THE PROPERTIES OF X-RAY-INACTIVATED BACTERIOPHAGE[1]

I. Inactivation by Direct Effect

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The potentialities of inactive viruses as a tool in virus research have been shown by Luria and Dulbecco (1949) in their study on the interactions between several ultraviolet-inactivated bacteriophage particles adsorbed to the same bacteria. Their finding that active phage may be produced in bacteria infected with two or more inactive particles indicated that inactivation occurs as the result of partial damage that blocks a specific step in virus multiplication.

It is probable that inactivating agents other than ultraviolet light can cause partial damage. More interesting is the possibility that these agents will cause different types of damage, which will block virus reproduction at different stages. We might therefore be able to reconstruct the successive steps in host-virus interaction by studying the stages in the synthesis at which the multiplication of the inactive phage is blocked. To test these possibilities we have begun to study bacteriophage inactivated by X-rays.

The inactivation of bacteriophage by X-rays was first studied by Wollman and Lacassagne (1940), who found that the X-ray sensitivity of different phages suspended in broth increased with the particle size as determined by ultrafiltration and centrifugation. These and later experiments on X-ray inactivation have been analyzed from the viewpoint of the "target theory" of radiation action, according to which ionizing radiation acts by producing ionizations ("hits") in localized structures or targets, within the biological object itself. Various authors (Wollman, Holweck, and Luria, 1940; Luria and Exner, 1941; Lea and Salaman, 1946) calculated the target volumes ("sensitive volumes") from the inactivation rates of bacteriophage suspended in broth and found them to agree relatively well with the sizes of the actual particles.

Because of concern with the validity of the target theory, little attention has been paid to the analysis of the properties of the inactive phage. The present work represents an attempt to determine the stages in phage multiplication that are blocked by X-ray damage.

MATERIALS AND METHODS

Cultures: All experiments employed the coli phages T1-T7 and their common host *Escherichia coli* strain B (Demerec and Fano, 1945). Use has also been made

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of their h mutants (Luria, 1945) and r mutants (Hershey, 1946), as well as of bacterial mutants resistant to one or more phages, as indicators for one phage in the presence of another (Delbrück, 1946). Phage stocks were lysates in broth or in synthetic medium M-9. Purified phages were prepared by differential centrifugation, using the multispeed attachment of an International refrigerated centrifuge, followed by resuspension in the M-9 medium, solution A, with the addition of 10^{-3} M Mg^{++} to stabilize the phage activity (Adams, 1949).[3] The general methodology used in work with these phages has been summarized by Adams (1950).

Media: Synthetic medium M-9, solution A—KH_2PO_4, 3 g; $MgSO_4$, 0.2 g; NH_4Cl, 1 g; anhydrous Na_2HPO_2, 6 g, dissolved in 900 ml of distilled water. Solution B—4 g of glucose dissolved in 100 ml of distilled water. Solutions A and B are sterilized separately and mixed in a 9:1 ratio before use. The pH of the final solution is 7.0.

The nutrient broth contained 0.8 per cent Difco nutrient broth plus 0.5 per cent NaCl. The nutrient agar contained 1.1 per cent Difco agar in 0.8 per cent Difco nutrient broth plus 0.5 per cent NaCl. Unless otherwise indicated, all experiments were done at 37 C and the plates were incubated at the same temperature.

Irradiation techniques: Two sources of X-rays have been used. One was an X-ray machine at the Memorial Hospital, New York City, which was kindly placed at our disposal by Miss E. Focht. The source consisted of two tubes placed face to face 30 cm apart, operating under 180 kV peak and 25 mA. The beam was filtered only by the glass wall of the tubes. The intensity of radiation at a point equidistant between the anticathodes was 1,940 *r* per minute. The second source was the X-ray machine at Indiana University, consisting of one tube operating under 200 kV peak and 20 mA. The beam was filtered only by the glass wall of the tube. The intensity of radiation was estimated indirectly as follows: With the X-ray machine at Memorial Hospital, the inactivation dose (e^{-1} survival; Lea, 1946) for phage T2 was determined to be 40,000 *r*. With the tube at Indiana University 30 minutes' irradiation at a distance of 13 cm from the anticathode gave one inactivation dose for T2. The intensity at this distance was therefore taken as 1,330 *r* per minute.

The phage was exposed to the X-rays in a cylindrical lucite container. The thickness of the phage layer was usually less than 5 mm; hence adsorption by the liquid was for all purposes negligible.

Ultraviolet light was provided by a General Electric germicidal bulb, 15 watts, alimented through a Sola stabilizer. The beam contains mainly radiation of wave length 2537 A. At a distance of 50 cm, the flux is 7.5 erg mm^{-2} sec^{-1}. For the photoreactivation experiments, the light source was represented by two parallel fluorescent lamps, 40 watts each, at a distance of 20 cm from the exposed material.

[3] In all experiments reported in this paper, the concentration of Mg^{++} added to synthetic media was 10^{-3} M.

