damages induced by ultraviolet light or alkylating agents (Wu et al., 1972).

Gene 58 mutants are of the DNA delay type and exhibit increased recombination (Yegian et al., 1970). Mutations in gene 58 apparently cause a defect that involves the same pathway as the x and y mutants (Hamlett and Berger, 1975). The complexities that have hindered an understanding of the molecular events of recombination are seen upon examining the sedimentation profiles of gene 58 or T4w mutants (Hamlett and Berger, 1975), since despite the obvious differences between these two phage types with respect to recombination and to patterns of DNA synthesis, they both exhibit abberant formation of concatemeric DNA lengths. The DNA of the w mutants is slightly shorter than mature phage length when examined on alkaline sucrose gradients (Hamlett and Berger, 1975), while the single stranded DNA lengths found in cells that are infected with gene 58 mutants are even shorter than those found in cells infected with the w mutants. Unlike the w mutants, the gene 58 mutants are able to very slowly form longer single stranded DNA lengths and by 40 minutes after infection there are indications that some concatemers have formed (Hamlett and Berger, 1975).

The DNA synthetic properties of T4x and T4y have not been thoroughly investigated. The point mutant T4x was the first nonlethal recombination deficient mutant to be isolated (Harm, 1963). Boyle and Symonds subsequently isolated

T4*y* on the basis of its ultraviolet sensitivity (1969). The lack of additive ultraviolet sensitivities in the double *xy* mutant first suggested that these two mutants were involved in a common repair pathway. This pathway was also shown to be distinct from the v-gene pathway, since the *xv* and *yv* double mutants are more ultraviolet sensitive than are the single mutants.

Similar investigative reasoning led Maynard-Smith and Symonds to examine functional survival of ultraviolet light-irradiated genes involved in DNA synthesis in the presence or absence of the *y* gene (1973). The results indicated that all the genes needed for DNA synthesis are also required for *y*-type repair. In other words, early gene survival after ultraviolet light-irradiation was independent of the *y* gene. These results, however, must be interpreted with caution. They are only valid if the *y* gene product functions prior to the expression of the other genes being tested. There is no evidence to show whether or not this is the case.

In addition to ultraviolet light sensitivity and recombination deficiency, T4*x* (Harm, 1964) and T4*y* (Boyle and Symonds, 1973) have been shown to be sensitive to gamma rays (Boyle and Symonds, 1969), and x-rays (Wallace and Melamede, 1972). T4*x*, *y* and *w* are also sensitive to alkylating agents (Drake *et al*., 1973, Hamlett and Berger, 1975). Both the DNA synthesizing ability and burst size of T4*x* has been found to be sensitive to mitomycin C

(Shimizu and Sekiguchi 1974). A modified form of excision repair, as has been found in *Escherichia coli*, may also be acting on mitomycin-induced damages in T4. Genetic analysis has shown that the repair mediated by *x*, *y* and 1206 is error prone (Drake, 1973) and readily leads to mutation induction.

Clearly, despite the many phenotypic traits associated with x-y repair, there is a tremendous void with respect to knowledge of the physical factors that are responsible for these traits. In recent work, Wakem and Ebisuzaki (1976) have shown that the *x* and *y* gene products are necessary for normal concatemer production. These observations do indicate the x-y pathway as a postreplicative recombination repair process. It may, in fact, be analogous to the rec pathway in *Escherichia coli*. Whether or not the analogy is accurate remains to be seen. There are many fundamental differences in the replicative processes of T4 versus *Escherichia coli*. The intimate involvement of recombination in T4 DNA synthesis is the most obvious one.

Recently, Dewey and Frankel (1975) have isolated a class of mutants that are capable of suppressing the lethality associated with gene 49 mutants. In addition, these suppressors eliminate the fast sedimenting DNA that is characteristic of gene 49 mutants. Hence, the suppressors were named *fds* or fast sedimenting DNA suppressors. Interestingly, the nuclease activity that is lacking in gene 49 mutants is not restored by the *fds* suppressors.

More interesting, however, are the map locations of the two fds mutants thus far isolated. FdsA is located between genes 41 and 42; the same place that T4x maps. FdsB is located between genes 24 and 25; the same place that T4y maps. Conkling and Drake have now shown that fdsA and fdsB are in fact the same as T4x and T4y (personal communication).

In conclusion, it may be seen that although the x and y gene products are not necessary for successful T4 replication, these proteins do appear to play a role in the normal processing of T4 DNA. It is possible that neither T4x nor T4y is completely devoid of its wildtype activities, hence they both appear to determine non-essential gene products. This possibility does not seem likely, especially since T4y is an amber mutant and is not as likely to produce a partially functioning protein as is T4x, which is a non-terminating point mutant. The roles that the x and y gene products play in T4 development are poorly understood. The elucidation of how and when these proteins function is the focus of the investigation to be presented.

In the initial experiments, the involvement of the x and y gene products in T4-directed DNA synthesis and the effects of DNA-damaging agents (ultraviolet light, x-rays, and mitomycin C) on DNA synthesis in mutant-versus wild-type-infected cells were studied. The reduced mutant-directed DNA synthesis that could be demonstrated under appropriate conditions then served as an assay for the

presence or absence of the _x_ and _y_ gene products.

The use of rifampin and chloramphenicol which inhibit transcription and translation, respectively, provided a means by which these activities could be examined for the _x_ and _y_ gene products. The phenotypes of the _x_ and _y_ mutants that were derived from the above experiments were subsequently correlated with the physical state of the T4-induced DNA via the use of both neutral and alkaline sucrose sedimentation analysis. In all, the experiments provide insight into the nature of T4-directed DNA synthesis, recombination, and repair suggesting some possible functions for the _x_ and _y_ genes.

## MATERIALS AND METHODS

### Bacteria and bacteriophage

*Escherichia coli* B and T4$^{+}$ (T4D) were originally supplied by A. Doermann. T4v, derived from T4D, was obtained from W. Harm; T4y, derived from T4D, was obtained from J. Boyle; and T4px, which was originally isolated as T4x by W. Harm from T4D, and extensively backcrossed to T4B by J. Drake, was obtained from him.

### Chemicals

Methyl-$H^3$thymidine (63.8 ci/m mole) and thymine 43 Ci/m mole was purchased from ICN. Mitomycin C, rifampin and chloramphenicol were purchased from Calbiochem and fresh aqueous solutions of the antibiotics were prepared before each experiemnt (usually 1 mg/ml). Concentrations of mitomycin C were determined by using an $E^{1\%}_{360nm}$ of 740 (Merck Index 1976). Mitomycin C experiments were performed under dim light due to the light sensitivity of this chemical.

### Media

Slants for bacterial cultures contained per liter final volume: 10 grams Bacto tryptone, 8g Bacto agar, 3g glucose, 8g NaCl, 2g sodium citrate. Overnight cultures were prepared in Hershey broth (H broth) that contained in grams per liter of final volume: 8 nutrient broth, 5 Bacto tryptone, 1 glucose, 5 NaCl. Diluting fluid contained in grams per liter final volume: 5 Bactotryptone, 5 NaCl and 2 $K_2HPO_4$. Plating agar contained in grams per liter final

volume: 13 Bacto tryptone, 12 Bacto agar, 1.3 glucose, 8 NaCl, 2 sodium citrate. Top layer agar contained in grams per liter final volume: 10 Bacto tryptone, 6 Bacto agar, 3 glucose, 8 NaCl, 2 sodium citrate. Supplemented M9 media contained per liter final volume: 100 ml 10x M9 salts, 40 ml of 20% (W/V) glucose, 10 ml 0.1 M $MgSO_4$, 10 ml 0.01 M $CaCl_2$, and 50 ml of 20% (W/V) Difco casamino acids. 10x M9 salts contained in grams per liter final volume: 60 $Na_2HPO_4$, 30 $KH_2PO_4$, 5 NaCl, and 10 $NH_4Cl$. Phosphate gelatin buffer (gel buffer) contained in grams per liter of $H_2O$: 3 $Na_2HPO_4$, 1.5 $KH_2PO_4$, 5 NaCl, 5 $K_2SO_4$, 0.25 $MgSO_4$, 7 $H_2O$, 0.01 gelatin, and 0.013 $CaCl_2$ that was added after autoclaving. (Adams, 1972).

Preparation of bacteria and bacteriophage

Overnight cultures of *Escherichia coli* B were prepared by incubation at 37°C with aeration in H broth. Daily cultures were prepared by a 100 fold dilution of the overnight stationary phase cultures into supplemented M9 medium. The bacteria were then incubated at 37°C with aeration until they reached a concentration of 2 x $10^8$/ml.

Bacteriophage stocks were prepared by eluting confluent plates and subjecting the lysates to differential centrifugation. The phage stocks were stored in gel buffer. For some experiments, phage were further purified by sucrose gradient sedimentation.

Lysis procedure

Cells were lysed for both neutral and alkaline sucrose

gradients by adding to the 0.3 ml reaction volume an equal volume of lysis mix, and immediately icing the mixture after gentle hand mixing. The 0.3 ml lysing mix contained 2 mg/ml of freshly prepared lysozyme and 0.05 M EDTA. After 15 minutes on ice, 0.066 ml of 10% Brij 58 was added (Barry et al., 1973). The samples were left on ice for an additional 10 minutes before loading onto the sucrose gradients.

Sucrose gradients

Neutral 5-30% sucrose gradients contained 0.02 M Tris HCL pH 8, 0.1% sarkosyl, 1 mM EDTA, and 0.15 M NaCl. Gradients (4.6 ml) were made three at a time using an LKB Multiperpex pump. The gradients were formed on top of a 0.4 ml pad that contained 20% sucrose dissolved in Angio-Conray (Malinkrodt). 0.2 ml samples were loaded along with 0.02 ml of $^{14}C$ labeled intact marker phage which sediments at 1000S. The gradients were spun for either 30 minutes or 1 hour, as indicated in the figure legends, in a Beckman Model L ultracentrifuge at 17,000 rpm. Either a six bucket Sw 50.1 or a three bucket SW 50L rotor was used (Hosoda et al., 1971).

Alkaline sucrose gradients were prepared as above, but contained 5-20% sucrose, 0.01 M EDTA, 1 M NaCl, and 0.1% sarkosyl over the Angio-Conray pad. The gradients contained enough NaOH to give a pH of 12.4. One tenth ml of 0.35 M NaOH was layered on the gradient, followed by the addition of 0.02 ml $^{14}C$ marker phage and 0.2 ml sample.

Alkali lysis of intact phage produces intact single stranded phage lengths of DNA. The sample layer was then gently mixed with a disposable pipet tip. The gradients were centrifuged in the same rotors as used for the neutral gradients and spun for 2 hours at either 30,000 or 35,000 rpm as indicated in the figure legends (Hosoda *et al.*, 1971). S values were determined by $\frac{D_1}{D_2} = \frac{S_1}{S_2}$ (D = distance).

Irradiation

For ultraviolet irradiation, a 30 ul phage suspension in gel buffer (0.5 to 1 x $10^{11}$/ml) was irradiated by a General Electric 15 W germicidal lamp (G 1578), powered through a Sola constant-voltage regulator, and filtered through a quartz-glacial acetic acid filter. The dose rate determined as described previously was 0.35 $Jm^{-2}$/second. (Wallace and Melamede, 1972). Under the above conditions, the $D_{37}$ for T4+ is 8 $Jm^{-2}$.

For X-irradiation, a 200 ul phage suspension in 5X citrate broth (1 to 5 x $10^{10}$/ml) was irradiated by a Picker X-ray source with a beryllium window. A setting of 60 kVp and 20 mA delivered a dose rate of approximately 90 Krads/min. as determined by ferrous sulfate dosimetry. Under the above conditions, the $D_{37}$ for T4+ is 45 Krads.

Incorporation of Labeled DNA Precursors

Log phase *Escherichia coli* (0.2 ml) at a concentration of 4 x $10^8$/ml were added to 4 ul of 1 x $10^{11}$/ml phage. All reaction mixes were brought up to final 0.3 ml volumes with

M9 medium. Labeling was carried out at a final concentration of 0.12 ug/ml or 25 ug/ml thymidine, or 25 ug/ml thymine. At the low thymidine concentrations, phage were adsorbed at 37°C and transferred to 20°C 1 minute prior to labeling (33 uCi $^{3}H$/0.12 ug thymidine/ml or 33 uCi $^{3}H$/0.16 ug thymidine/ml). After a 4 minute adsorption period, incubation was continued at 37°C.

In each of the above experiments, 20 ul samples collected on Whatman GFA filters. They were immediately precipitated batchwise in 10% trichloracetic acid (TCA), washed batchwise with 5% TCA, and then with 95% ethanol. After drying samples were counted in the scintillation fluid described below.

In experiments involving the use of rifampin, chloramphenicol, or mitomycin C, the drugs were added at the time and concentration indicated in the text.

Sample collecting and counting

Samples were collected from the bottom of the gradients by means of capillary tubes that were lowered into the centrifuge tubes. Fractions were collected from three gradients simultaneously onto 2.4 cm Whatman GF/A disks. The disks were then stacked in beakers of 10% TCA, and filtered in groups of approximately 20. The filters were first washed with 2 ml of 5% TCA followed by 15 ml of ethanol. The disks were then dried in a 37°C oven overnight and counted the following day using an ambient temperature scintillation counter made by Beckman. The

scintillation fluid used was either Aquasol (New England Nuclear) or Liquiscint (National Diagnostics).

## RESULTS

A number of the phenotypes which are associated with T4*x* and T4*y* mutants including decreased recombination frequency, decreased burst size and increased sensitivity to ultraviolet light could be due to abnormalities in the DNA metabolism of these mutants. Furthermore, the genetic correlation and temperal coincidence of DNA synthesis and recombination during T4 infection suggests a mutual interdependence. In order to find the suspected involvement of the *x* and *y* proteins in T4-directed DNA synthesis, thymidine incorporation was examined in wildtype- and mutant-infected cells.

### DNA synthesis measured under conditions of linear incorporation

DNA synthesis in T4+-, T4*x*-, and T4*y*-infected cells was measured by the incorporation of tritiated thymidine into the acid-insoluble fraction. Figure 1A shows that the rate of incorporation of this label is reduced in T4*x*- and *y*-infected cells to about 70% that of wildtype. Similar relative rates of incorporation were obtained for T4*x* using $^{3}H$ thymine (Figure 1B). We have consistently observed a 20 to 30% reduction in the DNA synthetic rate in the mutant-infected cells compared to wildtype infected cells when incorporation was measured with labeled thymidine or thymine at concentrations of 15 ug/ml or greater.

Because of the role of the x-y pathway in DNA repair, the DNA synthetic capacity of T4-infected cells after ultra-

violet or x-irradiation to the phage was examined. Both ultraviolet and x-irradiation produce a lag and a reduced final rate of DNA synthesis as measured by the incorporation of high concentrations of thymidine (20 ug/ml) in both wildtype- and mutant- (including T4v) infected cells (Figures 2 and 3). When the final slopes of the linear portions of the DNA synthesis curves obtained by measuring incorporation of $^{3}$H-thymidine, are compared at different doses, the results shown in Figure 4A and B are obtained. The rate of DNA synthesis induced by T4v is ultraviolet-inactivated (Figure 4A) at a slightly greater rate than that of wildtype or T4x; while that of T4y is inactivated at a lower rate. Shimizu and Sekiguchi (1974) observed that DNA synthesis in T4x-infected cells was sensitive to ultraviolet light. However, they were comparing DNA synthesis after irradiation to zero dose T4x controls which showed no synthetic difference from wildtype cells. In contrast, in the present studies, the zero dose controls already exhibited a 30% decreased synthetic rate compared to wildtype-infected cells. Thus, the discrepancy appears to lie in the measurement of DNA synthesis, not in relative ultraviolet sensitivity.

It might be expected that the kinetics of label incorporation into DNA after irradiation are complex. After irradiation, excision repair synthesis should precede replicative synthesis, and the recombinational repair component of the latter might be expanded. The total

measurement would include all three. Since $\underline{v}^+$-induced excision repair appears to occur prior to replicative synthesis (Sato and Sekiguchi, 1976), one might expect the increased ultraviolet sensitivity of T4$\underline{v}$ DNA synthesis as has been observed here and elsewhere (Ebisuzaki, 1966). On the other hand, if excision repair is expanded in the absence of x-y recombinational repair, one might expect a decreased radiosensitivity of DNA synthesis for the $\underline{x}$ and $\underline{y}$ mutants as was shown above.

Figure 4B demonstrates that the DNA synthetic capacities of T4$\underline{x}$ and T4$\underline{y}$ appear to be slightly less sensitive to inactivation by x-irradiation than wildtype. Thus, as found after ultraviolet irradiation, the absence of recombinational repair makes DNA synthesis more resistant to x-irradiation.

Figure 4C shows the effect of mitomycin C, a bifunctional alkylating agent (Iyer and Szybalski, 1963), on the DNA synthetic capacity of T4$^+$-, T4$\underline{x}$-, and T4$\underline{y}$-infected cells. It should be noted that in contrast to the above experiments where the free phage are irradiated, in this case phage-bacterium complexes were exposed to mitomycin C throughout infection. Although this drug affects wildtype DNA synthesis at higher doses, it is a more potent inhibitor of T4$\underline{x}$- and $\underline{y}$-induced DNA synthesis. Further, Figure 5 shows that T4$\underline{x}$ and $\underline{y}$ are also more sensitive to mitomycin C when viability is measured. Shimizu and Sekiguchi (1974) have shown T4$\underline{x}$-induced DNA synthesis and average

burst size to be sensitive to mitomycin C. It is interesting to note that the original studies of Sekiguchi and Takagi (1960), which compared the sensitivity of DNA synthesis to mitomycin C of uninfected cells to T4-infected cells, showed that although DNA synthesis in T4-infected cells was resistant to mitomycin C compared to host DNA synthesis, the resulting phage were not viable.

DNA synthesis measured by high specific activity pulses

In order to pinpoint more clearly the time during the infective cycle at which the alteration in DNA synthesis in T4*x*- and *y*-infected cells appears, high specific activity thymidine was administered at various times after infection, and the acid-insoluble fraction measured. Cells were infected at 37°C, placed at 20°C one minute before addition of $^{3}$H thymidine, and subsequently sampled every fifteen seconds. The results of such an experiment are shown in Figure 6. As can be seen, incorporation of label proceeds at the same rate in T4+-, T4*x*-, and T4*y*-infected cells until 12 minutes after infection at which time incorporation in the mutant-infected cells decreases dramatically. This phenomenon was more closely examined in cells infected and maintained at 20°C. For example, Figure 7 shows that incorporation of label at 20°C in wildtype-infected cells continues to increase at least until 60 minutes after infection, while in T4*x*- and T4*y*-infected cells the rate of incorporation increases until 45 minutes after infection and then decreases by 60 minutes after

infection. Figure 8 shows the incorporation of tritiated thymidine (65 Ci/mmole, 0.12ug/ml, 37°C) in $T4^+$-, T4x-, and T4y-infected cells that are either amber suppressor positive (*E. coli* CR63) or amber suppressor negative (*E. coli* B). Clearly, the amber y phenotype is suppressed in *E. coli* CR63, whereas $T4^+$ and T4x are unaffected. The reason that suppressed T4y synthesis is greater than $T4^+$ is unknown, however, this phenomenon was repeatedly observed.

