

BACTERIOPHAGE  $\phi$ X-174: ITS SENSITIVITY TO ULTRAVIOLET  
LIGHT AND GROWTH IN STARVED AND IRRADIATED CELLS

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I should like to dedicate this thesis to my parents.

## ABSTRACT

## Part I

Ultraviolet action spectra for inactivation of  $\Phi$ X-174 virus, its infective single-stranded DNA (SS), and an infective, intracellular and presumably double-stranded DNA (RF) have been determined. The biological activity of the irradiated DNA was measured using bacterial protoplasts.

The inactivation cross-section of the RF is nearly an order of magnitude less at all wavelengths than that of either the free single-stranded DNA or the intact virus, which have very similar cross-sections. Besides the difference in magnitude, the action spectrum of RF, when compared to that of SS  $\Phi$ X DNA shows several differences in band shape.

The similarity of the free SS and virus sensitivities to radiation in the range 240-302  $m\mu$  suggests that energy of these wavelengths which is effective in inactivation of the virus is that absorbed by the nucleic acid. Below 240  $m\mu$  the DNA is less sensitive than the virus; UV inactivation as a consequence of energy absorbed by the virus protein is a likely explanation of the higher viral sensitivity.

The quantum yield for inactivation of the single-stranded DNA is a function of wavelength in the range of wavelengths used, 225-302  $m\mu$ . This may be understood as a result of the variable fraction of absorbed energy localized in pyrimidines. This dependence of quantum yield on wavelength is altered in the case of the whole virus, presumably because of the important role of the protein at low wavelengths. The quantum yield of SS  $\Phi$ X DNA increases slightly with salt concen-

tration, reflecting the existence of some process which is enhanced on contraction of the polymer and the resulting stronger interactions between bases.

#### Part II

The ability of  $\Phi$ X bacteriophage-infected cells to release progeny after UV irradiation has been examined using both a host possessing host cell reactivation (E. coli C<sub>N</sub>) and one lacking it (E. coli C<sub>S</sub>). The UV sensitivity of both free  $\Phi$ X DNA extracted from infected cells and DNA irradiated in situ in the infected cell, as judged by their infectivity to bacterial protoplasts, is sufficient to account for the intrinsic sensitivity of the host-phage complex.

#### Part III

The burst of a starved bacterium infected with several  $\Phi$ X174 bacteriophage is usually found to contain descendants of only one of the parents; less often, two phage may multiply. Unstarved cells, in contrast, can support the growth of at least four phage. The unproductive phage seem to convert their parental single-stranded DNA into intracellular, double-stranded RF. These results are interpreted to mean that some factor required by  $\Phi$ X for the production of progeny is limited in starved cells.

#### Part IV

Evidence is presented that starved, UV-irradiated E. coli C which has lost its capacity to support  $\Phi$ X bacteriophage reproduction, has also become unsuitable for the synthesis of infective RF, though the incoming single stranded viral DNA is able to undergo the transition to an RF whose behavior on neutral density gradient analysis is the same as normal RF. Very alkaline conditions appear to

activate the inactive RF, releasing infective parental single strands as well as making infective those RF whose strands do not separate.

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Part I

THE SENSITIVITY OF THE INFECTIVE FORMS  
OF  $\phi$ X-174 TO UV LIGHT

### Introduction

$\Phi$ X is a minute bacteriophage, known to attack Shigella paradysenteriae, Escherichia coli, and Salmonella typhosa (54).

The virus particle contains one molecule (43, 44) of single-stranded DNA (44). This DNA molecule, extracted from virus and purified, is infective to bacteria made into protoplasts with lysozyme and versene (19, 20, 21, 34, 49); it is therefore the complete phage genome. The infective viral DNA is a ring (14, 15, 17) containing one discontinuity at which exonuclease action stops (15). The structure of this discontinuity, which may be the result of the joining of the ends of a  $\Phi$ X DNA molecule, is unknown, save that it is not strikingly more heat- (16) or alkali-sensitive (17) than the rest of the polynucleotide chain, and contains no sulphur (25). On entering its host, the viral single strand is transformed into a double-stranded structure, called RF, which is also infective to bacterial protoplasts (45), and is, like the parental single strand, a ring (8, 24).

The availability of an infective, single-stranded, well characterized viral DNA and the same DNA in a double-stranded form makes this system an attractive one for the study of the effects of UV. Presented here are the results of measurements of the sensitivity of the infectivity of  $\Phi$ X virus, single-stranded DNA (SS), and RF, to inactivation by monochromatic ultraviolet light (UV). The course of inactivation by UV may be described by the relation  $F = \exp(-\sigma_I D)$ , in which F is the fraction of the original infectivity remaining after exposure to D quanta/cm<sup>2</sup> of ultraviolet light. The quantity,  $\sigma_I$ , is called the inactivation cross-section and characterizes the sensitivity of the irradiated system. The product,  $\sigma_I D$ , is the average number



of damages per individual in the exposed population. Ultraviolet damages are distributed according to the Poisson distribution and only those units having received no damage retain their integrity. The measurement of  $\sigma_I$  at several wavelengths determines an action spectrum; a plot of  $\sigma_I$  versus wavelength is expected to resemble the absorption spectrum of the chromophore which absorbed the light quanta whose energy produced the measured effect. One may also assign absorption cross-sections by using the relation  $I/I_0 = \exp(-n\sigma_A l)$  in which  $I/I_0$  is the ratio of incident to transmitted quanta after traveling a length  $l$  of the solution and  $n$  is the concentration of the absorbing species in molecules per unit volume. The ratio of the inactivation to the absorption cross-section  $\sigma_I/\sigma_A$ , is the quantum yield or quantum efficiency; that is, the probability that an absorbed quantum will lead to the observed effect (26).

Conclusions about the mechanism of inactivation may be drawn from such experiments.

#### The Photochemical Basis of Inactivation

Many experiments have been performed on the constituents of DNA in an attempt to find photochemical alterations which might be responsible for biological damage.

Sinsheimer (42) found that derivatives of cytosine, including deoxycytidylic acid, form a photoproduct with high quantum yield. The photoproduct has a distinctive UV spectrum, and reverts to the corresponding parental cytosine compound spontaneously. The reversion rate is enhanced by heating and by acid or alkaline pH; recovery of the cytosine absorption spectrum is almost quantitative.

The photoproduct is widely surmised to be cytosine with water added across the 5, 6 double bond (40, 48), though proof is difficult because of quick reversion to the parental compound.

There is a recent report from Daniels and Grimison (9) that cytosine, on irradiation by 2537Å light, yields uracil and ammonia with appreciable efficiency. They suggest the deamination of cytosine by UV. Wacker (48), however, failed to detect deamination on irradiation of deoxycytidine.

The relevance of these results on model compounds to the inactivation of highly polymerized DNA is uncertain. Howard and Tessman (22) have found, after irradiation of S13 virus and its host, mutants attributable to C to U conversions, but because of the great degeneracy of the amino acid code (46) cytosine to uracil conversions in regions of the DNA containing structural information for proteins may rarely be lethal. Such changes might not change the amino acid specified by the affected codon at all, and even when an amino acid substitution did occur, the changed protein might be functional. Setlow and Boyce (37) irradiated  $\phi$ X virus under acid, neutral and alkaline conditions; the resulting action spectra show only small, if any, difference in sensitivity at 265 m $\mu$ , where pH-caused absorption changes in the cytosines would be small. One might have expected the acid and alkaline conditions to reduce the net formation of hydrate by enhancing reversion. Perhaps the reversal of photohydration in DNA is too fast at any pH to be detected by biological assays.

The other known photoproduct formed at biologically effective

doses is the pyrimidine dimer, first detected in frozen aqueous solutions of thymine by Beukers, Ijlstra and Berends (1) and thought in this case to consist of two thymines joined at the 5 and 6 positions of the heterocyclic rings to form a cyclobutane structure (3). The thymine dimer was quickly detected in the DNA of irradiated bacteria (47). Other combinations of pyrimidines may also form dimers. According to Setlow, Carrier and Bollum (39), irradiation of a polydeoxyinosine:polydeoxycytidine complex results in the formation of dimers between cytosine residues; this structure may deaminate to give uracil dimers. Sauerbier has observed the cytosine dimer in irradiated T1 DNA (32).

The dimerization reaction and the photolysis of dimers are both promoted by UV light; the fraction of thymine in dimer will, after large doses of UV, reach a steady state characteristic of the wavelength; largely dimers at long wavelengths because of the low absorption coefficient of the dimer in this region and mostly monomers at short wavelengths at which the dimer absorbs light (23). A sample suitably preirradiated at long wavelength in which many dimers have been formed, will undergo net breakage of dimers if reirradiated at a shorter wavelength.

Much of the evidence for the biological effect of dimers rests on the observation by Setlow and Setlow that some biological damages in transforming principle are reversed in such a wavelength switch experiment (38) and that these reversible damages overlap with the damages repaired by visible light in the presence of the photoreactivating enzyme (35). From the data of Wulff and Rupert (51), we know

that the photoreactivating enzyme undoes thymine dimers.

A somewhat different impression may be gained from Wulff (52), who has shown that 4.8 dimers/lethal hit are formed in the DNA of phage T4. One would expect, then, that at maximum only about 20% of these dimers could be lethal biological damages. Sauerbier's data (31) for the same phage show that the dimers formed during irradiation of free phage persist in the acid precipitable fraction of cells infected with irradiated phages. Thus, if this result may be interpreted as indicating that dimers persist in intracellular T4 DNA, at least 80% of the dimers have no lethal effect. Perhaps we may conclude, with the Setlows, that some biological damages are dimers, and with Wulff, that not all dimers are biological damages.

The discussion here is not exhaustive; however, for the purposes of this discussion, it seems fair to conclude that the chemical nature of the lethal ultraviolet damage is still not established. One may, in general, expect inactivation of the biological activity of DNA to be accompanied by a large excess of photochemical changes for each lethal hit because a) some photoproducts may be intrinsically harmless, b) some may be neutralized by biological repair mechanisms (see Discussion, RF section) and c) usually, a damage confined to one strand of a double-stranded DNA would not be fatal if the DNA could replicate afterwards, since replication would reconstitute an unaltered molecule.

## MATERIALS AND METHODS

### Irradiation

Irradiations were performed in a 1 cm x 1 cm x 4 cm quartz

cell in the Young-Thollon double prism monochromator described in Winkler, et al. (50). Techniques were generally as described by these authors except that the slit widths used in this work were characteristically smaller and thereby, the band of wavelengths passed, narrower. The experiments were done in a room whose temperature was controlled at 22°C and DNA samples were allowed to come to this temperature before irradiation. Since the DNA solutions were vigorously stirred by a quartz propeller during an experiment, suitable control experiments were performed which showed that the processes of handling and stirring the samples did not cause significant inactivation.

The solvent for most inactivations was 0.05 M tris, pH 7.5 or 8.1. Some experiments were performed with single-stranded  $\phi$ X DNA in high salt concentration; for these experiments the medium was 0.05 M tris plus the desired molarity (0.2 to 4.0 M) of NaCl. The presence of versene at  $10^{-3}$  M in either type of solvent did not change the sensitivity of SS.

The energy reaching the solution in the cell was measured with a photocell and photometer calibrated by comparing its reading with the incident energy determined with a thermopile and also by comparison to the rate of bleaching of malachite green leucocyanide (6) exposed to the UV beam. Readings were taken with the photocell placed behind the sample position before and after each irradiation, and the two measurements averaged to get the true dose rate. Corrections were commonly applied for the absorption of light by the sample (28) and for volume withdrawn in sampling.

### Virus and DNA

Single-stranded DNA was obtained from preparations of  $\phi$ X virus by the  $\text{Ca}^{++}$  or phenol procedures (44). RF was typically obtained from drop collection of an equilibrium density gradient of CsCl, into which had been put a lysate of infected cells. Usually lysates were of cells in which mature virus synthesis had been inhibited with chloramphenicol. The lysates were treated with phenol before banding (45). Preparations isolated without phenol treatment and preparations purified from lysates by selective precipitation and column chromatography (5) have also been used and found to have the same cross-sections.

In most determinations on RF reported here, the predominant DNA species in solution was E. coli DNA. No role in the inactivation is attributed to this DNA because experiments at different DNA concentrations gave consistent cross-sections.

The  $\phi$ X virus used was isolated as by Sinsheimer (43).

### Infectivity Assay

Assays of DNA were performed by the protoplast procedures described by Guthrie and Sinsheimer (19, 20), the later procedure being employed mainly in the spectra for single-stranded DNA. Release of phage from infected protoplasts is characteristically slow and incomplete (19), so osmotic shock or freezing and thawing or both were used to free the phage still bound in protoplasts at the end of the assay. In all experiments, a dilution curve was done on the unirradiated DNA sample being used and in all cases the assay was linear,

i. e., the phage output was proportional to the input concentration of DNA. The concentrations of single-stranded and RF DNA (assumed here to be as infective as SS) used in assays were in the range  $10^9$  to  $10^5$  particles/ml, and the number of output phage/input DNA particle ranged from 0.1 to 1.0 in different experiments.

It was established that the infectivity of irradiated DNA samples of both kinds (SS and RF) decreased upon dilution exactly as did an unirradiated standard; i. e., irradiated samples behaved exactly as populations of molecules which had reduced numbers of infective particles, without interference from damaged molecules. Therefore, the assay used in routine inactivation experiments was not a complete dilution curve, but an assay in duplicate or triplicate of the sample from the irradiation cuvette. Assays were carried out in dim light.

Absorption spectra presented here as "corrected for scattering" were obtained with the Beckman DU spectrophotometer and corrected by extrapolation from wavelengths where there was no absorption using the procedure suggested by Dulbecco (13). An exponential dependence of OD due to scattering at wavelengths  $> 350 \text{ m}\mu$  was observed in all cases, with an exponent of approximately 4.0. Spectra of the DNA were taken on the Beckman DK-2 recording spectrophotometer; no scattering correction was necessary.

The spectrum for  $\phi$ X protein presented in Figure 5 was obtained as follows: The 70 S-light component (7) from several preparative RbCl equilibrium density gradients was rebanded twice in RbCl (43) plus saturated sodium tetraborate ( $\rho = 1.31$ ). After the first centrifugation only the material lighter than "70 S heavy" component (7)

was selected for rebanding by puncturing the side of the gradient tube with a syringe and needle. This material was then rebanding and the sharp band called "70 S light" was collected in the same way. The 70 S light fractions were pooled and dialyzed into 1/20 saturated sodium tetraborate. The volume of these fractions was reduced to 0.2 ml by evaporation under vacuum and then the concentrated sample was layered over a 5 to 20% sucrose gradient containing saturated borate plus  $10^{-3}$  M EDTA. After centrifugation for 110 minutes at 38,000 rpm and  $5^{\circ}\text{C}$  in the SW-39 head of the model L ultracentrifuge, drops were collected and a single symmetrical peak at a calculated  $S = 66$  was found. Those fractions containing material of  $S_{20, w} = 50$  to 75 Svedbergs were pooled and dialyzed exhaustively against deionized distilled water. A sample was then diluted volumetrically into 0.05 M tris, pH 8.1 for the spectrum. Absolute absorption cross-sections are based on  $4.5 \times 10^6$  avograms of coat protein/virus and a specific absorptivity of 1.3/mg/ml protein at 275 m $\mu$ . This absorptivity is that of the 5S coat subunit of Carusi and Sinsheimer (7).

To isolate DNA from irradiated phage, extractions were performed by the phenol procedure detailed by Guthrie and Sinsheimer (20), with the following changes. The phage samples contained  $10^{10}$  -  $10^{11}$  PFU/ml in 0.05 M tris, pH 8.1. After irradiation, BSA (Armour, Fraction V) and denatured calf thymus DNA (Sigma) carrier (dissolved in the same solvent and heated to  $100^{\circ}$  for 10 minutes) were added to final concentrations of 6 and 1 mg/ml respectively. Sometimes a mutant of  $\phi\text{X}$  which could be plated selectively was added to each tube at this point; the recovery of infectivity from this virus was the same



in all samples. Only one extraction with phenol was performed and a wash of the phenol with saturated sodium borate was added to the first aqueous phase before extraction with ether.

## RESULTS

In the protoplast assay as usually performed the phage yield from the infected protoplasts is the quantity determined and used as a measure of the input DNA. It seemed possible that the sensitivity of the DNA particle to inactivation by ultraviolet radiation might not be reflected simply in the phage yield, because of sublethal damages which decreased the burst size but did not abolish phage production entirely. Therefore comparisons were made of the reduction of the ability to initiate an infective center and of the reduction of total phage production. Dilution and soft-agar assays of infected protoplasts were performed by methods described previously (20). Figure 1 shows that for both SS and RF these two functions have the same sensitivity.

These results, taken together with the single-hit kinetics of inactivation, mean that a complete loss of the ability to complete the infective process and release phage occurs as a result of a single photochemical alteration.

Comparison of the growth curves of irradiated and unirradiated  $\phi$ X virus in cultures synchronized by starvation (12) leads to this same conclusion for whole virus: the number of infective centers obtained upon infection at low multiplicity corresponds to the survival of plaque formation, and the times at which there is one phage per cell, the rates of phage synthesis, the times of lysis and the burst sizes are, within experimental variation, the same for the survivors of heavy irradiation

(8-9 hits at 260  $m\mu$ ) as for an unirradiated population. This is in contrast to the situation in T-phages, where the survivors of UV irradiation are observed to have extended latent periods (27, 36).

The action spectra for  $\phi$ X virus, single-stranded  $\phi$ X DNA in a solvent of low ionic strength, and that for RF are presented in Figure 2. Figure 4 is a comparison of the spectra for the single-stranded DNA in low and in high ionic strength media. The inactivation cross-section of SS irradiated in a solvent of low ionic strength is 16.6 x that of RF at 235  $m\mu$  and about 7 x that of RF in the 280-290  $m\mu$  region. The SS spectra are quite similar to that of the whole virus above 240  $m\mu$ , suggesting that DNA is the chromophore which absorbs most of the energy effective in inactivation. This last point is pursued somewhat further in Figure 3, which shows that DNA extracted from UV-irradiated virus declines in infectivity at the same rate as does the viral particle itself.

The single-stranded DNA is quite flexible, and responds to changes in ionic strength by expanding and contracting (44). In Figure 4 are data for the SS DNA in 0.05 M tris pH 8.1, and also in that buffer plus 0.5 M NaCl. No striking changes in cross-section are observed, though the polynucleotide chain should be in the one case extended, and in the other folded into a much more compact form.

In Figure 5 are absorption spectra for  $\phi$ X virus and its component DNA and coat protein. Also presented is the summed absorption of the coat protein and SS DNA. The measured spectrum agrees well with the sum of the spectrum of purified DNA and coat protein; the DNA must be packed into the virus particle much as it occurs in solution. I offer as a counterexample tobacco mosaic virus (TMV):

the spectra of its separated protein and RNA sum to a lower absorption coefficient than that of the intact virus. This result presumably reflects the ordered configuration of the intraviral RNA and its intimate association with the structural protein (4, 18). Figure 6 shows the quantum yields for the virus and SS as a function of wavelength. The pyrimidines are the most UV-sensitive of the constituents of DNA (for a review, see 41, 48); therefore, also plotted is the fraction of energy absorbed in the pyrimidines, calculated on the basis of spectra of nucleotides and the composition of  $\Phi$ X SS DNA, as given by Sinsheimer (44).

It can be seen that for SS the two quantities are related. The quantum yield and fractional absorption in pyrimidines fall in concert at low wavelength, both have minima at 240-250  $m\mu$  and a maximum at longer wavelength. However, the quantum efficiency of inactivation declines above 285  $m\mu$ , while the fractional light absorption in pyrimidines or thymine does not. A reason is suggested by the data in Figure 7, in which the quantum yields are replotted after normalization by the fractional absorption. The quantum efficiency for light absorbed by pyrimidines then, declines at longer wavelength. The dotted lines are replotted data of Deering and Setlow (11) showing the quantum efficiencies measured by following the destruction of the characteristic UV absorption of pTpT and polythymidylate; presumably as a result of UV-induced thymine dimerization (3). The apparent lack of correlation of pyrimidine absorption with quantum yield in the region above 260  $m\mu$  may then be a result of a true decline in the quantum efficiency of UV damage at longer wavelengths.

## DISCUSSION

 $\Phi$ X Virus

Looking first at the range of wavelengths 240 to 302  $m\mu$ , the pronounced similarities in the action spectra of  $\Phi$ X virus and SS (Figure 2) and of both to the absorption spectrum of  $\Phi$ X SS DNA (Figure 5) mean that quanta absorbed by the viral DNA lead to the observed inactivation of the virus particle. Significant disparities between the inactivation cross-sections of SS and  $\Phi$ X virus occur above 260  $m\mu$ , in the range in which the virus protein has an absorption maximum (Figure 5) but the data in Figure 4 indicate that at these wavelengths increases in the inactivation cross-section of the DNA itself occur when the DNA assumes a compact configuration, as it must in the virus particle. Therefore, on irradiation with UV light of wavelength 240-302  $m\mu$ , the lethal quanta are those which cause primary excitations in viral DNA. In Figure 3,  $\Phi$ X inactivation by 254 and 289  $m\mu$  light is accompanied by inactivation of the intraviral DNA. Thus, the action spectra indicate that quanta absorbed in the DNA are the cause of lethal damage and the data of Figure 3 show that the site of the lethal damage initiated by the absorption of light is in the DNA itself. There is no way to decide from this data what the lethal alteration might be.

The action spectra for SS and the virus particle diverge in the range 225 to 240  $m\mu$ , (Figures 2, 4). Reference to Figure 4 shows that such changes in the inactivation cross-section as may occur on folding of the  $\Phi$ X SS DNA in solution cannot account for the increased sensitivity of the virus particle. Therefore, absorption of light by a chromophore other than DNA is contributing to the inactivation of the

of the virus. Again, the data of Figure 3 show that the DNA extracted from irradiated virus is inactivated to the same extent as is the virus itself. These observations indicate that damage to DNA is again the final cause of inactivation by ultraviolet, but that the lethal quanta may find their way to the DNA through another absorbing species.

From the absorption spectra in Figure 5 one may identify the steep rise of absorption in the coat protein as the cause of the higher absorption of the virus at low wavelengths; around 235  $m\mu$ , about half the light absorption by  $\Phi X$  virus is attributable to the protein. The quantum efficiency of inactivation by light absorbed by the virus definitely decreases in the 240-225  $m\mu$  interval even though the quantum yield for free viral DNA increases (Figure 6).

One can unify the data by supposing that the primary event in virus inactivation by low wavelengths is sometimes the absorption of a quantum by the protein which then, through inefficient and unknown secondary reactions, leads to a lethal alteration in the DNA. The protein might, for instance, attack and form a link to the DNA.

Those conclusions are consistent with those reached by following morphological alterations in phage T4 in response to UV irradiation (50). Several other action spectra of  $\Phi X$  virus have previously been published (12, 29, 37). The cross-sections and quantum yields presented here are in good agreement with these other determinations. The wide agreement on the sensitivity of  $\Phi X$  virus suggests its use as a biological dosimeter.

#### The Free Single-Stranded DNA

The small ultraviolet hyperchromicity produced by pancreatic

DNAse digestion or heating of  $\Phi$ X SS (44) implies that the interactions between nucleotides are weaker than the interactions between base pairs in the more ordered DNA's. If the spectra of 5' mononucleotides are summed in the proportions found in  $\Phi$ X DNA, the calculated spectrum has the same maximum, minimum, and general shape as the observed spectrum of  $\Phi$ X DNA. One may rationalize, then, the calculation in Figure 6, in which the fraction of absorbed energy absorbed by pyrimidines, based on the spectra of mononucleotides, is plotted and compared with the variation of quantum yield with changes in wavelength. The relation of high pyrimidine absorption to high quantum yield, pointed out in RESULTS, is expected, since pyrimidines are by far the most UV-sensitive components of DNA (for a review, see 41, 48). The persistence of this property of pyrimidines in a highly polymerized DNA may be taken as evidence that quanta absorbed by purines are less effective than those causing excitation of pyrimidines, and thus that the energy of excitation is not fully available to all bases. Energy migration among purines or among pyrimidines would, naturally, not be detected.

The quantum yield for SS is a function of ionic strength; the more compact molecule is inactivated more efficiently than the extended SS by absorbed energy of wavelengths above 250  $m\mu$  (Figure 6). One possible explanation is that the collapse of the SS into a more compact, ordered structure would enhance the likelihood of any damage requiring interaction of different parts of the molecule. Dimer formation between two pyrimidine residues (3) might, for example, be enhanced by chain contraction. An explanation can be offered for preferential enhancement

of the quantum yield at longer wavelength; David's (10) data indicate that thymine containing dimers are only one, and not the major lethal photoproduct in 260 m $\mu$  UV-irradiated virus. Wulff's (53) investigation of dimerization in E. coli DNA shows that the initial rate at 235 m $\mu$  is about 1/3 of that at 254 m $\mu$ . Thus the influence of an enhanced rate of dimerization would be more evident in the spectral region in which it accounted for a larger fraction of the lethal damages; that is, at longer wavelengths where its rate may be enhanced relative to the rate of formation of other lesions. This argument prompts the suggestion that dimers may supply an even smaller fraction of the fatal photochemistry in the extended forms of the DNA in solution, and that David's (10) figure of 0.34 - 0.40 thymine dimers per lethal hit for  $\Phi$ X may be regarded as a maximum since it refers to DNA packed into phage particles. The chemical nature of the majority of lethal hits in  $\Phi$ X DNA is unknown, save for evidence from the work of Setlow and Boyce (37) that primary excitation of cytosine residues can inactivate the virus; it appears from their data that on changing the pH from neutral to acid, the action spectrum for the virus changes in the same ways as does the absorption spectrum of cytidine; the absorption spectrum of thymidine is not affected by this change in pH.

$\Phi$ X SS DNA (see below) and  $\Phi$ X virus (33) are not reactivated by the host cell reactivation system(s). Therefore, even though the amounts and types of UV-induced alteration of  $\Phi$ X DNA may be similar to those in other organisms, a greater fraction of the photochemical effect will be lethal to  $\Phi$ X. In this sense, these inactivation cross-sections are representative of the total rate of lethal damage.

RF

As expected, the action spectrum of RF in Figure 2 has the maximum, minimum, and general shape of absorption spectra of DNA.

The infectivity of RF is much more resistant to ultraviolet inactivation than SS at all wavelengths examined; the reasons for this difference are taken up in the following inserted note.



### The u.v.-Resistance of Double-stranded $\phi$ X174 DNA

The infective, intracellular form of bacteriophage  $\phi$ X174 DNA has been reported to be much more resistant to inactivation by u.v. irradiation than is the infective single-stranded DNA isolated from mature virus. The tenfold smaller inactivation cross-section of the RF† is consistent with its postulated double-stranded structure (Sinsheimer, Starman, Nagler & Guthrie, 1962).

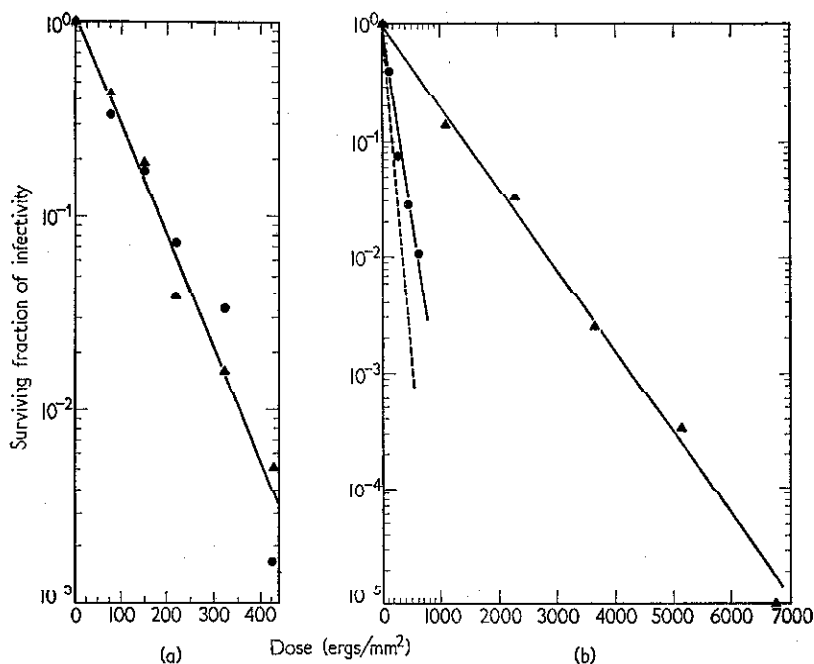


FIG. 1. (a) The u.v. sensitivity (258 m $\mu$ ) of single-stranded  $\phi$ X DNA isolated from mature virus and assayed on protoplasts of *E. coli* W6 and AB1886. —▲—▲—, assay organism W6; —●—●—, assay organism AB1886. Cross-section =  $9.0 \times 10^{-16}$  cm<sup>2</sup>.

(b) The u.v. sensitivity (270 m $\mu$ ) of RF isolated from infected cells and assayed on protoplasts of *E. coli* W6 and AB1886. —▲—▲—, assay organism W6; —●—●—, assay organism AB1886; - - - - - , single-stranded DNA, assay organism W6. Cross-sections: RF on W6 =  $1.2 \times 10^{-16}$  cm<sup>2</sup>; RF on AB1886 =  $5.5 \times 10^{-16}$ ; SS on W6 =  $8.7 \times 10^{-16}$ .

"Dark reactivation" mechanisms have been described (Garen & Zinder, 1955; Sauerbier, 1962), which, for bacteria and for bacteriophage containing double-stranded DNA, negate the effects of a large fraction of the u.v. damages. Bacterial mutants lacking these reactivation mechanisms have also been isolated (Hill, 1958; Howard-Flanders, Boyce, Simson & Meriot, 1962; Rörsch, Edelman & Cohen, 1963). By the use of such mutants as hosts in the protoplast assay system (Guthrie & Sinsheimer, 1963), it is possible to decide whether the greater u.v. resistance of the

† Abbreviations used: RF = the infective, intracellular form of  $\phi$ X174 DNA; SS = infective single-stranded  $\phi$ X174 DNA.

RF is, in part, due to the neutralization of some u.v. damage by dark-reactivation mechanisms, which may not be able to act similarly upon the single-stranded DNA.

In Fig. 1(b) the inactivation of RF irradiated in a double quartz prism monochromator (Winkler, Johns & Kellenberger, 1962) and assayed on protoplasts of *Escherichia coli* K12 W6 (Guthrie & Sinsheimer, 1963), a reactivating host, is compared to the inactivation of RF similarly irradiated and assayed on protoplasts of AB1886, a non-reactivating mutant of K12 (Howard-Flanders *et al.*, 1962). Plotted for comparison in Fig. 1(a) is the inactivation of single-stranded DNA assayed on the same hosts. The RF is more sensitive when assayed on the strain lacking the reactivation mechanism, while the single-stranded DNA has the same u.v. sensitivity on both strains. The difference between SS and RF, thus, is partially accounted for by reactivation mechanisms which decrease the sensitivity of the double-stranded form by a factor of about five.

Since the SS would become RF in the normal course of infection (Sinsheimer *et al.*, 1962), it must be that most damages to the single strand prevent the synthesis of a reactivable RF. This is consistent with a proposed mechanism for this class of dark reactivations, in which information in the complementary strand is used to replace an u.v.-altered sequence by excision and resynthesis of the tract of nucleotides containing the damage (Boyce & Howard-Flanders, 1964).

The residual 1.6 times difference between the cross-sections of RF and SS assayed on AB1886 may (a) be due to a lower rate of photochemical damage in the more ordered, double-stranded RF (David, personal communication; Wacker, Dellweg & Jackerts, 1962) or (b) be the result of a reduced lethality of u.v. damage in the RF due, conceivably, to redundancy of information in the complementary strands.

*Note added in proof.*

After this work was submitted a note by Jansz, Pouwels & van Rotterdam (1963) appeared in which similar experiments were reported.

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A point perhaps worth emphasis is that, though RF assayed on AB1886 is only 1/1.6 as sensitive as SS, the absorption coefficient of RF is expected to be somewhat less than 2-fold as large as that of SS. Therefore the difference in the quantum efficiency of inactivation for the two types of DNA, even when both are assayed on HCR<sup>-</sup> hosts, is still quite significantly different. This contrasts with the similarity of the quantum yield for formation of dimers in single- and double-stranded DNA (10). Repetitions of the inserted experiment above on RF in the range 248 m $\mu$  to 275 m $\mu$  have given data qualitatively similar to that presented.