EXPERIMENTAL RESULTS

Influence of the Suspending Medium on the Inactivation of Bacteriophage

The rate of bacteriophage inactivation by X-rays depends on the medium in which the phage is suspended (Luria and Exner, 1941). Figure 1 shows that bacteriophage T2 is inactivated at a faster rate in M-9 synthetic medium than in nutrient broth. The inactivation of phage in synthetic medium is interpreted (Luria and Exner, 1941) as being largely an indirect effect caused by agents produced in the surrounding medium by ionizations outside the phage. When phage is suspended in broth, the inactivation is not due to indirect agents, since these are neutralized by "protective substances" present in broth. The chemical basis for the protective action has been recently analyzed by Dale *et al.* (1949) for the indirect X-ray inactivation of enzymes and by Latarjet and Ephrati (1948) on bacteriophage.

The initial inactivation rate in nutrient broth cannot be decreased by increasing the concentration of broth or by adding other protective substances. There exists, therefore, an X-ray effect on phage against which no protection is given by foreign substances in the medium. This "unprotectable" inactivation is considered as due to a direct effect of X-rays (Luria and Exner, 1941), that is, to acts of absorption of X-ray energy within the phage articles.

In our investigation of the biological properties of X-ray-inactivated phage we have analyzed phage inactivated under various conditions of the surrounding medium. This paper is concerned with the properties of phage inactivated in broth ("direct effect"). Later papers will deal with the properties of phage inactivated by the "indirect effects."

Biological Properties of Phage Inactivated by "Direct Effect"

Survival curves. X-ray inactivation of T2 suspended in nutrient broth follows the pattern observed earlier by Luria (1944) and Latarjet (1948). The exponential inactivation curve (figure 2) indicates that the inactivation process is due to a one-hit mechanism (Wollman, Holweck, and Luria, 1940), which we assume consists of the production of one successful ionization.

When the average number of hits per particle is r, the proportion of active to total phage is e^{-r}. The dose necessary to give on the average 1 hit per particle ($e^{-1} = 37$ per cent survival) has been called the "inactivation dose" (Lea, 1947). The dose can be expressed in multiples of the inactivation dose, these values giving the average "number of hits" per particle. For T2 the inactivation dose is 40,000 r. This value is unchanged if the phage is suspended in 5 per cent gelatin instead of nutrient broth, gelatin being a "protective substance" (Luria and Exner, 1941).

For survivals lower than 10^{-2}, a downward bend becomes noticeable in the inactivation curve, suggesting the presence of a second inactivating mechanism. This bend almost disappears if the broth concentration is doubled, suggesting that the extrainactivation is caused by toxic agents produced by the radiation in the surrounding medium.

The inactivation rate of phage mutants T2r and T2hr was found to be identical with that of wild type T2. T4 and T6 showed equal radiosensitivity as T2, in agreement with earlier observations of Luria (1944). Exponential inactivation was also found for T1 and T7, with the "inactivation dose" for both being

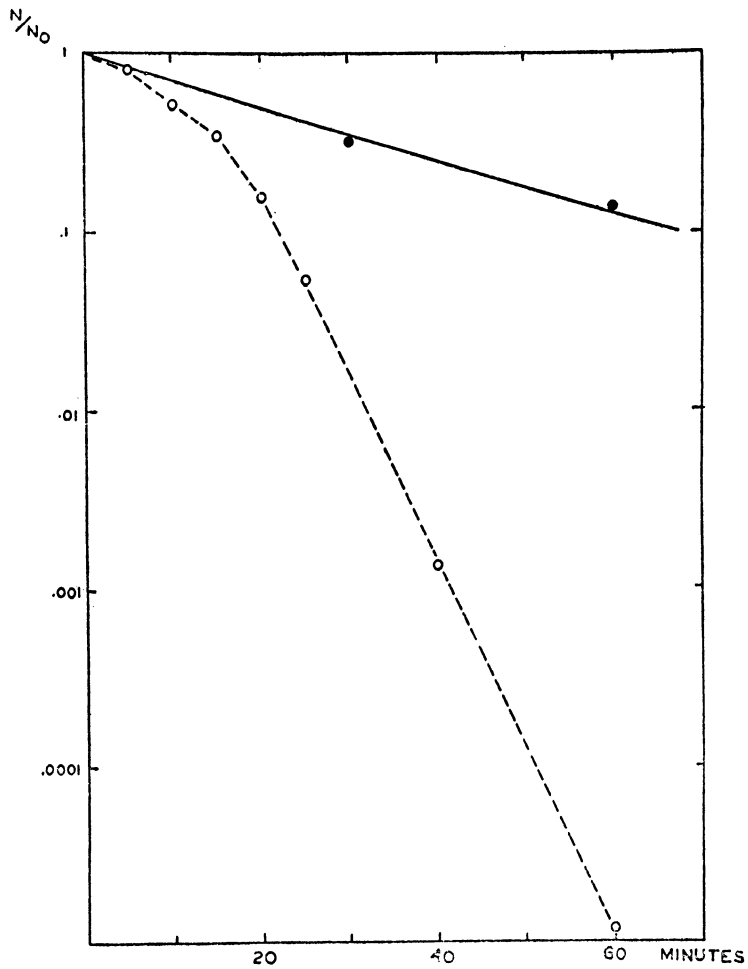

Figure 1. Inactivation of phage T2 by X-rays in different media. N/N_0 = fraction of active phage after irradiation. The X-ray dose is expressed in minutes of exposure. Solid line: Phage in nutrient broth. Broken line: Phage suspended in M-9, solution A.

approximately 85,000 *r*, confirming earlier experiments of Luria and Exner (1941) with T1 and of Luria (1944) with T7.