The large difference in incorporation of high specific activity label between wildtype- and mutant-infected cells fifteen minutes after infection at 37°C does not appear to reflect a difference in the amount of intracellular label but in the amount of incorporated label, as can be seen in Figure 9A. In these experiments, total cellular counts were measured after filtering the complexes through millipore filters, and acid-insoluble counts were determined in the usual manner. The difference between these two numbers was interpreted as reflecting the amount of soluble label. The latter amounts are approximately the same for $T4^+$-, x-, amd y-infected cells.

Apparently, wildtype-infected cells incorporated labeled thymidine very efficiently as compared to x-infected cells at the high specific activity (low thymidine concentrations) used in these experiments. When the labeled thymidine is diluted by cold thymidine in a manner which more closely resembles conditions used for linear

synthesis, the difference between wildtype and *x* incorporation is less pronounced (Figure 9B). Clearly, during T4 replication nucleotide pools are dynamic and complicated. Yegian *et al.* (1971) have suggested that the DNA delay mutants which manifest all the normal DNA intermediates have alterations in their intracellular nucleotide pools. It appears, however, that the difference in rate of incorporation of high specific activity label between wildtype and *x*- or *y*-infected cells reflects some difference in the replicative phenomenon since total incorporation of label levels off at the same time in both cases; and both are capable of taking up additional label, indicating similarity of thymidine processing in the pools (Figure 9C,D). If there were a slower rate of uptake into the precursor pools with *x*, one would expect continued incorporation, albeit at a slower rate.

Effect of rifampin and chloramphenicol on the manifestation of the T4*x* and *y* gene products

In an attempt to delineate the time of transcription of the *x* and *y* gene products, rifampin was administered at various times after infection at 37 C and subsequent incorporation of high specific activity thymidine was measured at 15 second intervals after administration at 15 minutes of infection. Figure 10 shows that there is no difference in the incorporation of label by wildtype-infected cells in the absence of rifampin addition or after the addition of rifampin 4 minutes after infection. Since the *x* and *y*

proteins are necessary for normal DNA synthesis later in infection, the T4*x* and *y* genes must be transcribed before 4 minutes after infection. In fact, a comparison of DNA synthesis between mutant- and wildtype-infected cells, indicates that the *x* and *y* transcripts have been made before 2 minutes of infection. Interestingly, mutant-induced incorporation after rifampin addition does not resume its normal, low level of incorporation until after 8 minutes of infection. Thus it is is possible that genes essential for mutant DNA synthesis are not transcribed until later in infection, between 6 and 10 minutes.

In order to elucidate translational events, similar experiments were performed using chloramphenicol added at 4, 8, or 12 minutes after infection and incorporation of labeled DNA precursor measured after a total of 15 minutes of infection. Chloramphenicol added at 4 minutes after infection completely inhibits incorporation at 15 minutes in wildtype-infected cells, while chloramphenicol added at 8 or 12 minutes has little or no effect on subsequent incorporation at 15 minutes (Figure 11A). Since this 15 minute incorporation is a measure of the functioning of the *x* and *y* gene products (among others), these data indicate that the *x* and *y* transcripts have been translated before 8 minutes. Likewise, addition of chloramphenicol at 4 minutes after infection completely inhibits *x*- or *y*-induced incorporation at 15 minutes. However, addition of chloramphenicol at 8 minutes after infection with T4*x* or *y* gives a

level of incorporation similar to that of wildtype, while incorporation by the mutant-infected cells in the absence of chloramphenicol is greatly reduced when compared to that of wildtype (Figure 11 B and C). This "rescue" was not observed (or only slightly so) when chloramphenicol was added 12 minutes after infection. It appears that prevention of protein synthesis in T4*x*- or *y*-infected cells at a critical time, 8 minutes after infection, allows for a subsequent rate of DNA synthesis which more closely resembles that of wildtype.

Effect of Mitomycin C on incorporation of high-specific activity thymidine

The effect of mitomycin C on incorporation of high-specific activity thymidine was measured at 8 and 15 minutes after infection. As shown in Figure 12, DNA synthesis at 8 minutes after infection in wildtype- and mutant-infected cells is reduced to a similar small degree by mitomycin C. In contrast, mutant-induced DNA synthesis at 15 minutes after infection is more sensitive to mitomycin C than is that of wildtype. When chloramphenicol is added at 8 minutes after infection, and incorporation of label measured at 15 minutes after infection, the increased "rescued" DNA synthesis observed in mutant-infected cells retains its 15 minute sensitivity to mitomycin C (Figure 13). Thus, even though the rate of DNA synthesis by the *x* and *y* mutants in the presence of chloramphenicol is similar to that of wildtype, it retains the mutant quality of being

sensitive to mitomycin C.

Complementation

In the following experiments, an attempt was made to measure gene complementation with respect to DNA synthesis. High specific activity thymidine was administered 15 minutes after infection at 37°C and incorporation into the acid-insoluble fraction was measured in cells infected with one or a combination of genotypes. Figure 14A shows that coinfection with T4+ and T4*y* gives wildtype phenotype, but coinfection with T4+ and T4*x* gives an intermediate phenotype. Coinfection with T4*x* and *y* shows little or no complementation. The *x* mutant used in these studies, T4*px*, has a point mutation (Drake, 1973) and could produce a protein which has partial function. For example, if the *x* gene product is directly involved with the DNA replicating complex the mutant protein might still bind to the complex, and thus act as an inhibitor. This possibility is supported by the observed inhibition of wildtype synthesis in the presence of the *x* mutant, and by the inability to observe significant complementation in T4*x* and *y* coinfected cells. In accord with the above hypothesis, Boyle and Symonds (1969) were also not able to demonstrate significant complementation of *x* and $x^+$ in survival of phenotype experiments. Inhibition of DNA synthesis would not be as likely with T4*y* since it is an amber mutant. It is also possible that the *x* gene product is required stoichiometrically, and therefore the apparent inhibitory effect of the *x* mutant on

wildtype DNA synthesis might be due to an insufficient amount of the x gene product.

Earlier experiments (see Figure 10) indicated that the x gene product was transcribed before 4 minutes. Thus, the addition of rifampin at 4 minutes to coinfected cells should distinguish between inhibitory products transcribed before and after this time. Results of such experiments are shown in Figure 14B. In the presence of rifampin cells coinfected with $T4^+$ and T4x mutant phage exhibit a still greater inhibition of DNA synthesis than was observed in its absence. Coinfection of $T4^+$ and T4y in the presence of rifampin exhibits a slight reduction in the wildtype level of synthesis. Also coinfection with both x and y mutant phages shows no complementation. These experiments support the notion that the defective x gene product is transcribed before 4 minutes and is responsible for the inhibition observed.

It was unlikely that the increased mutant DNA synthesis observed in 8 minute chloramphenicol-treated cells (see Figure 11) was caused by "defective x protein", since it also occurred with amber T4y. However, coinfection experiments were performed under these conditions as well. As can be seen from Figure 14C, where chloramphenicol was added 8 minutes after infection, and synthesis measured at 15 minutes, the standard coinfection pattern is observed. Coinfection of wildtype with x gives x phenotype, whereas coinfection with y gives wildtype phenotype. Again, little

or no complementation is observed with T4*x* plus T4*y*.

Sedimentation analysis of progeny DNA

In order to examine the physical characteristics of the DNA produced under the various conditions presented, sucrose gradient analysis was performed. Figures 15 and 16 show the neutral sucrose gradient sedimentation profiles of T4 DNA replicative complexes isolated at 8 or 15 minutes from $T4^+$-, T4*x*-, or T4*y*-infected cells. As can be seen in Figure 15, the 9 minute DNA replicative complexes from wildtype- and mutant-infected cells sediment as a broad peak slightly ahead of the 1000S marker. In contrast, DNA replicative complexes isolated from mutant-infected cells at 15 minutes after infection (Figure 16) sediment significantly more slowly, at about 700S, than those of wildtype which sediment just behind the 1000S marker at about 900S.

When DNA labeled at 8 minutes after infection with $^3H$ thymidine is chased with 500 ug/ml cold thymidine until 15 minutes after infection, the profiles, shown in Figure 17A are obtained. Again, mutant-induced DNA replicative complexes sediment more slowly than those induced by wildtype T4. Similar 15 minute profiles are obtained when the DNA is continuously labeled from 5 to 15 minutes of infection under conditions of high thymidine concentration (Figure 17B). DNA replicative complexes isolated from mutant-infected cells 10 minutes after infection are found in both the 900S and 700S size classes. Thus, it seems that the 8 minute replicative complex is modified in mutant-infected

cells in a manner different from that of wildtype-infected cells. This modification leads to the sedimentation patterns seen 15 minutes after infection. The accumulation of counts at the bottom of the gradient as shown in Figures 17A and B has not been investigated. It is not reproducibly observed and may reflect incomplete lysis or release of DNA from membranes.

In Figure 18 can be seen the increase in the formation of concatemers (single stranded DNA longer than the 73S parental single strand) in T4-infected cells from 8 until 20 minutes after infection. These profiles, and those obtained at 15 and 25 minutes after infection (same as Figure 18B), indicate that the T4*x* and *y* mutants are about 30% less efficient in concatemer formation than wildtype. Concatemer formation is determined by the relative number of counts sedimenting ahead of the $T4^+$ marker peak. No concatemer formation was observed in either mutant- or wildtype-infected cells 10 minutes after infection. This picture is similar to that reported by Wakem and Ebisuzaki (1976) using *vx* and *vy* mutants and by Hamlett and Berger (1975) for T4*w*, another non-lethal recombination deficient mutant of T4. Alkaline sucrose gradient profiles obtained from pulse-chase or continuous label experiments as described above appear identical to the 20 minute profile depicted in Figure 18B.

*Sedimentation analysis of chloramphenicol "rescued" mutant DNA*

The addition of chloramphenicol at 8 minutes after

infection was previously shown to allow mutant-infected cells to synthesize wildtype levels of DNA at 15 minutes after infection (Figure 9). Figure 19 shows that the addition of chloramphenicol at 8 minutes after infection in mutant- and wildtype-infected cells prevents the development of the characteristic 15 minute sedimentation patterns of DNA replicative complexes observed in neutral sucrose gradients. The wildtype DNA replicative complexes sediment slightly faster than those formed at 8 minutes, about 1200S, while those induced by the x and y mutants, decrease in sedimentation rate to about 900S from their normal 8 minute pattern (faster than 1000S). There has been some thus far unexplained variability in the S value obtained in chloramphenicol-treated infected cells. The alkaline sucrose sedimentation patterns of chloramphenicol "rescued" DNA from mutant- and wildtype-infected cells are shown in Figure 20. Clearly, addition of chloramphenicol at 8 minutes after infection prevents the formation of concatemeric DNA in both mutant- and wildtype-infected cells.

Sedimentation analysis of parental DNA

When labeled parental DNA, from wildtype or x and y mutant phage, is followed during T4 development, results similar to those depicted in Figures 15 and 16 with progeny DNA are observed. The characteristically different sedimentation patterns of labeled wildtype and x or y mutant parental molecules at 15 minutes after infection permits examination of possible complementation after coinfection

with a given labeled and a given unlabeled genotype. All six possible combinations among wildtype, T4x and T4y have been examined: Figure 21A depicts the sedimentation profiles where the labeled parent is T4+; 21B where it is T4x; and 21C where it is T4y. In Figures 21A it can be seen, that when T4x is present as cold phage with labeled T4+, the label sediments in both wildtype (900S) and mutant (700S) positions. When T4x is present as labeled phage with cold $T4^+$ (Figure 21B), most of the label sediments in the position characteristic of T4x. Thus, not only does T4x appear not to be complemented by wildtype T4, but it seems to interfere with wildtype development. In contrast, Figure 21C shows that labeled T4y is complemented by wildtype T4 and the DNA sediments in the wildtype position. In addition, as shown in Figure 21A, T4y does not appear to affect the sedimentation profile of labeled T4 wildtype. Neither coinfection with labeled T4x and unlabeled T4y (Figure 21B) nor coinfection with labeled T4y and unlabeled T4x (Figure 21C) gives any indication of complementation. These complementation data are in agreement with those previously obtained using DNA synthesis as an assay (Figure 14).

## DISCUSSION

DNA recombination is the essential process by which existing gene pools mix, enabling organisms to meet environtal demands. In T4, there has evolved a highly efficient, possibly unique, recombination process. The close link between recombination and replication in T4 has provided a sophisticated genetic tool. By using sensitive fine structure recombination analysis, it is possible to measure recombinants between adjacent nucleotide pairs (Benzer, 1964). However, the high frequency of recombination found in T4 has added to the complexity of elucidating the replication and recombination processes of this organism.

The genetic map of T4 is well defined; it contains about 170 genes (Wood and Revel, 1976). The availability of mutants spanning many of the known T4 genes has greatly assisted the elucidation of its replication, recombination and repair. Most of the genes that are involved in DNA replication have been found to be essential for phage survival (Epstein *et al*., 1963). Many of these essential gene products are produced early in infection. In their absence, there is no DNA synthesis (Epstein *et al*., 1963). By virtue of the interwoven replication and recombination process, most T4 mutants that affect recombination also affect replication (Bernstein, 1968). This interdependence is clearly seen in DNA arrest mutants which are unable to synthesize DNA after 12 minutes of infection. Recombination normally occurs at this time (Kozinski *et al*., 1967). All

of the DNA arrest mutants are recombination deficient (Bernstein, 1968; Wu et al., 1972).

T4x and T4y belong to a class of recombination deficient mutants other than those exhibiting DNA arrest (Harm, 1964; Boyle and Symonds, 1969). The x and y mutants produce viable phage. The recombination deficiency, DNA repair reduction, as well as the reduced burst size exhibited by the x and y mutants all suggest fundamental developmental abnormalities. The reduced thymidine and thymine incorporation shown in Figure 1 indicates that the mutant defects are manifest at the level of DNA synthesis. Since T4x and T4y are sensitive to ultraviolet light (Harm, 1963; Boyle and Symonds, 1969), x-irradiation (Boyle and Symonds, 1969; Wallace and Melamede, 1972), and T4x to mitomycin C (Shimizu and Sekiguchi, 1970), it was interesting to examine the influence of these DNA damaging agents on DNA synthesis.

After irradiating free phage, DNA synthesis directed by T4x and T4y is more resistant to ultraviolet light than is DNA synthesis directed by wildtype or T4v (Figures 2A and 4A). This finding indicates that excision repair may be expanded in T4x and T4y after ultraviolet-irradiation resulting in a protective effect on DNA synthesis. In contrast, neither $T4^+$ nor T4v exhibit expanded excision repair as evidenced by the relative increased sensitivity of DNA synthesis to ultraviolet-irradiation in these phage types.

X-irradiation inhibits DNA synthesis in wildtype, as compared to the x and y mutants, in a manner similar to that

found after ultraviolet irradiation. However, the effect is not as large as that found after ultraviolet irradiation. X-rays introduce a large number of single stranded breaks in addition to the other types of damages that are created (Ginoza, 1967). The single stranded breaks are likely to have damaged bases and/or sugars attached to one side of the nick, whether the nick was endonucleolyticly created or not. Thus, repair after x-irradiation is likely to occur along lines similar to those found after ultraviolet light irradiation.

In contrast to the effects mentioned above, DNA synthesis in T4*x* and T4*y* is sensitive to the action of mitomycin C. A consideration of the discussion of ultraviolet and x-ray effects in mutant-infected cells as compared to the wildtype-infected cells suggests that in the presence of mitomycin C damages, recombination is required to maintain lengths of DNA that are functional with respect to DNA synthesis. The *x* and *y* mutants are, therefore, unable to provide functional lengths of DNA appropriate for continued DNA synthesis. Mitomycin C is known to induce recombination (Holliday, 1968). The requirement for recombination that is indicated by the mutant sensitivity to mitomycin C may simply reflect the induction of a faulty pathway rather than the inability to repair the mitomycin C damages *per se*. For example, if mitomycin C damages are repaired by the concerted action of excision and recombination, as has been suggested for psoralen by Cole (1973),

then the endonuclease action would at least partly release one arm of the bifunctional crosslink. This structure might be responsible for induction of recombination. Alternatively, recombination intermediates such as noncovalently bound intermolecular hybrids may become stabilized by mitomycin C. The completion of recombination would thus be required for continued DNA synthesis. In the absence of completed recombination, the stabilized intermediates might impair the flexibility of the replication complexes or impair essential nuclease activity to the extent that DNA synthesis is stopped.

An alternate hypothesis suggests that mitomycin C-induced crosslinks may be indirectly responsible for the reduced rates of DNA synthesis found in T4x- and T4y-infected cells. When the replicating fork reaches a cross-linked nucleotide pair synthesis stops and is reinitiated at the next site for the initiation of Okazaki pieces (Hanawalt, 1975). A possible result of the mitomycin C induced replicative blocks is that single stranded lengths of DNA remain distal to them up until the point where the next Okazaki piece is started. There, single stranded regions would average one half of an Okazaki piece in length, and might be responsible for the induction of recombination by promoting hybridization. Kozinski and Felgenhauer (1967) have found single stranded regions to be necessary for interparental recombination. In the absence of the x or y gene products, the hybrids may not efficiently progress to

form recombinants. The overall replicating complex may thus become destabilized resulting in impaired DNA synthesis.

The 30% reduction of DNA synthesis found with T4*x* and T4*y* when compared to wildtype T4 is interesting, but of little value. The defect in DNA synthesis is not sufficient to provide the sensitivity that is needed for more thorough investigations into the nature of the *x* and *y* mutants, i.e., setting up an *in vitro* complementation system that would allow for the purification of the *x* and *y* gene products. In an attempt to define the time at which the *x* and *y* gene products function, the specific activity of the labeled thymidine DNA precursor was raised by lowering the concentration of exogenous cold thymidine. Unexpectedly, a disproportionately large decrease in mutant-directed thymidine incorporation is observed when measurements are made in the presence of low thymidine concentrations. This increased differential greatly enhances the usefulness of simple incorporation studies as an investigative probe. Possible explanations for the above-mentioned effect will be considered later in this text.

The appearance of differential rates of DNA synthesis between mutant and wildtype-infected cells is clear indication that the *x* and *y* gene products have functioned in the wildtype-infected cells. Thus, the *x* and *y* gene products have acted by 8 minutes post infection. A problem with this approach, however, is that it cannot specify whether the reduced mutant-directed DNA synthesis is an immediate result

of the missing proteins, or if it is the result of proteins that act subsequent to the time that the x and y proteins should normally have functioned. Insights into this question were gained from experiments in which rifampin (Figure 8) or chloramphenicol (Figure 9) were administered at various times after infection, followed by the use of the high specific activity DNA synthesis assay. The results of rifampin administration as early as two minutes after infection in order to block subsequent transcription, indicate that the x and y transcripts have been made. When chloramphenicol is used to block translation, the x and y proteins appear to be made between 4 and 8 minutes after infection.