Other differences in the action spectra of RF and SS are less well understood; the 260 m $\mu$ /235 m $\mu$  ratio of cross-sections is larger for RF than for SS, and the limb of the RF spectrum below 235 m $\mu$  seems to rise more rapidly.

Since the protoplast host used to assay the irradiated RF possessed the ability to reactivate ultraviolet damages (30) the cross-sections presented in Figure 2 presumably represent the rate at which non-reactivable damages are done.

These action spectra are the first known to me of the response to UV of a homogeneous, biologically active DNA.

Figure 1

The sensitivity of  $\Phi$ X DNA as measured by total phage yield and by ability to form an infective center. Circles, survival of final phage yield; squares, survival of ability to initiate an infective center. A broad band of wavelengths in the 280-285  $m\mu$  region was used in this irradiation.

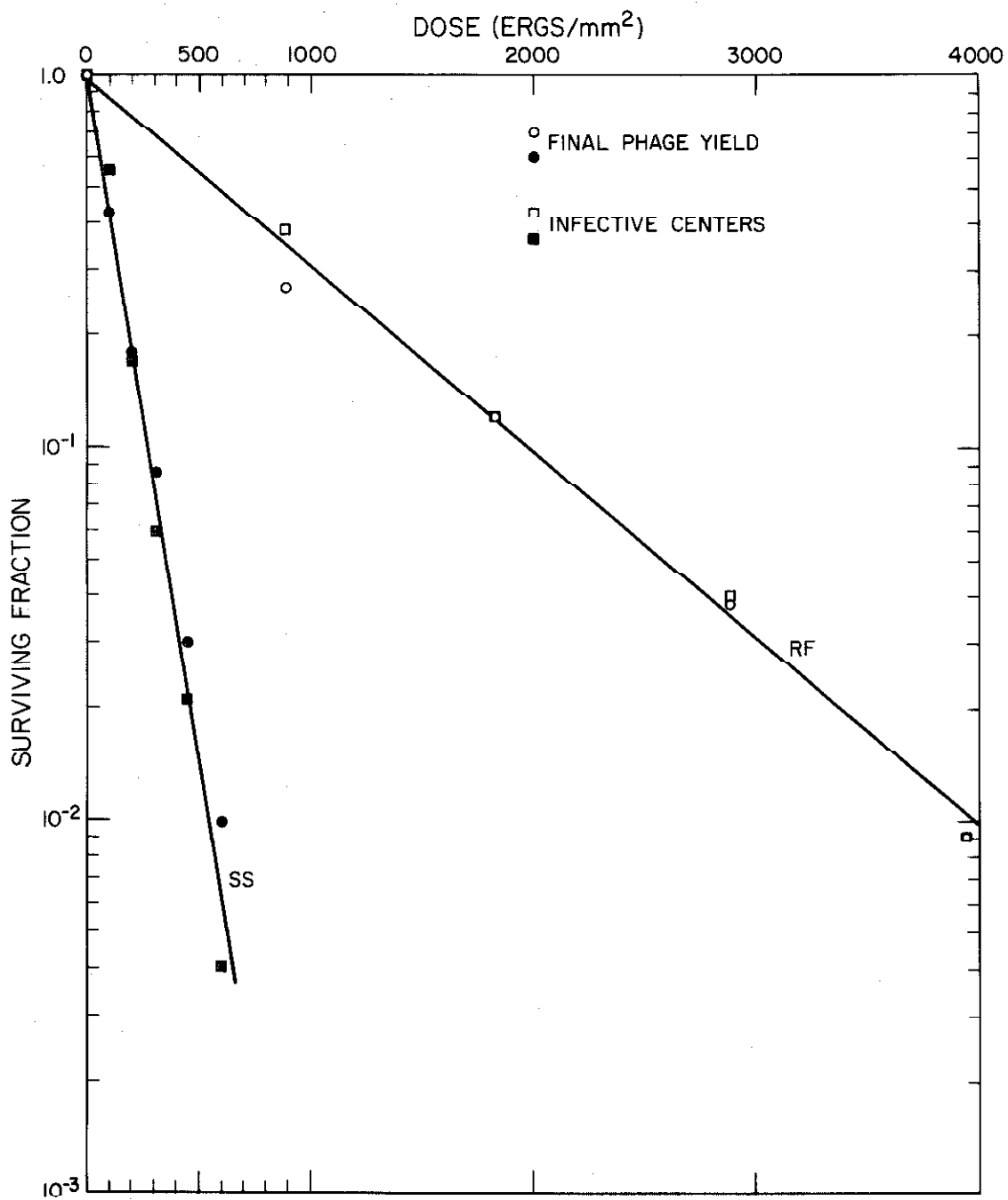


Figure 1

Figure 2

Action spectra for inactivation of  $\Phi$ X virus, free SS DNA and RF in tris 0.05 M, pH 8.1. Each point on the spectrum is the average of multiple determinations, ranging from 2 to 6 repetitions. Assays of the free DNA performed on E. coli K12 W6, a host cell reactivating host. O,  $\Phi$ X virus;  $\Delta$ , single-stranded DNA;  $\square$ , RF.

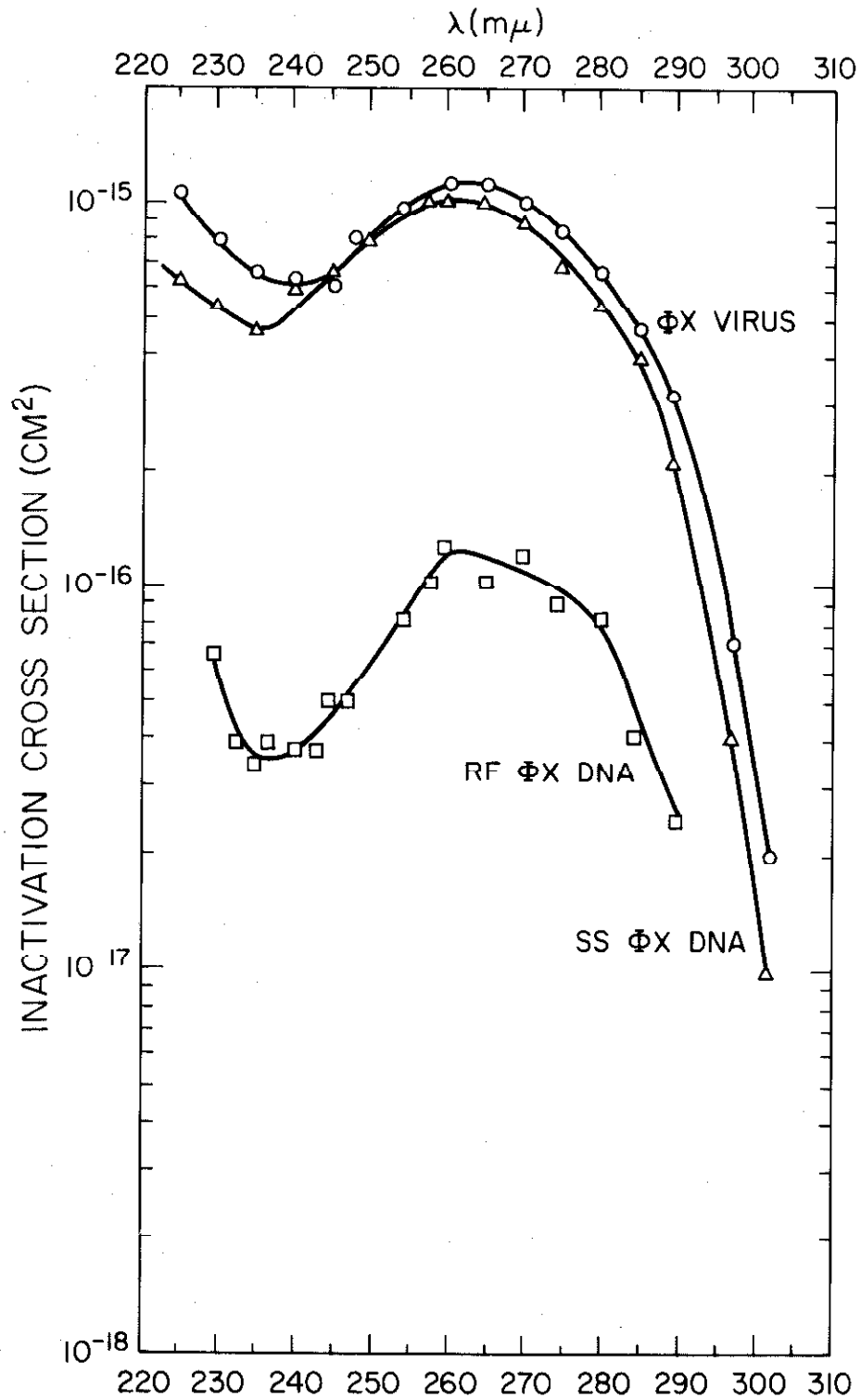


Figure 2

Figure 3

Comparison of survival of irradiated phage and the infective DNA extractible from them. O, survival of virus;  $\Delta$ , survival of DNA extracted from irradiated virus; ---, free DNA in 0.5 M NaCl, 235 m $\mu$ .



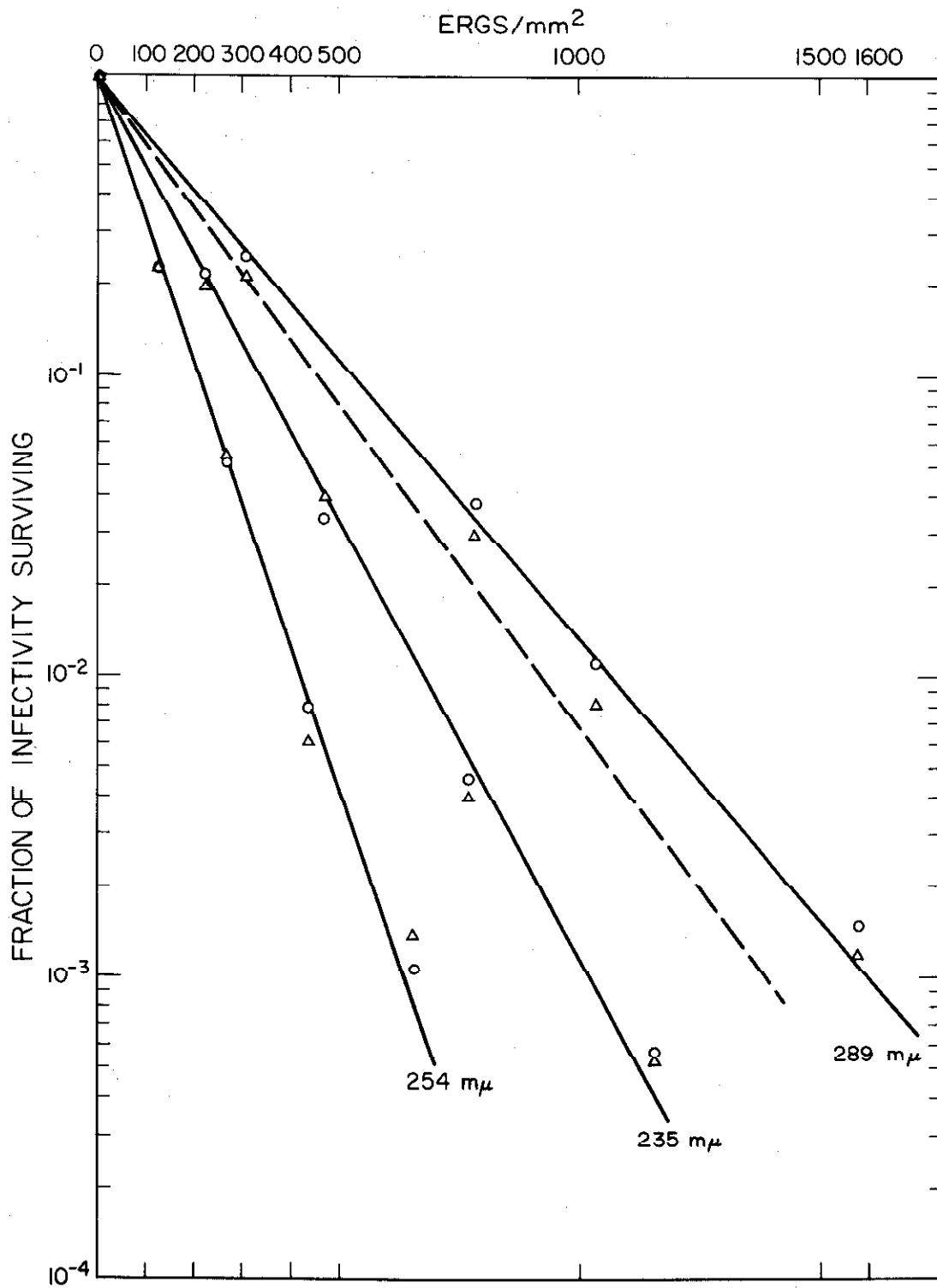


Figure 3

Figure 4

Action spectra of SS  $\phi$ X DNA at low ionic strength; 0.05 M tris, pH 8.1, and high; 0.5 M NaCl + tris.  
 $\Delta$ , tris; o, tris + 0.5 M NaCl.

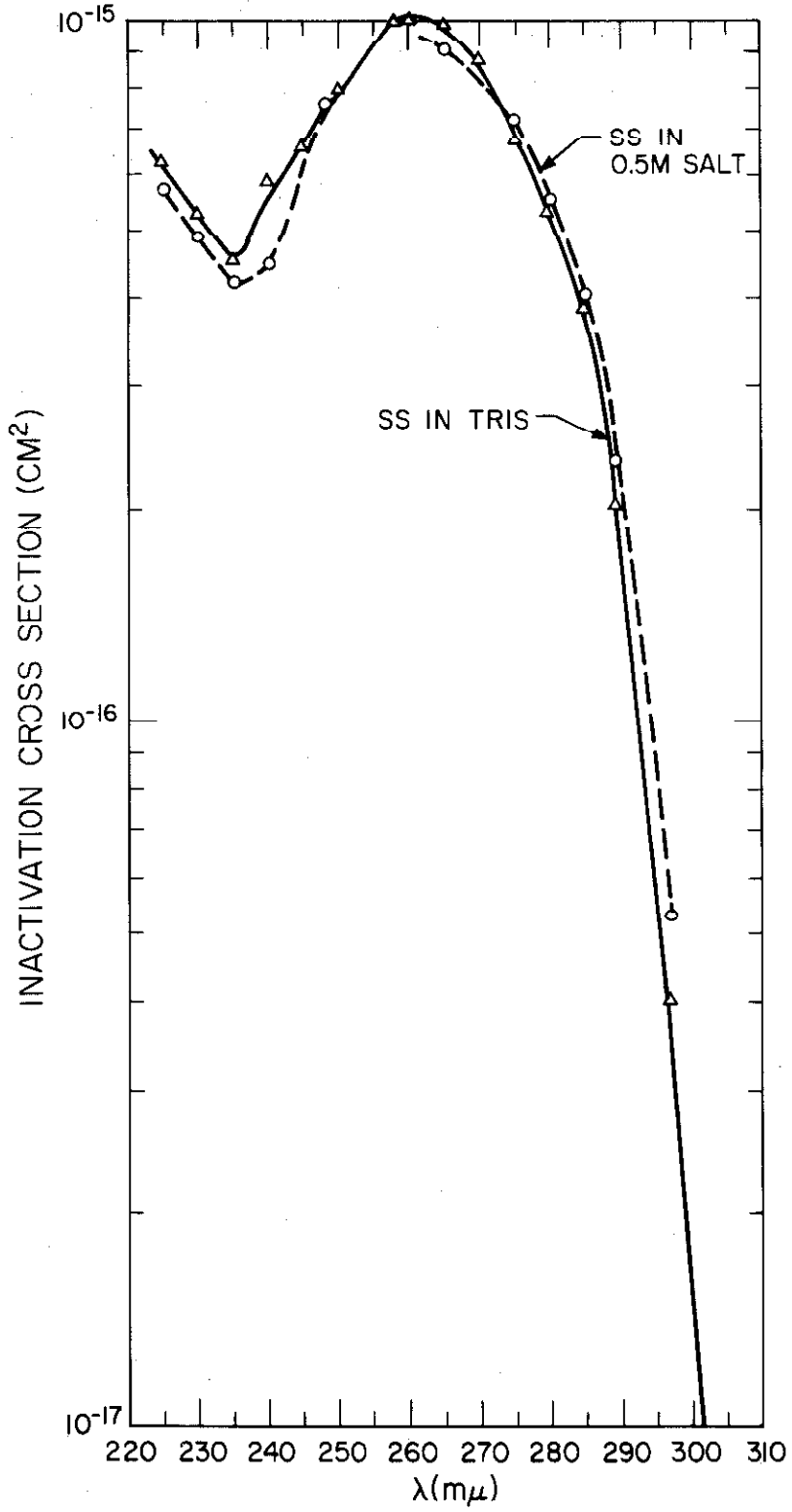


Figure 4

Figure 5

Absorption spectra for  $\phi$ X virus and its components, corrected for scattered light. The SS spectrum was taken in tris + 0.5 M NaCl at 20°C. The solvent for the protein was also 0.05 M tris, pH 8.1. The absolute value of the absorption cross-section of the DNA was arrived at by equating its  $\epsilon(P)$  in 0.2 M NaCl + 0.05 M tris pH 8.1, 37°C, to the value found by Sinsheimer (44) for mM phosphate buffer + 0.2 M NaCl at 37°C and pH 7.5.  $\square$ ,  $\phi$ X virus;  $\Delta$ ,  $\phi$ X DNA; o,  $\phi$ X coat protein; ---, sum of protein and DNA.

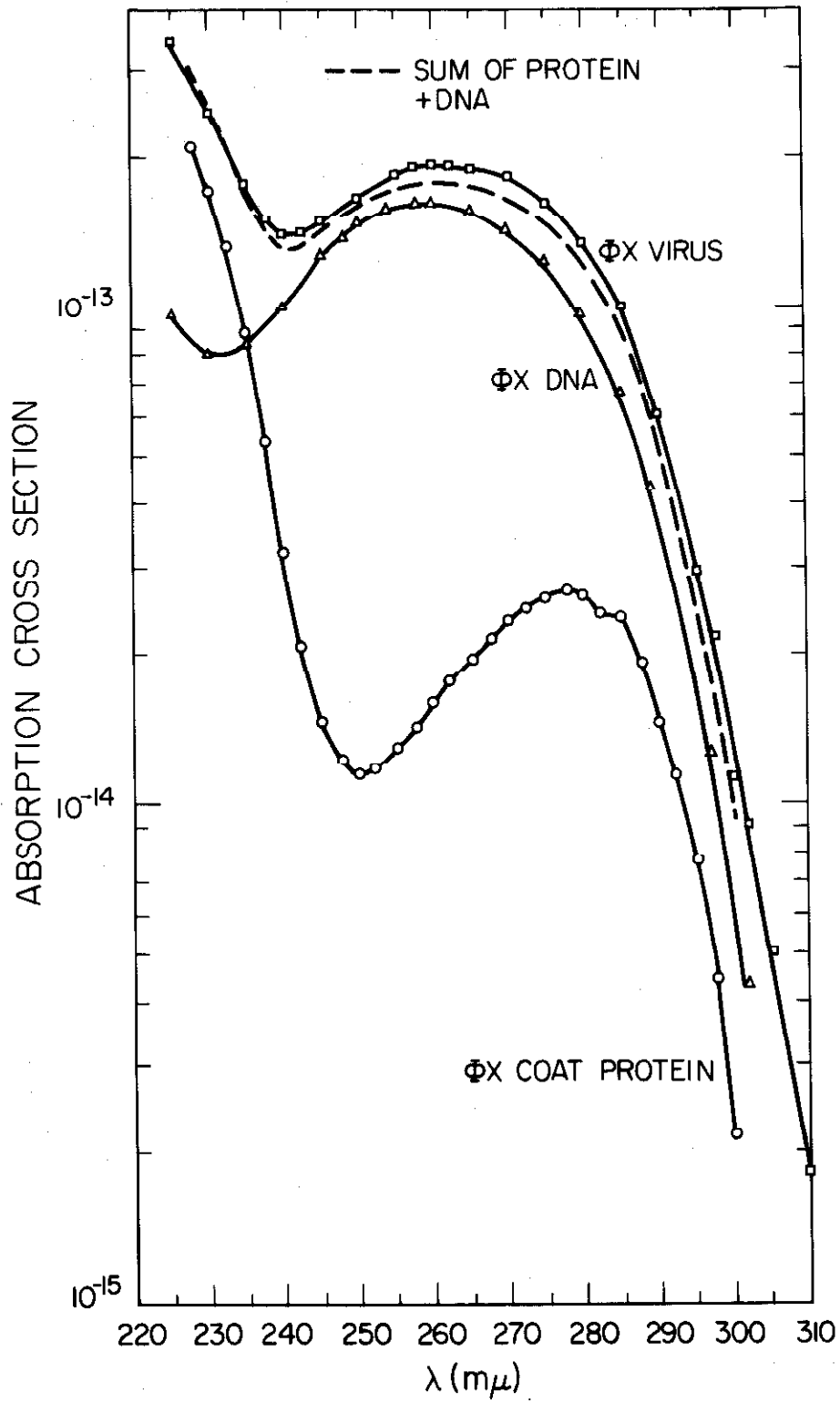


Figure 5

Figure 6

Quantum yields for inactivation of  $\Phi X$  virus and free  $\Phi X$  SS DNA, and calculated fraction of total absorbed light absorbed by pyrimidines. The fractional absorption was calculated from the relation fractional absorption in pyrimidines =  $(0.328 E_{\lambda}^T + 0.185 E_{\lambda}^C) / (0.246 E_{\lambda}^A + 0.328 E_{\lambda}^T + 0.241 E_{\lambda}^G + 0.185 E_{\lambda}^C)$  in which  $E_{\lambda}^C$ ,  $E_{\lambda}^A$ ,  $E_{\lambda}^T$  and  $E_{\lambda}^G$  are the extinction coefficients of the deoxymononucleotides of cytosine, adenine, thymine, and guanine respectively. Spectra for the nucleotides are from data partially presented by R. L. Sinsheimer JBC 208, 445 (1954). Though the spectrum for  $\Phi X$  SS in tris alone is not presented in Figure 5, it was measured and used to calculate the quantum yields in the central part of Figure 6.

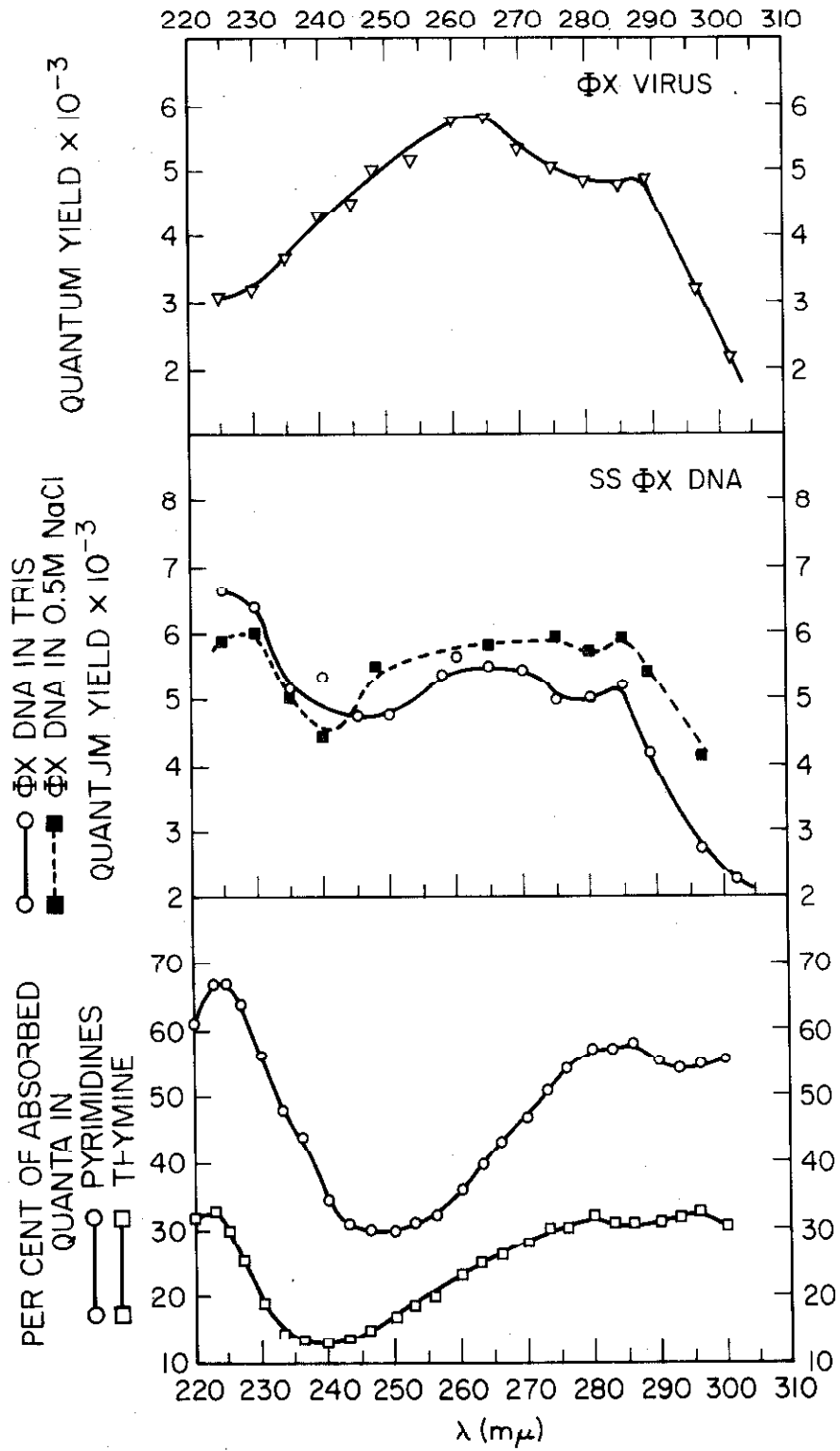


Figure 6

Figure 7

The variation of the quantum yield,  $\Phi$ , with wavelength after normalization by division by the fraction of absorbed light in pyrimidines,  $f^{C+T}$ , or in thymine,  $f^T$ . The data for pTpT and poly T are from Deering and Setlow, BBA 60, 526 (1963).



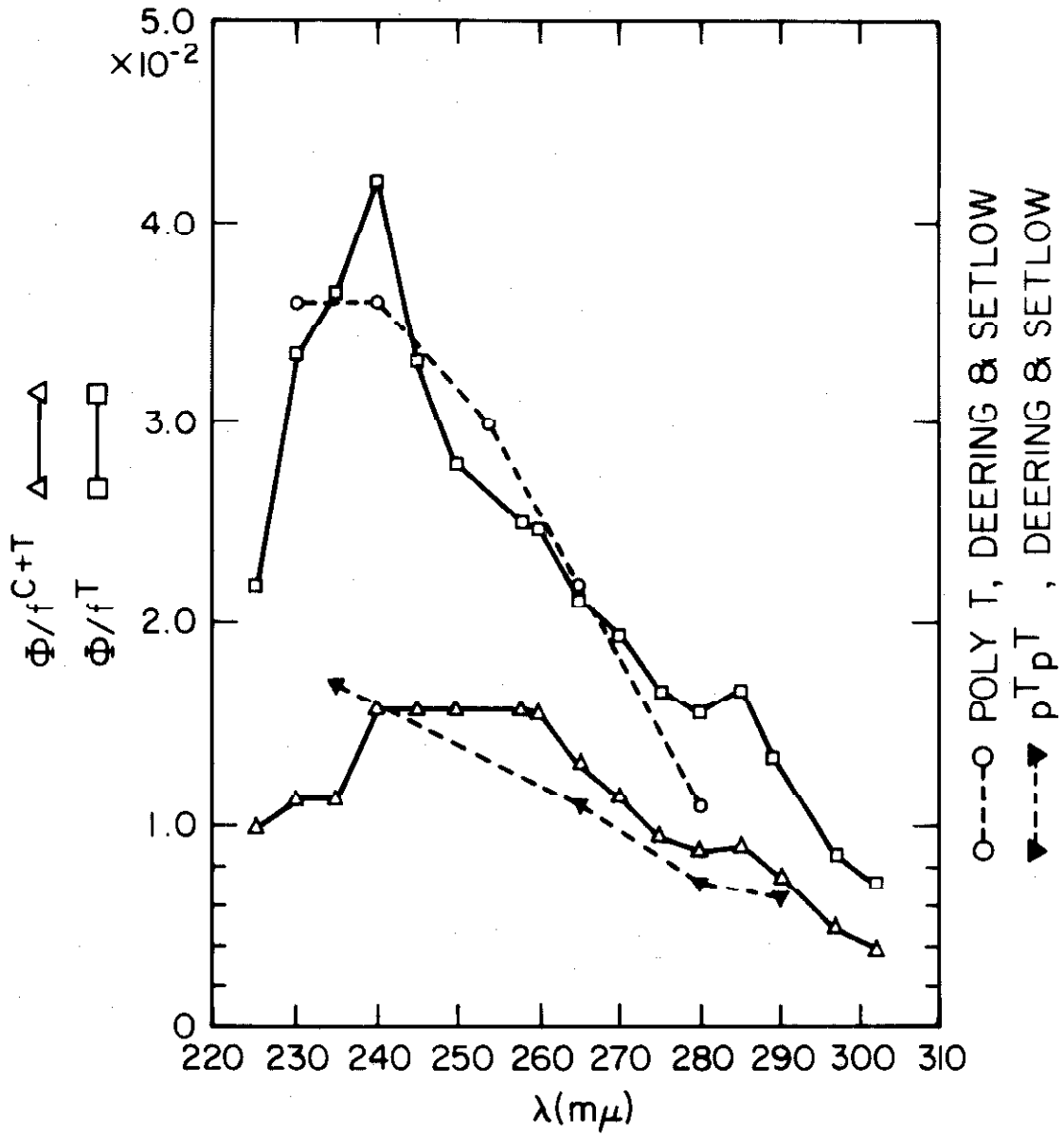


Figure 7

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Part II

THE SENSITIVITY OF THE  $\Phi$ X-E. coli C COMPLEX  
TO ULTRAVIOLET LIGHT

## INTRODUCTION

Irradiation of the complex of bacteriophage with its host bacterium was first performed by Luria and Latarjet (23) as a means of investigating the progress of the virus' intracellular development. The UV induced decline in plaque-forming ability of infected cells reflects the sensitivity of some vital part of the phage - bacterium complex: vital in the sense that, once the essential structure is damaged by UV, the release of viable progeny is prevented. Changes in sensitivity to UV during development might then reflect changes in the state of the vegetative phage.

This method of access to intracellular events has a unique appeal. It characterizes the intracellular state of the phage without disrupting the complex; the properties of the population are deduced by measuring the fraction which survive the treatment.

$\Phi$ X174 bacteriophage is a particularly suitable organism for this work as its DNA takes two known forms, easily distinguishable by their susceptibility to UV inactivation (35).

One of the salient points that emerges from the bulk of photobiological literature is that organisms have many ways in which they can modify the effects of UV-caused photochemical changes. The following paragraphs are a catalogue and brief description of these, and an attempt to decide on their relevance to the experiments described later.

Photoreactivation (PR). Kelner (22) discovered that some of the lethal damages resulting from ultraviolet irradiation of Streptomyces griseus conidia were nullified if the irradiated conidia were illumi-

nated with visible light. Dulbecco (9) extended this finding to bacteriophage; photoreactivation has since been observed in many systems.

Rupert, Goodgal and Herriot (30) found an enzymic activity in extracts of E. coli which could perform PR in vitro, when combined with transforming DNA and illuminated with visible light. Wulff and Rupert (40) subsequently showed that the enzyme acted on the UV-induced thymine dimer (2).  $\Phi$ X is photoreactivable, but PR was not desired in these experiments, so room lighting was selected to minimize it.

Photoprotection. Jagger (19) found that large doses of visible light reduced the susceptibility of E. coli to later inactivation by UV. It now appears that some fraction of PR may be a photoprotective effect, rather than true enzymic photoreversal of damage (20). In any case, photoprotection is not expected to be a factor in these experiments.