The probability that one ionization produces inactivation (ionic yield) can be determined by calculating how many ionizations occur within the phage particle for one successful hit. For these calculations it is necessary to know the particle size; from electron micrographs this has been estimated for phages T2,

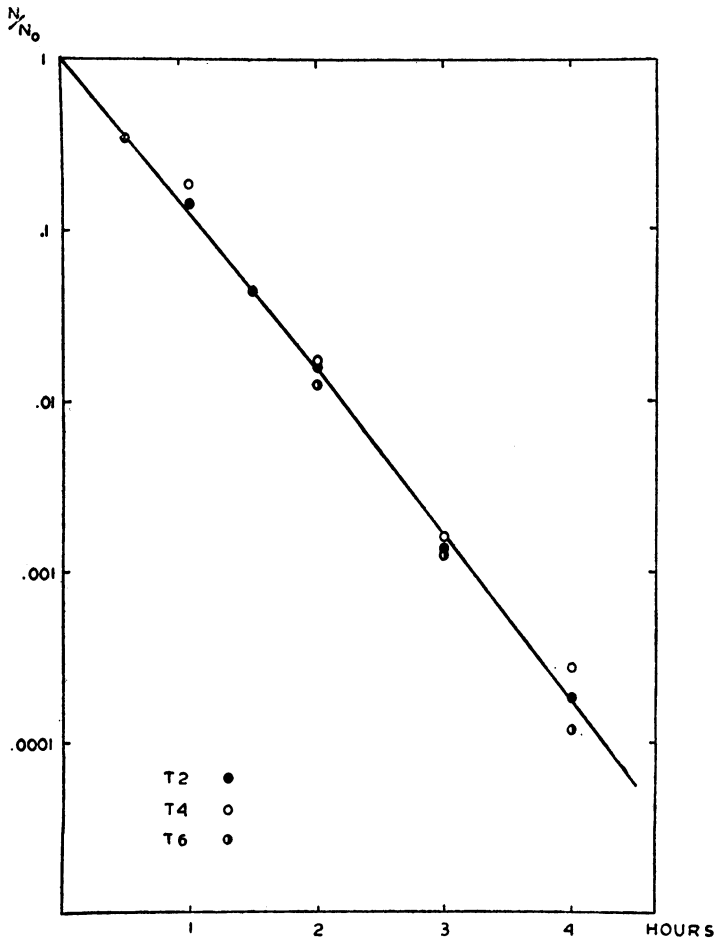

Figure 2. X-ray inactivation of various phages in nutrient broth. N/N_0 = fraction of active phage after irradiation. The X-ray dose is expressed in hours of exposure.

T4, and T6 to be 2.5×10^{-16} cm^3. Since the inactivation dose is 40,000 *r*, corresponding to 8×10^{16} ionizations per cm^3, the ionic yield is 0.05.[4]

[4] Earlier investigations have expressed ionization efficiency in terms of ionization clusters instead of individual ionizations. This was done because ionizations are not distributed at random but tend to occur on the average in groups of 3, so that when one ionization occurs within the phage, several others are also likely to occur. The use of clusters to express efficiency is justified only when a large fraction of the ionizations are effective. Then only one in a cluster is effective, the others being wasted. If, however, only 1/20 of the ionizations are effective, practically all of the ionizations in the cluster may have a chance of inactivating the phage and the probability of inactivation should be expressed in terms of single ionizations. This reasoning is valid only if all portions of the phage particle are equally radiosensitive; if the radiosensitivity is limited to a restricted region of the phage particle, then overlapping of "effective" ionizations should be taken into consideration.

The value 0.05 is considerably lower than the ones estimated for similar phages by earlier workers (see Lea, 1946), ranging between 1 in 3 to 1 in 6. This difference is due to the way in which the phage volume has been determined. We have used electron micrographic data, whereas earlier results were obtained using size determinations by ultrafiltration, which may be less accurate.

Adsorption. The adsorption of X-ray-inactivated phage (XRP) by bacteria cannot be measured directly by titrating the free particles after contact with bacteria, since the inactive phage cannot be titrated. We have determined whether inactive particles can be adsorbed by testing their ability to interfere with the adsorption of active phage by heat-killed bacteria (HKB), a method that permits the study of adsorption uncomplicated by virus multiplication.

Schlesinger (1932) showed that some phage strains are adsorbed by HKB, on which they cannot multiply, so that the adsorbed phage is lost and for all practical purposes inactivated. The adsorption of active phage on HKB is therefore measured as a loss of free phage. Schlesinger also showed that only a limited number of phage particles can be adsorbed by a bacterium before the available surface becomes saturated.