It should be noted that the early addition of rifampin, 2 minutes post infection, while demonstrating a differential rate of incorporation of thymidine between mutant- and wild-type-infected cells, does not allow for the rates of synthesis normally seen at 15 minutes after infection in the drug's absence. Thus, post 2 minute transcripts are required for the rates of synthesis that occur 15 minutes after infection, but the x and y gene products function in their absence. Rifampin cannot rescue DNA synthesis as can chloramphenicol added 8 minutes after infection. The above finding indicates that the protein which is responsible for reducing x- and y-directed synthesis (probably not the x or y proteins see later discussion) and is chloramphenicol inhibitable, is also transcribed 2 minutes after infection. This sequential translation of specific proteins

allows for chloramphenicol rescue. In contrast, coupled transcription does not allow for rifampin rescue.

Miller and Kozinski (1970) have found that the addition of chloramphenicol before 4 minutes of infection blocks the formation of a fast sedimenting replicating complex. Differences in the experimental conditions do not allow for comparative timing between Miller's experiments and those reported here to a resolution of one minute. Thus, it is possible that the x and y gene products might function early (by 4 minutes) in the establishment of the replicating complex, but not in a manner that is measurable via incorporation until after 8 minutes after infection. The chloramphenicol data presented here indicate that either chloramphenicol blocks development at a point prior to the need for the x and y gene products; or development is blocked after the time that these proteins act. In the latter situation, a subsequent protein is made that adversely affects the ability to synthesize DNA when the x and y proteins are absent.

The latter possibility finds support in the data from experiments that allow for the simultaneous examination of two phenotypic effects of the x and y mutations. Mitomycin C sensitivity of DNA synthesis and the reduced levels of thymidine incorporation both occur after 8 minutes of infection. Is the linkage between these two characteristics obligatory? The data of Figure 12 show that it is not. Mitomycin C sensitivity is independent of the levels of DNA

synthesis in T4*x*- and T4*y*-infected cells. Thus chloramphenicol administered 8 minutes after infection holds development after the time that the *x* and *y* proteins have functioned, as evidenced by the mutant sensitivity to mitomycin C, but before the expression of the proteins that are responsible for reducing the mutant-directed levels of synthesis.

The separability of mitomycin C sensitivity of DNA synthesis and the abnormal reduction of synthesis that is found during T4*x* and T4*y* infection is analogous to results that have been observed in the recombination deficient DNA arrest mutants. The reversal of the DNA arrest phenotype of mutants in genes 46, 47 and 59 by the addition of chloramphenicol between 6 and 13 minutes after infection is dependent upon the experimental conditions. Other phenotypes associated with the DNA arrest mutants may, or may not, be reversed depending on the characteristic examined, the experimental conditions used and the mutation involved (Hosoda *et al.*, 1971; Shah and Berger, 1971; Shalitin and Naot, 1971; Wu *et al.*, 1972).

Clearly, there are many similarities between the nonlethal recombination deficient T4 mutants and the DNA arrest mutants. It is of interest to note the effect that thymidine concentration has on DNA synthesis when measured in the nonlethal recombination deficient mutants. In the presence of low thymidine concentrations, they would appear as DNA arrest mutants. This misclassification seems in fact

to have recently been made. Dewey and Frankel (1975) have examined DNA synthesis in the fdsA and fdsB mutants in the presence of 5 ug/ml of thymidine. They found that these mutants exhibit the DNA arrest phenotype. Since the fdsA and fdsB mutants have now been associated with T4x and T4y, respectively (Cunningham and Berger, 1977), the results of Dewey and Frankel are consistent with those reported here using lower concentrations of thymidine during the labeling procedure. It is worth taking note at this point that the x and y mutants are not lethal, whereas the DNA arrest mutants are lethal.

Although the precise mechanisms are unknown, it does appear that the physical processes involved in recombination are occuring by about 12 minutes after infection (McCarty *et al.*, 1976). It is at this time (12 minutes after infection) that the DNA arrest mutants stop synthesizing DNA (Epstein *et al.*, 1963), whereas the DNA delay mutants show increased DNA synthesis (Yegian *et al.*, 1971; Mufti and Bernstein, 1974; Leung, 1975). The DNA arrest mutants exhibit decreased recombination under semi-restrictive conditions (Bernstein, 1968). The DNA delay mutants exhibit increased rates of recombination (Yegian *et al.*, 1971; Mufti and Bernstein, 1974; Leung, 1975). It appears that the increased rates of DNA synthesis found early in wildtype infection (up to 12 minutes) reflects an increase in the number of replicating forks (McCarthy *et al.*, 1976). In contrast, DNA replication measured later in infection

probably includes synthesis generated by recombinational events (McCarthy *et al.*, 1976; Shahn, 1968). The results of the thymidine incorporation studies presented here indicate that it is this latter type of synthesis that is deficient in the *x* and *y* mutants. In fact, the data indicate that the measurements of late DNA synthesis in the presence of low concentrations of exogenously added thymidine may in fact be a specific measure of recombination-directed DNA synthesis. If recombination-directed DNA synthesis can be measured by the incorporation of thymidine when the concentration of thymidine is low, then different DNA precursor pools may supply different DNA processing events. Wovcha *et al.* (1976) have recently described two independent pathways for providing DNA precursors in plasmolyzed cells of *Escherichia coli*. Ribonucleotides, thymidine and hydroxymethyl-cytosine are produced by one pathway. The second pathway requires the four deoxribonucleotide mono or triphosphates in addition to ATP. In the latter pathway, thymidine does not substitute for deoxythymidine triphosphate. Interestingly, both pathways require the standard T4-induced genes that are responsible for DNA synthesis. Since T4 DNA polymerase is required for both of the above pathways, they both must be capable of providing the deoxyribonucleotide precursors that are required by this enzyme. Alternatively, one pathway may be required for the other one to function. For example, recombination-directed synthesis may not be directly

dependent on T4 gene 43-directed DNA synthesis. A separate polymerizing apparatus using different substrates may exist. Yet, if replicative gene 43-produced DNA synthesis does not provide the necessary prerecombinational substrates, then there would be no recombination-induced DNA synthesis.

In a more conservative vein, the same basic replication apparatus may be responsible for both replicative and recombinational DNA synthesis. DNA precursors may, however, be supplied by different pathways. In the presence of low concentrations of thymidine a small capacity pathway may process the limited amounts of thymidine. When the thymidine concentrations exceed a critical level, the capabilities of the pathway are surpassed. The excess quantities of thymidine are shifted into the less favored precursor pathway which has a greater capacity for processing thymidine. It is the former of these two possible pathways that is presumably defective in the T4*x* and T4*y* mutants. Although the possible existence of two pathways for providing thymidine containing DNA precursors provides an interesting explanation for the apparent differential rates of DNA synthesis that is found in wildtype versus T4*x*-and T4*y*-infected cells; other explanations for this effect might be compatible with the data.

Clearly the dynamics of producing thymine containing nucleotide are complicated by the variety of pathways leading to dTTP production. Thymidine that is exogenously added may be incorporated into DNA by first being converted

to thymine, and then processed by conventional pathways (Rachmeler _et al_., 1971; Boyce and Setlow, 1962). Theoretically, thymidine may be directly phosphorylated to produce the monophosphate. This reaction, however, does not seem very probable _in vivo_ since thymidine is not thought to directly enter cells (Boyce and Setlow, 1962), and it must first be converted to the base (Rachmeler _et al_., 1961). It is possible that under the conditions of low thymidine concentrations used here, where incorporation is measured for short periods of time, that direct entry is observed. In fact, the leveling off of incorporation seen in Figures 9A, C, D may reflect the conversion of the thymidine to thymine with subsequent reduced rates of incorporation. The flow of thymine nucleotide intermediates is a dynamic process that may be limited by the supply and demands created by the rates of synthesis. It has recently been shown that in the absence of DNA synthesis, T4-infected cells expand thymidylate precursor pools indicating the lack of feedback regulation of pool sizes (Mathews, 1972). This conclusion is also indicated by the data of Figure 9O. Extending this line of thought , if the _x_ and _y_ mutants have only a small reduction in their ability to synthesize DNA, then an accumulation of thymidylate precursors would be expected in cells infected by these mutants. In the presence of high concentrations of exogenously added thymidine, the relative precursor accumulation in mutant-infected cells would be small compared to the contribution made by the

exogenously added thymidine. When synthesis is measured in the presence of low thymidine concentrations, the ratio of mutant surplus to input exogenously added precursor would be high. Thus, the mutant-infected cells would exhibit an artificially low level of thymidine incorporation. The relative rates of DNA synthesis that are measured by the incorporation of thymidine in the presence of high concentrations of thymidine should in fact be more accurate. It should be noted that Shimizu and Sekiguchi (1974), did not demonstrate a difference in the rates of DNA synthesis in T4 wildtype and T4*x*-infected cells when measurements were made using the diphenylamine assay for DNA synthesis.

Whether the rates of DNA synthesis that are measured in the presence of low concentrations of thymidine are representative of recombinational events or whether these measurements are the result of pool effects, a simple model that relates DNA synthesis in the *x* and *y* mutants to recombination and repair deficiencies is difficult to formulate. This difficulty is at least in part the result of the complex interactions that exist between the various phenotypes that are observed with these mutants and T4*w* and gene 58 mutants (Hamlett and Berger, 1975). T4*x*, T4*y* and T4*w* all appear to be involved in the same repair pathway as evidenced by the lack of additive sensitivities to ultraviolet light that is exhibited by multiple mutants of these genes. In contrast, when the recombination frequencies of the multiple mutants are examined, some combinations

produce cumulative effects. Hamlett and Berger (1975) have found that the combination of either a _w_, _x_ or _y_ mutant with a mutation in gene 58 exhibits the ultraviolet sensitivity of the most sensitive member of the pair. However, when recombination is examined in 58-_y_, 58-_w_ and _wy_ double mutants, additive frequencies of recombination are found. In contrast, 58-_x_ and _xy_ mutants do not have additive recombination effects.

Why should some of the recombination defects be cumulative when others are not? The information that is presently available does not favor any coherent picture that resolves these apparent inconsistencies. One simplistic interpretation, however, is that the repair of ultraviolet light-induced damages requires the successful, sequential action of the _w_, _x_, _y_ and gene 58 proteins. In other words, any of the above mutations knock out the entire pathway. On the other hand, events that induce recombination are numerous. The points of entry for any given pre-recombinational substrate may vary. In turn, the degrees of processing that are required for the completion of the recombinational events may also vary.

An additional consideration that must be incorporated into any model that attempts to explain the nature of the x-y involvement in T4 recombination is the relationship between the nonlethal recombination mutants and the lethal recombination mutants. Hamlett and Berger (1975) have shown that genes 46 and 47 affect the phenotypic expression

of gene 58 mutants. The arrest mutants block the increased rates of recombination that characterize mutants of gene 58. These observations will be considered more fully later in the discussion.

The examination of mutant- versus wildtype-directed DNA synthesis provides limited details of the molecular events that occur during T4 development. Sucrose gradient sedimentation analysis, in contrast, can help provide an accurate description of the molecular intermediates that characterize T4 infection. In an attempt to assess the role(s) of the x and y gene products, DNA replicating complexes were examined by neutral sucrose gradient sedimentation analysis. Prior to the onset of recombination, 8 minutes after infection, both the mutant- and wildtype-directed complexes sediment slightly ahead of the 1000S marker. At 15 minutes after infection, during the recombinational stage of T4 replication, wildtype-directed complexes sediment at about 900S, whereas x or y mutant-directed complexes sediment at about 700S. The alkaline sucrose gradient profiles of DNA isolated 15 minutes after infection indicate that concatemers have been formed in both wildtype and mutant-infected cells, although fewer are found in x or y mutant-infected cells. A similar but more dramatic decrease in the formation of concatemeric DNA has been demonstrated in T4w-infected cells (Hamlett and Berger, 1975) as well as in cells infected by the DNA arrest mutants (Hosoda et al., 1971; Hercules and Wiberg, 1971; Shah and

Berger, 1971; Shalitin and Naot, 1971; Wu and Yeh, 1972). Wakem and Ebisuzaki (1976) have recently reported similar findings with vx- and vy-infected cells.

Upon examination of DNA isolated at intermediate times, 10 minutes after infection, 900S replicating complexes may be found in both the wildtype- and mutant-infected cells. Thus a 900S intermediate is apparently modified in x- and y-infected cells into a 700S complex. Alkaline sucrose gradient profiles of mutant or wildtype replicating complexes, isolated 10 minutes after infection, do not reveal DNA of concatemeric lengths. Thus, a 900S complex may exist both prior to and concomitant with the completion of recombination as evidenced by the presence or absence of concatemer formation. "Chloramphenicol-rescued" DNA replicating complexes isolated from wildtype-infected cells 15 minutes after infection, subsequent to the addition of chloramphenicol after 8 minutes of infection results in an increase in the sedimentation rate of the wildtype replicating complex; whereas corresponding mutant-induced complexes continue to sediment at 1000S. As has been shown, the mutant-induced "chloramphenicol-rescued" intermediate can still support a high rate of DNA synthesis, but has the x and y mutant phenotype of mitomycin C sensitivity. Thus, it seems that the wildtype-induced 1200S intermediate is formed from the 1000S intermediate by proteins translated before 8 minutes after infection. In mutant-infected cells, the change from 1000S to a 700S intermediate is accomplished

by proteins translated after 8 minutes of infection.

Thus, the normal functioning of the recombination pathway in wildtype-infected cells leads to a progression from a 1000S DNA intermediate that does not contain concatemeric lengths of DNA to a 1000S DNA intermediate that contains concatemeric DNA. These intermediates are apparently responsible for the observed wildtype rate of DNA synthesis, the efficient production of genetic recombinants, as well as a normal burst size. In x or y mutant-infected cells, the 1000S intermediate is unstable and leads to an "aberrant" replicating complex of 700S. The 700S intermediate exhibits reduced DNA synthetic capacity and eventually results in a lowered recombination frequency and burst size, concomitant with increased radiation sensitivity. These data are consistent with the interpretation that gene products other than x or y are responsible for initiating into the "normal" T4 recombination pathway, but that the x and y gene products are responsible for a later step which ensures accurate and efficient functioning of this process.

What specific roles might the x and y gene products play in the recombination process? Kozinski has proposed (1967), that in addition to a nicking enzyme, an enzyme that would introduce "counter nicks" would be required to release the lengths of DNA from parent to recombinant DNA molecules. With the exception of these two presumptive enzymes, it seems that the most obvious proteins necessary for recombination have been accounted for. There is no evidence to

to identify the x or y gene products with either the nicking or counter-nicking enzymes. If the x or y proteins were to possess either of the two above-mentioned activities, there would have to be alternative ways to bypass the need for these proteins, since the x and y mutants are not totally lacking in recombinational ability.

In view of the degree to which recombination occurs in T4, it is not unreasonable to assume the existence of specialized proteins that might specifically facilitate the recombination process. Such proteins might aid in the formation of noncovalently bound "joint molecules" or in possibly stabilizing these structures, making the formation of covalent recombinants more likely. In the absence of these hypothetical proteins recombination would proceed with reduced efficiency, and with the production of aberrant recombination intermediates. If the reduction in the sedimentation rate of the T4x- or T4y-induced replicating complex is the result of a defective complex that lacks stability, then the supression of gene 49 mutants might be due to the packaging of appropriately sized pieces that are shed from the replicating complex. Thus, the essential genes involved in recombination might be responsible for generating certain obligate intermediates. The nonessential gene products might merely facilitate the functioning of the essential ones. For example, early recombinants may be produced normally, but the continued recombinational events necessary to maintain the 900S structure may not be produced

rapidly enough to replace the molecules that have matured. In other words, there may be two distinct phases to T4 recombination. One is obligatory as evidenced by the DNA arrest mutants. The other is secondary and is necessary for the efficient production of progeny. The latter process, however, is not essential for viability. What might be the relationship that exists between these two systems? If prematurely released, potentially viable DNA fragments are generated by the *x* and *y* mutants ( as possibly indicated by the gene 49 mutant suppression associated with the *fdsA* and *fdsB* mutants) then similar events should allow for the viability of gene 46-47 mutants when in the presence of *x* or *y* mutations. Since this suppression is not observed, it appears that the 46 and 47 mutants may suppress the proposed *x* and *y* mutant phenotype of shedding packagable material from the replicating complex.

## CONCLUSIONS AND FUTURE PERSPECTIVES

Two classes of properties associated with the x and y gene products have been presented: those that involve the incorporation of thymidine into DNA and those that affect the sedimentation properties of the DNA replicating complex. It is possible that some of either or both of the above classes of properties are the direct result of the x and y gene products. Specifically, chloramphenicol can prevent the normal reduction in the rate of thymidine incorporation found 15 minutes after infection in mutant-infected cells. Yet, chloramphenicol does not prevent the sensitivity of this incorporating ability to mitomycin C. It appears, therefore, that the rates of DNA synthesis found in the mutant-infected cells under the above conditions is a secondary result of the mutant gene products. An unknown, apparently primary event accounts for the sensitivity to mitomycin C.

Neutral sucrose gradient analysis of the DNA replicating complexes labeled after the addition of chloramphenicol indicates that both the wildtype and the mutant complexes sediment faster than they normally do at 15 minutes after infection. However, the wildtype complexes sediment as they do at 8 minutes after infection, whereas the mutant ones are more slowly sedimenting. Since these mutant-produced complexes are capable of high rates of synthesis, it appears that whatever is responsible for the decreased sedimentation rate may also be responsible for sensitivity

to mitomycin C. A possible explanation of the above data requires additional consideration.

Albert's group (Barry *et al.*, 1973) have demonstrated a functional multiprotein complex composed of T4 proteins that synthesizes DNA *in vitro*. Gene 42 protein, cytosine hydroxymethylase, is not one of the proteins in the complex of replication proteins. However, gene 42 protein is part of a complex that provides cytosine and thymine nucleotides to the replicating fork (Reddy *et al.*, 1977). Considering the existence of the two above protein complexes, it would make sense that there be some mechanism to retain precursor production in the vicinity of the replication apparatus. It is proposed that the *x* and *y* gene products represent a novel class of proteins that are involved with maintaining the physical proximity of at least certain DNA precursors to the replication apparatus. For example, the *x* and *y* protein might be responsible for binding the precursor complex to potential recombinational structures. In doing so, certain recombination intermediates may become stabilized, in particular to shearing forces generated by ongoing DNA synthesis. A less formal variation of the above proposal would assume that rapid DNA synthesis is necessary on certain prerecombinational structures (i.e., single stranded regions bounding heteroduplexes), in order to increase their stability. Appropriate location of the precursor aggregate in the latter case would thus fortuitously be responsible for maximum recombination.

A number of approaches may be used to determine the validity of the above working model. A particularly useful one would be to identify the *x* and *y* proteins on SDS polyacrylamide gels. Once this is accomplished, it should be possible to isolate replicating DNA complexes with proteins attached, thus establishing the presence or absence of the *x* and *y* proteins. In addition, the precursor complex as isolated by Mathews (Reddy *et al.*, 1977) could be examined for these proteins.