Multiplicity Reactivation (MR). Luria (24) first studied MR; if several UV-inactivated phage collectively infected the same bacterium, the individually sterile phages were found to cooperate and produce progeny. This process is particularly efficient with T-even coliphages, which are able to recombine efficiently (16). However, irradiated  $\Phi$ X virus, like the related minute phage Sl3 (36), is able to undergo MR at unobservable low levels, if at all. (Denhardt and Yarus, unpublished data) This observation may be related to the low levels of recombination found after mixed infection with genetically marked  $\Phi$ X (27, Mr. C. A. Hutchison, III, personal comm.).

However, it may be that MR takes place only after  $\Phi$ X has be-

come double-stranded and that UV damages in the SS prevent the appearance of a suitable RF. In Luria-Latarjet experiments on E. coli C infected with a multiplicity of 2-20  $\Phi$ X/cell, it is found that the inactivation curves are simply those expected for a number of independent infections at all stages of development (42). This result is provisionally taken to mean that interactions between different RF's (such as MR) are small enough to be neglected in considering the inactivation of  $\Phi$ X complexes. It is possible, however, that multiple infections do not provide a situation comparable to that occurring after replication in a single infected cell.

Ultraviolet Restoration (UVR). Weigle (37) found that UV-irradiated coliphage  $\lambda$  could be reactivated if grown in lightly irradiated host bacteria. Pre-irradiation of the host bacterium with doses larger than that required to elicit UVR will suppress the reactivation effect and phage grown in such bacteria will appear more sensitive again (21).  $\Phi$ X virus will undergo UVR, and consideration of the inactivation of host-phage complexes must take account of the complex effect of UV: it inactivates phage DNA, but also evokes, then suppresses a reactivation mechanism.

Host Cell Reactivation (HCR). Garen and Zinder (11) found some phages to be more sensitive to UV if assayed on a host which had itself been exposed to UV. Sauerbier (31) led the attempts to attribute this effect to inhibition of a cellular enzymic mechanism which could exorcise UV lesions. First Hill (17), then others (18, 28) found bacterial mutants which lacked HCR. Using these mutants Boyce and Howard-Flanders (3), and Setlow and Carrier (33) showed that the



mutants lacked the ability to remove the dimer of thymine from irradiated DNA. The HCR process, however, seems to have a broad range of application, since it also occurs after treatment of bacteria with a bifunctional alkylating agent, as shown by Hanawalt and Haynes (13). The original formulation of the mechanism of HCR (3, 33) called for removal of a section of the DNA strand containing damage and its replacement by complementary resynthesis using the remaining, undamaged strand. HCR does, indeed, require double-stranded DNA (29, 43) and the predicted product of HCR, a tract of DNA having an inserted, newly synthesized section in one strand, has been detected by Pettijohn and Hanawalt (26) in DNA subjected to repair.

$\Phi$ X virus or its single-stranded DNA, as expected, is not able to participate in HCR, but the double-stranded RF is (29, 43). Therefore, the inactivation of HCR<sup>+</sup> cells infected with  $\Phi$ X will presumably reflect the occurrence of the intracellular transition from the non-reactivable (SS) to the reactivable form of DNA (RF).

## MATERIALS AND METHODS

### Phage

Phage  $\Phi$ X174 used were isolated by the method of Sinsheimer (34). The lambda phage used in some experiments were  $\lambda_{b2b5C_I}$  and were a gift from Mr. E. T. Young, II.

### Bacteria

C<sub>N</sub>: E. coli C, BTCC No. 122. Unless otherwise noted, C<sub>N</sub> was used as seed bacterium.

C<sub>s</sub>: A mutant of E. coli C; it is UV-sensitive, and is incapable of reactivating UV damage in infecting bacteriophage; this strain

was a gift from Dr. Paul Howard-Flanders.

E. coli K12W6, the standard protoplast assay organism.

E. coli K12 AB1886, used as host in protoplast assay when HCR-minus property is desired; a gift of Dr. Paul Howard-Flanders.

#### Media for Assays

TKB is tryptone-KCl broth, containing 10 grams of (Difco) tryptone broth and 5 grams of KCl per liter of distilled water. This with addition of  $\text{CaCl}_2$  to  $1 \times 10^{-3}$  M is the usual growth medium for bacteria and is also used for dilutions of infective centers.

SB is starvation buffer, used for washing and starving cells in order to synchronize infection. It contains, per liter added water, 5 grams KCl, 1 gm NaCl, 1.2 gm (Sigma 121) tris, 0.1 gm anhydrous  $\text{MgSO}_4$  and 1 ml 1 M  $\text{CaCl}_2$ . It is adjusted to pH 8.1 with HCl.

Bottom and top agar are the tryptone media described by Sinsheimer (34), save that only 10 ml of bottom agar are used per plate, and no NaOH is added. Slants for carrying stock samples of bacteria were TKB + 2% (Difco) Bacto-agar.

When assay of infectivity in preparations of intracellular nucleic acid was required, the procedures and media of Guthrie and Sinsheimer (12) were followed, save that the ratio protoplast stock: DNA solution making up the assay mixture was 2:5, rather than 1:1. This change maximized the efficiency of infection by SS. All dilutions and assays of infective DNA were performed in 0.05 M tris, pH 8.1 (Tris).

The protoplast assay is inherently linear in its response to  $\phi\text{X}$  DNA; that is, the phage yield is proportional to the concentration of infective DNA. However, at high DNA concentrations, or in the presence of an inhibitor found in cell extracts (see Appendix),

proportionality fails. When such effects are expected, the unknown DNA is assayed at a series of increasing dilutions. This procedure is referred to as "performing a dilution curve." Infective titers quoted are always taken from dilutions of the DNA stock at which the yield of phage was proportional to the concentration of infective DNA, and are expressed as SS equivalents (SSE), that is, the number of SS molecules required to give the same phage yield when combined with the same protoplasts. All assays of irradiated material, whether of phage, inactivated DNA, or irradiated infective centers, were performed under yellow incandescent lights, to minimize photoreactivation (9). Plates were counted after 2-4 hours at 37°C.

#### Synchronization and Growth

Growth experiments were performed by inoculating TKB made  $10^{-3}$  M in  $\text{CaCl}_2$  with a sample of the desired host bacterium from a stock tryptone agar slant. When there were  $1-2 \times 10^8$  cells/ml, as judged by counting in a Petroff-Hauser counting chamber, the cells were centrifuged at ambient temperature for 10' at 3000 x g, re-suspended in SB recentrifuged, finally resuspended in SB at  $2-5 \times 10^8$  /ml and vigorously aerated for 90' at 37°C in order to starve them. To initiate the infection, phage were added to a final multiplicity of 0.05 to 0.2. After 5' adsorption, the lysozyme-versene technique (see below) released < 10% of the added plaque formers, assuring >90% eclipse. At this point, an equal volume of prewarmed TKB was added to initiate development. Samples were taken for measurement of infective centers, intracellular phage (see below) and for later irradiation. These last were diluted a hundred-fold into iced SB and

held for later irradiation. Under these conditions the complexes in iced SB were stable and controls showed that even in the several hours sometimes required to complete irradiations of all samples, the Luria-Latarjet curves did not change. The starvation synchronization system has been characterized further by Denhardt and Sinsheimer (5).

Eclipse and formation of intracellular phage were followed by adding 0.1 ml of culture to 0.9 ml chilled 0.05 M tris, pH 8.1 containing 250  $\mu$ g/ml (CalBiochem B grade) lysozyme: To this was added 1 ml EDTA, 0.8 gm/100 ml. After incubation at ice temperature for 15' - 1 hour, the samples were frozen and thawed twice before assay.

Dilution buffer for samples consisting of phage only was 0.05 M sodium borate.

#### Irradiations

Except as noted, irradiation of infective centers was carried out in one of two ways:

The dilute samples mentioned above were put into a 1 x 1 x 4 cm quartz cuvette and irradiated as described previously (41). Corrections were applied for the small residual light absorption in these suspensions, and for volume withdrawn in samples. A survival of 1.0 corresponds to  $2-5 \times 10^5$  infective centers/ml. Irradiated samples were diluted through iced TKB and plated immediately.

When large samples were required for the extraction of nucleic acid, 10-20 ml were withdrawn from the culture at an appropriate time and chilled in ice. These samples were irradiated at 12 inches from a double 8 watt unfiltered germicidal lamp, with constant swirling, in a petri plate 9 cm in diameter. The plate was chilled with ice during the

period of irradiation. The whole irradiation procedure, including the assays for plaque formers performed at each level of survival, could usually be completed in ten minutes.

#### Extraction of Intracellular Nucleic Acid

5-20 ml portions of the synchronized culture were centrifuged (3,000 x g, 10') at 4°C. They were washed by resuspension in an equal volume of iced 0.05 M tris pH 8.1 (Tris) recentrifuged, and finally suspended in 1-2 ml cold Tris. To this was added 0.1 ml (Sigma, grade I) lysozyme solution freshly made up in Tris at 2 mg/ml and 0.2 ml 40 mg/ml EDTA solution, made up in distilled water. After 15'-30' incubation in ice, a single freezing and thaw yielded a clear, highly viscous lysate. To this was added an equal volume of redistilled phenol previously equilibrated with saturated sodium borate at room temperature. The sample was shaken vigorously by hand or on the vortex orbital mixer (Scientific Industries, Inc., Springfield, Mass.) and allowed to come to room temperature. The phases were separated by centrifugation (1,000 x g, 5') and the aqueous phase drawn off with a Pasteur pipet. The phenol phase was re-extracted with one-half its own volume of saturated sodium borate and the top layer, after centrifugation, added to the first aqueous phase. The combined top phases were shaken with newly-opened (Mallinckrodt) non-anhydrous ether and centrifuged as above, 2 to 4 times, drawing off the aqueous (bottom) phase after centrifugation each time. At the last ether extraction, there was no material at the ether-water interface. The ether was removed by bubbling N<sub>2</sub> through the isolated aqueous phase.

In several experiments on cells infected with <sup>32</sup>P-labeled

parental phage, 50-100% of the radioactivity adsorbed was recovered in the final preparation, and recovery was the same among samples treated together.

## RESULTS

A typical one-step growth curve of  $\Phi X$  in starvation synchronized culture is presented for comparison with Luria-Latarjet curves (Figure 1). Most of the added plaque-formers are recovered as infective centers. Progeny phage appear in the first cells at about 9' after development begins; there is an average of one phage/cell at 11'-13' and lysis begins at 14'-16'. Growth curves in  $C_N$  and  $C_S$  are indistinguishable.

Luria-Latarjet curves for  $\Phi X$  developing in  $C_N$ , a host cell re-activating (31) (Figure 2) bacterium, and in  $C_S$ , a non-activating host (Figure 3) differ mainly in the course of inactivation at low doses of UV light. The ultimate slopes of their inactivation curves are similar and therefore the intracellular "target" seems the same for both types of complex.

It is convenient to consider the data in Figures 1, 2 and 3 in three sections. (A) The period of adsorption, -5' to 0': the changes in cross section are small for both types of cells and inactivation is exponential. At 0' over 90% of the phages are in eclipse (Figure 1). (B) A period of early development, characterized by the appearance of a point of inflection in the curves of  $C_N$  (HCR+) complexes (Figure 2) and by a "break" in the  $C_S$  (HCR-) (Figure 3) curves. Early development ends when the shouldered appearance of the  $C_N$  curves dominates the shape of the whole curve and when all  $C_S$  complexes have the

resistance of the more resistant fraction. At the end of early development, the final slopes of the  $C_s$  and  $C_N$  Luria-Latarjet curves have assumed their ultimate values: further development will not change them. The phage at this point have been developing 3 to 5 minutes at  $37^\circ\text{C}$ . (C) Finally, in the period between ca. 4 minutes and the time of lysis, the inactivation curves for both  $C_s$  and  $C_N$  become progressively more shouldered, while retaining the same final sensitivity. Around the time when growth curves show one phage per cell, that is when all cells become capable of maturing progeny, the inactivation curves attain their final shape, and change little, if at all, thereafter. Doses required to inactivate cells which have developed to this point should destroy all intracellular single-stranded DNA and progeny virus (Figures 2, 3). Therefore, the survivors must resynthesize mature virus before they lyse.

When starved E. coli  $C_s$  infected with several  $\phi\text{X}/\text{cell}$  is irradiated and lysis followed by measuring turbidity, it appears that most or all of the UV-inactivated fraction do not lyse, even if irradiation is performed as late as 11 minutes. This argues that premature lysis (of cells which would otherwise have made progeny to replace those inactivated by the UV) is not the cause of lethal damage to the late infective center. Lethal damages seem to suspend both lysis and the production of progeny. The fraction which survives irradiation late in the phage cycle lyses at roughly the same time as unirradiated cells. The concavity evident at this time near the origin of the  $C_s$  curves in Figure 3 is not always observed.

The characteristic changes in UV sensitivity during intra-

cellular development occur normally in the presence of chloramphenicol (CAM), an inhibitor of protein synthesis (39). A growth curve done on a culture of E. coli C<sub>N</sub> complexes (whose inactivation kinetics are in Figure 4, showed that infective centers were stable, even at 60 minutes) and that no intracellular phage appeared. Therefore, it appears that the evolution of UV resistance is much less sensitive to CAM than is the synthesis of proteins required for synthesis of mature phage or lysis of the infected cell. The same result has been obtained with E. coli C<sub>S</sub>.

We may now compare the sensitivity of  $\phi$ X DNA to that of the infected cell. Two types of experiments are presented. DNA is extracted from the infected cell at various times during development, then irradiated and assayed with bacterial protoplasts. In addition, the infected bacterium itself may be irradiated, and then the survival of DNA extracted from the irradiated complexes may be compared to the survival of the infective center itself.

Figure 5 shows the inactivation of DNA exposed to UV after having been extracted from infected cells allowed various amounts of development. The fraction of the extracted infectivity in the more UV-resistant state characteristic of RF varies in different experiments, (see Appendix) but it is clear that at 0', all extracted infectivity has the sensitivity of SS, and that intracellular development is accompanied by the appearance of infectivity with the characteristic UV resistance of RF (35). The complete sensitivity of 0' DNA is in accord with the observation that  $\phi$ X-infected starved cells are unable to form RF until nutrient is supplied them (7). The inactivation of infectivity in these



crude cell extracts is always observed to follow the course expected of a mixture of purified SS and RF. Replotted in Figure 5 for comparison with these data are the inactivation of the 0' complex and the ultimate sensitivity of the late complexes. Clearly, the inactivation of the intracellular SS could account for the sensitivity of the 0' complex, but the late complex is far more sensitive than is isolated RF assayed using protoplasts of E. coli K12W6.

The result of irradiating infected cells, then extracting and assaying the DNA is shown in Figures 6 and 7. The results resemble those obtained in Figure 5, in which irradiation took place after extraction; at the earlier time (Figure 6) the intracellular DNA is as sensitive as, or perhaps slightly more sensitive than, its infective center. At the later time, considering now the data obtained using E. coli K12W6 (HCR+) as host in the protoplast assay, one finds an appreciable fraction of the infectivity more resistant to UV than is the complex itself. Therefore, in vivo as well as in vitro,  $\phi$ X RF, as judged by assays on E. coli K12W6 protoplasts, is more resistant than is the mature complex.

RF formation is one of the first events in infection (in the experiment of Figure 7, parental phage had formed RF by three minutes, as judged by the appearance of parental  $^{32}$ P at the position characteristic of RF in the CsCl equilibrium density gradient) and the strand of RF complementary to the parental is believed to be the template for both progeny SS (7, 25) and messenger RNA (15). It seems likely that the effective phage DNA in the mature complex is all RF, and one may argue that if a phage structure is to be the most UV sensitive

entity in the infected cell, that structure must involve RF, since the destruction of any of its products would not inactivate the complex; the RF, if undamaged, would direct the synthesis of more product. The infectivity of free RF indicates that it can carry out all functions necessary for phage production (35). Thus, the apparent sensitivity of infective centers which contain RF is a puzzling and critical observation. This dilemma is resolved below.

The ultraviolet sensitivity of isolated RF is altered if the protoplast host is genetically incapable of repairing UV damage (29, 43). This is also true for  $\Phi$ X RF irradiated in vivo, then extracted (Figure 7). In agreement with results on isolated SS, the early DNA (Figure 7) has the same sensitivity whether the protoplast host is K12W6 (HCR+) or AB1886 (HCR-). This confirms the absence of a usable complementary strand in 0' DNA which, if it existed, could participate in host-cell reactivation (3, 33).

The HCR mechanism itself is sensitive to UV; UV-treated bacteria lose their ability to reactivate phages (11). Thus, the final slope of the Luria-Latarjet curves, attained after considerable irradiation of the complex, may be characteristic of the RF in the absence of HCR. The sensitivity of the infected cell would then, at low doses, reflect only inherently non-reactivable damages, but as irradiation progressed, fewer and fewer damages could be reactivated by the UV-inhibited HCR process. Finally, when the dose was high enough, the infective center would assume a sensitivity stable to further irradiation and characteristic of RF without access to HCR. Some of the examples of UV sensitivity increasing with dose that is, "shoulders" seen in the Luria-Latarjet experiments (Figures 2, 3) are believed to be results of this process of "double inactivation;" of both the phage DNA

and reactivation mechanisms applicable to it. At the right in Figure 7 are replotted the sensitivity of early and late complexes and for comparison, the sensitivity of free SS and also that of isolated RF DNA assayed on a host genetically incapable of HCR. On this basis, it is believed that the inactivation of the early complex is symptomatic of the inactivation of an intracellular SS; the ultimate sensitivity of the late complex is that of an intracellular RF deprived of HCR. However, if the inactivated RF is extracted from the infected, irradiated bacterium, it is still subject to reactivation by a capable protoplast (Figures 6, 7).

The inactivation of HCR is compared directly with the survival of  $\Phi X$  infective centers in Figure 8. Starved  $C_N$  and  $C_S$  were infected with a low multiplicity of  $\Phi X$  and then allowed 10 minutes development, chilled, harvested, washed, and resuspended in starvation buffer made 0.01 M with respect to  $Mg^{++}$ . These resuspended cells (concentration  $\sim 10^9$ /ml) were irradiated with 265 m $\mu$  light in 4 ml quartz cuvette, with vigorous stirring. At several times samples were removed and the survival of the  $10^1$   $\Phi X$  complex determined; then the irradiated cells were split into several portions and one was infected with unirradiated coliphage  $\lambda$ , the others with  $\lambda$  exposed to varied amounts of 265 m $\mu$  UV. Unadsorbed  $\lambda$  phage was removed with antiserum and the survival of the irradiated  $\lambda$  determined. The effect of this procedure is to measure UV inactivation curve for  $\lambda$  using as host pre-irradiated bacteria, whose exposure to UV is judged from the survival of  $10^1$   $\Phi X$  complexes among the cells. A large fraction of the ultraviolet damage to  $\lambda$  is reactivable (14). Therefore,  $\lambda$  adsorbed to unirradiated cells will be UV resistant; as the

irradiation of the cells progressively inhibits reactivation, the  $\lambda$  will appear more sensitive. The results in Figure 8 indicate that ability of irradiated cells to reactivate  $\lambda$  phage has decayed after the same doses required to reach the final slope of the  $10^1 \Phi X-C_N$  complex (cf. Figure 2). In contrast,  $\lambda$  assayed on E. coli  $C_S$ , which lacks HCR, starts and remains quite sensitive to UV. The decreased sensitivity of irradiated  $\lambda$  preadsorbed to lightly irradiated  $C_N$  is reproducibly observed and is an example of UVR (UV Restoration), in which a part of the UV damage to bacteriophage is remitted when a lightly irradiated host cell (21, 38) is used.

#### DISCUSSION

The UV sensitivity of  $\Phi X$ -E. coli complexes is probably not due in any measure to inactivation of some structure peculiar to the host cell. Extensive experiments by Denhardt and Sinsheimer (6) on the capacity of starved, irradiated, E. coli  $C_S$  and  $C_N$  to support  $\Phi X$  growth have shown that the capacity of the preirradiated bacterium to serve as a phage host is far more resistant to UV than is the complex. Further, these authors have extended this result to the infected cell by irradiating cells infected at high multiplicity with  $\Phi X$ , then testing by superinfection with  $\Phi X$ . In this case also, capacity was resistant. Therefore, the UV sensitivity of infected cells is not a consequence of the destruction of some sensitive structure which pre-exists in the host, or of some irreplaceable structure which becomes sensitive or is formed after phage infection.

The most sensitive entity in the cell is surely a nucleic acid.

The response of complexes to low doses of UV depends on the host's possession of an HCR mechanism, which is known to operate on nucleic acid (3, 29, 33); inactivated E. coli- $\Phi$ X complexes are photoreactivable (Yarus, unpublished data) and photoreactivation is performed by an enzyme which takes nucleic acid as substrate (30). The experiments recorded in Figures 5, 6 and 7, make it likely that intracellular  $\Phi$ X DNA may be considered a mixture of SS and RF and show that the UV sensitivity of these molecules in vivo, as judged by assays using HCR-minus protoplasts, unmistakably resemble the sensitivity of the 0' and late complex, respectively.

Denhardt and Sinsheimer (6) provide more evidence: The complex is sensitized to UV by the incorporation of BDU, a specific replacement for DNA thymidine. Further, the action spectra performed on 0' and mature  $\Phi$ X complexes are nucleic acid-like.

The changes in Luria-Latarjet curves during development are insensitive to CAM; so is the SS to RF transition and RF replication (35).

Let us now reconsider the three stages of the  $\Phi$ X Luria-Latarjet experiment as proposed in Results.

A) Adsorption, -5' to 0', a moderate decrease in sensitivity occurs; phages which were free at -5' are, at 0' in eclipse (Figure 1). A small decrease in sensitivity (~10%) may be expected on release of the SS DNA from the phage particle, since free SS is, around 260 m $\mu$ , slightly less sensitive to UV than is intact virus. A further small effect may be predicted because the bacterium contains UV-absorbing materials; when the adsorbed phage genome passes into the "shadow"

of the cell, its apparent sensitivity is reduced. (See the discussion by Benzer (1) ). The data of Figures 6 and 7 hint of an additional effect, since the DNA extracted from irradiated 0 min complexes appears somewhat more damaged than the complexes themselves; neither of the above rationalizations can comprehend the observation that the infective center has become more resistant while the DNA remained sensitive. It may be that some of the single strands damaged intracellularly at 0' will survive if allowed further time in the irradiated cell; disruption of the infective center or the process of DNA extraction stabilizes the lethal damage in some sense and precludes its reactivation by the protoplast assay host.

B) Early development; 0' to ~4'; marked in  $C_s$  by an increasing fraction of complexes which have assumed their ultimate resistance; in  $C_N$  by an increasing fraction which have assumed a shouldered in-activation curve and their final resistance or nearly so. This is the period during which the parental single-strand is directing the synthesis of its complementary strand, and becoming an RF (7, 35). It is natural to associate the two processes, and suppose that the shouldered fraction in  $C_N$  and the resistant complexes of  $C_s$  are those which have formed their RF. The form of the Luria-Latarjet curves suggests that even though the SS to RF transition requires about 4 minutes, the time of formation of an RF is much less than 4', but cells do not all initiate formation of their RF's simultaneously. At any moment in this period, some of the infected cells appear to contain an RF and others do not.

C) Late development; ca. 4 minutes to lysis: both  $C_s$  and  $C_N$

complexes become more shouldered with little, if any, change in ultimate slope. After 4' replicas of the parental RF are found in the cell (7, 35; Yarus, unpublished work) and usually, by 13' all cells are maturing phages.

As indicated in the data of Figure 8, the entire bend in the inactivation curve of the late  $C_N$  complex could be considered the consequence of the inactivation of a single intracellular RF, with concomitant suppression of the dark reactivation (HCR and UVR) mechanisms.

There is reason to believe that one, or a small number, of the RF in a cell is able to perform a necessary function which no other can (8, 38, 42). Having taken this as a working hypothesis however, one must account for the enlargement of the late shoulder: Why does it not appear full-blown at 4 minutes? Perhaps RF may not be fully reactivable until several minutes after its completion. In addition, some of this shoulder may be due to the accumulation of several potentially competent RF's in the cell after replication begins.

E. coli  $C_s$  was selected to lack reactivation (HCR); why then does its  $\Phi X$  complex still exhibit a shoulder? (The 12'  $C_s$  curve in Figure 3 portrays the most pronounced shoulder observed; many experiments show less pronounced bending.) Residual HCR is not likely since inactivation of the  $C_s$  complex is altered only slightly when it is assayed in the presence of 7.5  $\mu\text{g/ml}$  caffeine or 10  $\mu\text{g/ml}$  acriflavine, concentrations which abolish HCR of  $\lambda$  but do not affect UVR (Yarus, unpublished data). The initial concavity sometimes, but not always, seen in Luria-Latarjet curves on mature  $C_s$  complexes (Figure 3) suggests the induction of UVR.

Superposed on these effects of reactivation will be those of multiplicity; that is, if replication leads to the production of RF's able to perform all the functions of the UV-inactivated parental, all the competent RF's will have to be inactivated to inactivate the infective center. This will add to the shoulder, since no infective center would be inactivated until all their competent RF's were damaged. The observation of an increasing shoulder during the period of RF replication may be comfortably explained in this way. The extrapolate of the exponential portion of the late (12')  $C_s$  curve is 5-10, a number consistent with the expected number of replicas of RF (5). A relevant result is that of Denhardt and Sinsheimer (6), that mature complexes of  $\phi X$ , if inactivated by UV, may be re-infected successfully. This presumably indicates that an inactivated RF may be replaced by an undamaged one at least once.

### Summary

It seems most consistent to regard the non-linearity of the late  $C_N$  curves as a mixed effect of reactivation and replication of RF; that of the  $C_s$  experiment primarily as a result of replication. At least some of the replicas can complete the infective process after inactivation of the others by UV. On the exponential or "linear" part of the inactivation curves, inactivation consists in damaging an intracellular SS, or, after its appearance in the cell, an RF.



Figure 1

Growth of wild-type  $\phi X$  in E. coli C<sub>s</sub> at 37°C. Infection was synchronized by starving the host cells. Time is reckoned from the moment of broth addition; titers have all been corrected to the adsorption period, that is, before dilution by nutrient.

- BACT— is the concentration of starved cells
- Δ— titer after lysozyme-versene lysis
- O— infective centers

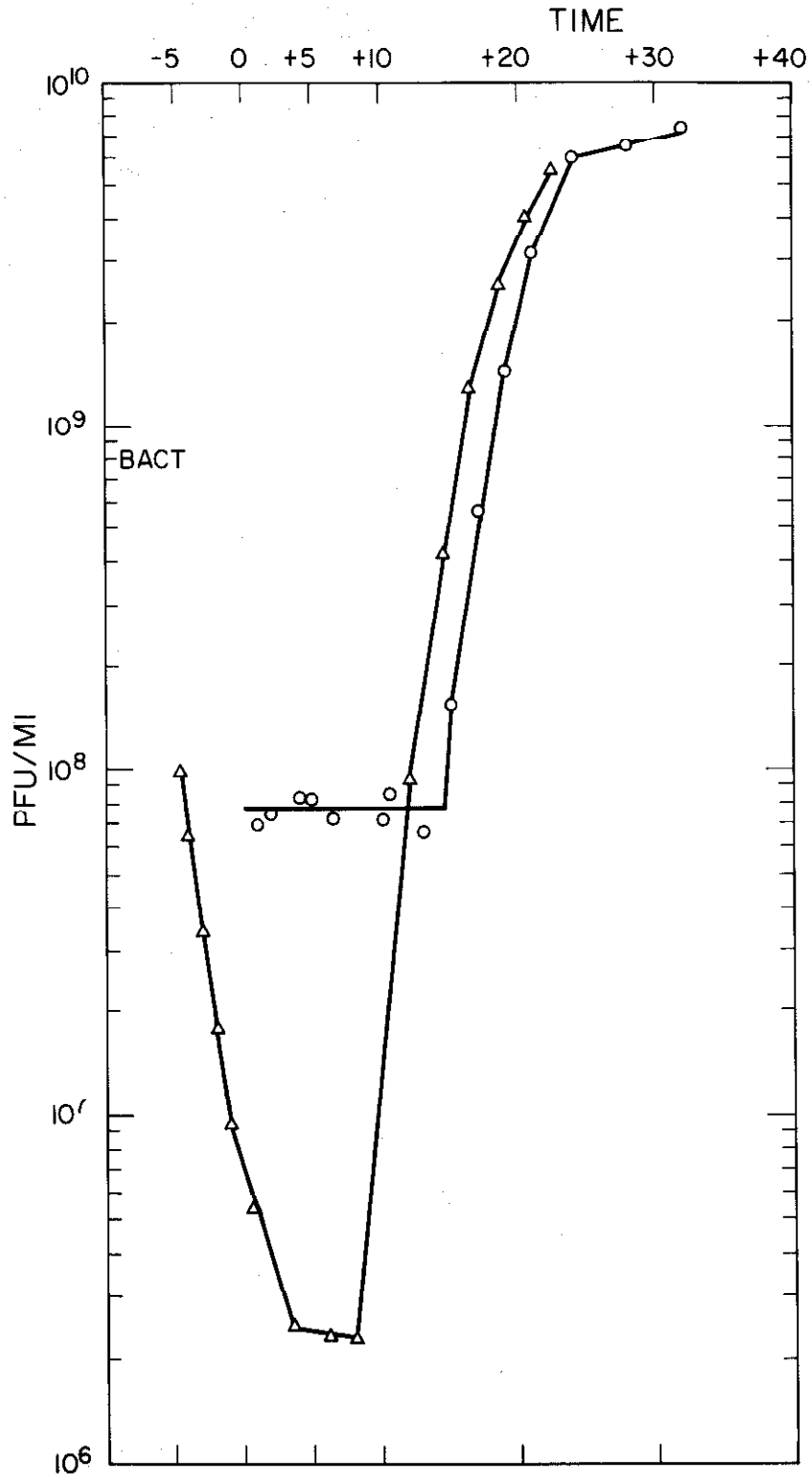


Figure 1

Figure 2

Inactivation of  $\phi$ X - E. coli C<sub>N</sub> complexes by ultraviolet light (260 m $\mu$ ). Individual curves were drawn from three independent experiments to compose this figure.

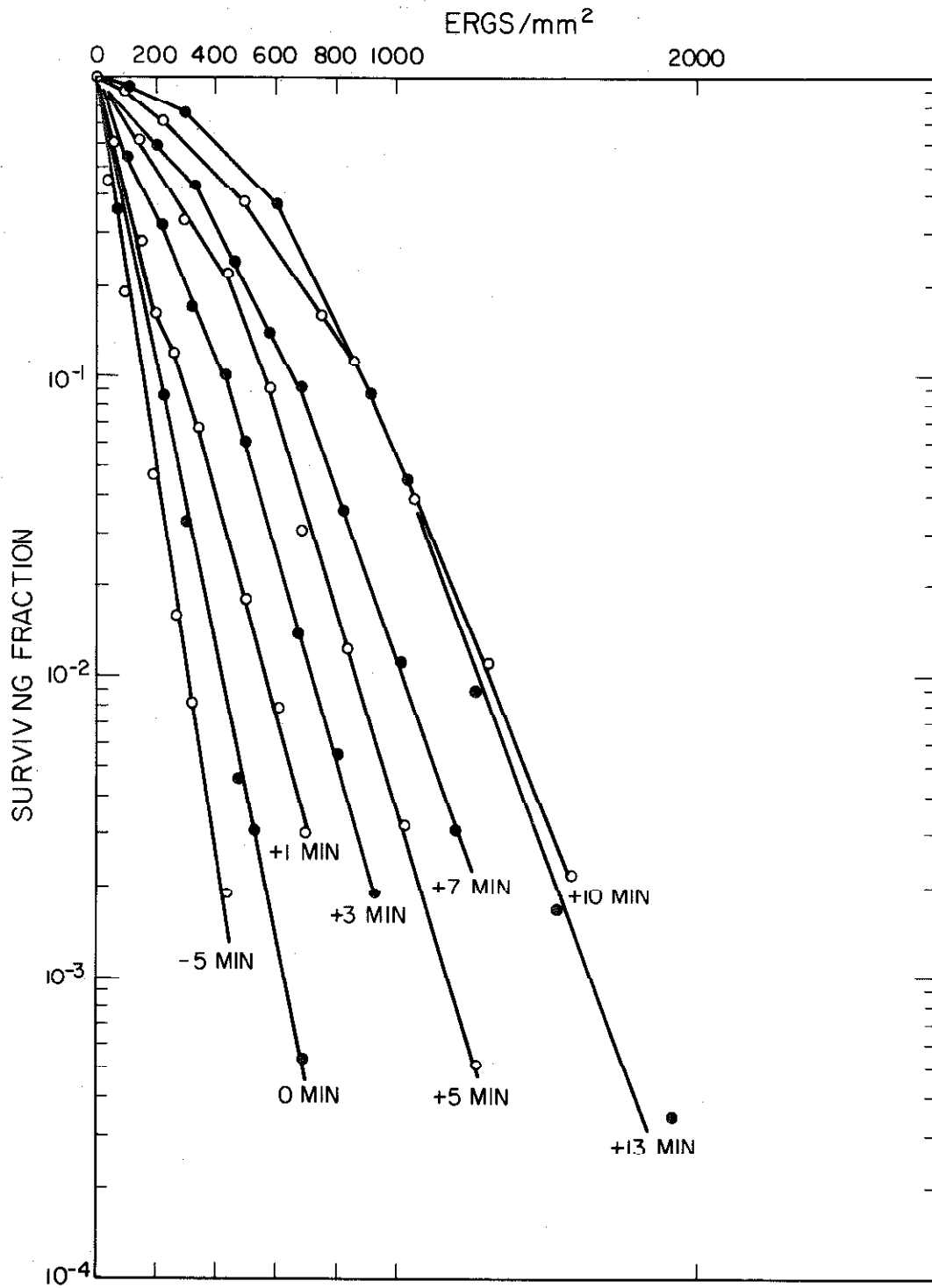


Figure 2

Figure 3

Inactivation of  $\phi X$  - E. coli  $C_s$  complexes by ultraviolet light (265  $m\mu$ ). Individual curves were drawn from two independent experiments to compose this figure.