We prepared HKB by heating broth cultures of bacteria in the logarithmic phase of growth at 65 C for 1 hour. No viable bacteria remained, and all ability to support phage growth was lost. After heat treatment the HKB were stored at 4 C; the adsorptive capacity remained constant, permitting use of the same preparation of HKB over a period of months.

The ability of active phage to adsorb on HKB was utilized to test the adsorption of XRP in the following way: The adsorption of active phage T2 by HKB was first measured, and a logarithmic rate of adsorption was obtained, approximately equal to the adsorption rate of T2 on living bacteria in the logarithmic phase of growth. The adsorption rate of T6 upon HKB was then tested and found to be logarithmic and equal to that of T2.[5] Next, the number of T2 particles adsorbed, before saturation of the bacterial surface manifests itself as a flattening of the adsorption curve, was measured and found to be approximately 300. Bacteria thus saturated with active T2 were then mixed with T6, and the adsorption of T6 was measured. T2 and T6 can be differentiated easily by plating on the bacterial indicator strain B/2, on which only T6 grows, and on B/6, on which only T2 grows. No adsorption of T6 was detected, indicating that saturation of HKB with T2, besides preventing further T2 adsorption, also prevented T6 adsorption.

Finally, we replaced the active T2 with XRP T2, to see whether the inactive particles could absorb on the HKB and prevent T6 adsorption. The results of such an experiment, shown in table 1, indicate that XRP T2 is as effective a coating agent as active T2, thus demonstrating the adsorption ability of XRP T2.

Bacterial killing ability of XRP. Following X-ray irradiation in broth, a fraction of the inactive T2 particles is still able to kill sensitive bacteria. The ability

[5] Of the seven phages of the T group, only T1 is not adsorbed by HKB. T2, T4, and T6 are adsorbed by HKB approximately as fast as by living bacteria. T3 and T7 are adsorbed by HKB at a slower rate.

of one inactive particle to kill was demonstrated by mixing various amounts of XRP with a constant amount of bacteria. If one particle is sufficient to kill, the fraction of surviving bacteria in the different mixtures should be e^{-x}, where x is the mean number of killing particles adsorbed per bacterium. The values of e^{-x} in different mixtures from an experiment of this type are shown in table 2.

TABLE 1

Prevention of T6 adsorption by XRP T2

HKB at a concentration of 2×10^7 cells per ml. The XRP T2 had received 8 hits. Platings were done on B/2 so that only T6 would form plaques.

TUBE NO.	CONTENTS AFTER FIRST INPUT	SECOND INPUT 2 HOURS LATER	T6 COUNT AT VARIOUS TIME INTERVALS			
			0	20 min	40 min	60 min
1	Active phage T2 plus HKB (300 phage particles per bacterium)	T6 (0.005 phage particles per bacterium)	272	267	261	242
2	Active phage T2 plus HKB (30 phage particles per bacterium)	T6 (0.005 phage particles per bacterium)	253	138	76	44
3	XRP T2 plus HKB (300 phage particles per bacterium)	T6 (0.005 phage particles per bacterium)	295	266	251	265
4	HKB alone	T6 (0.005 phage particles per bacterium)	251	120	62	26

TABLE 2

Survival of bacteria following infection with different amounts of XRP T2

Various amounts of XRP T2 (4 hits) were added to different portions of a bacterial suspension. After 9 minutes the mixtures were diluted and plated for colony count.

TUBE NO.	(a) CONCENTRATION OF XRP RELATIVE TO TUBE NO. 1	SURVIVING BACTERIA (e^{-x})	(b) x	(c) (b)/(a)
1	1	0.62	0.48	0.480
2	2	0.35	1.05	0.525
3	4	0.12	2.12	0.530
4	6	0.054	2.95	0.492
5	12	0.0061	5.10	0.425

The values of x (column b) are proportional to the amount of inactive phage (column c). In this regard, XRP behaves like ultraviolet-inactivated phage (UVP; Luria and Delbrück, 1942), since in both cases a bacterium can be killed by the action of one inactive particle.

A marked difference, however, between XRP and UVP is that the ability of the inactive particles to kill disappears quite rapidly with increasing X-ray doses. In figure 3 the fraction of killing particles remaining after various doses is plotted as a function of the dose. It is seen that the killing ability of a par-

ticle, like the ability to reproduce, is destroyed exponentially with the dose. The ratio of the two slopes is 1:3, indicating that for every three inactivating hits there is one hit that destroys the killing ability.

No difference with regard to the loss of killing ability was found between T2 and its mutants T2r and T2hr. Likewise the ability of T2h to kill B/2 was destroyed by one-third of the inactivating hits. T4 and T6 also behaved like T2, with one-third of the inactivating hits destroying the ability to kill. The same was true for T2 irradiated in 5 per cent gelatin.