It has been established by both Mathews' group (Stafford *et al.*, 1977) and that of Greenberg (Wovcha *et al.*, 1976) that there appear to be a number of independent precursor feeds that supply the replication forks. Through the use of plasmolyzed cells and various precursors (Tdr, dTMP, dTTP) a correspondence between a given precursor pathway and recombination might be established. Ultimately, through the use of *in vitro* complementation, it might be possible to develop an assay that would allow for the purification of the *x* and *y* proteins. *In vitro* the complementation of T4 replication proteins has been carried out by Barry *et al.*, (1973). Sadowski's group has successfully developed an *in vitro* recombination system using T7 bacteriophage (Sadowski and Vetter, 1976; Sadowski, 1977).

## SUMMARY OF OBSERVATIONS

1. DNA synthesis, as measured by the incorporation of tritiated thymine, or thymidine, is defective in T4x- and T4y-infected cells (i.e., 30% less than wildtype).
2. The degree to which DNA synthesis is impaired in T4x- and T4y-infected cells is a function of the thymidine concentration. Thus, the lower the thymidine concentration, the greater the impairment. (0.19 ug/ml = 50-90% impairment; 25 ug/ml = 30% impairment).
3. DNA synthetic capacity is less sensitive to inactivation by ultraviolet light irradiation or x-ray inactivation in the absence of x- and y-directed recombinational repair than in the presence of the x and y gene products.
4. Both DNA synthesis and viability of T4x and T4y are more sensitive to mitomycin C than are these two parameters in the wildtype-infected cells.
5. The defect in DNA synthesis found in T4x and T4y may be measured by twelve minutes after infection.
6. Transcription of the x and y genes occurs before 2 minutes after infection.
7. The x and y gene products are translated before 8 minutes after infection.
8. The absence of the x and y gene products is not directly responsible for the reduced rates of DNA synthesis found in cells infected with T4x and T4y.

9. The presence of chloramphenicol at 8 minutes after infection can prevent the synthesis of protein(s) responsible for reducing the ability of T4x and T4y to synthesize DNA.
10. DNA synthesis in mutant-infected cells measured after 15 minutes of infection in the presence of either high or low concentrations of thymidine is sensitive to mitomycin C.
11. The presence of the mutant x gene product in wildtype-infected cells inhibits wildtype levels of DNA synthesis (i.e., partial x dominance).
12. The replication complex of T4x- or T4y-infected cells sediments more slowly than does the replicating complex from wildtype-infected cells.
13. The presence of chloramphenicol at 8 minutes after infection prevents the breakdown of the mutant-directed DNA replicating complex.
14. Both T4x and T4y produce fewer concatemeric lengths of DNA than are produced in wildtype-infected cells.
15. Parental DNA associated with the DNA replicating complex sediments as does the respective progeny DNA.
16. The presence of the mutant x gene product can cause a reduction in the sedimentation rate of parental wildtype DNA in replicating complexes to a rate that is similar to that found in mutant replicating complexes.

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Figure 1. DNA synthesis in cells infected with $T4^+$ (O), T4x (□) and T4y (Δ) measured at 37 C°. (A) $^3H$ thymidine incorporation, 25 ug/15uCi/ml. (B) $^3H$ thymine incorporation, 25 ug/15uCi/ml. Both thymidine and thymine were added at 4 minutes after infection.

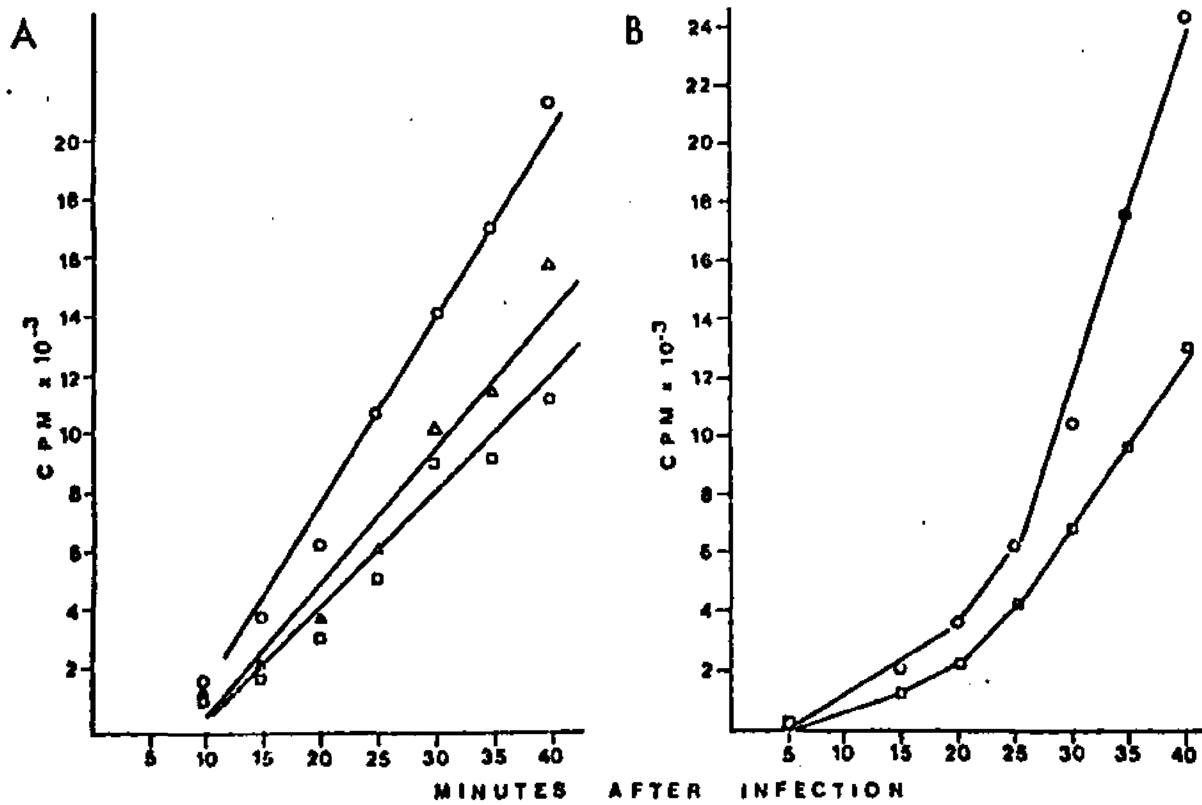

A
B
CPM x 10⁻³
MINUTES AFTER INFECTION

Figure 2. The effect of ultraviolet light on DNA synthesis in cells infected with T4$^+$ (A), T4*x* (B), T4*y* (C), and T4*v* (D). Free phage were irradiated at 0 $Jm^{-2}$ (0); 14 $Jm^{-2}$ (□); 28 $Jm^{-2}$ (△); and 42 $Jm^{-2}$ (▽). Incorporation of thymidine was measured as in Figure 1A.

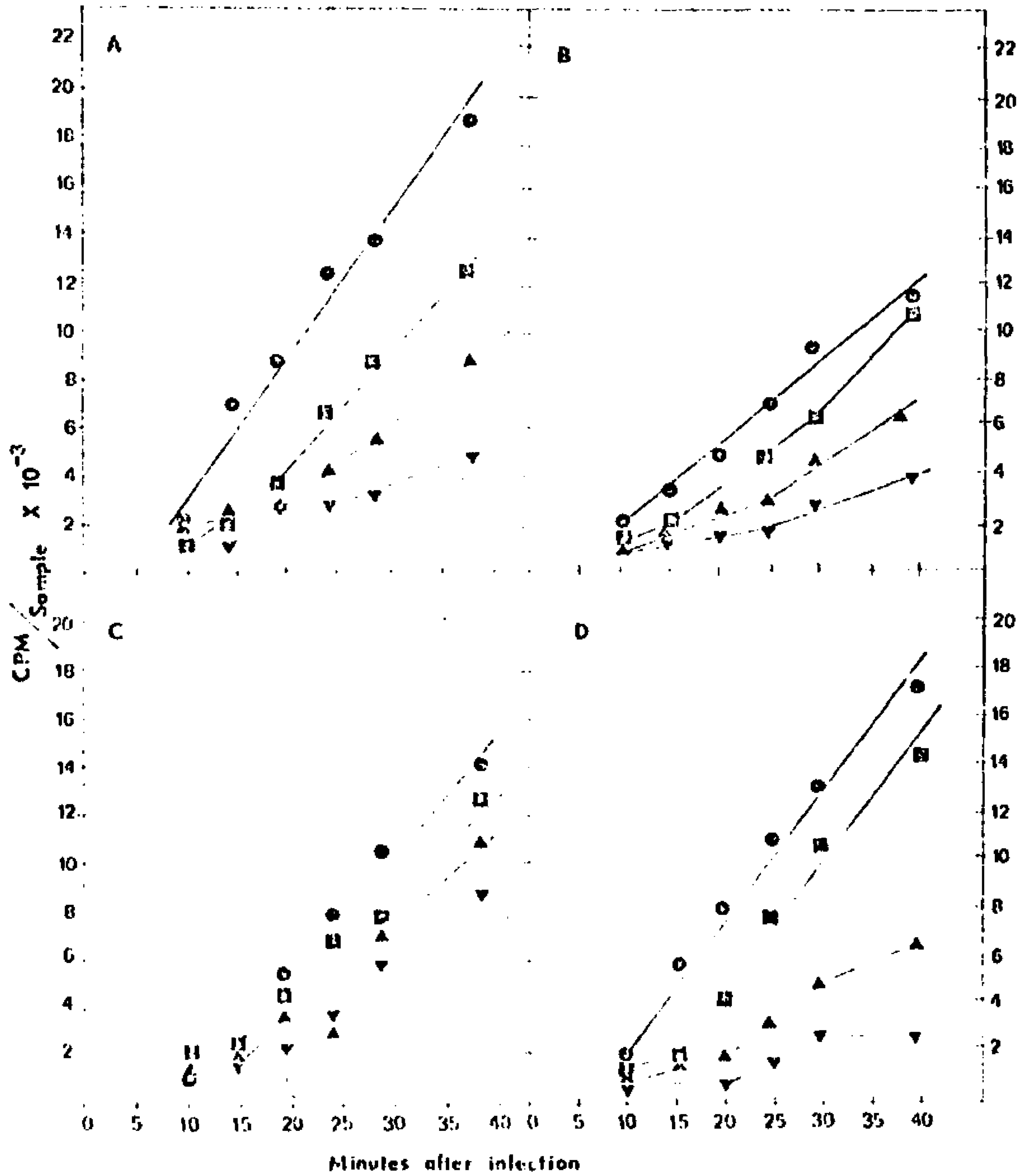
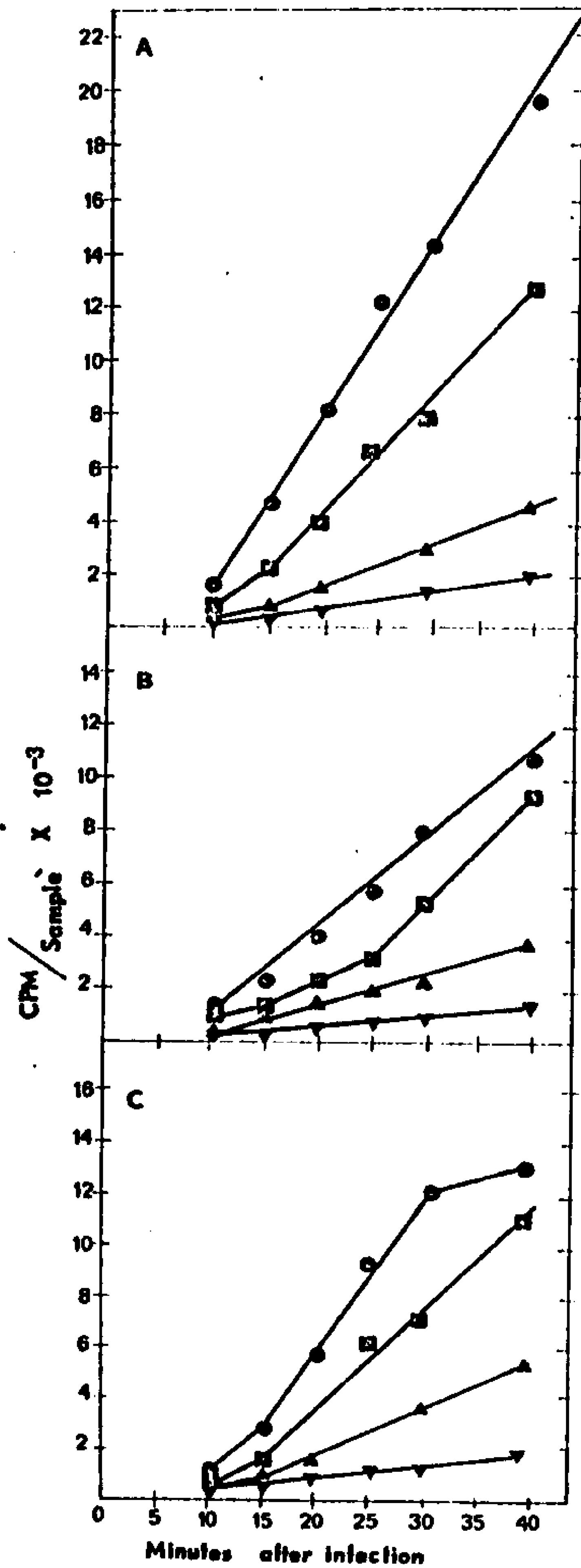

A
B
C
D
CPM/Sample X $10^{-3}$
Minutes after infection

Figure 3. The effect of x-irradiation on DNA synthesis in cells infected with $T4^{+}$ (A), T4*x* (B), T4*y* (C). Free phage were irradiated at 0 Krads (○); 45 Krads (□); 90 Krads (△); and 135 Krads (▽). Incorporation of thymidine was measured as in Figure 1A.


A
B
C
CPM/Sample X $10^{-3}$
Minutes after infection
0
5
10
15
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40
2
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10
12
14
16
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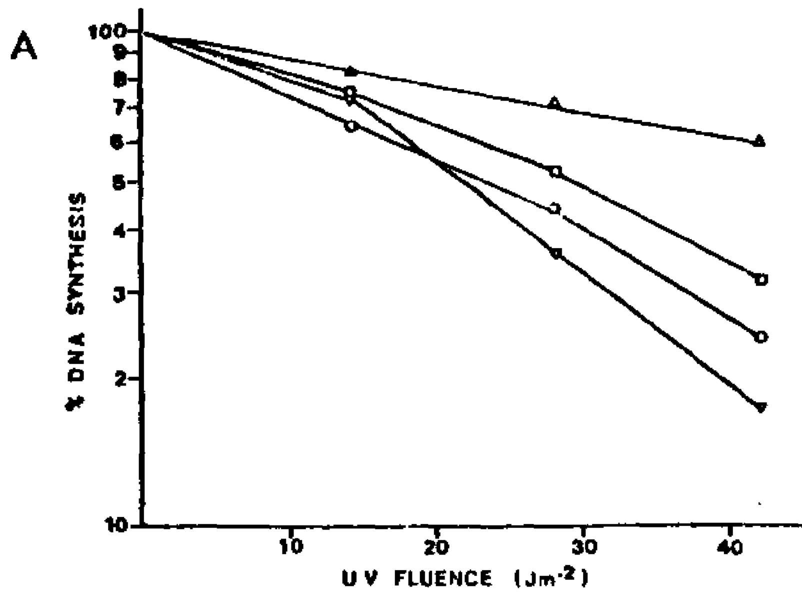
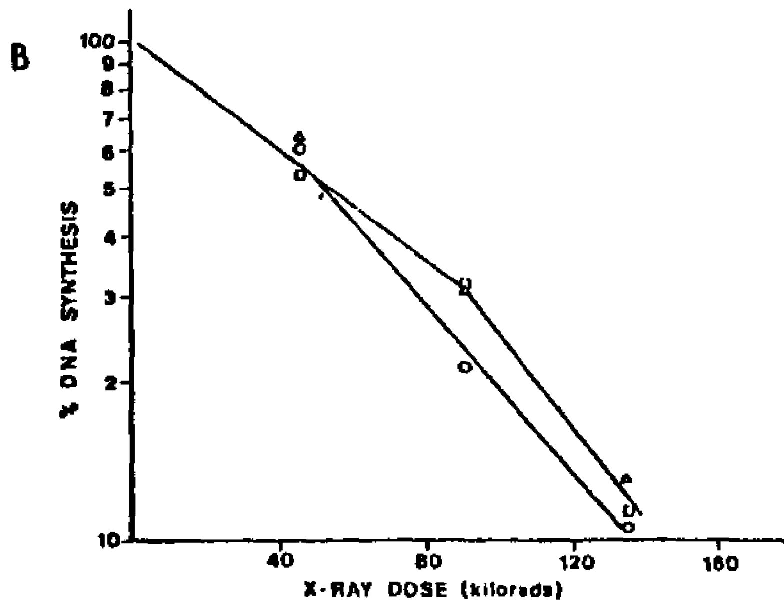
20
22

Figure 4. Inactivation of thymidine incorporation in cells infected with $T4^{+}$ (0): T4*x* (□), T4*y* (△), and T4*v* (▽) at 37°C as calculated from the final slopes of the DNA synthesis curves obtained in Figures 2 and 3. (A) Ultraviolet irradiation of free phage. (B) X-ray irradiation of free phage. (C) Mitomycin C inactivation of phage-bacterium complexes.

A




B




C

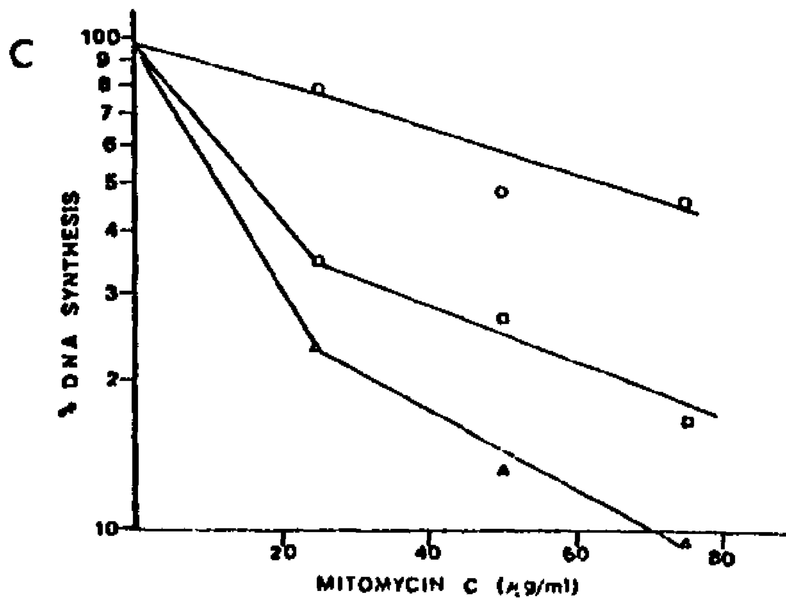


Figure 5. Mitomycin C inactivation of phage-infected cells. Infected complexes were exposed to mitomycin C for 10 minutes after infection prior to diluting and plating at 37°C. The entire experiment was performed at 37°C. Symbols: $T4^+$ (0), T4*x* (□), and T4*y* (Δ).