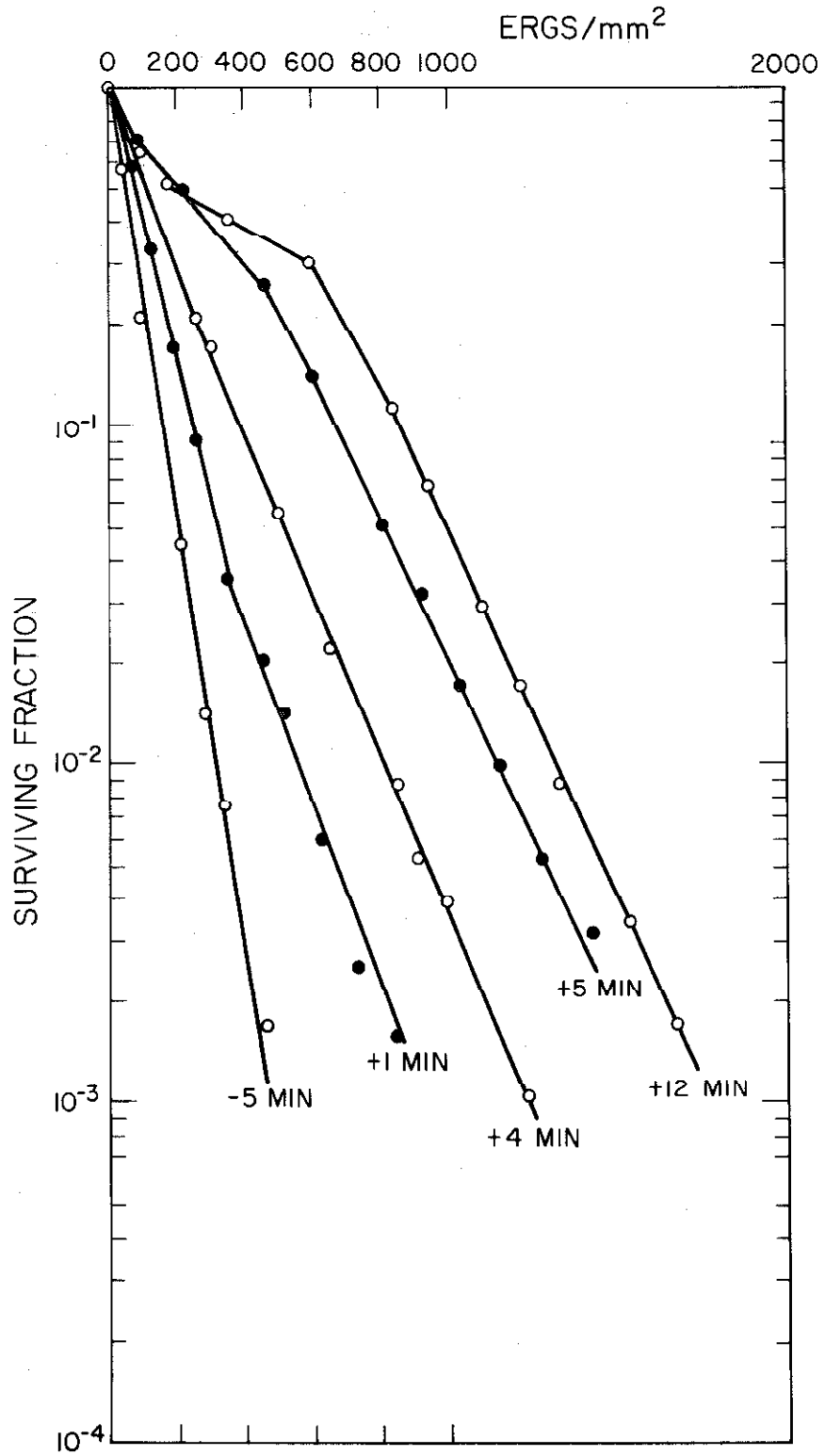


Figure 3

Figure 4

Sensitivity of E. coli C<sub>N</sub> complexes to ultraviolet light (260 mμ). Chloramphenicol (CAM) was added to starved cells five minutes before addition of phage. The concentration of CAM during adsorption was 30 μg/ml.; addition of the nutrient reduced it to 15 μg/ml.

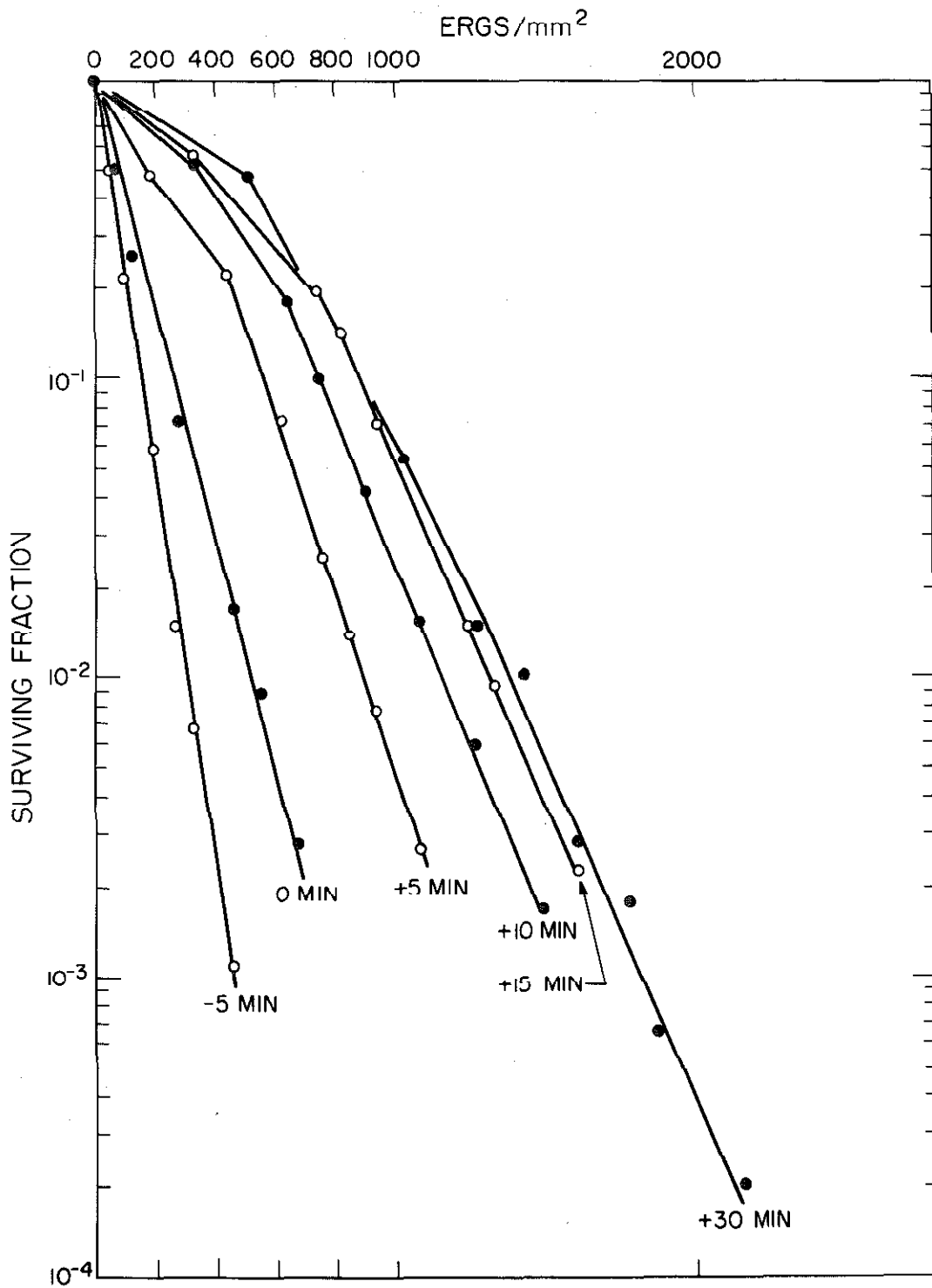


Figure 4



Figure 5

Inactivation of DNA extracted from developing E. coli  $C_N^+$  complexes and assayed with protoplasts of E. coli K12W6. Data have been drawn from several experiments. The ----- line represents the sensitivity of purified RF assayed on E. coli K12W6 (HCR+); the line drawn through the 0 min points is that derived from inactivations of SS extracted from virus (41). E. coli  $C_N$  was the host in these experiments, but similar curves result when  $C_s$  is used. The dot-and-dashed lines come from Figure 2. Data have been drawn from several independent experiments.

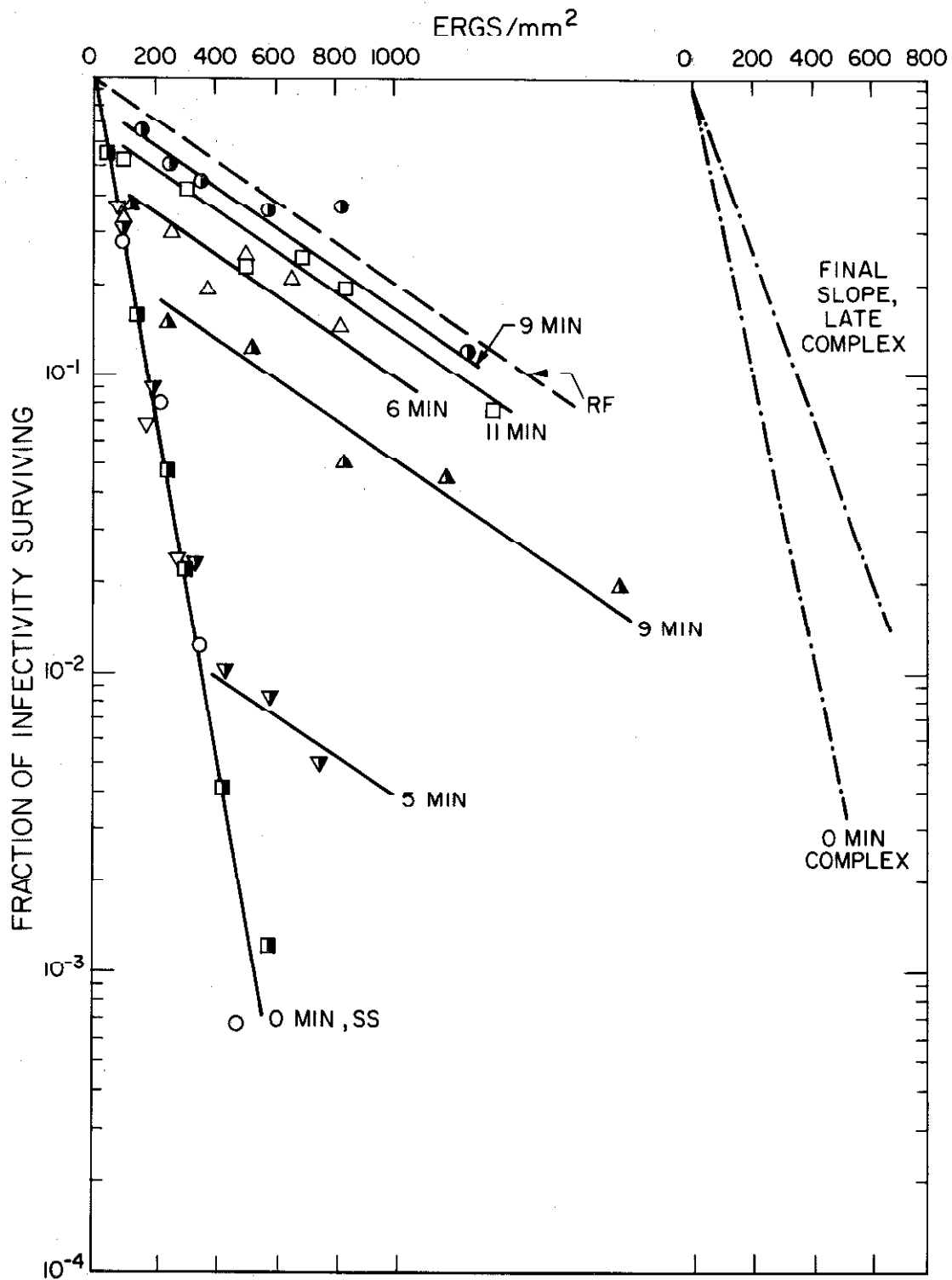


Figure 5

Figure 6

A comparison of the survival of irradiated  $\phi$ X E. coli C<sub>N</sub> complexes and that of the DNA extracted from them after irradiation. Each point on the curves for the DNA is the result of a dilution curve; the relative values plotted are derived from the section of the curves in which phage yield was proportional to DNA concentration. A small correction has been applied to these values to compensate for the recovery of <sup>32</sup>P parental label.

Open symbols: 0' complex and DNA

Filled symbols: 9' complex and DNA

—○— Survival of infective centers

—●—

Infectivity recovered from the irradiated complexes, divided by that recovered from the unirradiated (zero dose) sample. A germicidal lamp was used as the UV source. Infectivity was assayed using E. coli K12W6 protoplasts.

—◇—

—◆—

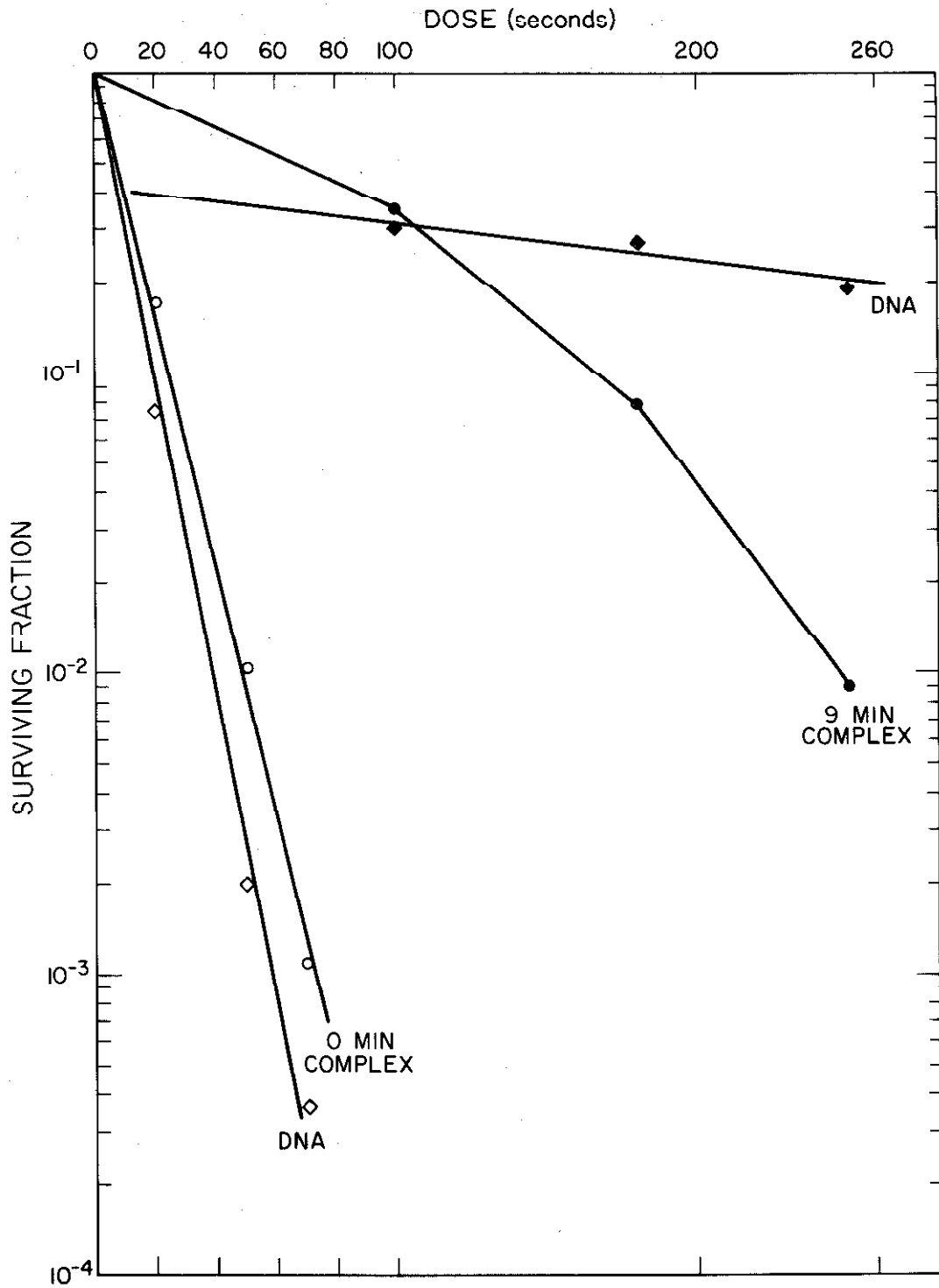
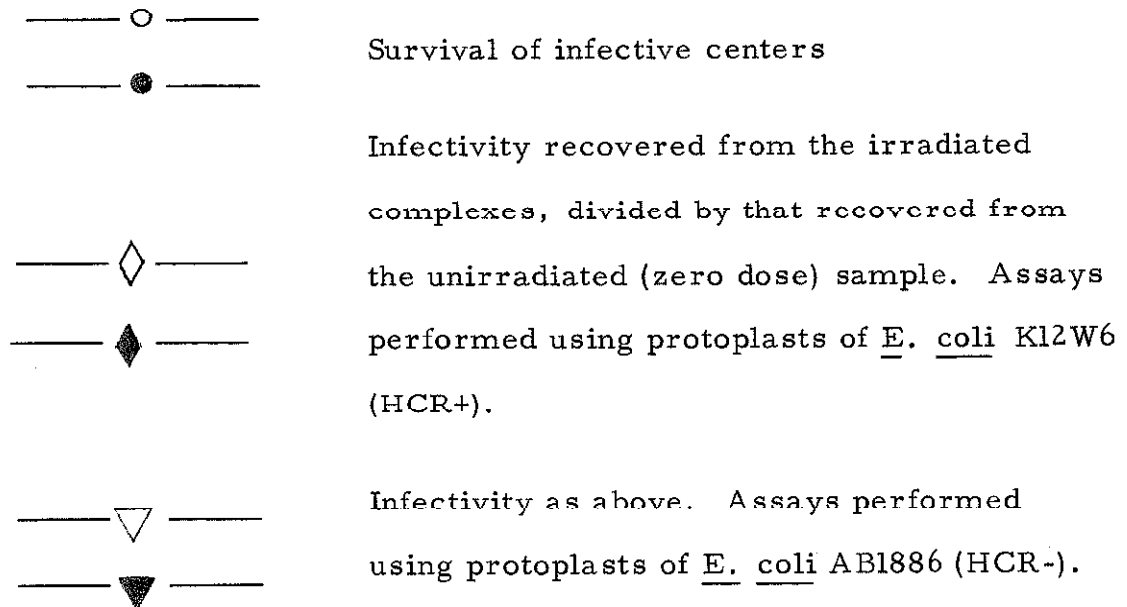


Figure 6

Figure 7

A comparison of the survival of irradiated  $\phi$ X-E. coli C<sub>s</sub> complexes and that of the DNA extracted from them after irradiation. 15  $\mu$ g/ml. CAM was present during development to prevent the formation of large amounts of SS DNA (35). Each point on the DNA curves is the result of a dilution curve; the relative values plotted are derived from the linear part of these curves.



A germicidal lamp was used as the UV source. Assays of DNA from 15 min. - old complexes on K12W6 were quite uneven, but each point is the average of two complete dilution curves, and the differences between the more heavily irradiated samples when assayed on the two hosts are certainly significant. The dash-and-dotted curves on the right are the sensitivities of the early and the late complex, taken from Figure 2, the sensitivity of free SS (41) and that of RF using E. coli K12 AB1886 (HCR-) (Yarus, unpub. data, cf. 39). All irradiations in this latter section of the figure were performed using 260 m $\mu$  light.

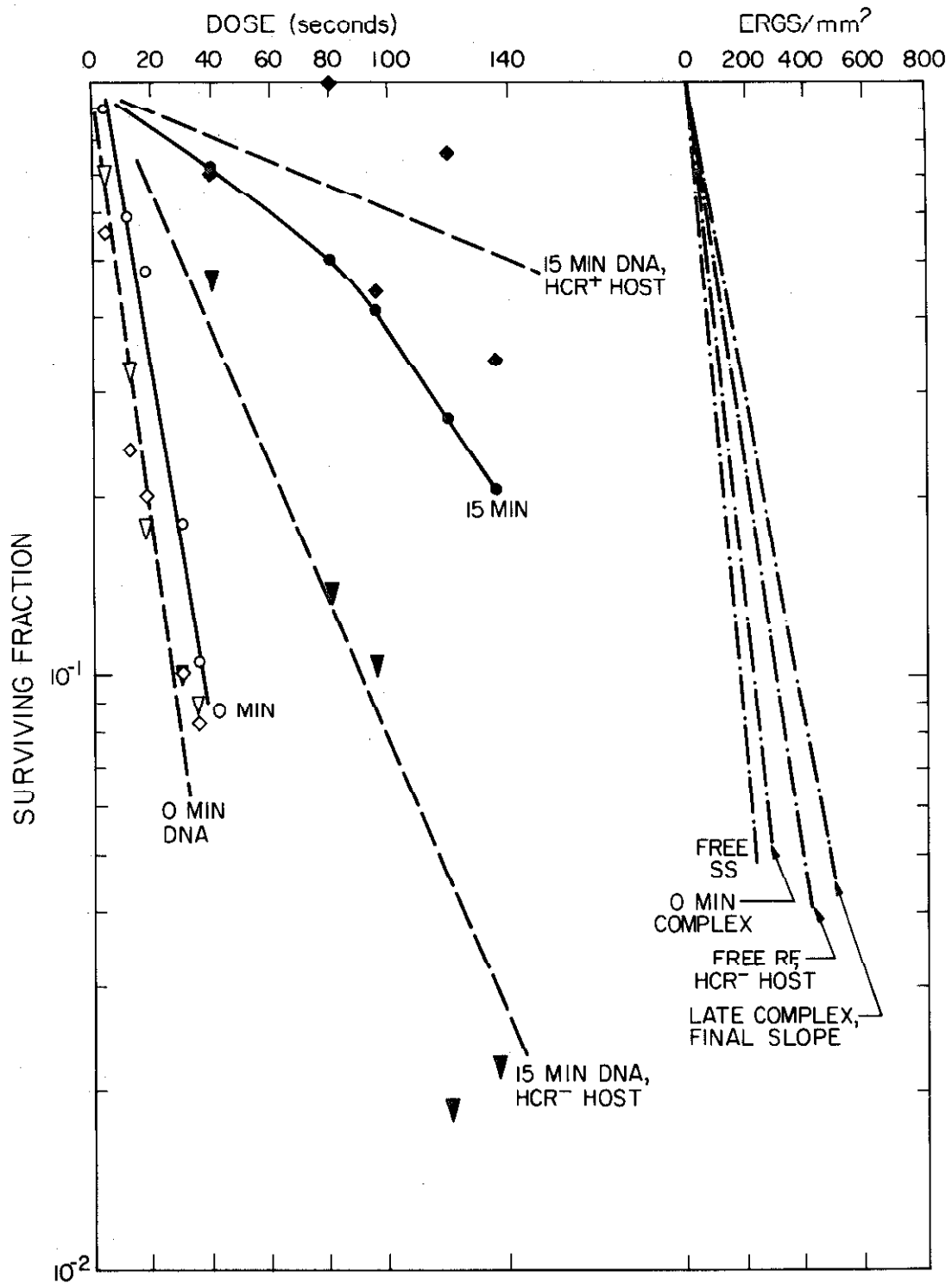


Figure 7

Figure 8

The UV sensitivity of coliphage  $\lambda$  assayed using irradiated cells. The numbers marking the curves (SF = ) are the fractions of the  $10^8$   $\Phi$ X complexes surviving the preliminary irradiation of the cells. Both the  $\lambda$  used for the secondary infection, and the cells themselves were irradiated with monochromatic 265 m $\mu$  light. The MOI of the primary infection with  $\Phi$ X was  $3 \times 10^{-3}$ ; that of the superinfection with  $\lambda$ ,  $2 \times 10^{-4}$ . Doses necessary to inactivate the  $\Phi$ X complex appreciably affect the capacity of the cells to support  $\lambda$  reproduction: such effects are accounted for by normalizing  $\lambda$  survivals to the titer of the un-irradiated  $\lambda$  sample adsorbed to the irradiated host.

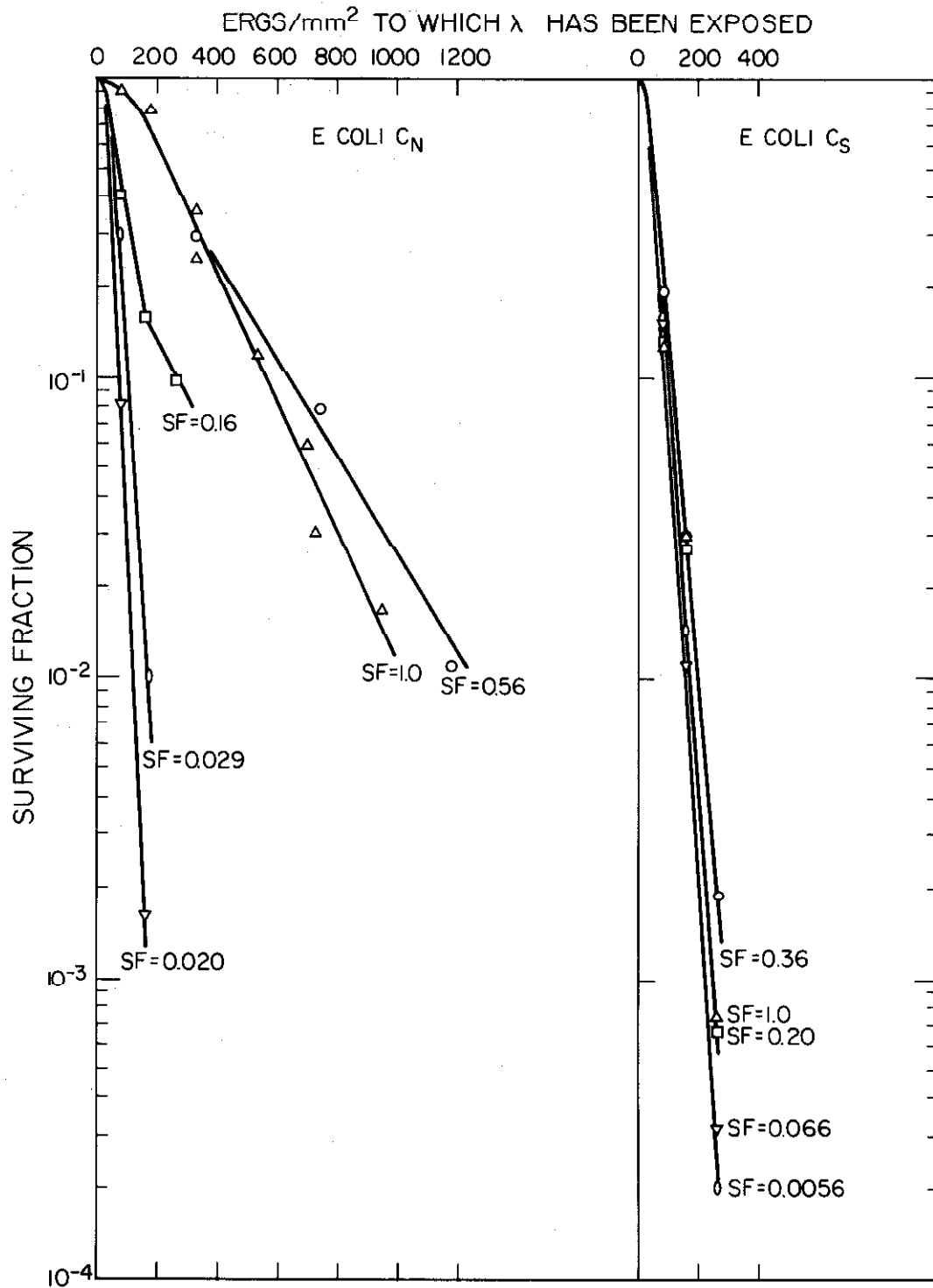


Figure 8



Appendix

The ratio of SS to RF observed when infected cells are extracted early in development is believed to vary for two reasons irrelevant to the purpose of this paper:

(A) Preparations of phage used are never 100% plaque formers; some of the inactive particles adsorb to cells without infecting them and release infective SS DNA when exposed to phenol. Because the protoplast assay system, under some conditions, is much more susceptible to infection by SS than by RF, these particles contribute disproportionately to the total infectivity.

(B) Assays of UV irradiated samples were performed at dilutions of DNA at which the response of the protoplast assay system is linear, that is, proportional to DNA concentration. However, it is now known (Figure 1) that the RF assay is specifically inhibited in the presence of native heterologous DNA. Therefore, when RF accounts for a small part of the total infectivity, dilution curves of cell extracts may become linear before the RF infectivity is fully freed of this inhibition. Estimation of the fraction of RF by UV irradiation would then yield a spuriously low result.

Figure 1, Appendix

The effect of added heterologous DNA on the apparent infectivity of  $\phi$ X-SS and RF DNA.

SS or RF at  $5 \times 10^8$  /ml was combined with various amounts of native or denatured (boiled 10') calf thymus DNA (Sigma) and then protoplasts were added as usual and the infectivity determined.

The notations on the curves indicate, first, the type of  $\phi$ X DNA, SS or RF, then the state of the added calf thymus DNA.

The slopes of the lower three curves may be taken to indicate that in the inhibitory event,  $\sim 1.2$  molecules of  $\phi$ X DNA are inhibited by one molecule of heterologous DNA. The heterologous DNA is present in great excess over  $\phi$ X DNA at all concentrations tested. Therefore, since only one molecule is required for inhibition, it must be that only a small fraction of the added heterologous DNA is able to inhibit the infection by  $\phi$ X.