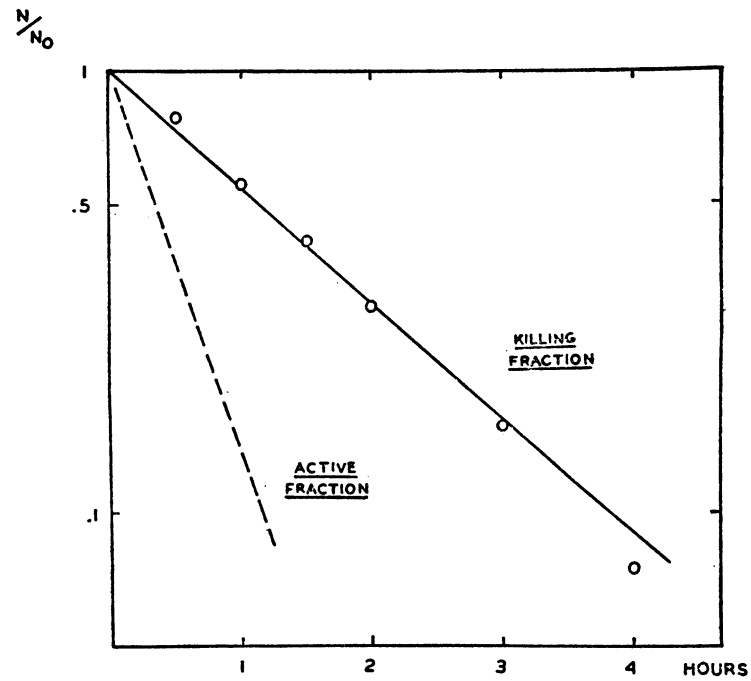

Figure 3. Loss of the bacterial killing ability of T2 as a function of X-ray dose. N/N_0 = fraction of phage able to kill bacteria after irradiation. The X-ray dose is expressed in hours of exposure. In order to compare the relative rates at which reproducing and killing abilities are lost the inactivation curve from figure 2 is also plotted (broken line). For this curve, N/N_0 = fraction of active phage after irradiation.

Since T2, T4, and T6 are genetically very similar (Delbrück, 1946), we wished to see how genetically unrelated phages behaved. The phages T1 and T7 were therefore investigated. Exponential loss of killing ability was again found, with approximately 1 out of 2.5 hits destroying killing ability. It thus appears probable that exponential loss of killing ability is a general characteristic of X-ray-irradiated phage.

We conclude, therefore, that the X-ray irradiation produces two distinct types of inactive phage, one that can kill bacteria and one that cannot. Since both types of particles can adsorb on sensitive bacteria, irradiation by X-rays provides a method for separating the ability to adsorb from the ability to kill bacteria.

Mutual exclusion of T1 by XRP T2. Delbrück and Luria (1942) found that, following infection of a bacterial cell with two unrelated phages, only one phage type can multiply. This "mutual exclusion" (Delbrück, 1945*b*) is well illustrated by the unrelated phages T1 and T2; if a bacterium is infected simultaneously by both phages, only T2 grows and T1 multiplication is completely suppressed; even the infecting T1 particles are lost. Luria and Delbrück (1942) also found that the T2 particle does not have to multiply in order to exclude T1; ultraviolet-inactivated T2, which had lost its ability to reproduce, was still able to exclude T1 completely.

TABLE 3

Exclusion of T1 by XRP T2

Culture of *E. coli* B in logarithmic phase = (B).

Four minutes at 37 C were allowed for adsorption of phage. An intermediate dilution was then made into anti-T1 serum and kept 4 minutes at 37 C. This serum treatment gives 7 per cent survival of the unadsorbed phage. All plaque counts below are corrected for this fraction. After further dilutions samples were plated on B/2 before lysis.

The multiplicity of infection for active T2 was determined from the survival of bacteria, assuming a Poisson distribution. Multiplicity of T2 = x.

The "expected" values are based on the assumption that T1 is liberated only by cells without any "killing" T2.

TUBE NO.	CONTENTS	T1 COUNT ON B/2					
		Exp. 74—x = 1.75		Exp. 77—x = 3.2		Exp. 82—x = 3.0	
		Found	Expected	Found	Expected	Found	Expected
1	Active T1 + (B)	330		332		390	
2	Active T1 + active T2 + (B)	66	58	15	14	52	20
3	Active T1 + inactive T2 (3 hits) + (B)	104	172	96	104	141	143
4	Active T1 + inactive T2 (6 hits) + (B)	206	234	160	205	160	240
5	Active T1 + inactive T2 (8 hits) + (B)	285	298	198	264	290	320

We have tested for this excluding ability in XRP. Bacteria were mixedly infected with active T1 and XRP T2. Anti-T1 serum was added to neutralize unadsorbed phage (Delbrück, 1945*a*), and the infected bacteria were plated before lysis on B/2. Under these conditions, T1 plaques are produced only by infected bacteria that liberate T1. Exclusion of T1 is detected by a decrease in T1 count below the value in a control without XRP. We found that exclusion of T1 by XRP T2 was less complete than with active T2, as shown in table 3. This means that some of the irradiated T2 particles had lost the ability to exclude T1. The fraction of particles able to exclude T1 decreases with increasing dose at a rate approximately equal to the rate at which the frequency of particles with bacterial killing ability decreases. This can be demonstrated by calculating the number of bacteria that are expected to liberate T1 if T1 mul-

tiplies only in bacteria that lack any "killing" T2 particle. Comparison of these expected values with the experimental data reveals a relatively close agreement (table 3). Although this agreement does not prove that the excluding ability is limited to those particles that kill, since the results are compatible with the assumption that the two properties are inactivated independently at equal rates, the latter interpretation seems unlikely.