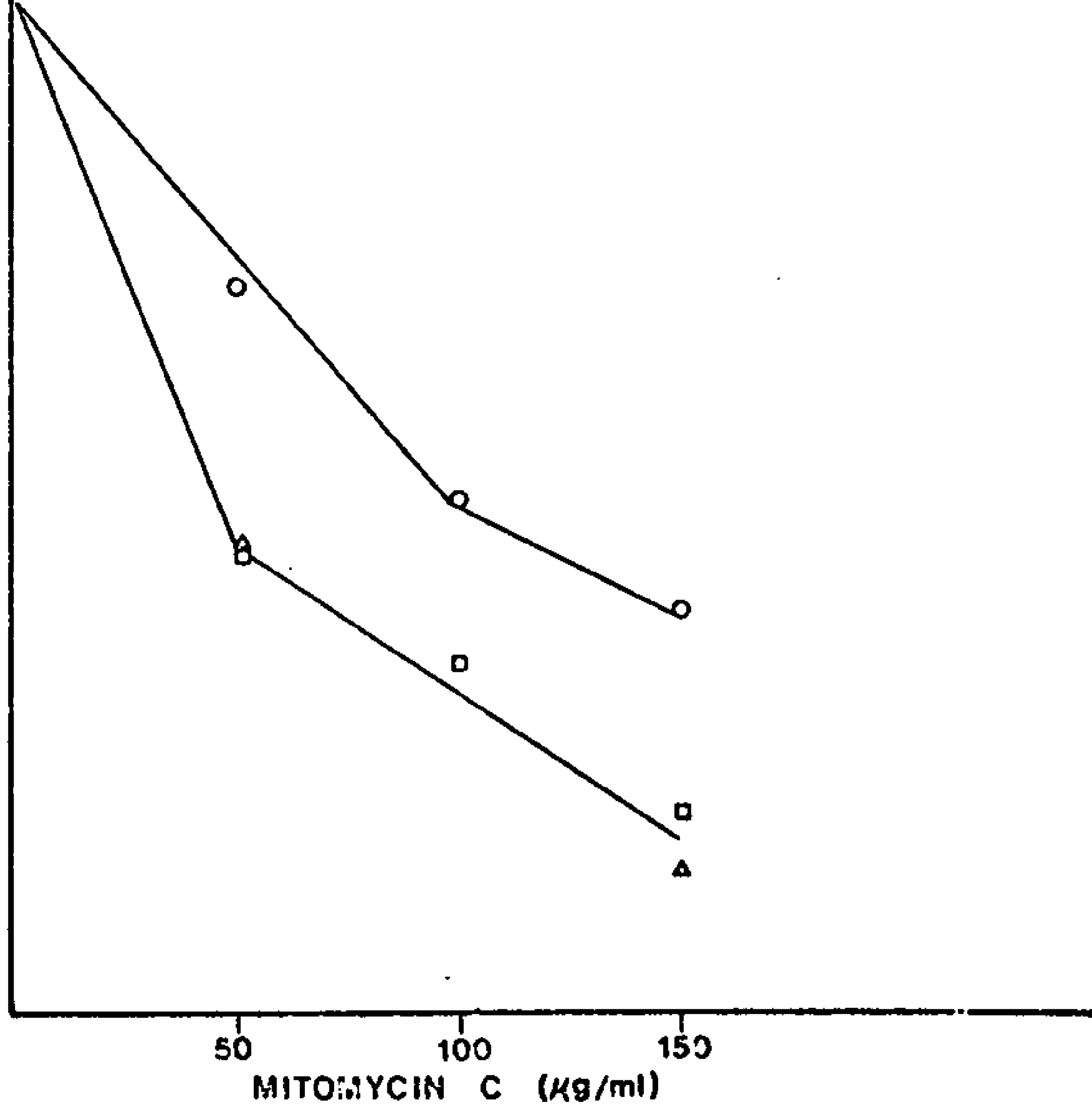
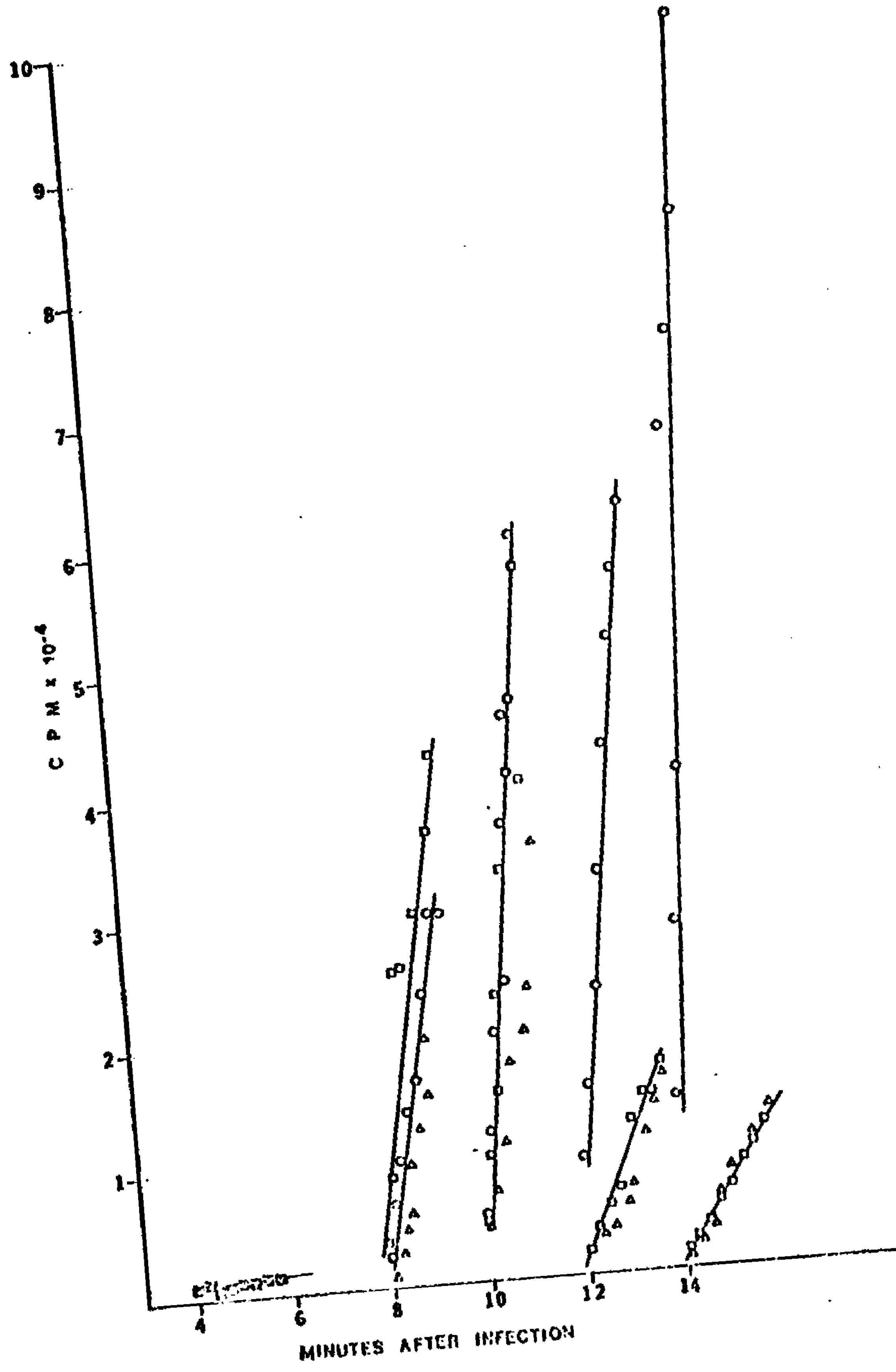
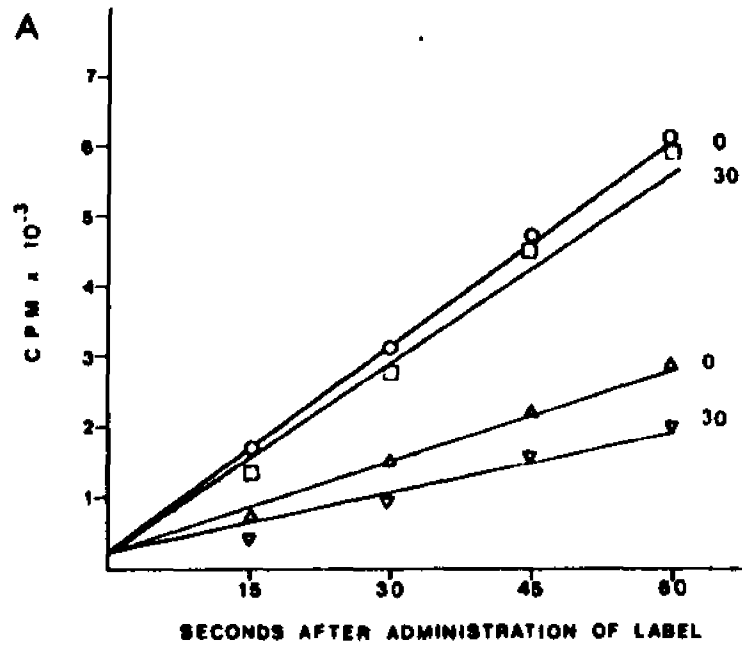

% SURVIVAL PLAQUE FORMING ABILITY
50
100
150
MITOMYCIN C (μg/ml)

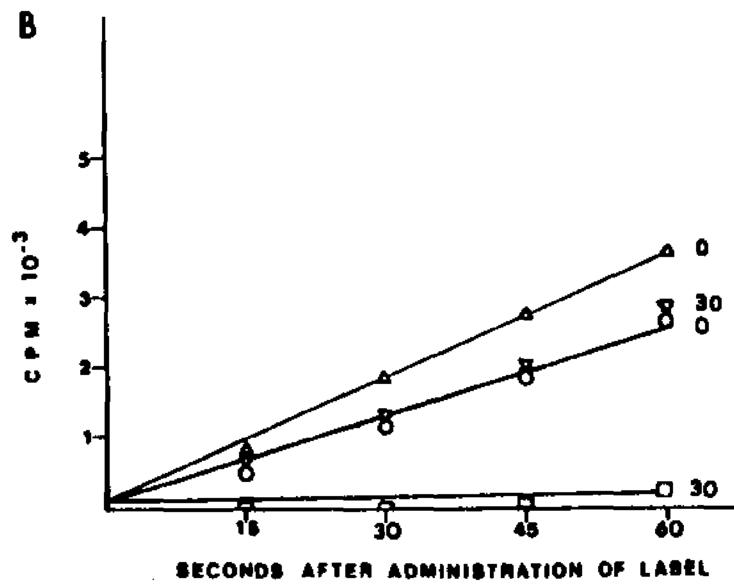
Figure 6. DNA synthesis at 20°C as measured by incorporation of high specific activity thymidine (0.12 ug/33uCi/ml) in the absence of additional cold thymidine (final concentration 0.12 ug/ml). Cells were infected with T4$^{+}$ (0), T4*x* (□) and T4*y* (△) at 37°C and transferred to 20°C one minute prior to labeling. Fifteen second samples were taken after administration of label at 4, 8, 10, 12 or 14 minutes after infection.



C P M x 10⁻⁴
10
9
8
7
6
5
4
3
2
1
4
6
8
10
12
14
MINUTES AFTER INFECTION

Figure 7. DNA synthesis at 20°C as measured by the incorporation of high specific activity thymidine in the absence of additional cold thymidine (final concentration 0.12 ug/33uCi/ml). Cells were infected and labeled at 20°C at the times in minutes indicated in the Figure. (A) $T4^+$, (B) T4x, and (C) T4y.


A
CPM x $10^{-3}$
7
6
5
4
3
2
1
0
30
0
30
15
30
45
60
SECONDS AFTER ADMINISTRATION OF LABEL


B
CPM x $10^{-3}$
5
4
3
2
1
0
30
0
30
15
30
45
60
SECONDS AFTER ADMINISTRATION OF LABEL

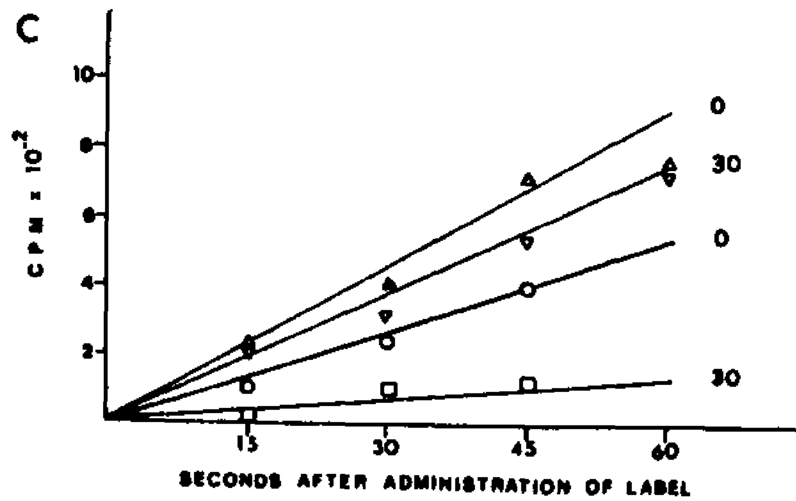

C
CPM x $10^{-2}$
10
8
6
4
2
0
30
0
30
15
30
45
60
SECONDS AFTER ADMINISTRATION OF LABEL

Figure 8. Effect of amber suppressor E. coli CR63 on thymidine incorporation. E. coli B or CR63 were infected with $T4^{+}$, T4x, or T4y at 37°C. At 14 minutes post-infection, cells were transferred to 20°C for one minute prior to labeling. Cells were labeled using final concentrations of 0.12 ug/33uCi/ml. Open symbols: E. coli B; closed symbols: E. coli CR63. $T4^{+}$: 0, ; T4x: □ , ■; and T4y △, ▲.

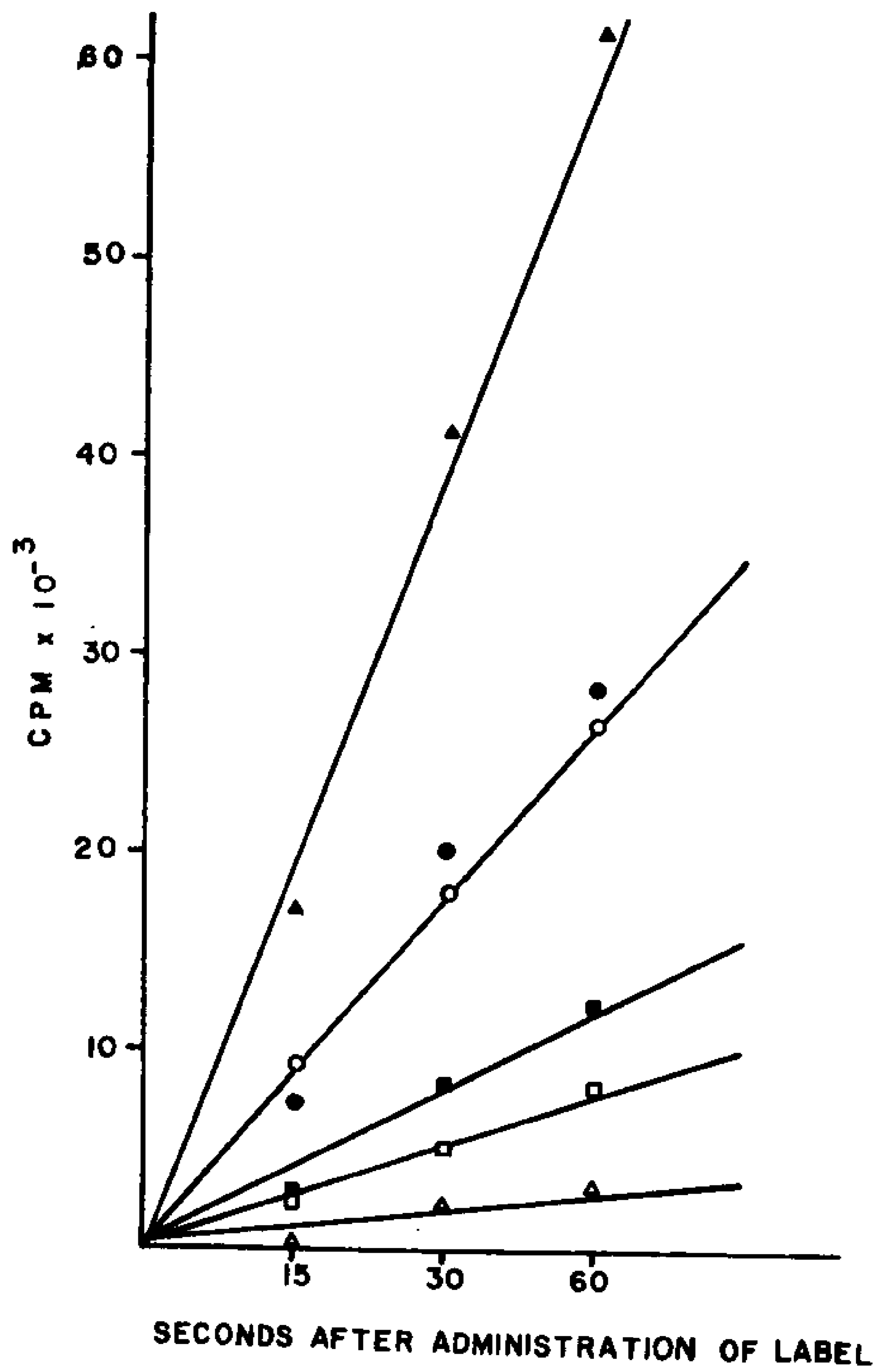
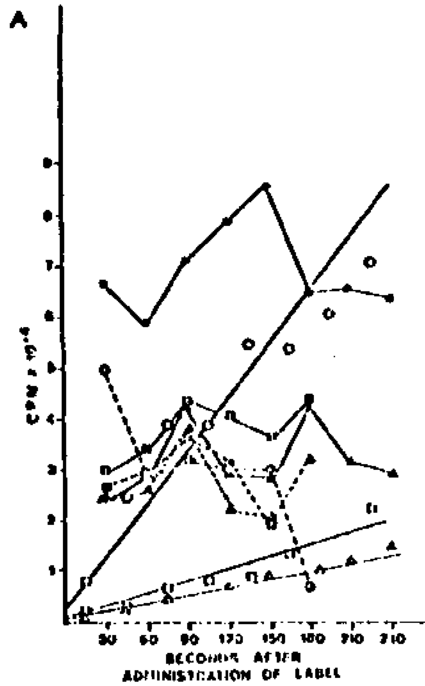
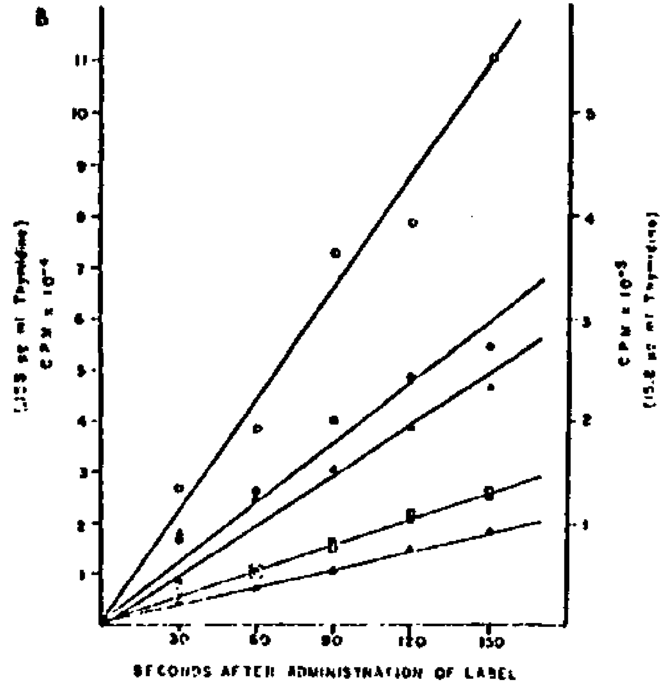
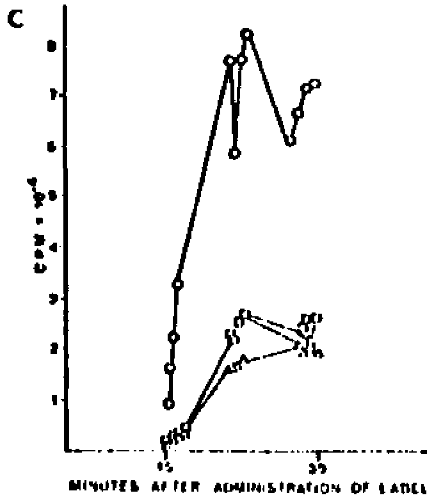