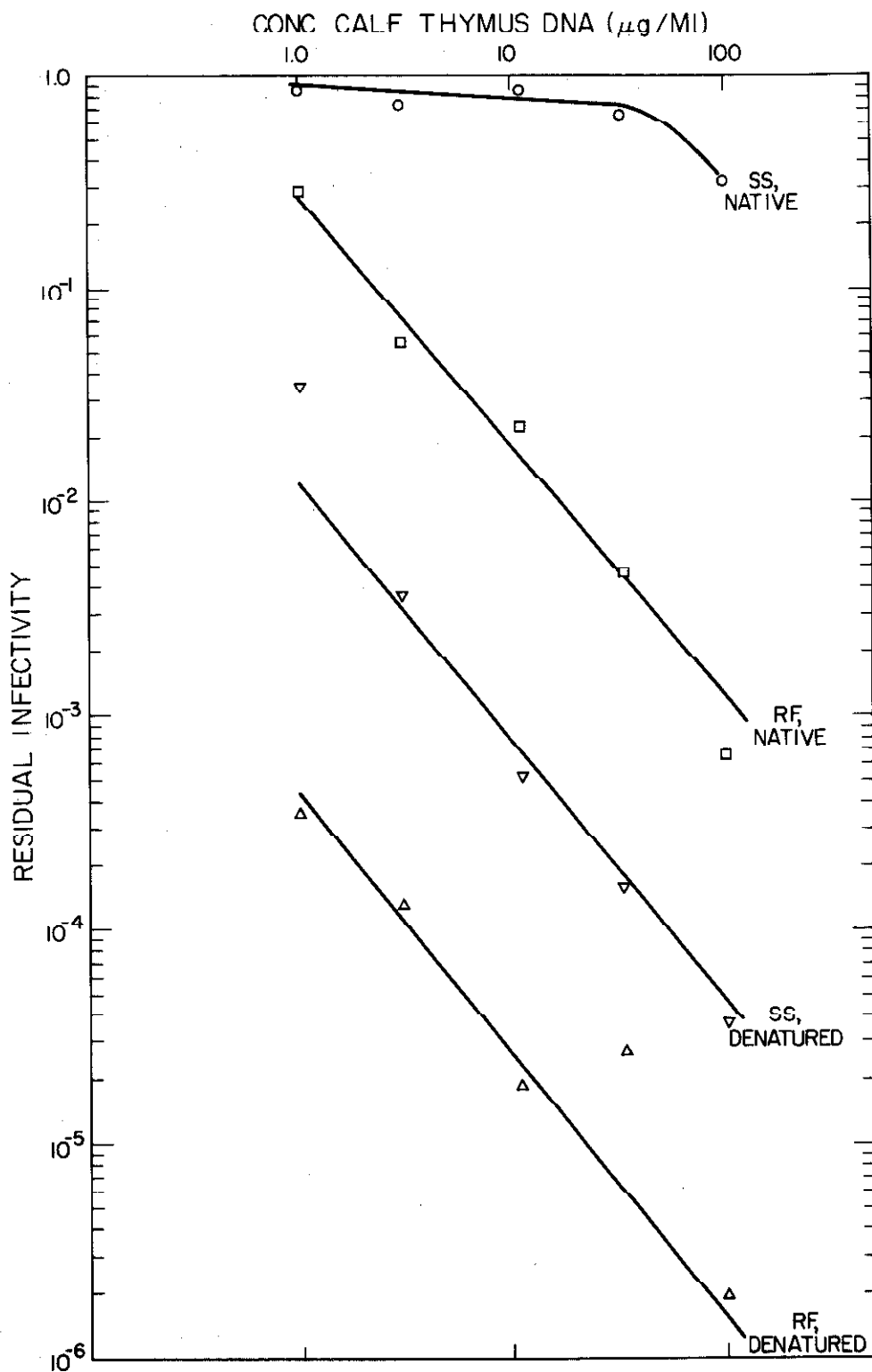


Figure 1, Appendix

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Part III

A LIMIT ON BACTERIAL CAPACITY FOR  $\phi$ X GROWTH

## INTRODUCTION

One of the first features of phage growth to emerge from quantitative studies of the phage life cycle was the strong exclusion between phage or different types. Delbrück and Luria (2) found that E. coli infected with phages T1 and T2 always yielded only T2. Delbrück later coined the phrase "mutual exclusion," to apply to the occurrence of pure yields after mixed, simultaneous, infection. Mutual exclusion appears to be based on incompatibility; the more closely related are the pairs of phages chosen for test, the smaller the tendency to exclude. Complete exclusion is the rule for the dissimilar T1 and T2 (2), partial exclusion for the related, but distinguishable coli phages T2, T4, and T6 (4), and no exclusion at all in mixed infection by mutants of the same viral strain, as Hershey (12) and Delbrück and Bailey (4) showed in founding the study of genetic recombination in phage. Dulbecco (8) later considered in detail the case of infection by similar phages and concluded that 10 T2 can contribute to the progeny of one infected bacterium. However, his original experiments were performed in such a way as to allow the occurrence of temporal exclusion (see immediately below), which probably led him to underestimate the hospitality of E. coli.

There are conditions in which identical phages do not propagate successfully in the same cell. Dulbecco (9) showed that if T2 infected cells were allowed an interval for development, before reinfection, they quickly became almost completely refractory to superinfection by more T2.

Development of the first phage seemed a prerequisite for this

phenomenon, since incubation of cells infected in buffer produced little exclusion (9). He associated this "temporal exclusion" with the observation of Lesley, et al. (15) that T2-infected cells quickly acquired the ability to degrade the DNA of superinfecting phage. French, et al. (11) could inhibit degradation of the DNA of the superinfecting phage without relieving the exclusion; it seems, therefore, that degradation is a concomitant, and not the cause, of temporal exclusion.

The purpose of Part III is to report a restriction on  $\phi$ X growth which, unlike temporal exclusion, is confined to cells starved in buffer and not accompanied by degradation and, unlike mutual exclusion, is expressed among phage differing by only one or two mutational steps, or in some experiments, among phages which are completely identical.

## MATERIALS AND METHODS

### Bacteriophage

$\phi$ X wild type (WT) is the virus described by Sinsheimer (17).

$\phi$ X  $H_A H_B$  has an extended host range: it will form plaques at 30 or 40°C on E. coli  $C_{AB}$  in the presence of glucose, or on E. coli  $C_1$ , hosts on which  $\phi$ X WT will not. Its isolation and properties are described by Dr. Dietrich Pfeiffer (16) who supplied our samples of this phage.

$\phi$ X TS $\gamma$  and TS41 are two temperature sensitive mutants which multiply in E. coli  $C_N$  at 30°C, but not at 40°C.  $\gamma$  and 41 were isolated and assigned to the same cistron by Mr. C. A. Hutchison, who kindly supplied samples of these phages.

$\phi$ X TS  $\gamma h$  is a double mutant; it contains TS $\gamma$  and also  $h$ , a mutation conferring the ability to form plaques on E. coli  $C_1$ . It was



constructed and supplied by Mr. C. A. Hutchison.

A mixture of WT,  $H_A H_B$ ,  $\gamma$  or 41, and  $\gamma h$  may be scored on a single agar plate, using as indicator bacteria a mixture of 5 E. coli  $C_N$ : 1 E. coli  $C_1$ . The phage are spread in soft agar with the mixed indicator (3), incubated for 2-3 hours at 30°C, then shifted to 40°C for 2-3 hours before scoring. Under these conditions WT forms plaques which are large and turbid with fuzzy edge,  $H_A H_B$  gives a large, clear, fuzzy-edge plaque,  $\gamma$  or 41 a small, turbid plaque with crisp edge, and  $\gamma h$  a small, crisp-edged, clear plaque.

Stocks of  $H_A H_B$ , 41,  $\gamma$  and  $\gamma h$  are prepared using E. coli  $C_N$  growing logarithmically at an appropriate temperature: a culture in TKB +  $1 \times 10^{-3}$  M  $CaCl_2$  which has reached  $10^8$  cells/ml is inoculated with the contents of a plaque. After clearing of the culture, the debris is pelleted by centrifugation and 2-5 ml of 0.05 M sodium tetraborate added to the pellet. Several hours at 2-4°C are allowed and then the suspension of debris is centrifuged. The supernatant fluid is the stock. The clear supernatant resulting from a 50 ml culture usually contains  $2 \times 10^{10}$  -  $2 \times 10^{11}$  plaque forming units of virus/ml and is stable to storage at 4°C for a period of months.

$^{32}P$  labeled WT were made as described by Denhardt and Sinsheimer (7);  $D^{15}N^{32}P$  WT as by Sinsheimer (18).

#### Bacterial Strains

E. coli  $C_N$  is BTCC No. 122. It possesses the ability to perform host cell reactivation ( $HCR^+$ ), and is used as seed bacterium unless otherwise indicated.

E. coli  $C_S$  was isolated by Dr. R. P. Boyce. It lacks the ability to perform HCR ( $HCR^-$ ).

E. coli C<sub>1</sub> is resistant to WT, but susceptible to  $\gamma$ h and H<sub>A</sub>H<sub>B</sub>. It was selected from a lab strain of E. coli C by Mr. C. A. Hutchison, III.

### Media

Top and bottom agar for the phage assay are the tryptone media described by Sinsheimer (17), save that no NaOH is added to bottom agar.

SB is starvation buffer. It is used for washing cells, when required, and for prolonged incubation of washed cells in order to starve them. A liter of distilled water is added to 5 gm KCl, 1 gm NaCl, 1.2 gm Tris, 0.1 gm MgSO<sub>4</sub> (anhydrous) and 1 ml 1 M CaCl<sub>2</sub>. The pH is adjusted to 8.1, and the buffer autoclaved.

TKB is KCl-tryptone broth. It is the standard cell-growth and dilution medium. A liter of distilled water is added to 10 gm (Difco) bacto-tryptone and 5 gm KCl. Cells to be used as indicator or as hosts in a growth experiment were grown in TKB +  $1 \times 10^{-3}$  M CaCl<sub>2</sub>. However, E. coli C<sub>1</sub> was usually grown in TKB +  $5 \times 10^{-4}$  M CaCl<sub>2</sub> to avoid aggregation of these cells which occurs at higher Ca<sup>++</sup> concentrations.

Samples containing phage alone were diluted in sodium tetraborate solution, 0.021 M.

3XD (10) is the preferred growth medium when cells are to be converted to protoplasts. It contains, per liter of deionized distilled water: 3.0 gm NH<sub>4</sub> Cl, 0.3 gm MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.3 ml 1 M CaCl<sub>2</sub>, 24 ml glycerol, 15 gm (Difco) casamino acids, 0.03 gm gelatin, 0.9 gm KH<sub>2</sub>PO<sub>4</sub> and 2.1 gm Na<sub>2</sub>HPO<sub>4</sub>; the last two are added after the water.

PA is a rich medium used for growth of infected protoplasts.

It contains, per liter of deionized distilled water: 10 gm (Difco) casamino acids, 10 gm (Difco) nutrient broth, 1 gm. glucose, 100 gm sucrose. PAM medium is PA plus 1.5 ml/100 ml medium of a 10% (w/v) solution of anhydrous  $\text{MgSO}_4$ .

BSA, when required for stabilization of protoplasts, is a dilution of Armour Bovine albumin, supplied by them as a sterile 30% solution.

### Growth Experiments

The starvation synchronization system has been described by Denhardt and Sinsheimer (5). Briefly, cells grown to  $1 \times 10^8$ /ml in TKB +  $1 \times 10^{-3}$  M  $\text{Ca}^{++}$  are washed and resuspended in SB and aerated vigorously for 90 min at  $37^\circ\text{C}$ . Phage is then added, and after a suitable time for adsorption and eclipse (usually 5 min at  $37^\circ\text{C}$ ), an equal volume of prewarmed TKB is added to initiate development. When temperature mutants  $\gamma$ , 41, or  $\gamma\text{h}$  are among the virus used, growth experiments are performed at  $30^\circ\text{C}$  and a longer time is allowed for adsorption. Time is reckoned from the addition of TKB. Times before this are given a negative sign; -7 min means 7 min before TKB was added. Using this system at  $37^\circ\text{C}$ , the first intracellular progeny appear at 9 min, eclipse period is 11-13 min and the minimum latent period, 15-16 min. At  $30^\circ\text{C}$ , these times are 15, 21 and 28 min respectively. Mean burst sizes are somewhat variable but seldom lie outside the range 50 to 300. The rate and extent of phage adsorption to starved E. coli C is indistinguishable from that to the same cells before starvation.

Starved cells prepared in this way and infected with a small ratio of plaque-forming particles/bacteria yield an infected cell for

each plaque former added. It seems, therefore, that every bacterium to which a potentially active phage adsorbs, becomes infected: the restricted growth described below is not the result of an exceptional requirement that several  $\phi X$  infect a cell in order that one among them may yield progeny.

Synchronization with  $CN^-$  is attained by adding KCN to a final concentration of 0.009 M; then 10 minutes later at 30°C, phage are added and after 10 further minutes for adsorption, a  $1/10^5$  dilution is made to start development. The timing of the events of  $\phi X$  growth under these conditions is similar to that seen with starved cells.

Infected cells to be converted to protoplasts were chilled, washed at ice temperature and resuspended in 0.5 M sucrose. They were then subjected to the lysozyme-EDTA procedure described by Guthrie and Sinsheimer (14), save that the concentrations of lysozyme and versene were doubled and all operations were performed in ice-cooled tubes.

When high multiplicities of phage were used, the medium in which growth took place contained anti- $\phi X$  serum,  $K = 2$  to  $3 \text{ min}^{-1}$ , to inactivate unadsorbed phage. Sometimes, in addition, starved infected cells were also filtered onto a Millipore HA filter and washed with SB. The filter was then immersed in an equal volume of SB and swirled with a vortex orbital mixer to resuspend the infected cells. Recovery of cells from the filter is good ( $> 50\%$ ), but not complete.

Very high phage/cell ratios result in the disappearance of potential infective centers. This effect becomes appreciable around 15-20 plaque-formers/cell. The affected cells do not reappear as

colony formers and are probably heavily damaged. In order to avoid selection against cells infected with many phage, total multiplicities in these experiments were kept well below these values. The observed recovery of most of the colony forming cells as infective centers after exposure to a high MOI assures that starved cells receiving  $> 1 \Phi X$  do survive and yield progeny.

Multiplicities of infection (MOI) quoted here are obtained by dividing the number of colony formers into the titer of phage eclipsed just prior to the time when TKB + anti  $\Phi X$  serum was added. Eclipse was followed by taking small samples of the combined phage and bacteria and lysing by treatment with lysozyme and versene. This procedure has been described elsewhere (16). The titer of eclipsed phage is taken to be the difference between the number of plaque-formers observed on lysis of a sample taken immediately after phage is added and the number remaining in a lysed sample taken at the end of the period of adsorption. This method yields smaller estimates of the effective multiplicity than are found by measuring adsorbed virus.

#### Single Burst Experiments

Infective centers are chilled after some time of development by dilution into a large volume of TKB held at ice temperature. They are held on ice until a convenient time and after further dilution, if required, 0.25 to 0.5 ml volumes are distributed into a number of small tubes with a semi-automatic pipet (Becton, Dickerson and Co., No. 1250). These tubes were then put into a water-jacketed incubator (National Appliance Co., Portland, Ore., No. 3312), kept at  $30^{\circ}\text{C}$  or  $40^{\circ}\text{C}$ , depending on the experiment, for at least three hours. The

tubes were then chilled to 4° C and held until assay, which was performed by combining 200 ml of melted soft agar and 30 ml of the indicator mixture at 42-44°C and delivering 2.3 ml portions of this into the single burst tubes using a semi-automatic pipet mounted on a stand. The contents of the tube were then poured on a plate. In this way, 100 single burst tubes could be assayed in 20 minutes or less; the seed bacteria survived this incubation at 42-44°C without harm.

At the outset of this work, any doubtful plaque in a burst was picked with a 1-1/4" straight pin and re-scored on mixed indicator at 30 and 40°C. After some experience it became possible to dispense with this procedure and score by inspection.

In these experiments the fraction of total yields which were of a given type corresponded approximately to the proportion of that phage among the input; that is, all types of phage used seem to be selected in accordance with their abundance. All the expected different types of bursts appear; the restriction of the number of genotypes released (see below) is not a reflection of the exceptional vigor of one of the mutants chosen.

#### Irradiation of Infected Cells

Dilute suspensions of infected cells in SB were exposed to monochromatic UV from a quartz prism monochromator and assayed under conditions selected to minimize photoreactivation. The techniques of irradiation and dosimetry may be found in Part II of this thesis.

#### Calculations

To construct a UV inactivation curve for a cell containing several vegetative centers, data on the inactivation of singly-infected cells

at a similar time in development are used. Each of the measured survivals (SF) is the probability of a phage yield after exposure to a measured amount of UV. Therefore, the probability of inactivation of a single vegetative center is

$$(1 - SF)$$

If a cell contains X independent vegetative centers, the probability that they will all be inactivated is

$$(1 - SF)^X$$

This is taken to be the probability that a multiply infected bacterium will be inactivated. The probability that at least one vegetative center will have survived is then

$$1 - (1 - SF)^X$$

Inactivation data gathered from experiments at low multiplicity is treated in this way and compared with the results of infections at high multiplicity in Figure 1.

To predict, assuming no limitation on phage reproduction, the fraction of cells which yield one, two, three, or four types of phage after simultaneous exposure to several of each kind, the probability of having been infected is taken to be  $P_1 = (1 - \exp(-m_1))$  in which  $m_1$  is the eclipsed multiplicity of phage 1.

The product of all the P's is taken to be the probability of a burst containing all genotypes. The probability of each of the combinations of phages containing three types is taken to be the product of the P's for each of the included types times  $(1 - P)$  for the missing type. For instance, bursts lacking type 4 phage should occur from  $[P_1 \times P_2 \times P_3 \times (1 - P_4)]$  of the cells. Similarly, bursts containing

only 1 and 2 should occur in  $[P_1 \times P_2 \times (1 - P_3) \times (1 - P_4)]$  of the cells and pure bursts of  $P_1$  in  $[P_1 \times (1 - P_2) \times (1 - P_3) \times (1 - P_4)]$  of the cells. The probability for each conceivable type of triple yield, for instance, is summed to give the total fraction of cells which would release 3 genotypes.

In order to compare these calculations to observed distributions when the total multiplicity of infection (MOI) is small, it is sometimes necessary to normalize all calculations to the fraction of cells expected to yield phage of any kind.

The theoretical curves in Figure 3 are graphs of the function

$$\sum \left[ \binom{w}{k} / \binom{h+w}{k} \right] \left[ \frac{e^{-\mu_h} (\mu_h)^h}{h!} \right] \left[ \frac{e^{-\mu_w} (\mu_w)^w}{w!} \right]$$

$w > 0, h \geq 0$

$\binom{w}{k}$  is  $w! / [(w-k)! k!]$ ; which is the number of ways of selecting a group of things of number  $k$  from among a total of  $w$  things. Take  $h$  and  $w$  to be the number of  $h$ -type and  $w$ -type phages adsorbed to a bacterium;  $[\binom{w}{k} / \binom{h+w}{k}]$  is then the probability that a bacterium which can allow  $k$  phages to multiply will select at random from among  $h$   $h$ -type and  $w$   $w$ -type phage, a group of size  $k$  which does not include an  $h$ -type.

This expression is not defined when  $(h+w) < k$ , but, of course, in this situation all phages will multiply.

The other two terms are the probabilities that a culture infected with an average multiplicity of  $\mu_h$   $h$ -type and  $\mu_w$   $w$ -type phage will yield cells infected by exactly  $h$   $h$ -type and  $w$   $w$ -type. Their product is the probability that a cell receives exactly  $h$  and  $w$  of the two phages. The sum above, then, gives the probability that cells



infected with an average of  $\mu_h$  and  $\mu_w$  phage will yield bursts containing only w-type, if each bacterium will support the growth of a number of phage, k.

The above reasoning follows Dulbecco (8) exactly. At low total multiplicities, this expression must be normalized to the fraction of cell which yield bursts of any kind

$$[ 1 - e^{-(\mu_w + \mu_h)} ]$$

in order to compare it with the observed fraction of pure bursts.

## RESULTS

In Figure 1 are typical results of the irradiation of starved E. coli  $C_N$  infected with several  $\Phi X$  per cell. Considering first the curves at 0 min, that is, after phage have adsorbed and are in eclipse, but before the beginning of intracellular development, we may see that the inactivation of multiply infected cells follows the course expected for several independent infections of the same cell. The infective center formed after infection at low multiplicity is inactivated exponentially according to the dashed line in Figure 1. Infection with high multiplicity yields an infective center whose ultimate sensitivity is the same, but which now has a small shoulder near the origin. The shoulder, however, reaches its maximum size at a rather low ratio of phage/cells. The extrapolate of the exponential part of these latter curves is 2.5 to 3, even at a multiplicity of 21 (Figure 1). A similar situation exists in the case of  $C_s$ , whose young, singly infected complexes are also inactivated exponentially (cf. Part II, this thesis), and have two distinguishable sensitivities (Figure 2). After infection with

several phage per cell, inactivation follows the course expected of cells containing an average of 1.5-2 vegetative phage.

Also shown in Figure 1 is the inactivation of complexes allowed various times of development. The dotted lines and solid circles are curves calculated assuming that the multiply infected complex contains 3 copies of the structure observed in singly infected cells at a similar time in development. The observed and calculated curves agree satisfactorily at all times.

Previous studies of the sensitivity of the  $\Phi$ X-E. coli C complex have led to the conclusion that inactivation of the complex results from inactivation of the intracellular phage DNA (6, Part II, this thesis). Secondly, in the Luria-Latarjet experiment, survivors are required to release progeny; that is the function whose sensitivity is tested by irradiation. Therefore, these experiments imply that only a limited number of those phages infecting a bacterium are capable of some function required for the production of progeny. The capable viruses appear to be inactivated independently.

A particularly simple example of the restriction indicated by the limited extrapolate of the UV curves would be a restriction on the production of progeny itself. That is, it could be that only a few among the phages potentially able to grow in a cell are selected to reproduce themselves.

If this interpretation is correct, then the release of progeny of one type of phage would be a function of the number of other phages with which it had to compete. An experiment based on this idea is shown in Figure 3. A fixed number (1.5/cell) of  $H_A H_B$ , a host range

mutant of  $\Phi X$ , was used to infect starved E. coli  $C_s$  in the presence of varying amounts of the wild type phage. The data is compared to theoretical curves calculated by the method of Dulbecco (8) in which it is assumed that the phage which are to produce progeny are selected at random from among those which infect a bacterium, and that the distribution of phage among bacteria is described by the Poisson distribution. The infected cells in this experiment were plated on a mixture of indicator bacteria, one of which is susceptible to both  $H_A H_B$  and WT and the other only to  $H_A H_B$ . Plaques corresponding to infective centers which released any  $H_A H_B$  may therefore be distinguished from those which yielded only WT. In addition, infected bacteria from several of these infections were diluted and distributed into tubes so that there would be only one infected cell in a tube; these tubes were plated on the mixed indicator after lysis and the types of phage released scored. Both types of data are presented in the figure and are consistent with the interpretation proposed for the irradiation experiment: only a small number of the phages infecting a starved cell are selected to propagate themselves.

A more direct demonstration of this limitation on phage growth is presented in Figures 4 and 5. Here the cell is infected with several phages of each of three or four genotypes. Under these conditions of infection, the Poisson distribution may be used to predict that if all phages can multiply, most cells will yield all genotypes. However, the conclusion does not depend on a detailed quantitative argument, because the limitation on growth is so effective. Infected cells are diluted and distributed into tubes so that most tubes in which phage will appear will

have contained only one infected cell. The predominant class of cells does not release all genotypes; instead, only one genotype. In addition, some cells appear to support the growth of two types of phage. The small number of bursts, observed in some experiments, which contain three parental types are no larger than the number expected as a result of chance dispensation of two or more infected cells into the same tube. The issue of a starved cell, then, are usually the descendants of only one of the viruses which infect it.

For succinctness, we may say that most starved cells have a "maturation number" of one, since the ability to produce mature progeny has been the property tested.

A low maturation number is not a general property of  $\phi X$  growth. Depicted on Figure 6 is the result of a single burst experiment on log phase *E. coli* C<sub>g</sub> infected in KCN and also without synchronization of any kind. As a control, a portion of the same culture was washed, starved, and infected with the same phage mixture. As usual, the majority of starved cells yield pure bursts. However, cells grown in broth and infected without synchronization, or cells infected in the presence of KCN, give yields little different from the prediction made assuming unlimited maturation number. The observation of a distribution which contains a number of quadruple and triple bursts in reasonable agreement with that predicted validates the procedures used to make the predictions and lends credence to the difference between the calculated distribution and that observed in starved cells.

E. coli C appears to be a doublet in the phase microscope; that is, the predominant type of cell is a rod with a constriction in the

middle. It is unlikely that this doublet structure is related to the restricted maturation, however, since conversion of infected cells to protoplasts, after which almost all bacteria appear to be undifferentiated spheres, has no effect on the genotype distribution (Figure 7).

No part of the evidence so far bears on the stage of phage growth at which the limit on maturation is impressed. To focus on the same point in a slightly different way, how far do the debarred phage proceed? Experiments intended to determine whether they form RF are summarized in Table 1. RF is distinguishable from the parental SS by virtue of the density change accompanying its formation (18). Therefore, the appearance of parental label at the density characteristic of RF in the neutral equilibrium density gradient was taken as the criterion of RF formation. The amount of RF formed by the labeled phage was compared in the presence and absence of a high multiplicity of unlabeled virus. It appears that the minority phage do form RF; this, of course, implies all that must come before: normal adsorption, eclipse, and access to intracellular enzyme. Normal adsorption and eclipse can be observed directly when the minority phage is distinguishable genetically, as in experiment II. In addition, the amount of parental  $^{32}\text{P}$  found with washed complexes is unaltered in the presence of unlabeled phage.

#### DISCUSSION

It is clear from these results that in the majority of cells only one, or less often, two, of the phage DNA's are selected to give rise to progeny. The others probably reach the stage of RF. The absence

of such a strict limitation in unstarved cells suggests that the starvation procedure has limited the ability of the host to provide something  $\Phi X$  requires. In this view, the restriction is a feature of growth in unstarved cells also, but the number of  $\Phi X$  allowed to reproduce is sufficiently large that it is not detected in these experiments.

Perhaps the starved E. coli cell contains a "site" whose possession is essential for the production of progeny. An RF without a site is destined to be sterile.

Two interesting possibilities may be distinguished for the quality conferred on the possessor of a site. The distinction might be an "active" one, that is, a site might be a co-factor in some obligatory reaction in the process of maturation, and its occupant would be the only vegetative phage able to perform this reaction. On the other hand, it could be "passive" and shield its possessor from inhibition. These ideas bear an unmistakable resemblance to the most venerable idea in the study of exclusion, that of the "key enzyme," proposed by Delbrück and Luria (2) to explain the incompatibility between T1 and T2 growth in the same cell. Though this idea was later replaced (3) with another when it was found that several like phage could multiply in a cell, the idea of an active site or key enzyme arose naturally in the course of these experiments on  $\Phi X$ .

The existence of this restriction on RFs formed during a synchronized infection raises the possibility that only one or a small number among the replicas of RF may be capable of completing the infective process in singly infected, starved cells. Denhardt and Sinsheimer (7) observe that complexes of starved E. coli C and

heavily  $^{32}\text{P}$  labeled  $\phi\text{X}$  are still sensitive to the  $^{32}\text{P}$  disintegration in the parental strand of an RF, even when RF replication in unlabeled medium has occurred. It appears from this result also that at least one necessary function is reserved to a single RF, the RF containing the parental SS, the first RF formed in the infected cell. The distinction between a successful and an unsuccessful infection, between RFs which will give rise to progeny and those which will not, remains obscure. The known steps intervening between appearance of the parental RF and appearance of progeny are RF replication, production of specific RNA and phage proteins, and synthesis of progeny single strand. The incompetent RF might fail at any of these.

The extrapolate of the inactivation curves of early complexes of  $C_s$  is usually 1.5, in good numerical agreement with other measurements of its maturation number presented here.  $C_N$  complexes, on the other hand, reproducibly yield extrapolates of 3; yet, the single burst experiment (Figure 3) indicates that only one type of phage is matured in most cells. My inclination is to accept the lower estimate; the single burst experiment is the more direct of the two. It may well be that irradiation itself (in the  $\text{HCR}^+$  strain) allows a second RF to assume a "site" after inactivation of the parental molecule, whereas in the course of normal growth, the original RF would have remained unique. Denhardt and Sinsheimer (6) were able to re-infect  $\phi\text{X E. coli C}$  complexes after inactivating the first infection with UV. This suggests that the replacement of the inactivated RF can occur at least once.

Formation of RF by the minority phages in starved cells distinguishes limited maturation from a type of temporal exclusion oc-

curing after  $\phi$ X infection (13). If  $\phi$ X-infected cells are allowed even a small amount of development before superinfection, the superinfecting phage do not appear among the progeny and are unable to complement a genetic defect in the primary infection. In this case (temporal exclusion) the superinfecting SS DNA is recovered as SS on phenol treatment and does not appear in RF (13). The smaller fraction of quadruple bursts among the progeny of an unsynchronized infection than among that of cells infected in KCN (Figure 6) may reflect temporal effects of this kind.

Since this type of temporal exclusion occurs in starved cells, it must be that exposure to two kinds of phage yields large numbers of mixedly infected cells, as assumed in the analysis of the experiments described here.

Presumably, starved cells allowed enough time in broth would become indistinguishable from unstarved cells. The minimum latent period at 30°C in this system is about 28 min, the mean latent period ca. 42 min. The excluded RF are unable, ipso facto, to propagate themselves in this time, though this might be because phage infection itself blocks progress back to the unstarved state.



Table 1

Expt.	Host	Infection Procedure	Minority Parent	Majority Parent	Time DNA Harvested	Method DNA Prep.	Cts/min in RF	% of Control
Ia	Starved <u>E. coli</u> C <sub>N</sub>	Minority parent alone, -5 min, 37 C	$\phi$ X <sup>32</sup> P WT eclipsed MOI = 0.027	$\phi$ X WT	12 min (37 C)	Complexes lysed with lysozyme-EDTA and	2192	100
Ib	"	Minority parent and majority parent simultaneously, -5 min	"	$\phi$ X WT eclipsed MOI = 6.4	"	banded without further treatment	1738	79
Ic	"	Maj. parent -10 min, min. parent, -5 min	"	$\phi$ X WT eclipsed MOI = 6.4	"	"	1719	79
IIa <sup>†</sup>	Starved <u>E. coli</u> C <sub>s</sub>	Minority parent alone, -15 min, 30 C	$\phi$ X D <sup>15</sup> N <sup>32</sup> P WT eclipsed MOI = 0.45	$\phi$ X WT	19 min (30 C)	Complexes lysed with lysozyme and EDTA,	165	100
IIb	"	Minority parent -15 min, maj. parent, -8 min	"	$\phi$ X TS $\gamma$ eclipsed MOI = 3.3	"	banded without further treatment	148	90
IIc	"	Minority parent alone, -15 min, 30 C	"	"	"	Lysed complexes above treated with	120	100
IId	"	Minority parent, -15 min, maj. parent, -8 min	"	$\phi$ X TS $\gamma$ eclipsed MOI = 3.3	"	phenol before banding	129	107

Table I (Continued)

† In this experiment (II), the disappearance of the minority parent after addition of the TSy could be measured: 5 consistent, independent dilutions and platings of WT infective centers with and without TSy showed that 57% of the WT infective centers observed when WT was used alone appeared when TSy was also present. After addition of TSy 97% of the colony formers appeared as infective centers. Therefore, the WT infective centers which did not appear in the presence of  $\gamma$  were not killed, but were recovered as pure  $\gamma$  bursts. Though entirely satisfactory in this respect, the fraction of WT infective centers which become  $\gamma$  infective centers is lower than expected.

Figure 1

Inactivation of starved E. coli  $C_N$  infected with high multiplicities of  $\Phi X$  and inactivation curves predicted for limited infection.

----- O min complexes infected with low multiplicity of  $\Phi X$ ;

———○——— observed inactivation of  $C_N$  complexes. MOI = 7.2.

The curve drawn through the O min data is a "3 hit" curve.

———●——— Predicted inactivation for 3 supposed vegetative phage per bacterium, calculated as described in Materials and Methods. Data were drawn from an inactivation performed on complexes of similar age infected at low multiplicity. The calculated curve for each time is beside the corresponding observed curve.

———△——— Observed inactivation of O min  $C_N$  complexes MOI = 6.4

———□——— Observed inactivation of O min  $C_N$  complexes MOI = 9.3

———▽——— Observed inactivation of O min  $C_N$  complexes MOI = 11.4

———◇——— Observed inactivation of O min  $C_N$  complexes MOI = 21

The time beside each curve is the amount of development allowed.

Irradiation performed using 260 m $\mu$  light.