Thus XRP consists of two classes of particles differing in interfering ability, the noninterfering particles being probably those that also have lost their bacterial killing ability.

Suppression of active T2 multiplication by mixed infection with XRP T2. When bacteria are mixedly infected with active phage and XRP, with low ratios of either phage to bacteria, no suppression of active phage reproduction results. This was shown by experiments in which bacteria were infected with one active particle, one inactive "killing" particle, and several inactive "nonkilling" particles, and were plated before burst to determine the number of bacteria that

TABLE 4

Liberation of T2r$^+$ by bacteria infected with active T2r$^+$ plus XRP T2r

Bacteria (B) were mixedly infected with T2r$^+$ active and XRP T2r and then plated before lysis. The number of plaques containing T2r$^+$ was determined and compared with a control containing bacteria infected with T2r$^+$ only.

TUBE CONTENTS	MULTIPLICITY OF ACTIVE T2r$^+$	MULTIPLICITY OF XRP T2r		T2r$^+$ COUNT
		"Killing"	"Non-killing"	
Active T2r$^+$ + (B)	0.06	—	—	198
Active T2r$^+$ + XRP (4 hits) + (B)	0.06	0.12	0.36	196
Active T2r$^+$ + XRP (6 hits) + (B)	0.06	0.50	2.50	206
Active T2r$^+$ + XRP (8 hits) + (B)	0.06	0.50	5.50	210

yield active phage. This number was compared with the number of bacteria that liberated phage in a control mixture with active phage only. The results of such an experiment, shown in table 4, indicate that one or several XRP particles, either "killing" or "nonkilling," do not suppress active phage production.

The result is different, however, when large numbers of inactive phage particles are adsorbed by each bacterium. Bacteria were mixed simultaneously with active T2r (ratio of phage to bacteria = 0.02) and with inactivated T2r$^+$ (ratios of phage to bacteria ranging from 1 to 100). The XRP had received 8 hits; under these conditions 7 per cent of the particles retained killing ability. Following adsorption, antiserum against T2 was added to neutralize unadsorbed phage (Delbrück, 1945*a*), samples were plated before lysis, and the number of bacteria that yielded T2r was determined; suppression of active T2r was detected by a decrease in the number of bacteria yielding T2r, the plaques of which are easily recognizable.

The results of a typical experiment are given in table 5 and indicate that the

degree of suppression increases with increase in the multiplicity of infection with XRP. The amount of suppression varies from 20 per cent when 10 XRP particles are adsorbed per bacterium to 94 per cent when 100 XRP particles are adsorbed.

The degree of suppression is not strongly influenced by the multiplicity of the active phage T2r; an increase from 0.9 to 9 did not markedly change the result. This suggests that the active and inactive particles are not competing with each other and that the suppression is due to an unspecific effect of a large excess of adsorbed phage. Suppression might, therefore, be related to the "lysis from without" phenomenon (Delbrück, 1940), in which adsorption of a large number of phage particles per bacterium causes prompt lysis of bacteria without permitting phage growth.

To test this possibility, XRP T2 was mixed with bacteria at a ratio of phage to bacteria of 200. The mixture was incubated at 37 C, and the degree of bacterial turbidity was observed. Within 5 minutes the turbidity decreased markedly,

TABLE 5

Suppression of active T2r by mixed infection with high multiplicities of XRP T2r+

The XRP T2r+ had received 8 hits. Ten minutes of adsorption on bacteria (B) give 80 per cent adsorption of the active phage. Adsorption of the XRP was assumed equal to that of the active. Following adsorption, an intermediate dilution was made into anti-T2 serum to neutralize unadsorbed phage. Platings were made before lysis and the T2r plaques were counted.

TUBE CONTENTS	MULTIPLICITY OF ACTIVE T2r	MULTIPLICITY OF XRP T2r+		T2r COUNT	SUPPRESSION OF T2r, %
		"Killing"	"Non-killing"		
Active T2r + (B)	0.02	—	—	260	0
Active T2r + XRP T2r+ (1:10^2) + (B)	0.02	0.07	0.93	259	0
Active T2r + XRP T2r+ (1:10) + (B)	0.02	0.7	9.3	190	23
Active T2r + XRP T2r+ (1:2) + (B)	0.02	3.5	46.5	54	79
Active T2r + XRP T2r+ pure + (B)	0.02	7.0	93	16	94

indicating that the bacteria were being lysed; by 10 minutes the turbidity was reduced to that of the suspending broth. Surprisingly, XRP particles are much more effective than active phage in causing this type of lysis. Over 3 to 4 times as many active particles are necessary to produce a comparable amount of lysis.