60
50
40
30
20
10
CPM x 10$^{-3}$
15
30
60
SECONDS AFTER ADMINISTRATION OF LABEL

Figure 9. Parameters affecting apparent DNA synthetic rates: (A) Permeability of cells infected with T4$^{+}$, T4*x* and T4*y* to labeled thymidine as determined by measuring free intracellular label. Cells were infected for 14 minutes at 37°C. One minute after transfer to 20°C, they were labeled with 0.12 ug/33 uCi/ml thymidine. Total intracellular label was determined by placing 20 ul aliquots onto Millipore filters and washing with 2 mls of M9 medium. Symbols: T4$^{+}$ (●), T4*x* (■) and T4*y* (▲). From the same tubes at staggered 30 second intervals incorporated counts were determined by collecting 20 ul samples onto GFA filters and precipitating with TCA. Symbols: T4$^{+}$ (○), T4*x* (□), T4*y* (△). Free intracellular label was determined by subtracting total intracellular label from incorporated label. Symbols: T4$^{+}$ (◐), T4*x* (◧), T4*y* (◭). (B) The effects of varying thymidine concentrations on the incorporation of labeled thymidine into TCA precipitable material were examined by labeling with 33 uCi/ml of thymidine in the presence of either 0.158 ug/ml or 15.8 ug/ml final concentration thymidine. Counts for the latter were corrected for the difference in specific activity. Symbols: T4$^{+}$ (○), T4*x* (□), T4*y* (△) 0.158 ug/ml thymidine; T4$^{+}$ (●), T4*x* (■), T4*y* (▲), 15.8 ug/ml thymidine. (C) Cells were grown and infected as in (A), except that the label was followed for 15 minutes after its administration. Symbols: T4$^{+}$ (○), T4*x* (□), T4*y* (△).

Figure 9 (Con't.). (D) Cells were grown and infected as in (A), except that an additional 33 uCi/ml of label was added 10 minutes after the addition of label at 15 minutes post-infection. Symbols: $T4^{+}$ (○), T4*x* (□), T4*y* (△).


A
CPM x 10⁻⁴
SECONDS AFTER
ADMINISTRATION OF LABEL


B
SECONDS AFTER ADMINISTRATION OF LABEL


C
CPM x 10⁻⁴
MINUTES AFTER ADMINISTRATION OF LABEL

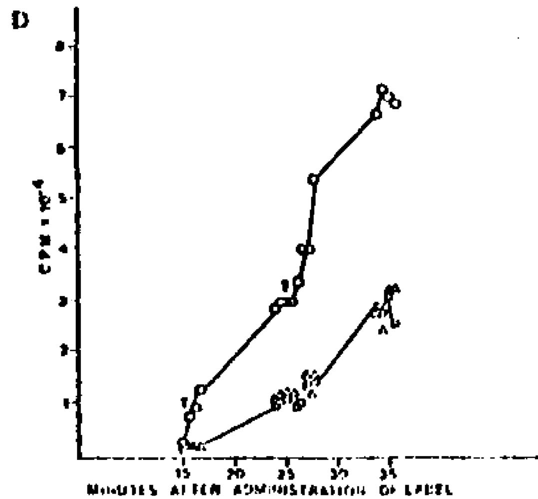

D
CPM x 10⁻⁴
MINUTES AFTER ADMINISTRATION OF LABEL

Figure 10. The effect of rifampin on DNA synthesis measured by incorporation of 0.12 ug/33uCi/ml thymidine (final concentration 0.12 ug/ml) at 20°C. The complexes were incubated at 37°C until 14 minutes after infection, at which time they were switched to 20°C. Label was added at 15 minutes after infection and samples taken at 15 second intervals thereafter. Rifampin (200 ug/ml final concentration) was added at the indicated time after infection.

Symbols: $T4^{+}$ (○), T4x (□) and T4y (△).

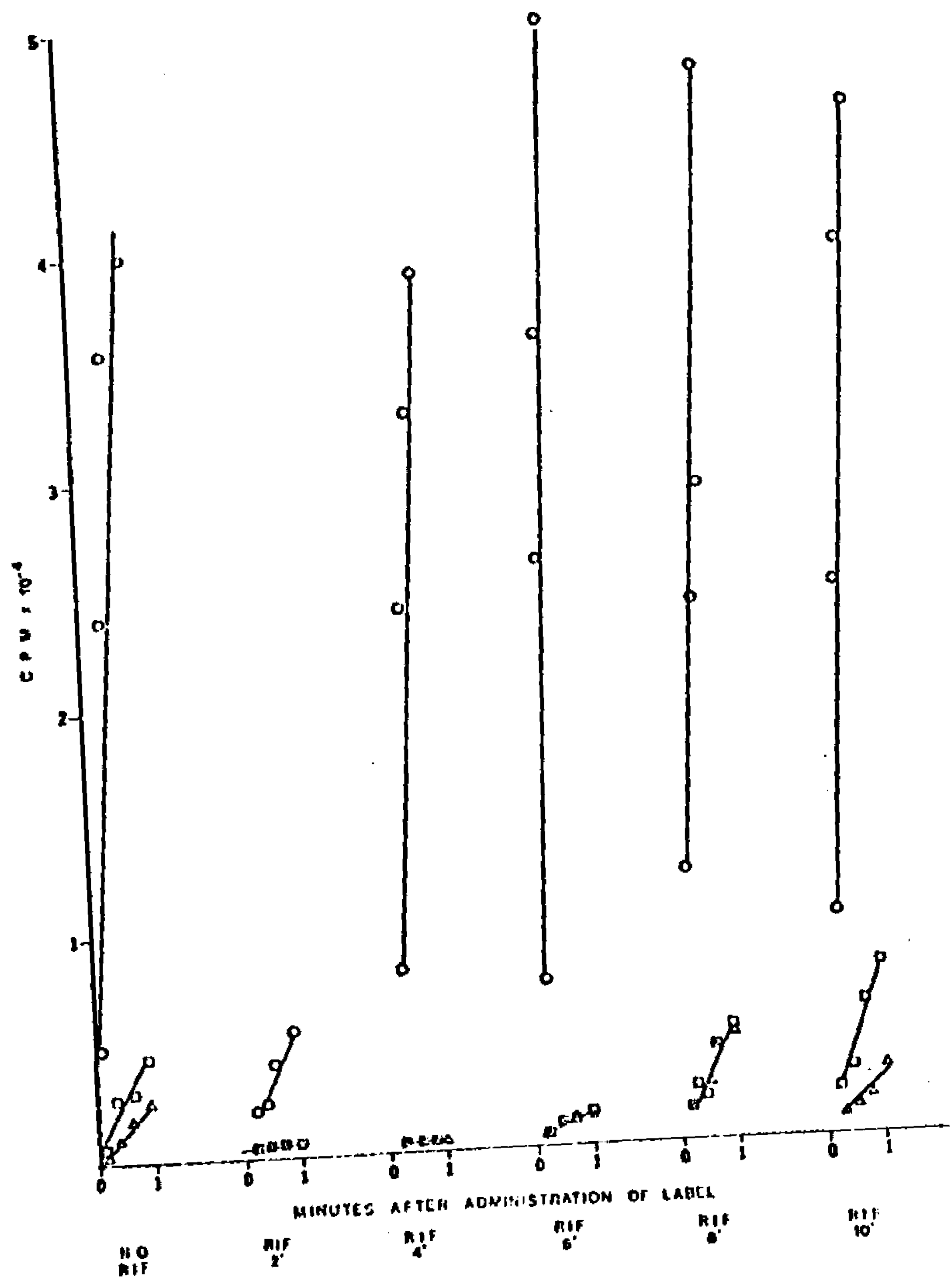
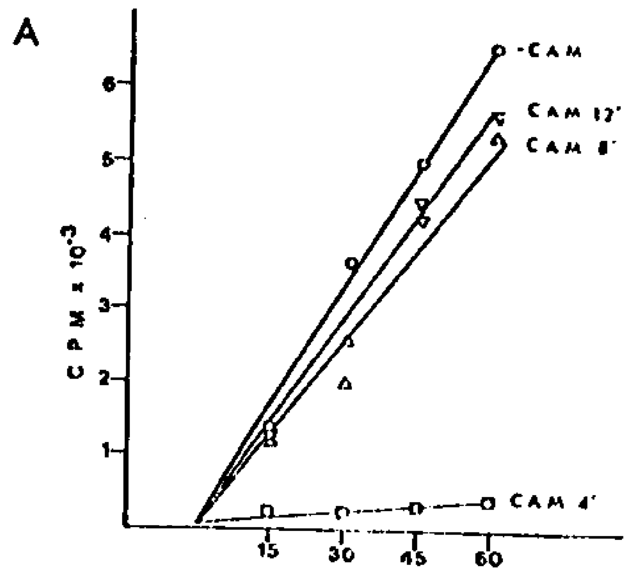
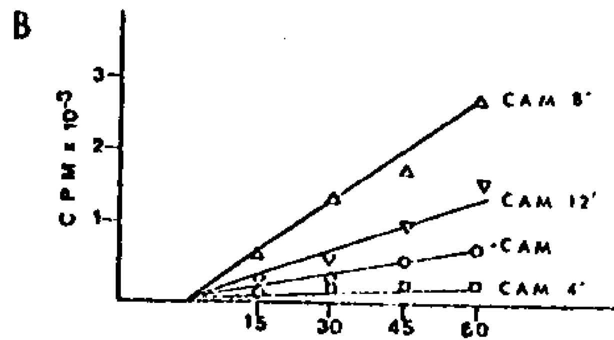

CPM x 10⁻⁴
5
4
3
2
1
0 1
MINUTES AFTER ADMINISTRATION OF LABEL
NO RIF
RIF 2'
RIF 4'
RIF 6'
RIF 8'
RIF 10'

Figure 11. The effect of chloramphenicol on DNA synthesis as measured by incorporation of thymidine at a final concentration of 0.12 ug/33Ci/ml at 20°C. The label was given 1 minute after 14 minutes of post-infection incubation at 37°C. Chloramphenicol (200 ug/ml final concentration) was added at the indicated times after infection. (A) $T4^{+}$, (B) T4x. Symbols: no chloramphenicol (O), chloramphenicol 4 minutes (□), chloramphenicol 8 minutes (△), chloramphenicol 12 minutes (▽).


A
C P M x 10-3
6
5
4
3
2
1
-CAM
CAM 12'
CAM 8'
CAM 4'
15
30
45
60


B
C P M x 10-3
3
2
1
CAM 8'
CAM 12'
-CAM
CAM 4'
15
30
45
60

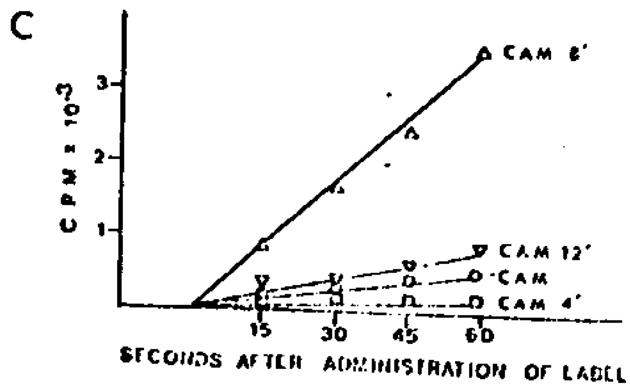
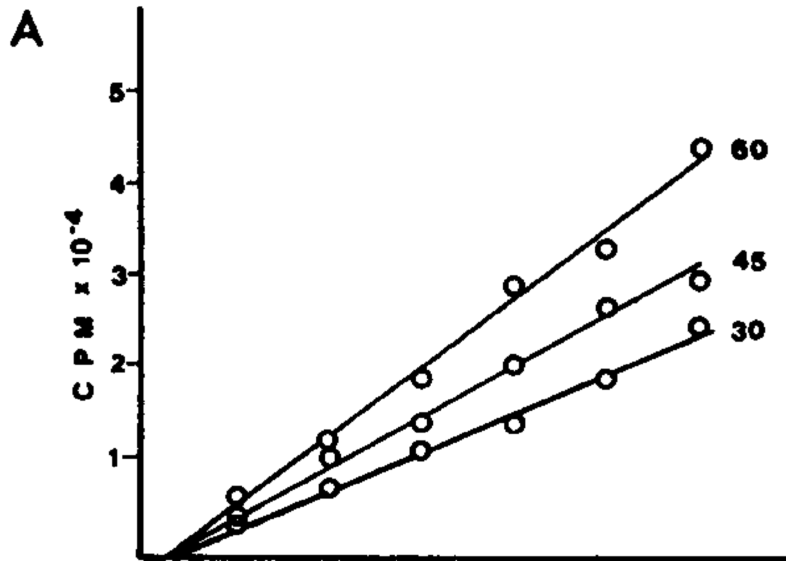
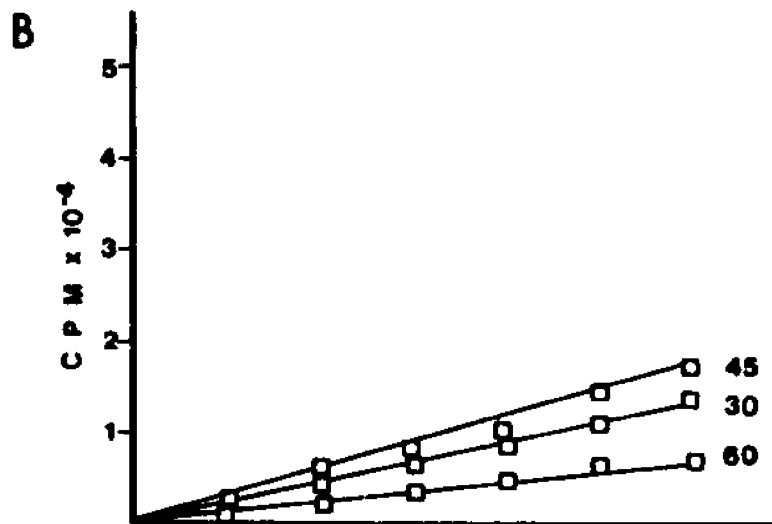

C
C P M x 10-3
3
2
1
CAM 8'
CAM 12'
-CAM
CAM 4'
15
30
45
60
SECONDS AFTER ADMINISTRATION OF LABEL

Figure 12. Effect of mitomycin C on DNA syntheais measured at 8 and 15 minutes after infection with $T4^+$, T4x and T4y. Infected cells were incubated at 37°C until 1 minute prior to labeling, at which time they were switched to 20°C. Cells were labeled with tritiated thymidine (0.12 ug/33uCi/ml). When present, mitomycin C was at a final concentration of 30 ug/ml as indicated in the Figure. (A) $T4^+$, (B) T4x, (C) T4y. Infected cells labeled at 8 minutes: minus mitomycin C (△), plus mitomycin C (▽). Infected cells labeled at 15 minutes: minus mitomycin C (O), plus mitomycin C (□).