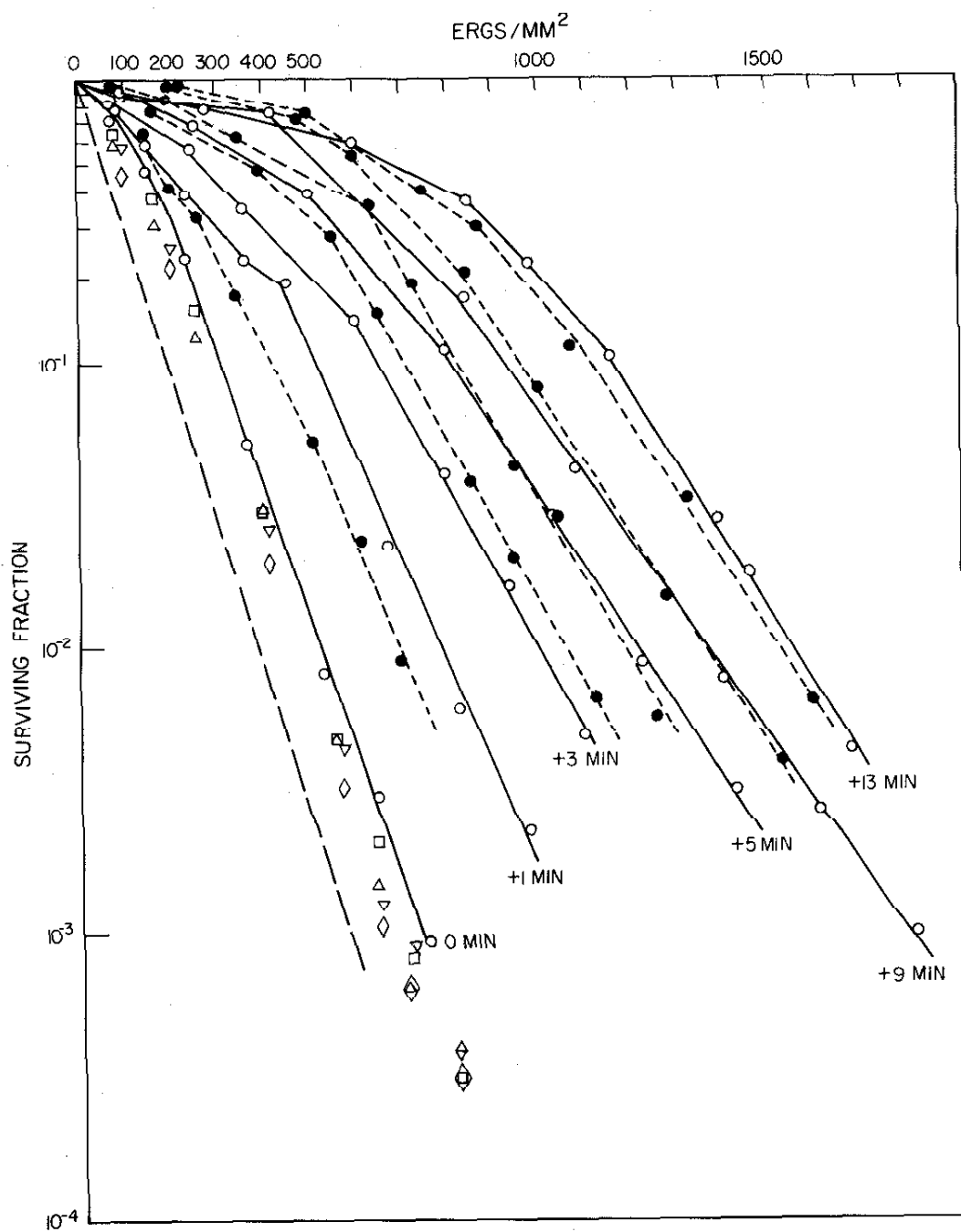


Figure 1

Figure 2

Inactivation of starved *E. coli* C<sub>s</sub> infected with high multiplicity of  $\Phi$ X. No calculated curves are presented because of the simple form of the curves.

————— O ————— MOI = 0.07  
————— □ ————— MOI = 2.0  
————— Δ ————— MOI = 3.6  
————— ▽ ————— MOI = 8.5

Complexes were harvested for irradiation after 3 min in presence of nutrient, but curves indicate that development was slower than usual. Irradiations were performed using 254 m $\mu$  light.

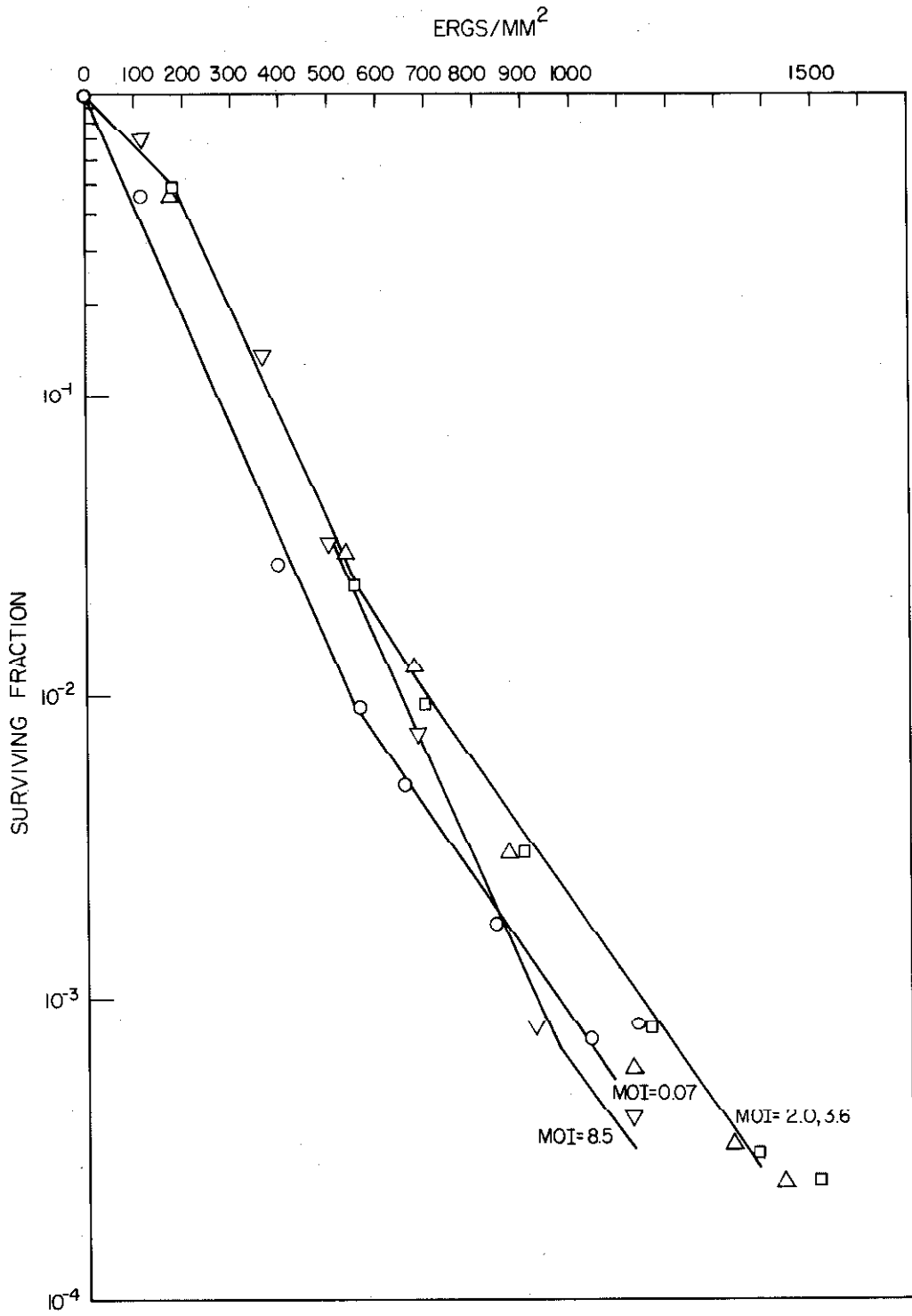


Figure 2

Figure 3

Occurrence of pure yields of wt as a function of multiplicity. Starved  $C_s$  were infected with a fixed multiplicity of  $1.5 \phi X H_A H_B$ /cell and a varying multiplicity of  $\phi X$  wild type. Progeny were scored

○ by plating on mixed indicator

□ by observation of single bursts

The solid lines are calculated assuming that each bacterium will allow the propagation of 1, 2, or 3 parental phage. (See Materials and Methods.)

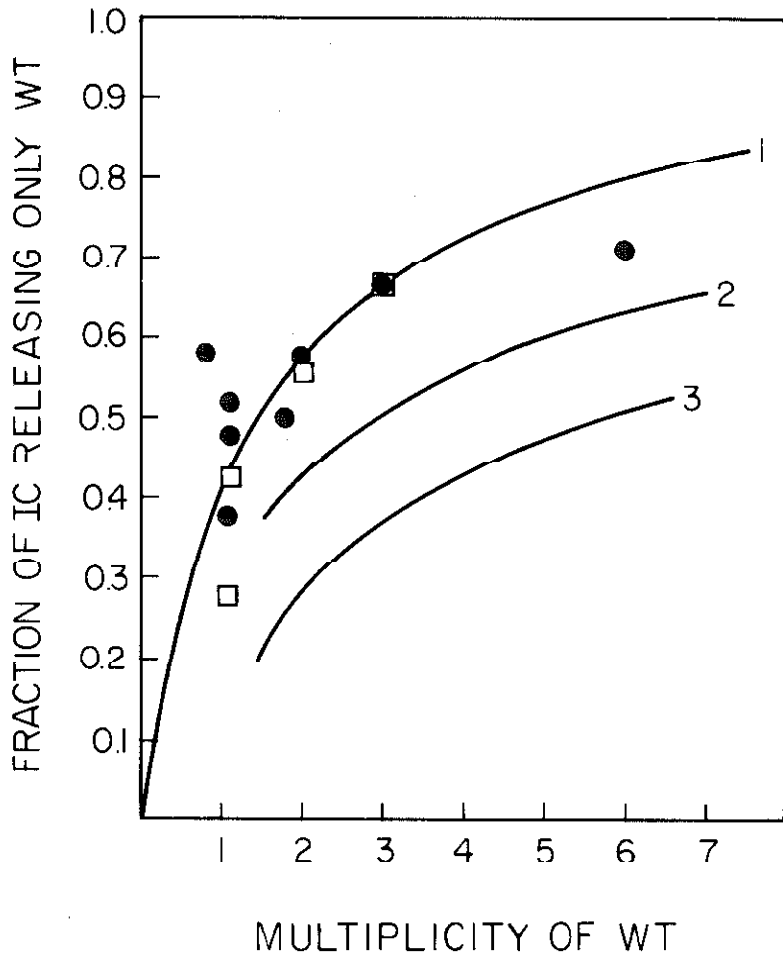


Figure 3



Figure 4

The number of different types of progeny released by single starved E. coli C<sub>N</sub> bacteria infected with

φX γh MOI = 1.7

41 MOI = 2.0

WT MOI = 3.6

The observed distribution is based on 53 bursts, 51 of which were scored among 130 tubes and 2 of which were found among 100 tubes at a higher dilution. A Luria-Latarjet curve taken using the 0 min complexes had an extrapolate of ~ 3. Mean burst size in culture was 75, in single burst tubes, 50. 83% of the colony forming bacteria were recovered as infective centers.

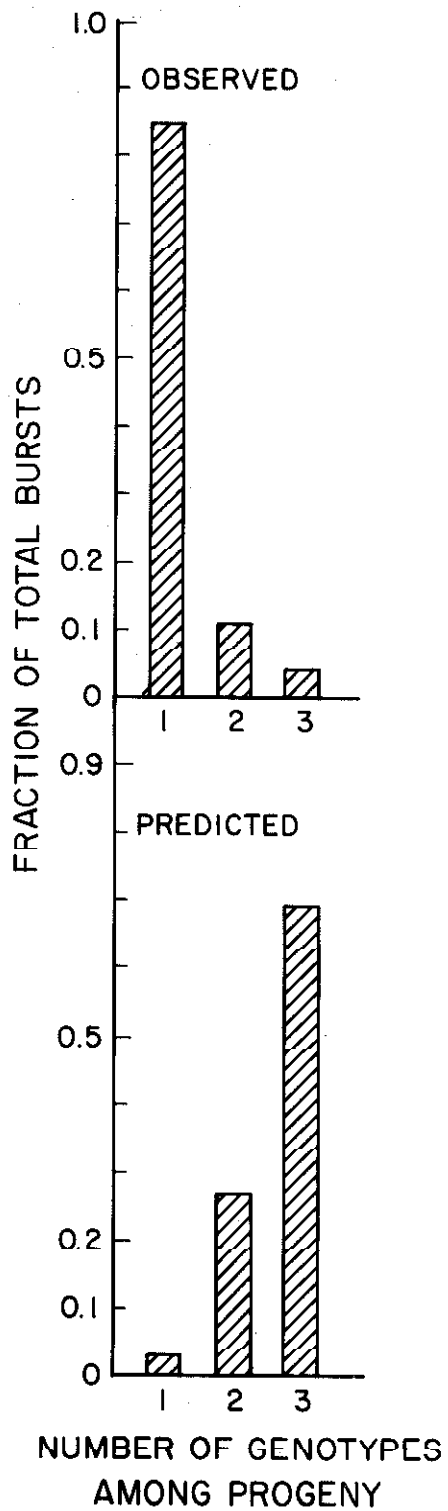


Figure 4

Figure 5

The number of different types of progeny released by single starved E. coli C<sub>s</sub> bacteria infected with

$\phi X \gamma_h$	MOI = 1.6
H <sub>A</sub> H <sub>B</sub>	MOI = 1.3
$\gamma$	MOI = 5.7
WT	MOI = 2.5

The observed distribution is based on 51 bursts observed among a total of 400 tubes. A Luria-Latarjet curve performed using these 1 min complexes had an extrapolate of  $\sim 1.5$ . Mean burst size in culture was 63, in single bursts, 92. The infected cells were washed by filtration after phage adsorption, the number of infected cells recovered from the filter was  $> 60\%$  of the original number of starved cells.

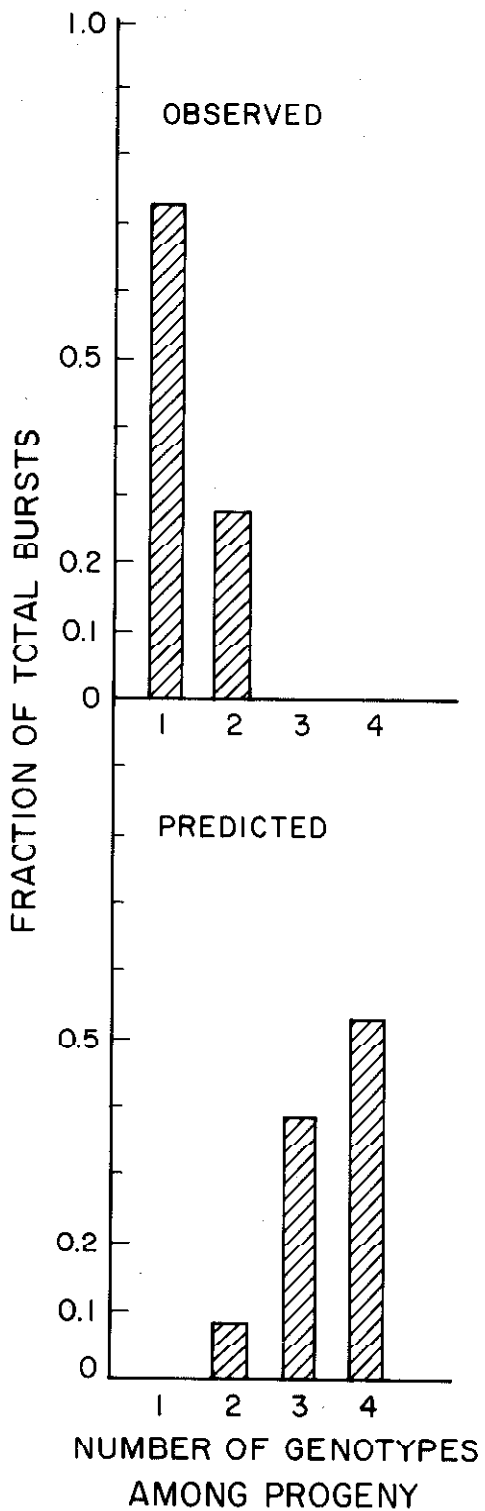


Figure 5

Figure 6

The number of different types of progeny released by single infected E. coli C<sub>s</sub> infected with

$\Phi$ X $\gamma$ h    MOI = 0.4

$\gamma$         MOI = 0.8

WT        MOI = 2.4

H<sub>A</sub>H<sub>B</sub>    MOI = 1.2

(A) A portion of the cells were starved and infected just as in the experiment of Figure 5. Twenty-six bursts appeared among 100 tubes in this portion of the experiment. 86% of the starved cells appeared as infective centers.

(B) Distribution of genotypes predicted from Poisson distribution, supposing no limitation on the number of phages which can propagate in a cell.

(C) The phage mixture was added to cells growing logarithmically; no attempt was made to synchronize infection. 55 bursts were scored among 194 tubes. 98% of the colony forming cells were recovered as infective centers.

(D) KCN was added to logarithmically growing cells to a final concentration of 0.009 M. 10 min later the phage mixture was added, and after 10 min for absorption, cells were diluted 10<sup>5</sup>-fold to initiate growth. 35 bursts were scored among 183 tubes. 45% of the colony formers were recovered as infective centers.

Mean burst sizes were: culture, starved cells 150, and single bursts 120; culture, unsynchronized infection, 240 and single bursts, 200; culture, KCN synchronized, 90 and single bursts, 87.

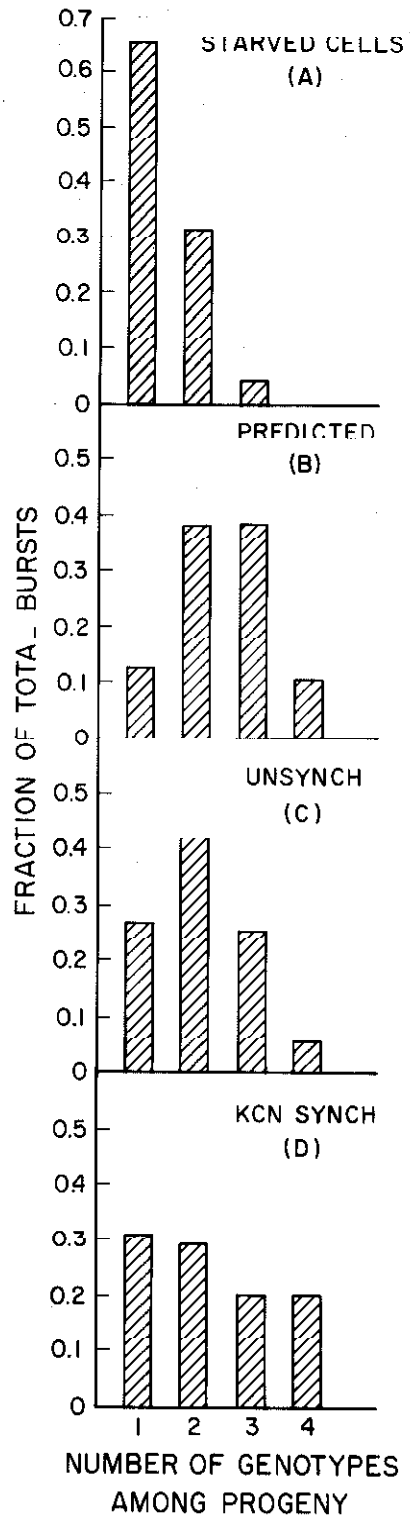


Figure 6

Figure 7

The number of different types of progeny released by single infected E. coli C<sub>s</sub> after conversion to protoplasts. C<sub>s</sub> was grown in 3XD medium, starved as usual, infected, and 10 minute-old complexes harvested and converted to protoplasts by the method of Guthrie and Sinshimer (14).

$\phi X$  H<sub>A</sub>H<sub>B</sub> MOI = 1.4

$\gamma$ h MOI = 0.7

$\gamma$  MOI = 0.8

WT MOI = 1.1

(A) Starved, infected cells harvested after 10 min development, diluted into PAM medium + 0.5% BSA and distributed into tubes without conversion to protoplasts. 17 bursts, found among 200 tubes were scored in this part of the experiment. At the end of phage adsorption, the cells were washed by filtration and 70% of the original number of starved cells were recovered from the filter as infective centers.

(B) Calculated distribution of genotypes, assuming no limit on maturation.

(C) The cells used in (A) were treated with lysozyme and versene at 0°C, diluted in PAM medium + 0.5% BSA, and distributed into tubes. 22 bursts appeared among the 200 tubes scored in this part of the experiment. 5 ml. of infected cells containing  $1 \times 10^8$  infective centers ml. were concentrated into one ml. of 0.5 M sucrose for treatment with lysozyme. After 5-fold dilution with PAM medium, which is a part of the protoplast procedure, one microscopically visible cell body was recovered for each cell in the original suspension. More than 90% of these had the spherical shape of protoplasts. The number of fertile

tubes observed after dilution and distribution into tubes suggested a titer of  $\sim 6 \times 10^7$  infected protoplasts/ml. in this diluted suspension. Therefore, a large fraction of the infected cells gave rise to a protoplast which yielded phage. The diluted cells gave an average burst size of 67, the protoplasts, 84. A concurrent single step growth curve yielded a burst of 68. A Luria-Latarjet experiment performed on these complexes at 0 min had an extrapolate of  $\sim 1.5$ .



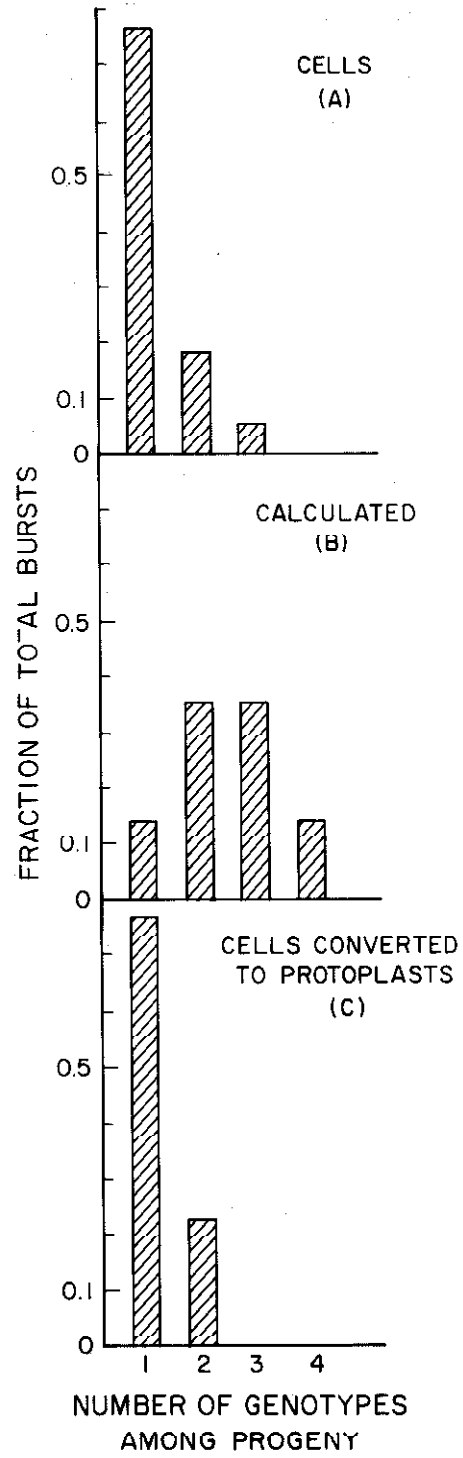


Figure 7

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Part IV

THE SYNTHESIS OF RF IN AN IRRADIATED BACTERIUM

## INTRODUCTION

The natural goal of radiobiology is an understanding of the effects of electromagnetic radiation on exposed organisms. Recent progress in the ultraviolet photochemistry of nucleic acids has only sharpened a complementary, biological question: What are the physiological effects of UV damage? Put more directly, why do the relatively restricted photochemical changes caused by small doses of UV cause large effects on the organism? As a preliminary to the description of a peculiarity of phage growth in an irradiated host cell, the effects of UV on bacteria are briefly reviewed below.

However complex may be the events initiated when an organism is exposed to ultraviolet light, the gross properties of inactivation itself have a certain simplicity. The numbers of viable bacteria and bacteriophages decrease exponentially at a constant rate with applied dose over a wide range of UV doses. This may be taken to mean that inactivation is due to the absorption of a single quantum by a structure which is the same in all members of the population. By determining rate of inactivation at several wavelengths, the absorbing structure may be characterized further; the rate of exponential inactivation of bacteria (21) and bacteriophages (46) varies with wavelength as does the absorption of light by nucleic acid. Therefore we can assume that the inability of a phage or bacterium to propagate itself is the result of a single damage to a purine or pyrimidine containing material, presumably the nucleic acids, DNA or RNA, themselves. Since the DNA of bacteriophages and bacteria serves as the repository of their genetic information, damage to DNA would be a certain way of

interrupting their multiplication. Considerations like these have focused attention on cell nucleic acids as the receptors for biologically effective UV quanta.

Studies of the UV sensitivity of bacterial mutants reveal two general sorts of modification which affect the sensitivity of colony-forming ability.

Three different sites on the bacterial chromosome are known whose alteration affects the reactivation of photochemical damage in DNA (19, 33), presumably altering the cell's ability to excise the damage and resynthesize an undamaged molecule. It has been suggested that this is done by copying the undamaged region in the complementary strand (2, 37). In the absence of this mechanism, called host cell reactivation (HICR), a cell's ability to form colonies and its synthesis of nucleic acids is more susceptible to inhibition by UV (25, 36) (see below) and certain irradiated bacteriophage appear much more sensitive to UV when the reactivationless cell is used as host (18).

On the other hand, very small doses of UV seem to have an effect on cell division per se. E. coli B, subjected to doses of UV far smaller than those necessary to kill the cell, is prevented from dividing for an extended period; long filamentous cells are formed (8, 9). The affected bacteria, however, continue RNA and DNA synthesis at the normal rate (see below). A permanent bar to division could presumably prevent colony formation and thus be scored as a lethal damage. In this experiment however, much of the inhibition of division must have been reversible, since the effect is observable at doses at which there is no lethality. It later became clear that some lethal damages in E. coli B are due to blocked cell division.

A second group of bacterial mutants affecting UV sensitivity was found which has an altered susceptibility to filament formation after exposure to UV (20, 34), or to antibiotics and dyes which affect the cell wall (34). A bacterium bearing the allele which confers resistance to filament formation is more resistant to UV, though the presence of this allele has no effect on the UV-sensitivity of DNA, RNA, or mass synthesis (see below) (34) or on the sensitivity of a UV-irradiated bacteriophage grown in these cells (21). Phenotypic resistance to filament formation and to UV is also conferred on a cell in the presence of pantoyl lactone (32). The UV-caused delay in cell division has an action spectrum resembling the absorption spectrum of a nucleic acid (6), and, as is implicit in the observation of long filamentous cells, does not seem to depend on gross inhibition of the synthesis of cell wall material (32). Otherwise, the cause of this effect is still obscure.

The UV-killed bacterium still respire normally, and if not too heavily irradiated, will continue mass synthesis at a normal rate for some time (23). Heavy doses of UV may lead to lysis. However, nucleic acid synthesis, and particularly DNA synthesis, is particularly susceptible to inhibition by UV (17, 22, 23).  $HCR^+$  cells, if irradiated with a dose of the order of that giving 37% survival, will all apparently cease synthesizing DNA for a period which increases with the dose, and then will begin synthesis at a rate comparable to that observed in the unirradiated control. The cessation of DNA synthesis in all cells, and its apparent resumption at a rate comparable to that of unirradiated cells suggests strongly that inhibited replication of DNA as such is not

a common source of lethal damage in HCR<sup>+</sup> cells.

Non-reactivating (HCR<sup>-</sup>) mutants cease DNA synthesis after doses of UV which are expected to form only a few pyrimidine dimers per bacterial genome, and do not appear to begin again, at least up to 90 minutes after irradiation (36).

Such in vitro evidence as is available makes it appear unlikely that photoproducts in DNA are directly responsible for the stoppage of DNA synthesis. Bollum and Setlow (25), using calf-thymus polymerase, which requires denatured primer, found that doses roughly 10<sup>4</sup>-fold larger than those required to affect DNA synthesis in HCR<sup>-</sup> cells were required to suppress the priming ability of bacterial, phage, or calf thymus DNA, whether irradiated as a denatured molecule or not. Admittedly, a different result might be found for DNA and enzyme irradiated together or for the replication of a double-stranded DNA. Perhaps, though, DNA replication stops in vivo not because of the damages themselves, but rather because of the single strand breaks or gaps which may be opened in DNA during HCR. I would suppose, for this purpose, that the reactivationless mutants examined for degradation can break, but not degrade their DNA.

As a result of the recognition of the HCR mechanism, our knowledge of what happens during the hiatus in DNA synthesis has been considerably extended. Apparently, the UV lesions in the DNA are excised from the strand of DNA in which they occur (2, 37) though pyrimidine dimers, at least, remain inside the bacterial cell (36). Along with excision of the damages themselves, a considerable fraction of the DNA itself may be degraded. The apparent amount varies:

Hanawalt and Setlow (17) find that E. coli 15 T<sup>-</sup> exposed to 400 ergs/mm<sup>2</sup> at 265 mμ releases no <sup>32</sup>P to the medium; Boyce and Howard-Flanders (27) report the appearance of about 10% of the DNA of E. coli K12 as acid soluble, but intracellular fragments after 960 ergs/mm<sup>2</sup>; later the same authors (3) report that the same organism released 26% of its DNA to the medium after 500 ergs/mm<sup>2</sup>. Pettijohn and Hanawalt (30) found that E. coli TAU-bar, after exposure to about 500 ergs/mm<sup>2</sup>, lost 2/3 of its prelabeled acid precipitable DNA when incubated for three hours after irradiation in the presence of bromouracil. Shuster and Boyce (38) observed a loss of up to 35% of the acid precipitable prelabeled DNA of E. coli TAU after exposure to 1040 ergs/cm<sup>2</sup>. These doses should yield a small number (< 10) lethal hits/organism. Extensive degradation is therefore elicited by doses comparable to those which are biologically effective.

In experiments in which degradation of the DNA in HCR<sup>+</sup> cells was detected, little or no release of label from the DNA of HCR<sup>-</sup> cells occurred (2, 15). Degradation, then, is associated with HCR, and may begin by the removal of one strand in the tract of DNA being attacked (2, 37). Degradation is accompanied by resynthesis, at least part of which consists of the replacement of only one strand of the repaired tract of DNA (30). In the experiment of Pettijohn and Hanawalt (30), it seems that only a small part of the degraded DNA was replaced. If it is usually true that the HCR process cannot repair all of its deprecations, then even types of damage subject to repair may become lethal. It is even conceivable that all photochemical changes produced by biological doses of UV are intrinsically repairable, and lethality of



some of them signifies miscarriage of the repair.

The degradation of a large fraction of the pre-existing bacterial DNA and possible resynthesis by a new mechanism prompts caution in interpreting the kinetic details of resumption of DNA synthesis in irradiated cells. Furthermore, doses of UV comparable to the lethal dose derange the sequence of DNA replication in HCR bacteria. Normally each section of the bacterial chromosome replicates once in every generation; if a randomly selected region of the chromosome is labeled by giving a short pulse of radioactive precursor, this labeled portion will not replicate until a time corresponding to the doubling time of the culture has passed. This may be detected by switching to a density label after the pulse. Using this method, Hewitt and Billen (17) showed that in some bacteria the portion of the bacterial chromosome just replicated would replicate again immediately on resumption of DNA synthesis after UV; whereas, in unirradiated cells, the radioactive DNA did not become density labeled for a generation. This presumably means that UV irradiated cells can initiate new points of replication in some cells and that the resumption of DNA synthesis in irradiated bacteria is not a direct reversal of an ultraviolet-produced inhibition.

Doses of UV which are barely sufficient to stop DNA synthesis appear to make RNA and protein synthesis linear-in-time without reducing their rate. More UV decreases the rate of bulk RNA synthesis; this is primarily an effect on ribosomal RNA synthesis since most of the RNA in a bacterium is ribosomal. In experiments in which different types of RNA were separated using preparative velocity sedimentation (25) or chromatography (39), it was found that

23S ribosomal RNA synthesis was most sensitive to UV, 16S ribosomal RNA was less sensitive and transfer RNA and the rapidly labeled RNA fraction usually supposed to contain messenger RNA, least sensitive of all. UV doses of several hundred to several thousand ergs/mm<sup>2</sup> are required for suppression of RNA synthesis; these doses are several times larger than those required for an equivalent suppression of DNA synthesis or colony forming ability in HCR<sup>+</sup> cells. The appearance of gaps in the DNA would be a sufficient explanation for a decreasing rate of RNA synthesis.