At lower multiplicities of XRP the bacteria do not lyse immediately; instead, a number of them assume a spherical shape, detectable by microscopic observation. If a 20-fold excess of XRP is added to bacteria, the following picture is observed: The cells retain their normal shape for about 5 minutes; then some begin to assume a spherical shape, approximately 25 per cent of the cells being rounded at 20 minutes. Longer observations indicated no increase in the proportion of swollen cells, lysis of which then occurs gradually.

A 25 per cent fraction roughly corresponds to the fraction of bacteria in which active phage production by the amount of XRP used in this experiment would be suppressed. These experiments thus suggest that the suppression of active

phage reproduction by XRP is due to lysis from without of some of those bacteria that adsorb large numbers of inactive phage.

The inactive samples used in these experiments contained a small fraction (7 per cent) of phage particles that retained the ability to kill bacteria by single infection, and it was possible that the suppression was caused either by the adsorption of a large number of particles, both "killing" and "nonkilling," or by the adsorption of a small number of "killing" particles. It was shown above, however, (see table 4) that one "killing" particle does not suppress reproduction of active phage of the same type. Since with a large number of "nonkilling" particles some suppression occurs even when the multiplicity of "killing" particles is less than one (table 5), it is likely that suppression can be caused by "nonkilling" particles.

In summary, infection of a bacterial cell with 10 or more XRP particles can cause "lysis from without." If a bacterium is infected with both active and inactive particles, "lysis from without" results in the suppression of active phage production. Following adsorption of a large number of XRP particles, the bacteria swell, assume spherical shapes, and lyse. The time interval before lysis is a function of the number of adsorbed particles, decreasing to less than 5 minutes with a multiplicity of 200. The ability to lyse bacteria from without is not destroyed by an X-ray dose sufficient to destroy the "killing" ability.

Cytological observations on bacteria infected with XRP. Using preparations stained by the HCl-Giemsa technique (Robinow, 1944) to study "chromatinic" material, Luria and Human (1950) have investigated the cytology of bacteria infected with UVP. They observed bacteria infected with phage T2 that had received heavy UV doses, so that little multiplicity reactivation (Luria, 1947) occurred. Following infection, the "nuclear bodies" break down and the chromatinic material becomes distributed throughout the bacterial cell. At the same time the cells become swollen, though they retain their normal rod shape. It was suggested that the inactive phage killed the bacteria by destroying their genetic apparatus.

The cytology of bacteria infected with XRP was studied in collaboration with Mrs. Mary L. Human. Bacteria were infected with XRP T2, and, after various incubation times, stained for "chromatinic" material. A multiplicity of 2 "killing" particles was employed, so that the majority (85 per cent) of bacteria would be killed. Since the inactive phage had received 8 hits, very few bacteria would liberate active phage either because of multiplicity reactivation (see below) or of residual actual phage.

Within five minutes after infection, the nuclear bodies began to lose their clear outline, and by 20 minutes the chromatinic material was distributed throughout the cell. The intensity of the chromatinic staining gradually decreased, and the cells became markedly swollen in appearance. Thus the cytological picture of bacteria killed by low multiplicities of XRP is similar to that of bacteria killed by UVP.

Although a multiplicity of two "killing" particles was used, the total number of particles adsorbed per bacterium, both "killing" and "nonkilling," was 25.

Some of the cells should, therefore, be "lysed from without." This expectation was confirmed by finding a second type of infected bacteria in addition to the type described above. These cells are easily distinguished by their spherical shape in contrast to the normal rod shape of the other infected cells. Lysis from without occurs without phage liberation. It was of interest, therefore, to find that these cells, though they become spherical in shape, still contain distinct "nuclear bodies" 20 minutes after infection. This observation supports the hypothesis of Delbrück (1940) that "lysis from without" is basically different from normal lysis.

Lysis inhibition by XRP. Phages T2, T4, and T6 differ from their r mutants in that the latter produce rapid lysis of turbid bacterial cultures, whereas the wild type (r^+) produces very delayed lysis. Doermann (1948) has shown that this lysis inhibition is caused by the phage particles themselves. The r^+ phages liberated from the first lysed bacteria are adsorbed by the remaining bacteria and inhibit their lysis. One r^+ particle added as early as 3 minutes following primary infection by r^+ can cause lysis inhibition. $T2r^+$, $T4r^+$, and $T6r^+$ can cross-inhibit lysis; for instance, following primary infection by $T6r^+$, a secondary infection with $T2r^+$ causes lysis inhibition. The r mutants do not inhibit lysis by second infection.

We wished to see whether XRP that had lost the ability to kill bacteria could still inhibit lysis. If so, this would dissociate lysis inhibition from the phage mechanisms directly connected with synthesis of new phage.

Bacteria were mixed with active T6 at a low ratio of phage to bacteria, so that single infection would result. After 3 minutes had been allowed for adsorption, the mixture was diluted to stop further adsorption. A second infection with XRP T2 (12 hits) was made 4 minutes later. The multiplicity of the secondary infection was 5, so that less than 1 per cent of the bacteria remained uninfected. Since only 2 per cent of the XRP retained "killing" ability, less than 10 per cent of the bacteria were infected with "killing" T2 particles. Five minutes were allowed for the second infection, after which the mixtures were again diluted to stop adsorption. At various intervals aliquots were plated on B/2, to obtain a one-step growth curve of T6 (Ellis and Delbrück, 1939).