A
CPM x 10-4
5
4
3
2
1
60
45
30


B
CPM x 10-4
5
4
3
2
1
45
30
60

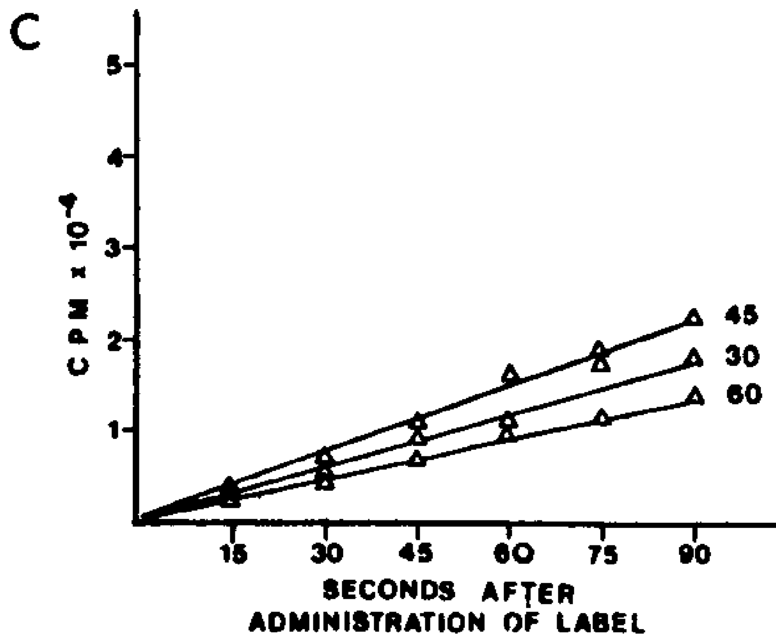
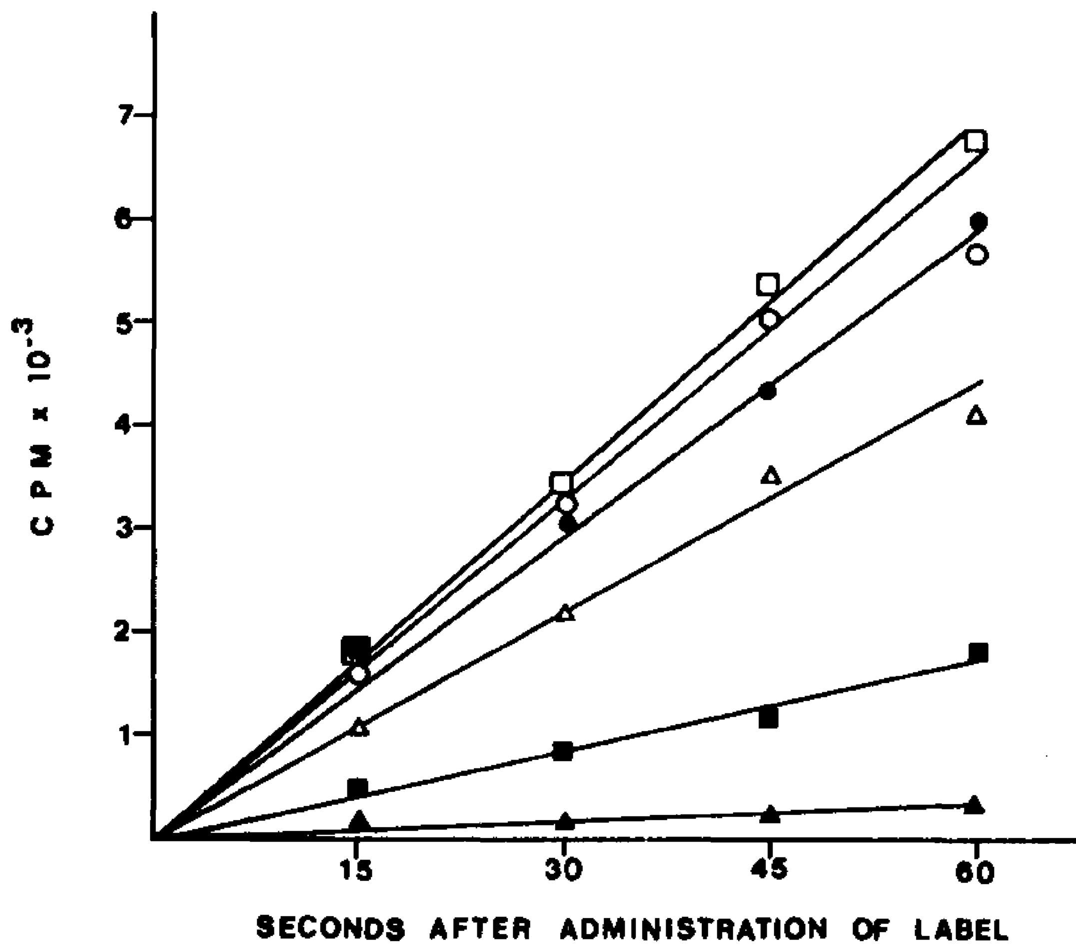

C
CPM x 10-4
5
4
3
2
1
45
30
60
15
30
45
60
75
90
SECONDS AFTER
ADMINISTRATION OF LABEL

Figure 13. Effect of mitomycin C on chloramphenicol rescued synthesis. $T4^{+}$-, T4*x*-, and T4*y*-infected cells were incubated at 37°C and treated with chloramphenicol (200 ug/ml final concentration) at 8 minutes after infection. One minute prior to labeling, cells were shifted to 20°C and $^{3}H$ thymidine was added (0.12 ug/33uCi/ml). Final mitomycin C concentration was 30 ug/ml. Symbols: $T4^{+}$ (○); $T4^{+}$ + mitomycin C (●); T4*x* (□); T4*x* + mitomycin C (■); T4*y* (△), T4*y* + mitomycin C (▲).



7
6
5
4
3
2
1
C P M x 10-3
15
30
45
60
SECONDS AFTER ADMINISTRATION OF LABEL

Figure 14. DNA synthesis in cells coinfected with mutant and wildtype phage. (A) Infected cells were grown for 14 minutes at 37°C and then transferred to 20°C for 1 minute prior to the addition of high specific activity thymidine (0.12 ug/33uCi/ml). (B) Cells were infected and labeled as in (A), except that 200 ug/ml (final concentration) of rifampin was added 4 minutes after infection. (C) Cells were infected and labeled as in (A), except 200 ug/ml (final concentration) of chloramphenicol was added 8 minutes after infection. The total input M.O.I. of each phage was 10. Symbols: $T4^+$ (O), T4*x* (□), T4*y* (△), $T4^+$ + T4*x* (■), $T4^+$ + T4*y* (▲), and T4*x* and T4*y* (▼).

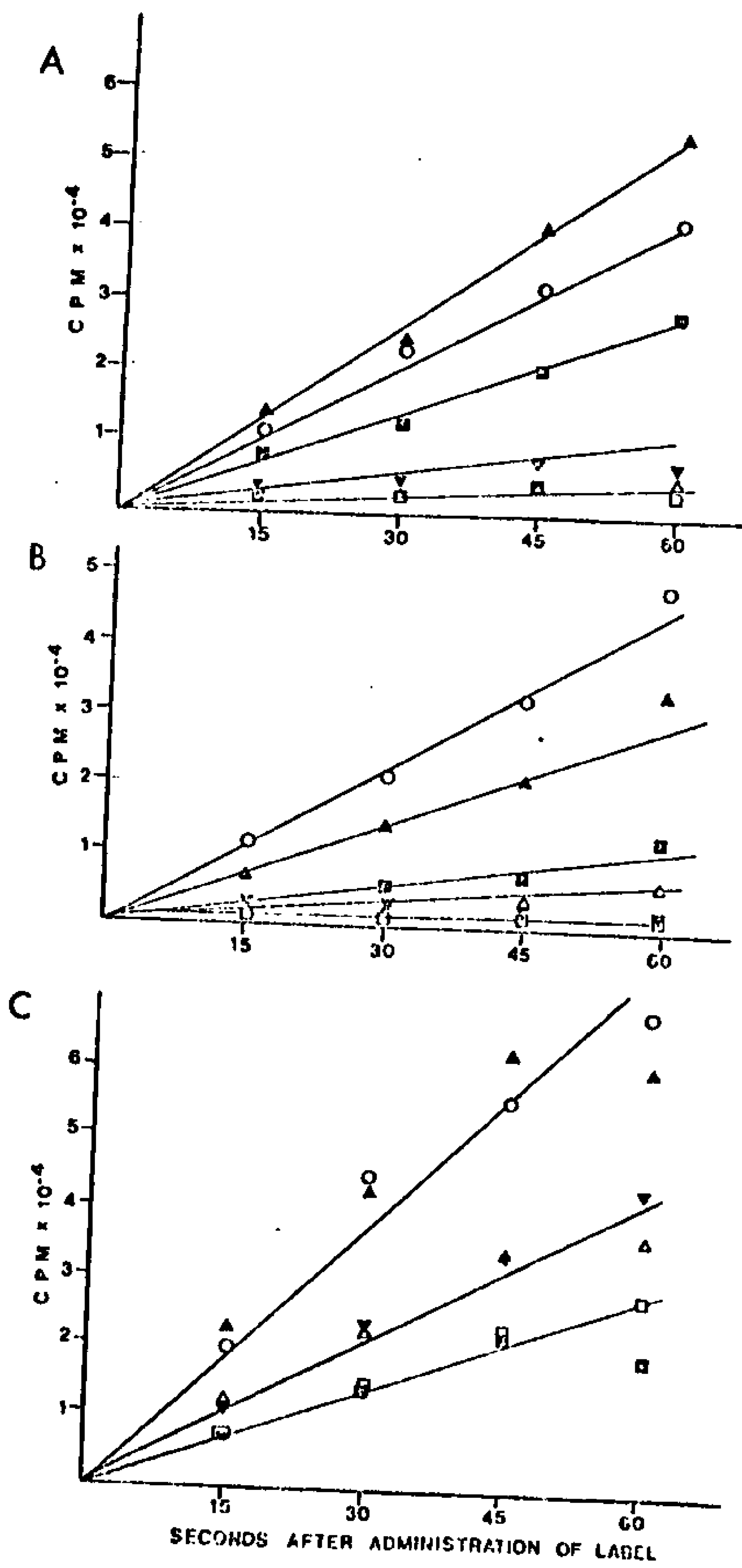

A
C P M x $10^{-4}$
15
30
45
60
B
C P M x $10^{-4}$
C
C P M x $10^{-4}$
SECONDS AFTER ADMINISTRATION OF LABEL

Figure 15. Neutral sucrose gradient analysis of progeny T4 DNA labeled 8 minutes after infection. *E. coli* B cells were grown and infected with T4 wildtype, T4*x*, or T4*y* at a M.O.I. of 5 at 37°C as described in Materials and Methods. Seven minutes after infection the phage-bacterium complexes were transferred to 20°C for 1 minute prior to labeling with $^{3}H$ thymidine (0.19 ug/33uCi/ml) for Materials and Methods using lysozyme, EDTA, and BrdU 58. Samples were centrifuged for 20 minutes at 17,000 rpm at 4 C through 5-30% neutral sucrose gradients. Symbols: $T4^{+}$ (O); T4*x* (□); and T4*y* (Δ).

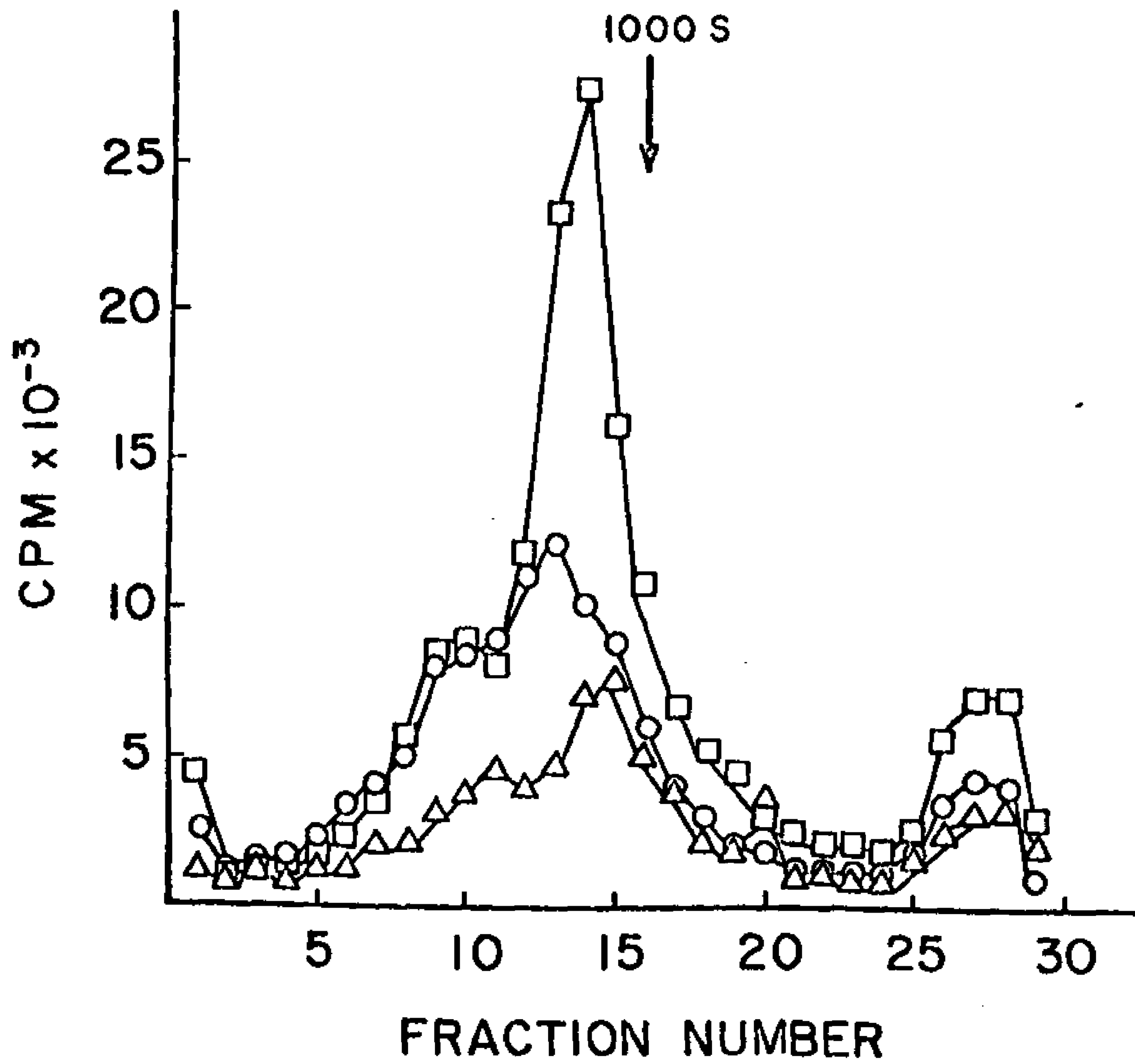
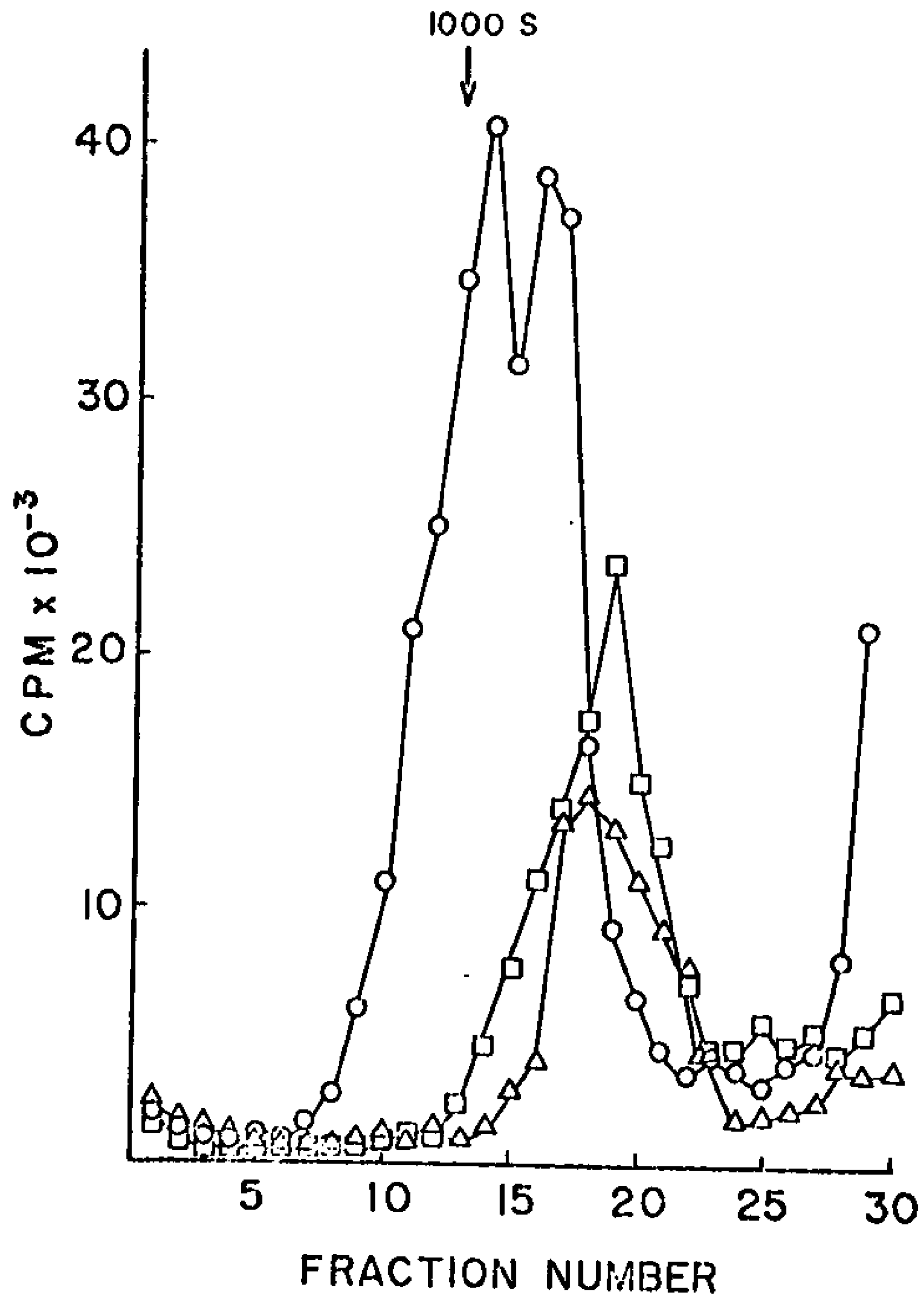

1000 S
CPM x $10^{-3}$
25
20
15
10
5
5
10
15
20
25
30
FRACTION NUMBER

Figure 16. Neutral sucrose gradient analysis of progeny T4 DNA labeled 15 minutes after infection. *E. coli* B cells were grown and infected with T4 wildtype, T4*x* or T4*y* at a M.O.I. of 5 at 37°C as described in the Materials and Methods. Fourteen minutes after infection the phage-bacterium complexes were transferred to 20°C for 1 minute prior to labeling for 1 minute with thymidine (0.19 ug/ 33 uCi/ml). The complexes were lysed as described in Materials and Methods using lysozyme, EDTA, and BrdU 58. Samples were centrifuged for 45 minutes at 17,000 rpm at 4°C through 5-30% neutral sucrose gradients. Symbols: $T4^{+}$ (O); T4*x* (□); and T4*y* (△).



1000 S
40
30
20
10
CPM x $10^{-3}$
5
10
15
20
25
30
FRACTION NUMBER

Figure 17. Neutral sucrose gradient sedimentation analysis of T4 DNA isolated after pulse labeling followed by a cold chase or after continuous labeling. (A) Cells were prepared and labeled as in Figure 7. At 8 minutes after infection, after labeling for 1 minute, the cells were transferred back to $37^{o}C$ and chased with 500 ug/ml of cold thymidine until lysis at 15 minutes. (B) Cells were labeled from 4-15 minutes post infection at $37^{o}C$ with $^{3}H$ thymidine (25 ug/33 uCi/ml). In (A) and (B) cells were spun for 60 minutes at 17,000 rpm at 4 C through 5-30% neutral sucrose gradients. Symbols: $T4^{+}$ (O); T4x (□); and T4y (△).