The apparatus of protein synthesis, irradiated in vitro, seems insensitive to UV: the amino acid acceptor and transfer activity of transfer RNA (11, 43) ribosomes (43), the template function of synthetic messengers (13, 43); all require inactivation doses of UV several orders of magnitude larger than are biologically effective in inhibiting protein synthesis (17). The enzymatic activities of many proteins are also resistant (35), but the activity of enzymes which interact with nucleic acids or a purine or pyrimidine cofactor might be more sensitive. For these reasons we may tentatively view the inhibition of protein synthesis in vivo (17) after UV irradiation as a consequence of the suppression of RNA synthesis, rather than of some separate sensitivity of protein synthesis per se.

Where irradiation experiments are performed in the usual fashion, induced enzyme synthesis appears more sensitive than bulk protein synthesis, in fact, nearly as sensitive as is colony formation (1, 28). This effect appears to be a result of accumulation of catabolites in the irradiated cell; if cells are irradiated, then shifted to a nutrition-

ally poor medium before induction, production of  $\beta$ -galactosidase has the same UV sensitivity as gross protein synthesis (29).

Several thousands of ergs/mm<sup>2</sup> of germicidal UV are required to reduce the number of *E. coli* which will support  $\phi$ X reproduction by a factor of 10 (8). These doses abolish colony forming ability. The phage infecting such a culture find cells which have ceased RNA and DNA synthesis; the DNA of the cell contains several thousand pyrimidine dimers and would be expected to be extensively degraded in the period following irradiation, if the cell is HCR<sup>+</sup>. However, the oxidative metabolism and ability of such a cell to support protein synthesis should be only slightly affected, though synthesis of bacterial proteins would presumably have largely ceased.

The subject of this report is an unexpected property of the RF synthesized by unirradiated  $\phi$ X virus in such an irradiated cell.

## MATERIALS AND METHODS

### Media

TKB is tryptone-KCl broth (44).

SB is starvation buffer (9).

D-TGL is the defined medium described by Sinsheimer (40). It is used for preparation of D<sup>15</sup>N labeled  $\phi$ X. The only modifications in the above recipe were: the D<sub>2</sub>O (99.8 mole % D<sub>2</sub>O, Bio-Rad Labs, Richmond, California) was not distilled before use, and the pH was adjusted by use of 0.17 gm (Sigma) tris-HCl and 0.03 gm tris-base per 20 ml medium. This eliminates the addition of acid to the D TGL. Cells grown in this medium were aerated using air which had been passed through anhydrous CaCl<sub>2</sub>, then a bubbler tube containing pure D<sub>2</sub>O.

Media for the soft agar assay of whole virus were those of Sinsheimer (41), save that no NaOH was added to the bottom agar, and only 10 ml of bottom agar was used in each plate.

Media for the protoplast assay of infective DNA were those of Guthrie and Sinsheimer (14).

PPM is phage protective medium containing, per liter of deionized, distilled H<sub>2</sub>O: 0.1 gm of (Armour) bovine serum albumin, 0.04 gm disodium versenate, and 7.5 gm sodium tetraborate.

TWB is used for the storage of infective DNA. It contains 5 µg./ml (Armour, from their 30% sterile solution) bovine serum albumin, and 40 µg./ml disodium versenate in 0.05 M (Sigma, grade 121) tris, pH 8.1. Fractions of the CsCl equilibrium gradients, consisting of 1 ml TWB and several drops of the CsCl solution, retained their biological activity over a period of weeks.

### Bacteria

E. coli C<sub>N</sub> and E. coli C<sub>S</sub> have been described (44). They are HCR<sup>+</sup> (host cell reactivating, UV-resistant) and HCR<sup>-</sup> (non-reactivating, UV-sensitive) respectively, and are equally susceptible to  $\Phi$ X.

E. coli CR is a hybrid of E. coli CR 34 and E. coli C416 whose only requirement is thymine. It is susceptible to  $\Phi$ X.

E. coli K12 W6 is the usual host in the protoplast assay (14). It is resistant to  $\Phi$ X, performs HCR (HCR<sup>+</sup>) and was obtained from Dr. J. Weigle.

E. coli C<sub>1</sub> is susceptible to  $\gamma$ h, but not to wild type. It was selected from a lab strain of E. coli C<sub>N</sub> by Mr. C. A. Hutchison, III.

Virus

The wild type virus (WT) is that described by Sinsheimer (41).  $\gamma h$  is a double mutant, containing the temperature sensitive  $\gamma$  marker, and also  $h$ , which confers the ability to form plaques on E. coli  $C_1$ . It was constructed by Mr. C. A. Hutchison, III.

Preparation and Properties of  $D^{15}N^{32}P \ \Phi X$ 

A  $D^{15}N^{32}P$  lysate is prepared by infecting E. coli  $C_N$  as described by Sinsheimer (40). The resulting suspension of  $D^{15}N^{32}P \ \Phi X$  and bacterial debris are then subjected to a procedure resembling that of Hall and Sinsheimer (15) in order to purify the phage.

Specifically, E. coli  $C_N$  is adapted to D-TGL medium (40) by growing to saturation at  $37^\circ C$  five or six times, diluting 1/100 at each subculture. These cells are used to inoculate a large culture, containing a total of 20 ml D-TGL. When this culture reaches  $2-3 \times 10^8$  cells/ml, a multiplicity of 0.1/cell  $D^{15}N \ \Phi X$  is added, and simultaneously, 2 mc carrier free  $^{32}PO_4$  (Nuclear Consultants Corp., St. Louis, Mo.) which has been dried over KOH and  $CaCl_2$ , and redissolved in a small volume of D-TGL. Foaming, a sign of lysis, is usually first observed 90 minutes after infection. At the first indication of lysis, disodium EDTA dissolved in  $D_2O$  (Bio-Rad Labs, Richmond, Calif.) is added to a final concentration of  $1 \times 10^{-3} M$  EDTA in order to slow readsorption of virus to debris. After 30 minutes - 1 hour further aeration the lysate is chilled. At this point there are usually  $2 \times 10^{10}$  PFU/ml. For each 10 ml of culture, 2.35 ml 44% (w/v) polyethyleneglycol (Carbowax 6000, Union Carbide Chemicals), 0.77 ml 20% (w/v) dextran sulfate (Dextran sulfate 500, Pharmacia, Uppsala, Sweden) and 0.5 ml

5 M NaCl is then added and the tube inverted several times to mix the solutions (15). After 20 min centrifugation at 2°C and 120 x g, the lysate-polymer solution resolves into two clearly defined phases with a sharp line of bacterial debris at the interface. The bottom phase is very small, usually accounting for about 5% of the total volume. The disc of interfacial material obtained by centrifugation contains > 99% of the phage and usually has considerable structural strength; it may be fished out in a Pasteur pipet. It is transferred to 1 ml of PPM and shaken into suspension. After an hour at 4°C, the debris is separated by centrifugation and re-extracted with 1 ml PPM. The supernatants of these two extractions of the debris now contain the virus; they are combined and dialyzed against 2 changes of 100 x volume PPM. The dialyzed virus suspension is combined with 100 µg amino acid mixture (NBC), and 0.65 gm CsCl (Harshaw Chemical Co., optical grade) per ml and the resulting white flocculent polymer precipitate, which contains no phage, centrifuged off. The supernatant solution, which has a density of 1.42 grams/ml, is spun in the SW-39 rotor of the Beckman model L ultracentrifuge 40 hours at 4°C (27). The cellulose nitrate tube containing the centrifuged virus suspension is punctured at the bottom and the contents collected dropwise into 15-20 tubes containing PPM. Several broad, partly resolved bands of counts are found, but only one sharp peak of infective virus at a density of ca 1.47. Most of the plaque formers were usually in one fraction, which was dialyzed against 100 volumes PPM. This is the phage stock; it usually contained over  $10^{11}$  plaque-forming virus, about 1/3 the total in the lysate, and had a specific infectivity of 1 to  $2 \times 10^{-5}$  CPM/PFU. Another centri-

fugation did not usually change this figure.

In Figure 1 below it appears that all the  $^{32}\text{P}$  in a phenol extracted virus stock has the density characteristic of single-stranded  $\phi\text{X}$  DNA. Preparative sucrose gradient velocity sedimentation (26) of extracted DNA also yields only one band, which sediments as does a standard sample of the viral SS DNA. Therefore, all the phosphorus in a  $\text{D}^{15}\text{N}^{32}\text{P}$  stock is in  $\phi\text{X}$  DNA particles.

The newly prepared phage contain  $0.3$   $^{32}\text{P}$ /phage particle, which results in one disintegration/min/ $10^5$  particles. Assuming that the gas flow counter used here detects 40% of the  $^{32}\text{P}$  disintegrations, a preparation containing only phage, all of which were infective would have a specific activity of  $4 \times 10^{-6}$  CPM/PFU. Therefore, it is estimated that this method yields virus 20-40% of which are plaque-formers.

The biologically active fraction, as calculated above, corresponds roughly (see below) to the fraction of the radioactivity which remains adsorbed to infected cells, washed by centrifugation. Therefore, the inactive majority are so because they do not adsorb to susceptible cells, though the active fraction eclipses faster and to a greater extent in starved cells than does  $\text{H}^{14}\text{N}$  WT virus. Perhaps these last two observations may be taken to indicate that  $\text{D}^{15}\text{N}^{32}\text{P}$   $\phi\text{X}$  has an altered adsorption and eclipse mechanism which can either yield quick eclipse or an abortive adsorption. The adsorbed fraction is a reproducible characteristic of each preparation of  $\text{D}^{15}\text{N}^{32}\text{P}$   $\phi\text{X}$ .

However, there are inactive particles among those which adsorb also: even late in the phage latent period an extract of cell DNA shows, on centrifugation to equilibrium in a CsCl gradient, two bands of radioactivity. One is at the density position expected of

$D^{15}N^{32}P$  SS which has acquired a complementary  $H^{14}N$  strand, and the other band of radioactivity is at the position expected of free  $D^{15}N^{32}P$  SS (40). The 20-60% of the adsorbed counts still single-stranded late in phage growth are probably not destined to participate in intracellular growth at all, even though this DNA is as infective per  $^{32}P$  atom as is the bulk SS DNA of the phage stock. Luria-Latarjet curves on  $D^{15}N^{32}P$  virus-infected cells suggest a homogeneous sensitivity to ultraviolet light, unlike that which would be expected if a large fraction of the potential infective centers contained an undeveloped parental single strand. This single-stranded fraction does not appear if the crude lysate of an infected culture is banded without phenol treatment. They are therefore bound to protein; perhaps these virus get no farther than irreversible adsorption to the cell. The adsorbed inactive fraction was a reproducible characteristic of samples prepared together. The existence of these phage requires that the fraction of counts adsorbed be greater than the fraction of biologically active virus, but the estimate of the active fraction has not been determined precisely enough to quantitatively confirm this. Some  $^{15}N^{32}P$  phages adsorb, but remain single-stranded also (40), though the fraction of those which do seems smaller than observed here for  $D^{15}N^{32}P$  phage.

$D^{15}N^{32}P$   $\phi X$  show the same eclipse period, latent period, and burst size as unlabeled virus. They are distinguished only by their more efficient eclipse.

#### Infection and Treatment of Infected Cells

All experiments presented were performed using starved cells to synchronize the phage infection (9). A volume of prewarmed TKB



equal to that of the starved cells is added to initiate development, and times are reckoned from this moment. A 7 min complex has had access to the nutrient for 7 minutes. Using this system, 90-99% of the  $D^{15}N^{32}P$  phage added had entered the eclipse phase in the 5 minutes ( $37^{\circ}C$ ) allowed for adsorption of phage to the cells in SB. The first cells to mature progeny do so at 8-9 minutes; there is an average of one mature phage/infected cell at 11-13 minutes, the first cells lyse at 15-17 minutes and mean time of lysis is around 25 minutes. In a typical experiment on cells whose capacity was 0.06 that of unirradiated cells, the minimum eclipse period was 14 minutes, one phage per infective center had accumulated at 24 minutes, the first cells lysed at 26 minutes, and half the burst size of 10 had been released at 38 minutes.

Methods of DNA extraction have been detailed in Part II of this thesis. Briefly, complexes chilled after an appropriate period of development were washed, concentrated by centrifugation and lysed using lysozyme, versene and freezing. At this point, a sample was taken for measurement of adsorbed radioactivity. The clear lysate may be combined with CsCl and centrifuged or may then be extracted with phenol before centrifugation. Phenol is removed using ether and the ether removed by aeration or bubbling with  $N_2$ .

In the experiment presented in Figures 2 and 3, the nucleic acids in the lysate or the phenol extract were precipitated before centrifugation by adding 0.1 x volume 3 M NaAc, pH 5.5 and 1 x volume cold isopropanol. After several hours at  $4^{\circ}C$ , the precipitate was separated by centrifugation and then redissolved in 0.05 M tris,

pH 8.1 before CsCl centrifugation.

Eclipse of parental phage was followed and intracellular progeny measured by lysing small samples of the culture with lysozyme, versene, and freezing (42).

When irradiated cells were required, the starved bacteria were exposed to the output of two 8 watt germicidal lamps at a distance of 12". They were swirled in a 9 cm ice-cooled petri plate during irradiation, which typically required ca. 4 minutes exposure to UV to reduce the capacity to 1/10 that of untreated cells. By comparison to the data of Denhardt and Sinsheimer (8), this is equivalent to an effective dose of ca. 4500 ergs/mm<sup>2</sup>. Doses of this order reduced the number of colony forming cells to less than 10/ml.

#### Equilibrium Density Gradients

A 1.9 ml sample to be analyzed at pH 8 was combined with 0.1 ml of 4% disodium versenate and 5  $\mu$ l of a preparation of  $\gamma$ h single-stranded DNA. CsCl (Harshaw Chemical Co.) was added to give a density of 1.72 and the solution covered with paraffin oil. Centrifugation was performed using the SW-39 rotor of the Beckman model L ultracentrifuge. After an initial 6-8 hours at 37,000 RPM, 10<sup>o</sup>C, angular speed was lowered to 30,000 RPM and a further 40 hours of centrifugation allowed.

The cellulose nitrate tubes were punctured and the drops caught in small vials containing 1 ml TWB. Sometimes alternate samples were caught in vials and on aluminum planchets. The planchets are dried and counted directly in a Nuclear Chicago low background gas flow counter. If all drops had been collected into vials, a fraction of

each vial was spread on a planchet, dried and then counted. Sometimes, especially in rebanding a gradient fraction, it was necessary to work with very low levels of radioactivity. However the background of radioactivity unrelated to that in the sample was usually 1.4 - 1.8 counts per minute, so even a fraction of a count per minute could be detected. The low counts were reproducible, in multiple countings, and the regularity of the  $^{32}\text{P}$  distribution speaks for itself.

In alkaline gradient experiments, nalgene tubes (Nalge Co.) were used instead of cellulose nitrate and a special device, which bored into the bottom of the thick walled tube with a hollow needle, was used to collect drops. Details of the alkaline treatments are given in legends to the figures.

Biological assays of fractions were performed by making a 1/10 to 1/1000 dilution in 0.05 M tris pH 8.1. The higher dilutions were used when fractions were expected to contain E. coli DNA, which is an inhibitor of the protoplast assay. The infectivity profiles presented here have been assayed at least twice at different dilutions (in those regions where E. coli DNA occurs) to make sure that the samples were diluted sufficiently to avoid inhibition. One half ml of the diluted gradient fraction was combined with 0.2 ml of protoplast stock (14); otherwise the procedure of Guthrie and Sinsheimer was followed (14). Assays of gradients containing  $\gamma\text{H}$  SS as a density marker were carried out entirely at 30°C.

The hybrid  $\text{D}^{15}\text{N}-\text{H}^{14}\text{N}$  RF has the same density as  $\text{H}^{14}\text{N}$  SS (Figure 1). Therefore, if progeny SS synthesis has taken place in a culture, the infectivity in the hybrid region must be analyzed to distinguish that due to the first RF from that of progeny SS. This is done

in two ways:

A) Samples containing hybrid are pooled, diluted as for assay, and irradiated with germicidal UV light. SS is 8-fold more susceptible to UV inactivation than is RF (44). A mixture of SS and RF, therefore, is inactivated quickly at first, until only RF remains; then inactivation proceeds at the rate characteristic of RF. The more resistant limb of the inactivation curve is extrapolated to zero dose, and its extrapolate taken to be the amount of RF in the original sample.

B) It has been remarked elsewhere (44) that heterologous native DNA added to a sample containing infective  $\Phi$ X DNA will specifically inhibit the RF, leaving the infectivity of SS unaffected, or nearly so. Diluted gradient samples were therefore assayed several times in the absence and presence of 50  $\mu$ g./ml (Sigma) calf thymus DNA, as were standard samples of RF and SS. The amount of RF could then be computed from the apparent difference between the inhibited and uninhibited titers.

Infectivity is quoted in single-strand equivalents (SSE); that is, the number of SS required to yield the same phage titer as the unknown, if added to the same protoplasts. A standard sample of SS derived by phenol treatment of virus was assayed at several dilutions along with the gradients; the phage yield was always proportional to DNA concentration, which was calculated by assuming that an optical density of 1.0 at 260  $m\mu$  at room temperature in 0.05 M tris, pH 8.1 represented a concentration of  $1.2 \times 10^{13}$   $\Phi$ X SS/ml.

Unlabeled  $\gamma$ h SS was used as a marker in all gradient experiments. The yield of the protoplasts infected with a mixture of  $\gamma$ h and WT from such an experiment was plated on a mixture of 9 E. coli C<sub>1</sub>:

1 E. coli  $C_N$  and incubated at  $30^\circ\text{C}$  to assay  $\gamma\text{h}$  specifically. The small amount of  $C_N$  does not allow WT to form plaques, but does increase the plating efficiency of  $\gamma\text{h}$ . To assay only WT, the seed bacterium was E. coli  $C_N$  alone, and the plates were developed at  $40^\circ\text{C}$ . If plates are put in the incubator within 10 minutes, less than  $10^{-6}$  of the  $\gamma\text{h}$  present can form a plaque at  $40^\circ\text{C}$ .

## RESULTS

Figure 1 shows the distribution in a  $\text{CsCl}$  equilibrium gradient at pH 8 of radioactivity and infectivity from a phenol extracted  $D^{15}\text{N}^{32}\text{P}$  phage preparation. From left to right, one sees first the large peak of  $D^{15}\text{N}^{32}\text{P}$  WT SS infectivity and at the same position a matching peak of  $^{32}\text{P}$ ; there is no  $^{32}\text{P}$  at any other position. Next is the  $\gamma\text{h}$   $\text{H}^{14}\text{N}$  SS added as a density marker; this is also the position of the  $D^{15}\text{N}-\text{H}^{14}\text{N}$  RF hybrid formed after infection with these phage. To the light (right) side of the hybrid peak at the position marked by the labeled arrows are the replicas (light RF) of the parental SS-containing RF and practically superposed, the host's DNA (E. coli).

Figure 2 and 3 present the distribution of infectivity and radioactivity in the  $\text{CsCl}$  equilibrium gradient after infection of starved E. coli  $C_s$ . The eight sets of profiles characterize the state of the DNA from irradiated and unirradiated cells, after a short and long period of infection, extracted with and without phenol treatment. In order to succinctly present these data, only the section of each gradient containing the hybrid  $\Phi\text{X}$  RF, and the fully light RF is presented: the position of the unlabeled  $\gamma\text{h}$  SS infectivity peak, which marks the density of the normal hybrid, is indicated by an arrow.

Starved infected cells allowed five minutes in nutrient synthesis the parental-containing RF, and perhaps one replica (6, and Figure 2A, 2B, 3A, 3B). Despite generally delayed phage growth in irradiated cells (see Methods) this is as true of UV survivors as of unirradiated cells. The replicated RF was not recovered in the five minute samples if treatment with phenol was omitted (Figure 2A, 2B, and 3A, 3B). The unexpected observation is that while the irradiated cells contain a well-formed parental RF, as evidence by the appearance of parental  $^{32}\text{P}$  in a sharp band at hybrid density, it is less infective than that formed in normal cells.

Immediately after the 5' DNA sample was taken chloramphenicol (CAM) was added to prevent lysis and synthesis of SS (40); Figures 2C, 2D, 3C, 3D are then intracellular DNA from cells which have developed 15 minutes more in the presence of CAM.

There is internal evidence that the CAM has had its expected effects; the infectivity of the hybrid-progeny SS position is the same at 5 and 20 minutes, indicating that no SS has been made. The recovery of counts is equivalent, indicating none has been lost by lysis. (Figure 2A, B, C, D). At 20 minutes, there are clearly several replicas of the parental RF, as expected, but the infectivity of the material from the irradiated cells is still low. The CAM causes the death of irradiated cells which otherwise would have released phage, though unirradiated, infected cells are stable under these conditions. Thus, at 20 minutes 40% of the infected cells observed at 5' in this experiment had survived.  $C_{\theta}$  complexes made with irradiated cells die exponentially on addition of CAM, at a rate which increases with the

dose of UV to which the cells have been subjected. This effect may complicate the interpretation of the late time data in Figures 2 and 3, but the conservation of the hybrid infectivity, and the appearance of infective replicas, suggest that the CAM effect may be irrelevant in this context. Accordingly, it seems that in irradiated cells, a non-infective parental RF is formed and that even if allowed further time in the irradiated cell, it does not become infective or produce infective replicas. In this experiment, it appears that the infective RF formed in irradiated cells have replicated to nearly the same extent as those formed in unirradiated cells. This observation was not reproducible: usually replication was suppressed by UV irradiation. In Figures 2D and 3D it seems that the light RF is somewhat closer to the hybrid peak than it is in the unirradiated samples. Whether this is an indication of increased density or only an irregularity caused by the presence of irradiated coli DNA is not known. Similar results have been obtained using *E. coli*  $C_N$ , and in cells not inhibited with CAM and allowed more time before collection of the DNA. These experiments are summarized in Table 1. The suppression of infectivity in the experiment of Figures 2 and 3 is about the same as the suppression of capacity. It is as if those cells which could not support phage reproduction were so because of their inability to support the synthesis of a competent RF. This point is pursued in Figure 4, which presents the massed results of several similar experiments on  $HCR^+$  and  $HCR^-$  cells.

In section A of Figure 4 is shown the effect of irradiation on the retention of parental radioactivity; the radioactivity adsorbed to the host cell was measured using washed complexes harvested at various

stages of growth. Since growth curves show that eclipse of the parental phage is unaffected by irradiation of the host, it is concluded that phage DNA is stable in irradiated cells; it is not, for instance, extensively degraded and the phosphorus exchanged with the medium.

Section B, Figure 4, exhibits the ability of labeled parental virus to form RF in irradiated cells. The quantity plotted is the recovery of radioactivity in the RF band of a neutral density gradient, normalized to the recovery of counts as RF in unirradiated cells. It is concluded that irradiation of the host cell produces no bar to the SS to RF transition.

Finally in C, of Figure 4, is the relative infectivity of the hybrid RF formed in irradiated cells. That is, the infectivity per  $^{32}\text{P}$  atom was computed and normalized to that of the RF formed in the unirradiated control of each experiment. The data are somewhat scattered, but as shown below, the infectivity of RF made in UV-damaged cells is labile. It seems, in any case, that most cells whose capacity is damaged must be unable to serve for the synthesis of an infective parental RF.

Figures 5 and 6 show two experiments whose purpose was to extract the parental SS from the non-infective hybrid by alkaline denaturation and separation of strands. Specific infectivities measured at each stage of these experiments are shown in Table 1.

Figure 5 shows the density distribution of radioactivity and infectivity when the hybrid section of a neutral density gradient like those in Figures 2 and 3 was pooled and centrifuged to near-equilibrium in alkaline CsCl (42). RF, under these conditions denatures and increases



in density, separating into several partially resolved components roughly centered on the density of viral SS(4). Therefore, the band under the  $\gamma$ h marker DNA is progeny single-strand, and the products of denaturation of any fully light RF which might have overlapped the hybrid density. As expected, there is no parental material at this density. The peak evident at this position in Figure 5A, is smaller and less evident in Figures 5B and 5C. Though all three cultures contained  $< 0.005$  progeny phage per infected cell when DNA was harvested at 8 minutes, the unirradiated cells, in which the first mature progeny virus usually appears at 9 minutes, already contain some progeny SS; the lessened central peak in the patterns of 5B and C is presumably a reflection of the delayed growth in the UV-irradiated cells (see Methods).

There is a peak of parental material flanking the unlabeled SS DNA on either side. To the right (ca. 0.04 gm/ml lighter) side, there is parental DNA which is lighter than unlabeled SS. This would seem to be RF which either has not denatured or has been able to renature during centrifugation. It is known that a fraction of RF in which both single strands are closed rings is more resistant to denaturation than those in which one of the strands is open (4). That some RF should be native is nevertheless puzzling since the alkaline conditions used should have denatured all classes of unlabeled RF. In any case, the patterns are independent of the origin of the RF; that from irradiated and control cells behaves similarly.

The identity of the left-most (heaviest) band is less clear. Parental material at this position, 0.04 - 0.05 gm/ml, heavier than

unlabeled SS could be either released SS or the other components observed on denaturation of unlabeled RF. One might expect the order of the components of the band of denatured RF to be changed by the density label in the parental strand, and, in particular, that the released SS would move to a relatively heavier position than it usually occupies. However, no structure is observed in the radioactivity profile; probably the rather low resolution in this experiment has lumped together all the forms of denatured hybrid including released SS. Both bands of parental material from irradiated cells are at least as infective as those from unirradiated cells (Figure 5, Table 1). Therefore, it appears that alkali treatment has not only made infective that parental material which was in hybrid denatured by alkali but that also the portion of RF which is still native has regained infectivity.

Hybrid RF from E. coli C<sub>s</sub> was denatured by short exposure to high pH, then reneutralized and banded at pH 8; the result is presented in Figure 6. Again, there are two bands of parental material. The heavier is about 0.04 - 0.05 gm/ml heavier than the marker: this is the position of free D<sup>15</sup>N<sup>32</sup>P SS. The light band must be so because it has retained its complementary strand. But it is also heavier than the density marker  $\gamma$ h, whereas undenatured hybrid has, at neutral pH, the same density as unlabeled SS. The density difference ( $\sim 0.015$  gm/ml) between it and the  $\gamma$ h suggests that it may be RF which has denatured but not undergone strand separation.

As in the experiment of Figure 5, the products of alkaline treatment of the originally defective RF are at least as infective as are those of normal hybrid (Figure 6, Table 1). Indeed, the defective hybrid

seems to yield a consistently more infective double stranded molecule, though the extracted single strands all appear to have the same specific infectivity. (See Table 1, Columns 5 and 6.)

The DNA of these complexes was collected after ten minutes development, a time at which many unirradiated cells contain mature progeny virus and progeny SS DNA. This presumably accounts for the large peak of infectivity at the right side of the pattern in Figure 6A; this peak is absent in 6B and 6C; the irradiated cells have again apparently not begun the synthesis of infective single strands. The heavy SS recovered when the cell extract is first centrifuged (Column 4, Table 1) serves as an internal control on the procedures for determination of specific infectivity. Its infectivity is, in fact, relatively similar in all samples.

The identification of the lighter of the two parental bands in Figure 5 and 6 as double-stranded was confirmed by taking samples from each of the six peaks and irradiating with germicidal UV. All were predominantly resistant when assayed on K12 W6, as is purified RF (40). This presumably indicates the presence of a complementary strand (45), which is required for the HCR mechanism which makes RF UV-resistant. Pouwels and Jansz have previously reported that denatured RF is UV-resistant (31).

Figures 5 and 6 also show that the RF from irradiated cells is as susceptible to denaturation and strand separation in alkali as is normal hybrid RF.

## DISCUSSION

Starved, irradiated cells which lack the capacity to support the full course of phage growth, appear to also lack the ability to transform the viral DNA into a competent RF. Recovery of infective parental material from non-infective RF suggests that the defective RF may contain an unaltered strand of parental DNA. This would imply that the defective RF is unable to replicate. Denhardt and Sinsheimer (10) have shown that the RF containing the parental SS replaces its complementary strand in the course of normal replication. Therefore, if the defective RF could replicate, the parental material would acquire a normal complement and presumably, become infective and initiate a normal cycle of growth. While the non-infective RF might or might not replicate in the original irradiated host it seems reasonable to suppose that it would, if it could, in the bacterial protoplast used to assay its infectivity. However, the RF does not seem to become infective or yield infective replicas at any stage of growth in the irradiated host, and it is not infective to protoplasts. This line of argument suggests an RF in which the complementary strand synthesized in the UV-damaged cell has a flaw which precludes replication. Alkaline conditions would be supposed, then, to free the parental information from the effect of its incompetent complement.

On the other hand, the appearance, after alkali treatment, of infective material which has the density and UV-resistance characteristic of double stranded RF is more easily understood if the lesion itself is reversed by exposure to high pH. The observed susceptibility to HCR, presumably required for the UV resistance of RF, implies

the availability of the information in both strands of the alkali-treated RF (45). Having allowed that the lesion introduced by an irradiated host may be inherently susceptible to alkali, it could have affected either strand or both.

The RF which is made in the preirradiated bacterium appears to have the same density as that made in normal cells and, within the limits of analysis possible in the preparative equilibrium gradient, to be as uniform in density as normal hybrid. This suggests a complete, complementary structure for the defective RF. Taken together with the reactivation by alkaline treatment, this limits the alteration to relatively small changes in the structure of the RF. The observation (Figures 5, 6) of similar denaturation and strand separation in RF from both irradiated and unirradiated cells eliminates both adventitious alkali-stable cross-linking of the DNA strands and abnormal closure of the  $\phi$ X RF ring (4, 5, 24). Among the conceivable reversible lesions at this point, one numbers the attachment of a small protein or nucleic acid to the defective RF or a small derangement in secondary structure which might deny the defective RF access to normal replicative and transcriptive mechanisms.

The incorporation of UV-damaged nucleic acid precursor into the first RF is perhaps worth consideration; but the supply of usable nucleotide in starved cells is so minute that the first RF is not formed until nutrient is supplied (10). Most of the nucleotide material in the first RF probably arises, therefore, long after UV irradiation is over, and even such as may exist in starved cells will have to compete with the comparatively large amount of normal precursor which is formed or supplied when broth is added.

It is unexpected that loss of capacity in UV irradiated cells should be accompanied in some measure, by loss of ability to serve as host for synthesis of infective RF. This may indicate a role for a host structure which is not a material precursor in the synthesis of phage DNA. Denhardt and Sinsheimer (8) have examined the sensitivity of the capacity of starved E. coli  $C_N$  and  $C_S$  to UV of different wavelengths: the action spectrum of the exponential inactivation of capacity resembles a protein rich in tyrosine or tryptophan. The only protein which may be confidently presumed to interact with SS during the transition to RF is the polymerase whose intervention supplies the complementary strand. This enzyme seems to pre-exist in the host, since RF synthesis and replication occur in the presence of chloramphenicol (40). It is appealing to suppose that the large doses of UV required to inactivate bacterial capacity may alter the properties of the bacterial enzyme required by  $\phi X$  and therefore give rise to altered RF.

The observed occurrence of non-infective RF after similar irradiation of both  $HCR^+$  and  $HCR^-$  cells is consistent with the proteinoid action spectrum of capacity for  $\phi X$  growth (8) and with the observation that the presence of the HCR character has little effect on the UV-sensitivity of the capacity function (8).