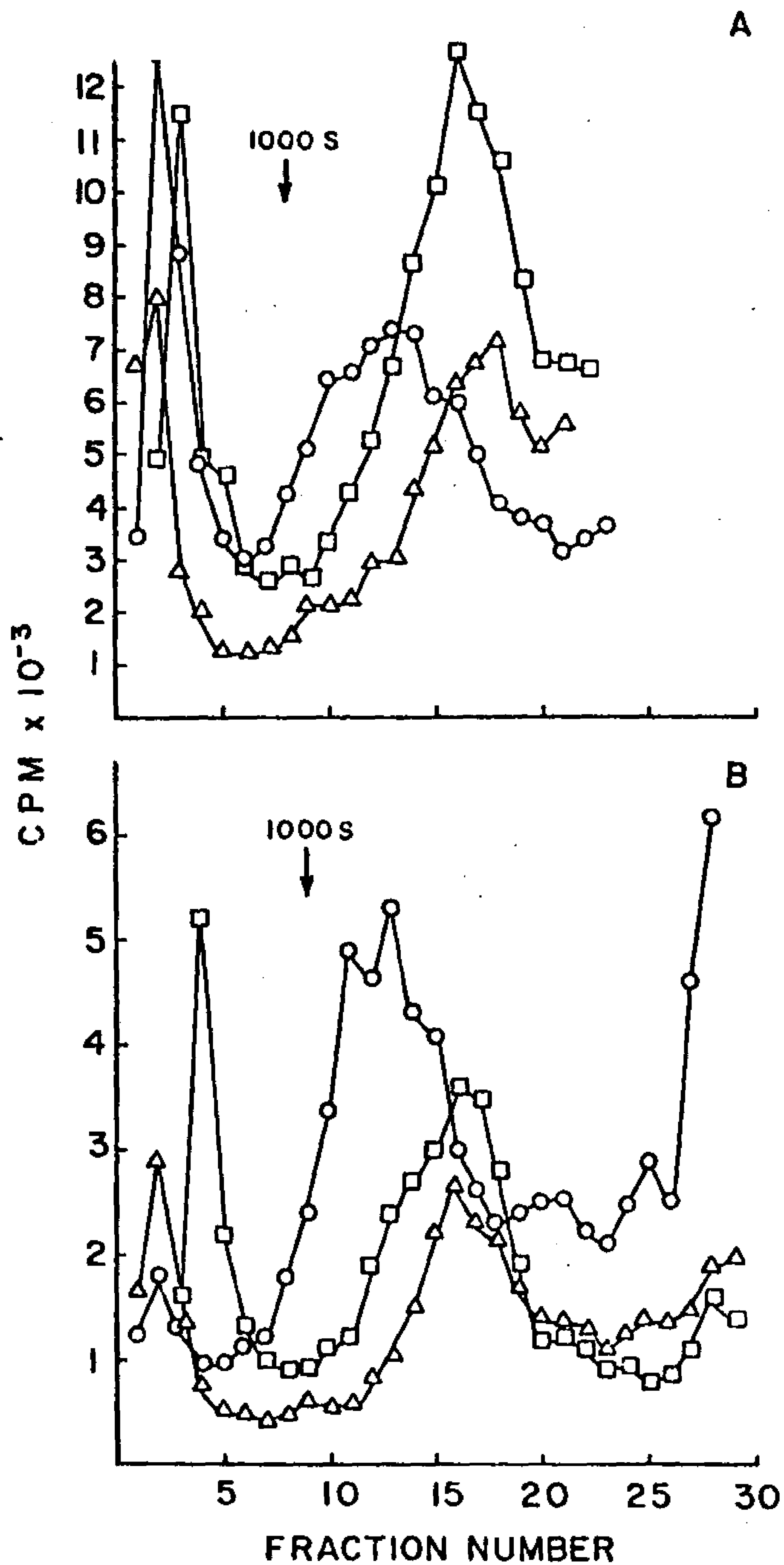

A
1000 S
B
1000 S
CPM x $10^{-3}$
FRACTION NUMBER

Figure 18. Alkaline sucrose gradient analysis of progeny T4 DNA labeled 8 and 20 minutes after infection. Cells were transferred to 20°C at 19 minutes after infection 1 minute prior to labeling for 1 minute at 20°C. DNA was labeled with 0.19 ug/33 uCi thymidine/ml. After loading, the lysed samples were made basic by the addition of 0.1 ml of 0.35N NaOH. Samples were centrifuged for 2 hours at 35,000 rpm at 4 C through 5-20% alkaline sucrose gradients. (A) Lysed at 8 minutes after infection. (B) Lysed 20 minutes after infection. Symbols: $T4^{+}$ (O); T4x (□); and T4y (▲).

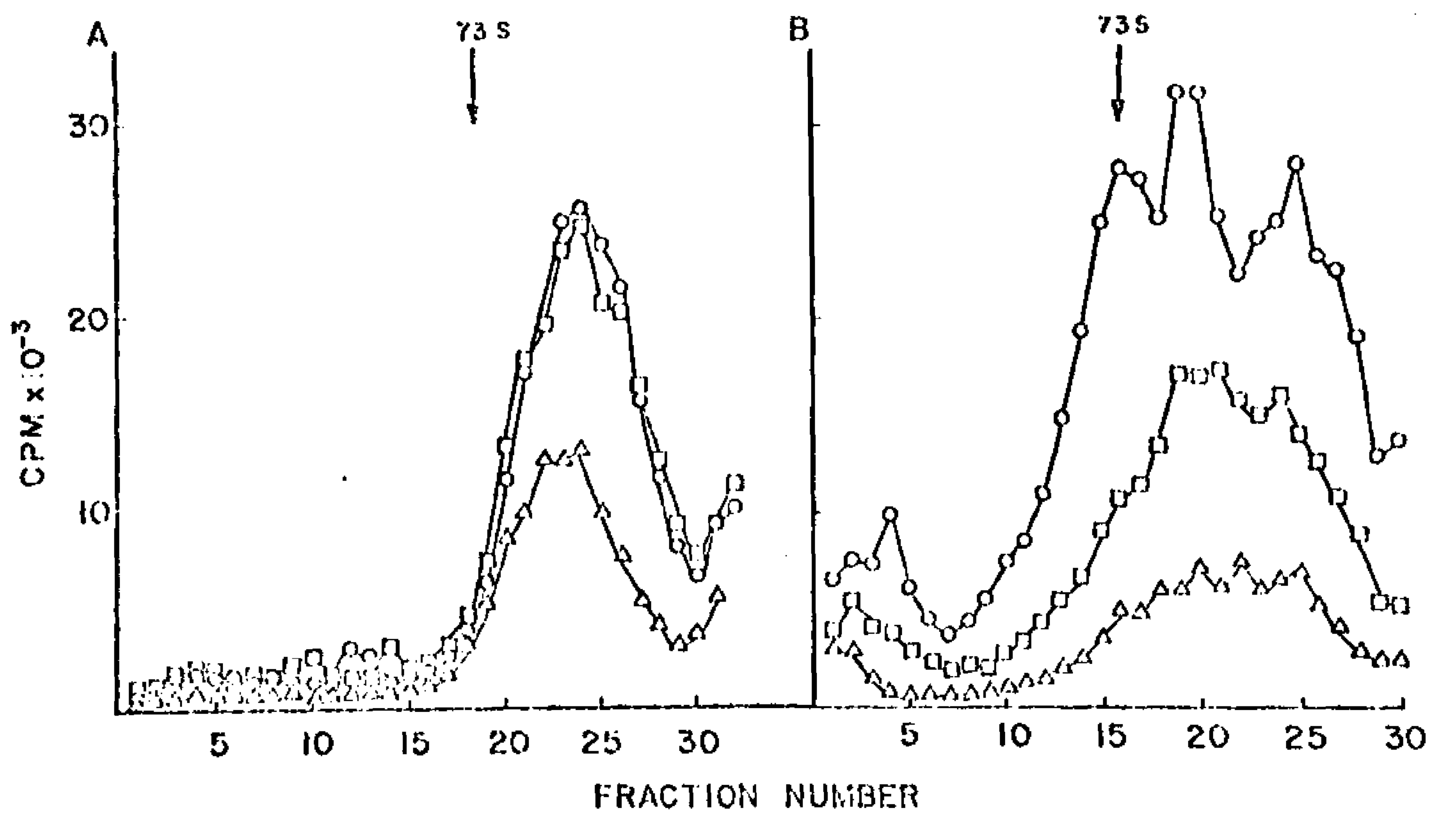
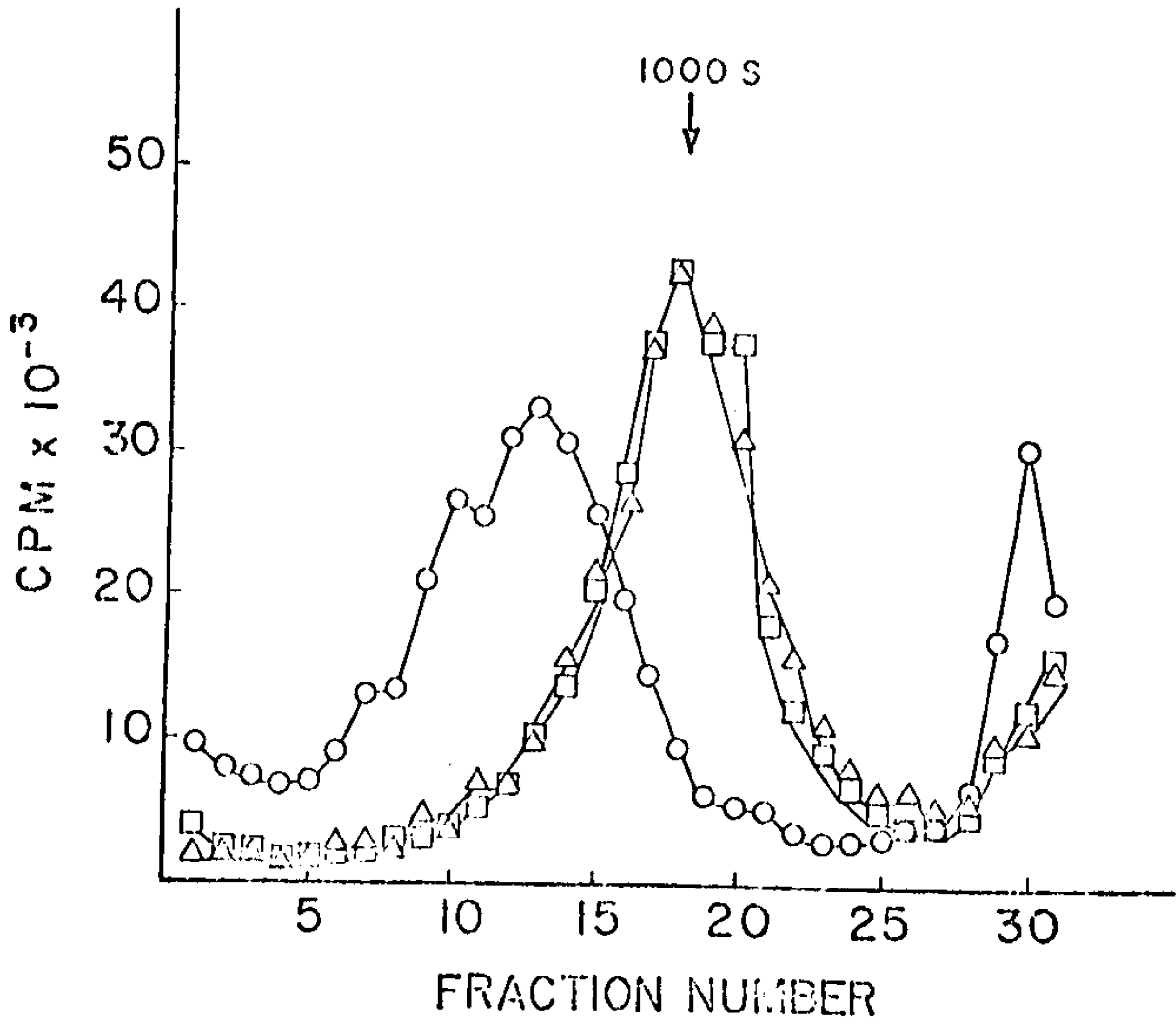

A
73 S
B
73 S
CPM x 10^-3
30
20
10
5
10
15
20
25
30
FRACTION NUMBER

Figure 19. Neutral sucrose gradient analysis of "chloramphenicol rescued" progeny T4 DNA labeled 15 minutes after infection after pretreatment with chloramphenicol at 8 minutes after infection. Cells were prepared and labeled at 15 minutes after infection as described in Figure 6, except that at 8 minutes they were treated with chloramphenicol (200 ug/ml). Samples were spun for 25 minutes at 17,000 rpm at 4 C through 5-30% neutral sucrose gradients. Symbols: $T4^{+}$ (O); T4x (□); and T4y (△).



1000 S
50
40
30
20
10
CPM x 10⁻³
5
10
15
20
25
30
FRACTION NUMBER

Figure 20. Alkaline sucrose gradient analysis of "chloramphenicol rescued" progeny T4 DNA labeled 15 minutes after infection after pretreatment with chloramphenicol at 8 minutes after infection. Samples were prepared and labeled as described in Figure 18. Samples were spun for 2 hours at 35,000 rpm at 4 C through 5-20% alkaline sucrose gradients. Symbols: $T4^+$ (O); T4x (□); T4y (△).

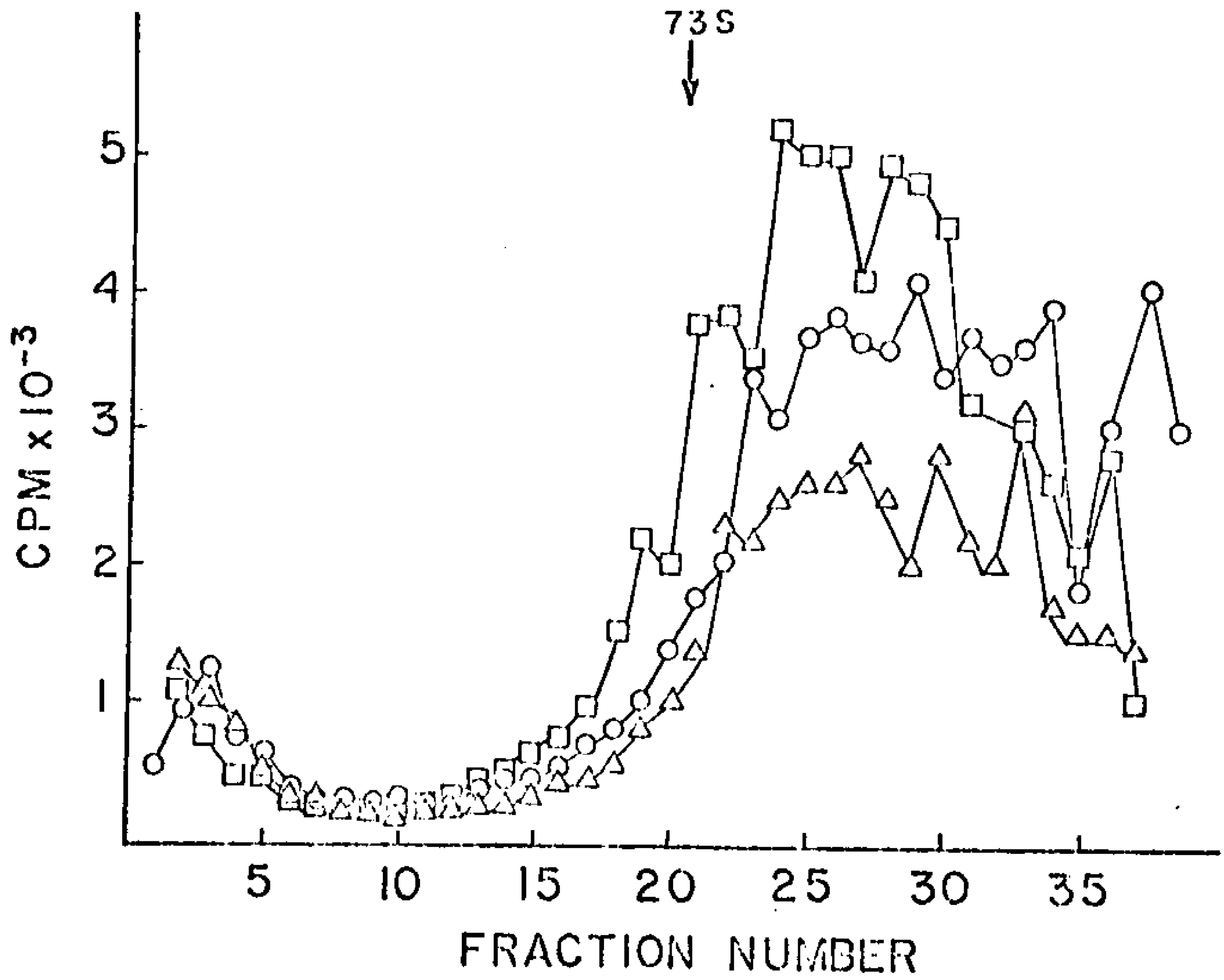
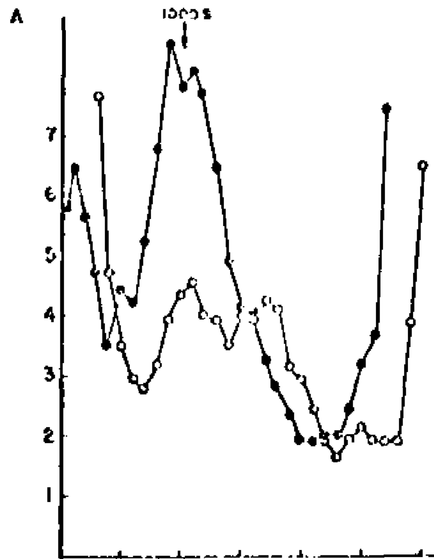
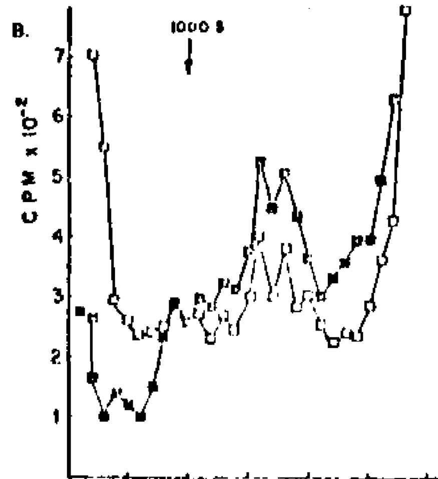
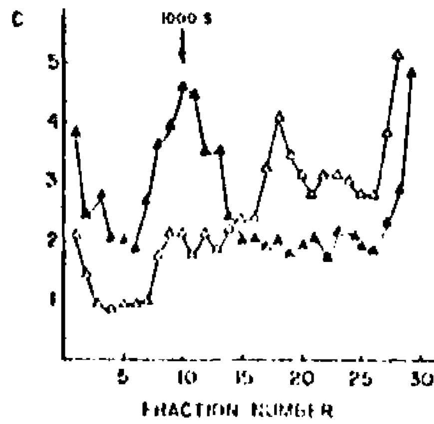

73S
CPM x 10^-3
5
4
3
2
1
5
10
15
20
25
30
35
FRACTION NUMBER

Figure 21. Neutral sucrose sedimentation analysis of parental DNA isolated after 15 minutes of infection. Labeled parental T4$^{+}$, T4$\underline{x}$ and T4$\underline{y}$ were prepared as described in the Materials and Methods. Cells were coinfected with the 6 possible combinations of labeled and unlabeled T4$^{+}$, T4$\underline{x}$ and T4$\underline{y}$. Complexes were lysed after 15 minutes of infection as described in Materials and Methods. Samples were spun on 5-30% neutral sucrose gradients for 1 hour at 17,000 rpm at 4 C. Symbols: (A) labeled T4$^{+}$ and unlabeled T4$\underline{x}$ (O), labeled T4$^{+}$ and unlabeled T4$\underline{y}$ (●); (B) labeled T4$\underline{x}$ and unlabeled T4$^{+}$ (□), labeled T4$\underline{x}$ and unlabeled T4$\underline{y}$ (□); (C) labeled T4$\underline{y}$ and unlabeled T4$\underline{x}$ (△), labeled T4$\underline{y}$ and unlabeled T4$^{+}$ (△).


A
1000 S
7
6
5
4
3
2
1


B.
1000 S
7
6
5
4
3
2
1
CPM x 10^-2


C
1000 S
5
4
3
2
1
5
10
15
20
25
30
FRACTION NUMBER