Table 1

THE EFFECT OF ALKALI TREATMENT ON  $\phi$ X DNA FROM IRRADIATED CELLS

<u>E. coli</u> C <sub>N</sub>		MOI = 0.8		DNA Collected at 8 Min by Phenol Treatment	
		First, Neutral, Gradient, Cell DNA		Second, Alkaline, Gradient: Figure 5	
CAP	Specific Inf. Hybrid	SF $\ominus$	Sp. Inf. Heavy SS	Sp. Inf. of denatured hybrid and recovered SS	Sp. Inf. of alkali treated RF $\dagger$
1.0	$1.2 \times 10^6$ SSE/CPM	1.0	$1.4 \times 10^6$ SSE/CPM	$2.5 \times 10^6$ SSE/CPM	$1.1 \times 10^6$ SSE/CPM
0.45	$5 \times 10^5$	0.41	$1.6 \times 10^6$	$6.1 \times 10^6$	$5.4 \times 10^6$
0.19	$2.9 \times 10^5$	0.24	$1.3 \times 10^6$	$4.1 \times 10^6$	$4.4 \times 10^6$

<u>E. coli</u> C <sub>s</sub>		MOI = 0.6		DNA Collected at 10 Min by Phenol Treatment	
		First, Neutral, Gradient, Cell DNA		2nd, Neutral, Grad. on alkali treated DNA: Figure 6	
CAP	Specific Inf. Hybrid*	SF $\ominus$	Sp. Inf. Heavy SS	Sp. Inf. of alkali recovered SS	Sp. inf. of alkali treated RF
1.0	$1.3 \times 10^6$ SSE/CPM	1.0	$2.9 \times 10^6$ SSE/CPM	$5.7 \times 10^6$ SSE/CPM	$7.3 \times 10^6$
0.15	$5.4 \times 10^5$	0.41	$2.3 \times 10^6$	$4.9 \times 10^6$	$1.4 \times 10^7$
0.03	$3.1 \times 10^5$	0.24	$2.1 \times 10^6$	$5.8 \times 10^6$	$1.5 \times 10^7$

\* The values quoted here are averages of determinations by both the SS-specific protoplast assay and UV inactivation procedures described in Methods.

$\ominus$  In this column, the specific infectivities are normalized to those observed in the unirradiated cells.

+ This RF is denatured.

† This RF appeared native, though centrifuged in alkali (see text).

Table 1 (Continued)

The absolute values quoted here for the specific infectivity are higher than the theoretical value for fully active virus ( $2.5 \times 10^5$ ) because: A) phage were used in which some of the  $^{32}\text{P}$  had decayed. B) radioactivity is measured on samples which contain large amounts of CsCl, C) the SS standard used in these assays was later found to have contained many inactive molecules; this leads to an overestimate of the infectivity of unknowns compared to it.



Figure 1

Distribution of radioactivity and infectivity after CsCl equilibrium sedimentation of phenol-extracted  $D^{15}N^{32}P$  phage. Unlabeled  $\gamma$ h SS was added as a density marker.

——  $\Delta$  —— WT infectivity to protoplasts of E. coli K12 W6  
——  $\square$  ——  $\gamma$ h infectivity  
—— O —— radioactivity, measured with an end-window gas  
flow counter.

A total of sixty-six 2-drop fractions was collected alternately onto planchets and into vials. The arrows indicate the position of other species found on centrifugation of an extract of cells infected in an unlabeled growth medium. At the time of this experiment the  $D^{15}N^{32}P$  phage were 4  $^{32}P$  half-lives old.

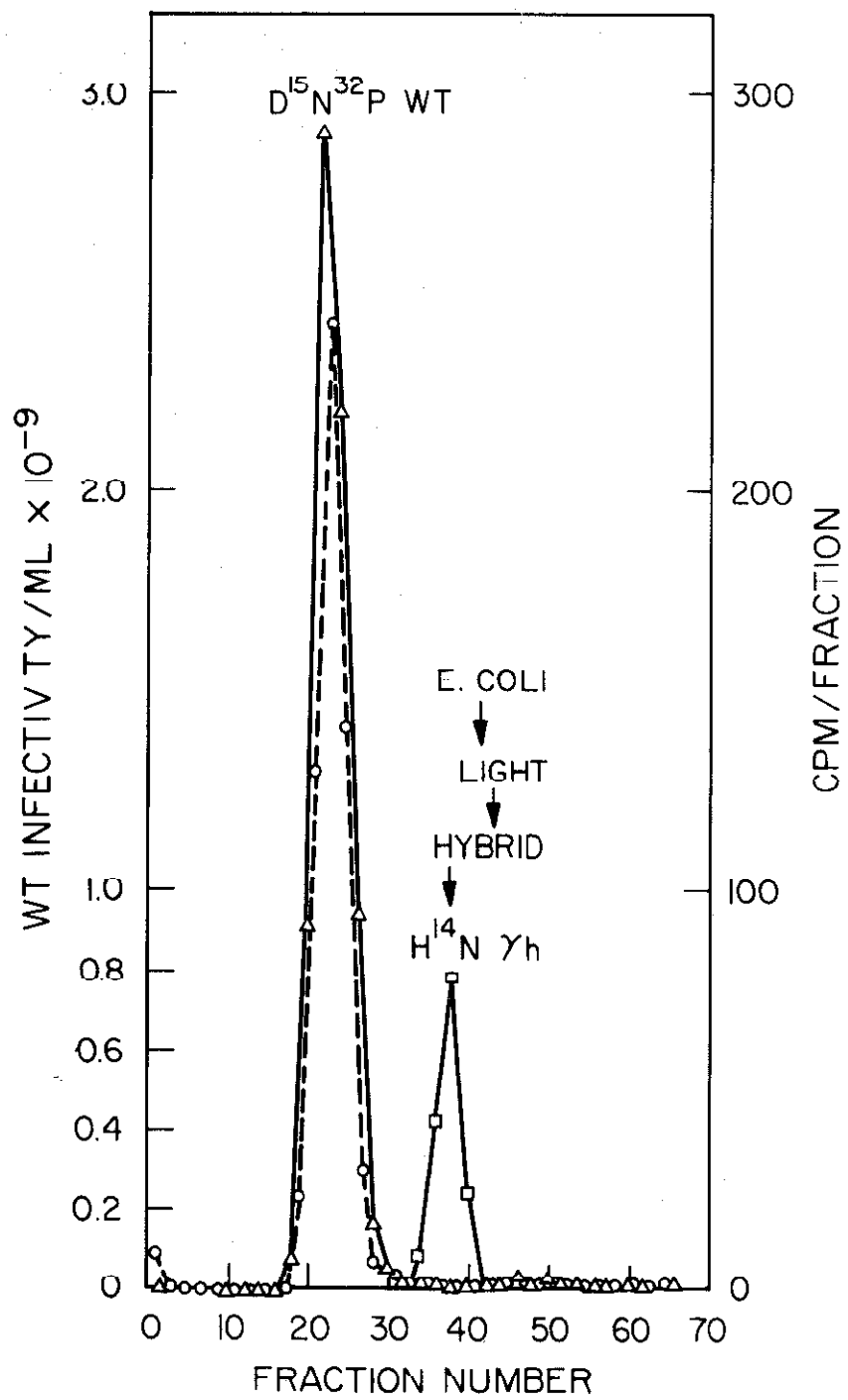


Figure 1

Figure 2

Phenol-treated extracts of starved E. coli C<sub>s</sub>, centrifuged to near-equilibrium in CsCl at pH 8.

- (A) 5 minutes growth at 37°C, unirradiated cells, A total of 84 2-drop fractions was collected.
- (B) 5 minutes growth, starved cells irradiated with germicidal UV, capacity survival = 0.19. A total of 90 2-drop fractions was collected.
- (C) 20 minutes growth, last 15 minutes in the presence of 30 µg/ml chloramphenicol, unirradiated cells, 108 2-drop fractions were collected.
- (D) 20 minutes growth, starved cells irradiated as in (B). 98 2-drop fractions were collected.

——— Δ ——— WT infectivity/ml in the fraction vial

----- O ----- Counts per minute per fraction

Fractions were alternately caught on planchets for the radioactivity assay and in vials for assay of biological activity. Multiplicity of infection was 0.25.

The few counts found at an atypical, light density are almost surely being physically trapped there by E. coli DNA. Degradation and reincorporation of the <sup>32</sup>P into DNA lacking density label seems unlikely, in general, because of the great excess of <sup>31</sup>PO<sub>4</sub> in the medium; and specifically, because the heavily irradiated bacteria of Figures 2B, 2D, 3B, 3D do not synthesize bacterial DNA. This "trapping" seems to be relieved somewhat when vigorous agitation is used to shear the E. coli DNA during extraction. This raises the problem of the identity

Figure 2 (Cont'd.)

and the density of the peak identified here as light RF which accumulates at the same position as the host DNA. However, when a fraction from the light RF region of Figure 2C was rebanded, a single sharp peak of infectivity at the expected position was observed.

These same samples were analyzed before phenol treatment. The results are in Figure 3.

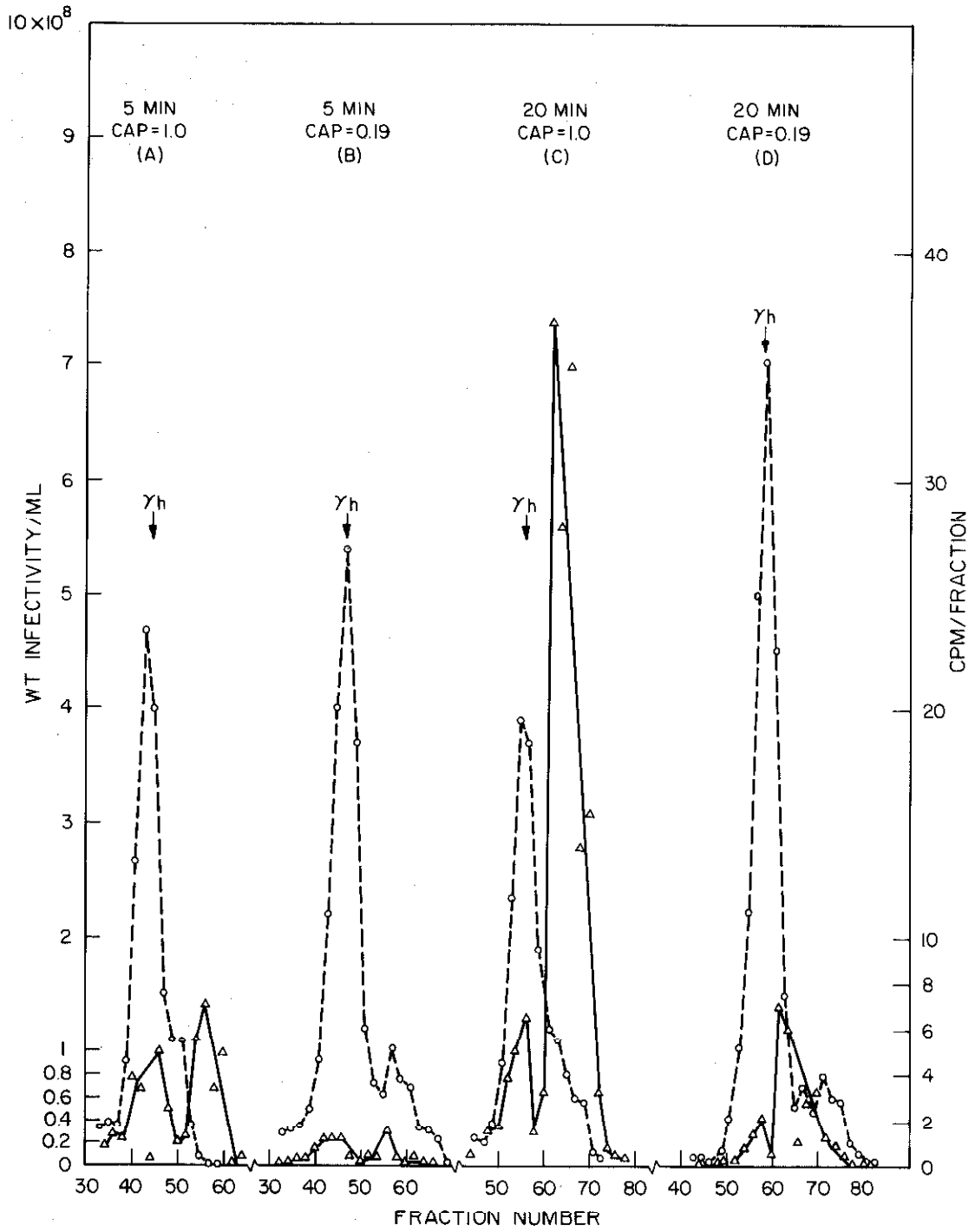


Figure 2

Figure 3

Radioactivity and infectivity from lysed E. coli C<sub>s</sub> banded in CsCl at pH 8 without phenol treatment.

- (A) 5 minutes growth at 37°C, unirradiated cells. 77 2-drop fractions were collected.
- (B) 5 minutes growth, cells pre-irradiated with germicidal UV to A capacity survival = 0.19. 106 2-drop fractions were collected.
- (C) Unirradiated cells, 20 minutes growth, the last fifteen minutes in the presence of 30 µg./ml chloramphenicol, 86 2-drop fractions were collected.
- (D) Pre-irradiated cells, 20 minutes growth, the last fifteen minutes in chloramphenicol. 92 2-drop fractions were collected.

Alternate fractions were caught on planchets and in vials. At the time of first infection, the D<sup>15</sup>N<sup>32</sup>P stock used was 2.5 <sup>32</sup>P half-lives old.

——— Δ ——— Wt infectivity/ml in the fraction vial  
 ——— ○ ——— Counts per minute per fraction

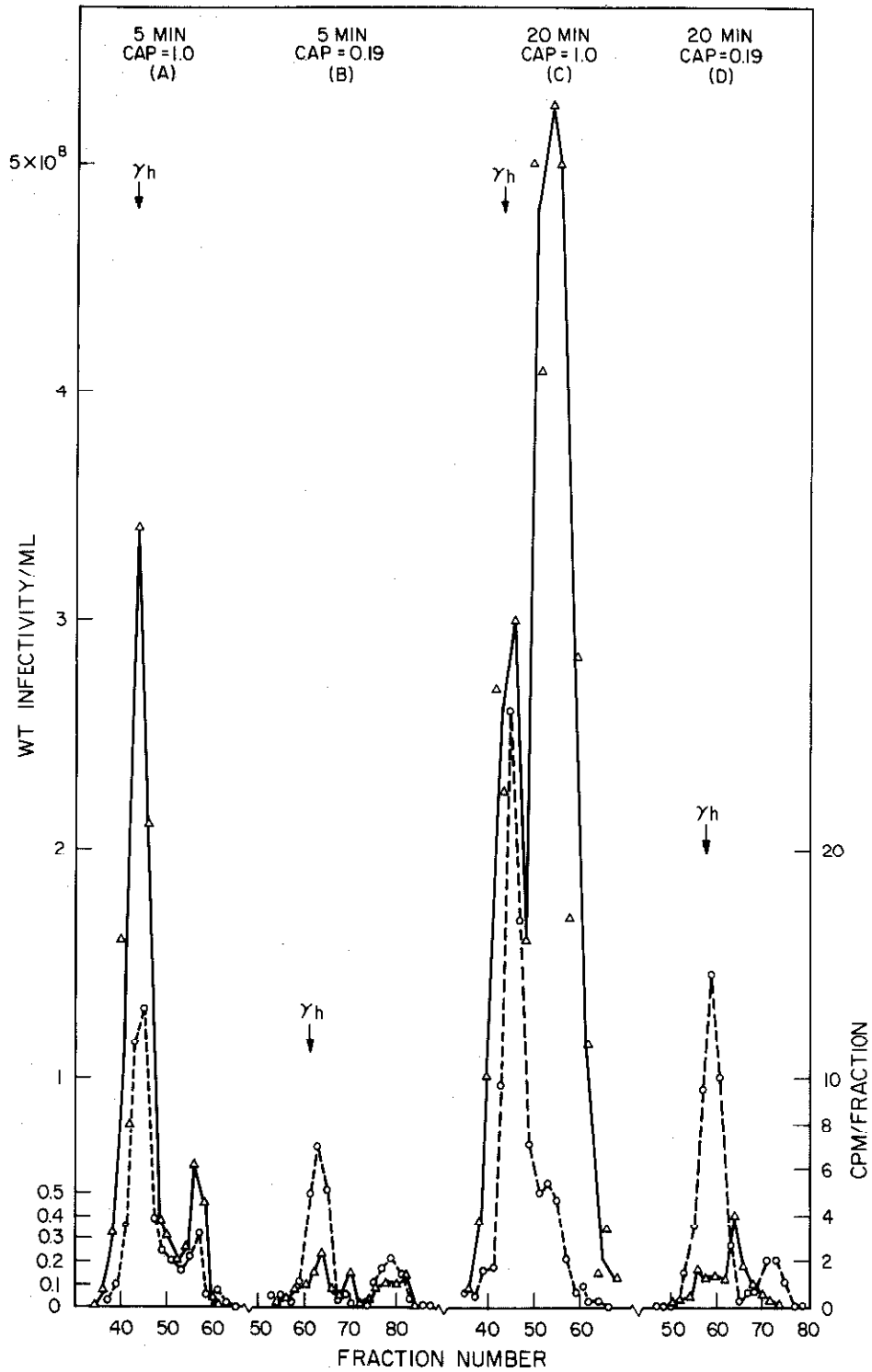


Figure 3

Figure 4

The effect of UV irradiation on the fate of viral DNA. The different symbols refer to different experiments. The 45° dashed line indicates, by definition, the response expected of a function which has a sensitivity to UV equal to that of capacity itself.

(A) Complexes of phage and irradiated host were harvested after various amounts of development and washed by centrifugation. A sample of the washed cells was assayed for radioactivity. The quantity plotted is radioactivity attached to cells/radioactivity attached to unirradiated cells.

(B) The parental  $^{32}\text{P}$  appearing at the position of RF in a neutral CsCl equilibrium gradient was normalized by that added to the gradient tube. The quantity plotted is this recovery as RF further normalized by the recovery of  $^{32}\text{P}$  as RF in the unirradiated control in each experiment.

(C) The specific infectivity of the material appearing at the hybrid RF position after infecting starved cells is here plotted after normalization to the specific infectivity of the hybrid synthesized in unirradiated cells.

- ▲- E. coli C<sub>s</sub>, MOI 0.25, No Phenol, 5 Min Development
- ▲- " " " " " " " " 20 " " (15 M' CAM)
- " " " " " " " " Phenol extraction, 5'
- " " " " " " " " " " 20' (15' CAM)

Partial data from the experiment represented by these first four symbols is presented in detail in Figures 2 and 3.



Figure 4 (continued)

- E. coli C<sub>s</sub>, MOI = 1.0, Phenol Extr., 8' Development
- " " " MOI = 0.6, " " 10' "
- ⊙- E. coli C<sub>n</sub>, MOI = 0.8 " " 8' "
- Δ- " " " MOI = 0.022, No Phenol, 12' "
- " " CR 34/C416 MOI = 0.4, DNA

extracted by treating complexes with 2.5% EDTA at 65°C after 15' development in the presence of 7.5 μg/ml chloramphenicol.

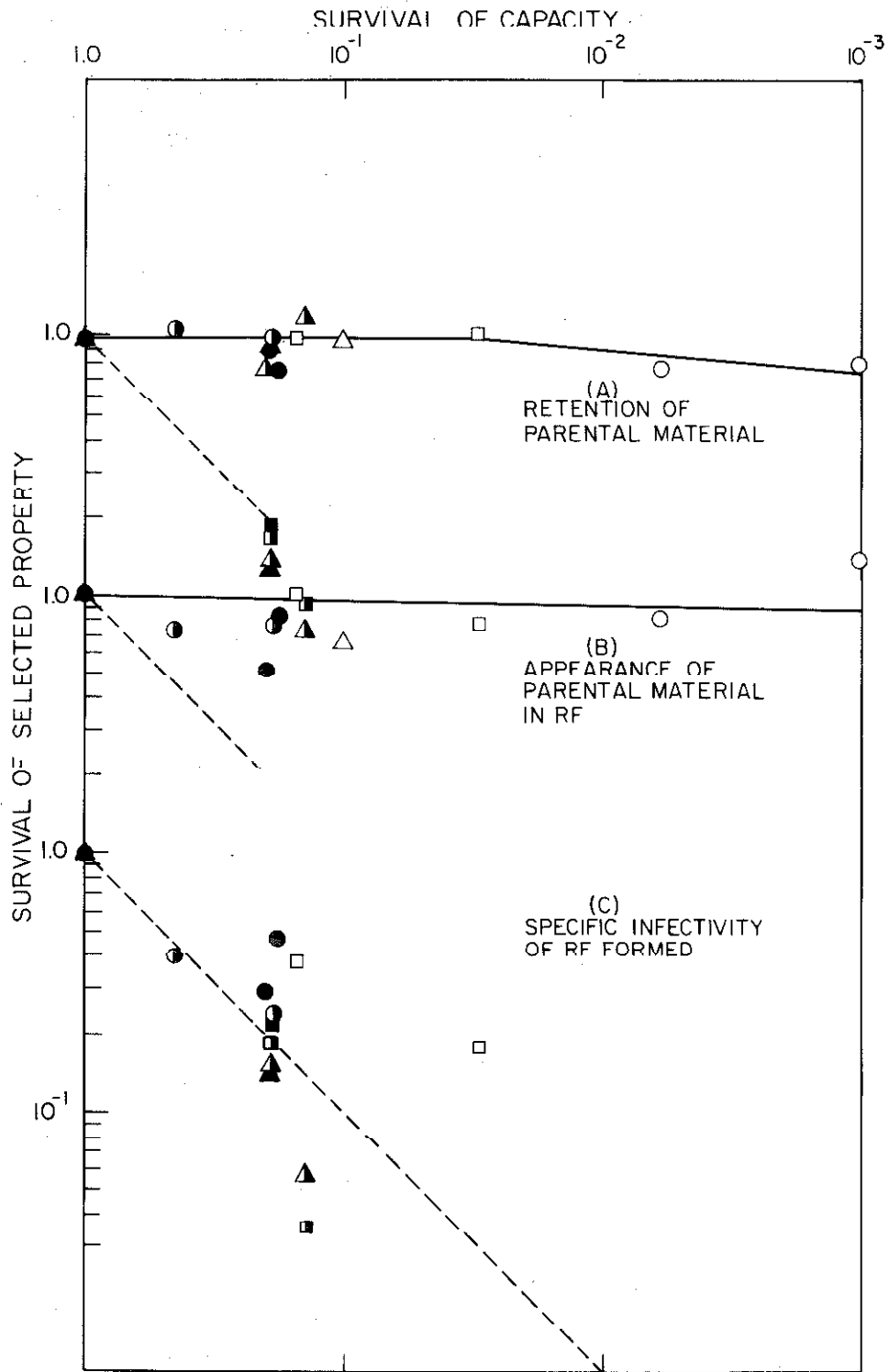


Figure 4

Figure 5

Distribution of infectivity and radioactivity on recentrifugation of hybrid RF in alkaline CsCl.

Two fractions from the peak of the hybrid region of a neutral CsCl gradient containing phenol-treated cell extract were combined with 1/5 their volume of 0.5 M,  $\text{Na}_3\text{PO}_4$  and allowed 10 minutes at room temperature before addition of CsCl to a final density of about 1.74 gm/ml. The apparent pH before addition of CsCl was 12.2, afterwards 12.7, as measured using the No. 40308, type E-2 Beckman electrode and model G pH meter.

The 40 hour centrifugation was carried out at 5°C and 37,000 RPM in the SW-39 rotor. Eight drops per fraction were collected into vials only; half of each 1 ml fraction was spread on a planchet for the radioactivity assay.

(A) Hybrid from unirradiated, starved E. coli  $C_N$  recentrifuged in alkali. A total of 31 fractions were collected.

(B) Recentrifuged hybrid from starved E. coli  $C_N$  pre-irradiated to a capacity survival of 0.45. A total of 35 fractions was collected.

(C) Recentrifuged hybrid from starved E. coli  $C_N$  pre-irradiated to a capacity survival of 0.19. A total of 31 fractions was collected.

Other data on this experiment are cited in Table 1. At first infection, the phage used for this experiment was one  $^{32}\text{P}$  half-life old.

-Δ- WT infectivity/ml in fraction vial

-O- Counts per minutes/ml, fraction vial

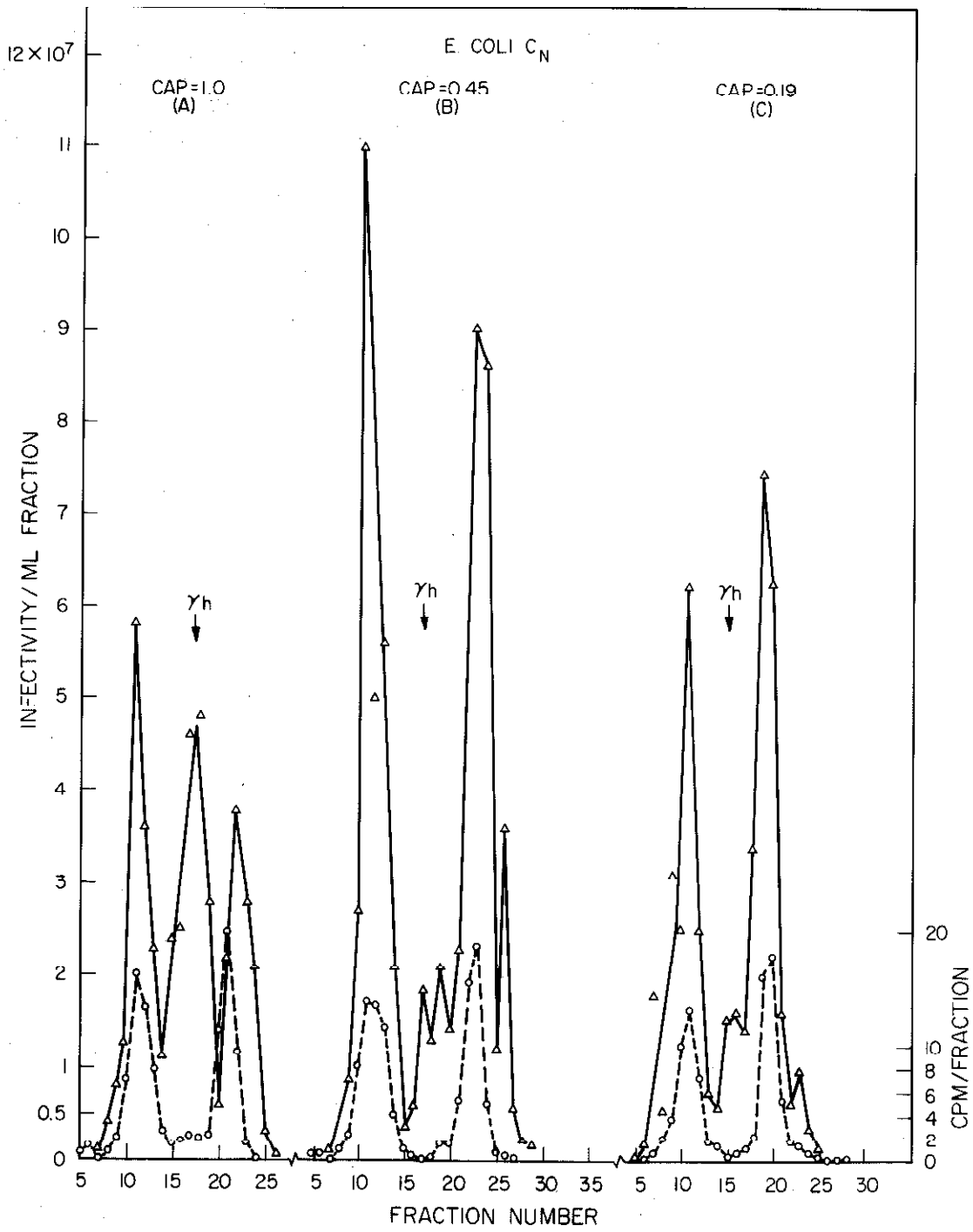


Figure 5

Figure 6

Distribution of infectivity and radioactivity on recentrifugation at pH 8 of alkali treated hybrid RF. Two fractions from the peak of the hybrid region of a neutral CsCl gradient containing a phenol extract of treated E. coli C<sub>s</sub> complexes were pooled and combined with 1/5 their volume of 1M NaOH. After 15 minutes at room temperature, 1/5 volume of 1 M HCl was added to bring the pH back to 8. Addition of alkali produced an apparent pH of 13.2, measured as described in the legend of Figure 5.

Centrifugation was carried out as for other neutral samples.

(A) Re-centrifuged hybrid region from a gradient containing DNA of unirradiated cells, 28 3-drop fractions were collected.

(B) Re-centrifuged hybrid region from a gradient containing DNA from irradiated E. coli C<sub>s</sub>; capacity survival 0.15. 28 3-drop fractions were collected.

(C) Re-centrifuged hybrid region from a gradient containing DNA from irradiated E. coli C<sub>s</sub>, capacity survival 0.03. 30 3-drop fractions were collected.

Two-thirds of each 1 ml. fraction was spread on plachets for the radioactivity assay. Other data on this experiment are cited in Table 1. The D<sup>15</sup>N<sup>32</sup>P phage used here were 3 <sup>32</sup>P half-lives old.

——— Δ ——— Wt. infectivity/ml in fraction vials.

——— O ——— Counts per minutes/ml in fraction vials

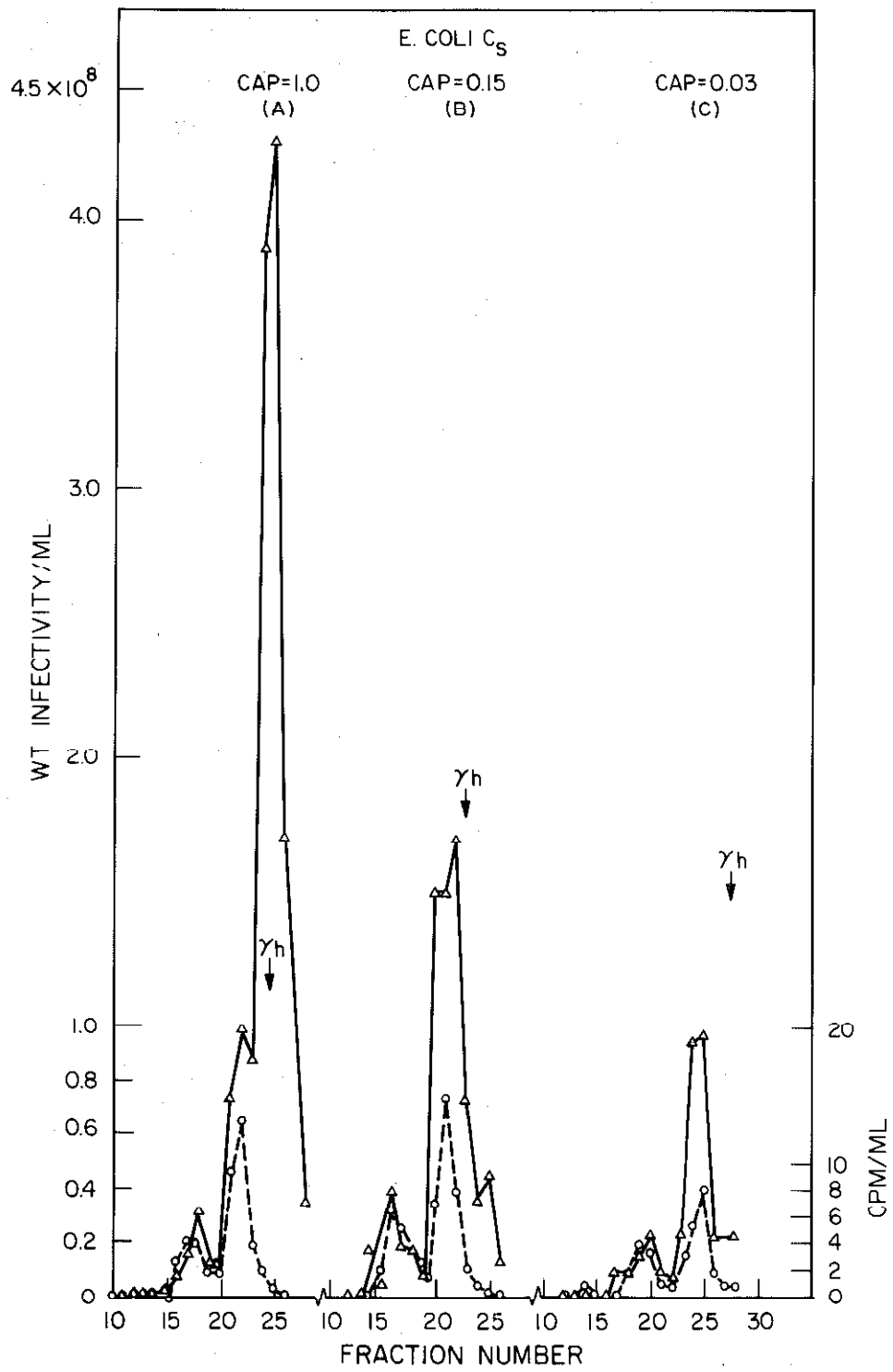


Figure 6

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