This curve was compared with a curve obtained when the second infection was with active T2, and with a curve obtained with primary infection with T6 only. Two experiments showed that T6 liberation from bacteria also infected with XRP T2 was delayed 8 minutes beyond the normal latent period of T6 (26 minutes), and that this delay was equal to that produced by active T2. We conclude, therefore, that XRP T2 can inhibit lysis as effectively as active phage T2.

Thus, phage particles do not need the ability to reproduce in order to inhibit lysis; this was confirmed by the finding that UVP also can inhibit lysis. Moreover, the "killing" ability is apparently not necessary in order to inhibit lysis; the ability to adsorb is probably the only requirement.

Photoreactivation (PHTR). Dulbecco (1949*b*, 1950) observed reactivation of UVP by exposure to visible light in the presence of bacterial cells (photoreactiva-

tion = PHTR). His observation represented an extension of the original finding of Kelner (1949*a*,*b*) that visible light could reactivate spores of actinomycetes and cells of *Escherichia coli* killed by ultraviolet light. We have tested whether PHTR of X-ray damage occurs by exposing XRP to visible light. Since Dulbecco (1950) has shown that the UVP must be adsorbed to bacteria for PHTR to occur, we have applied the photoreactivating light to XRP in the presence of bacteria. XRP was plated with bacteria, and the plates were immediately incubated under fluorescent light at room temperature. The dose of visible light was sufficient to cause maximum PHTR of UVP (Dulbecco, 1950).

Table 6 summarizes experiments with phage T2, which definitely show the existence of some PHTR of XRP. In contrast to UVP, the amount of PHTR is small; in the dark, there remains an inactivation corresponding to 7.6 hits with an X-ray dose that gives 8 hits after maximum PHTR. As a result, the difference between plaque counts before and after PHTR is very small, necessitating many repetitions of each experiment to demonstrate PHTR beyond doubt. Our data are still too incomplete to determine the ratio of the damage in maximum light to the damage in the dark.

Experiments to determine the influence of variables such as the amount and wave length of photoreactivating light have not been attempted, because of the very small amount of PHTR. Storage of inactive phage for weeks at 4 C has no effect on its susceptibility to photoreactivation. XRP T4 and T6 are also capable of PHTR, with T4 showing about half as much PHTR as T2, whereas T6 slightly exceeds T2

Since PHTR of XRP is so small and can be detected only under optimum experimental conditions, assays of XRP done under ordinary laboratory light are indistinguishable from those done in the absence of photoreactivating light. It has therefore been possible to do experiments with XRP without taking special precautions to exclude visible light.

In summary, we can say that PHTR of XRP exists but is very small in amount. Concerning the difference between X-rays and ultraviolet light, we should not forget that approximately one-half of the energy of X-rays is dissipated in the form of excitations (similar to those produced by ultraviolet) as opposed to ionizations. It is therefore possible that the small amount of photoreactivable damage in XRP corresponds to damage similar to that produced by ultraviolet light.

Multiplicity reactivation. In 1947, Luria reported that assays of UVP are dependent on the concentration of the inactive phage when first mixed with bacteria. Bacteria were mixed with various concentrations of UVP, and after a few minutes the mixtures were diluted enough to bring the total dilution of the irradiated phage samples to a constant value, after which aliquots were plated for phage count. Since plating was done before lysis, each plaque represented an infected bacterium liberating active phage. The plaque counts were found to be higher when bacteria had first been mixed with more concentrated phage. Luria showed that this increase in plaque count is due to the production of active phage in a fraction of the bacteria infected with two or more inactive particles (multiplicity reactivation).

We have repeated this experiment using XRP instead of UVP. XRP T4 at various dilutions was mixed with bacteria and allowed to adsorb. Before lysis, the samples were diluted and plated with an excess of sensitive bacteria. Following incubation, plaque counts were made to determine the number of bacteria that liberated active phage. The results shown in table 7 indicate that the number of

TABLE 6

Photoreactivation of XRP T2

All experiments were performed with the same irradiated samples, whose residual titers remained constant throughout the series of experiments. After plating, all plates were incubated for 15 hours at room temperature, some in light, some in darkness.

NO. OF X-RAY "HITS" FROM PLAQUE COUNTS IN DARKNESS	EXPERIMENT NO.	PLAQUE COUNT IN LIGHT (a)	PLAQUE COUNT IN DARKNESS (b)	(a)/(b)
0	64	260	246	1.06
	68	391	441	0.87
	70	328	339	0.97
	76	2,266	2,279	0.99
	Σ	3,245	3,305	0.98
3	64	277	285	0.97
	68	313	280	1.12
	70	246	243	1.01
	81	1,488	1,419	1.05
	Σ	2,324	2,227	1.04
6	64	310	241	1.29
	68	339	269	1.26
	70	301	214	1.41
	79	1,661	1,328	1.25
	84	1,160	908	1.28
	Σ	3,771	2,960	1.28
8	64	278	196	1.48
	68	336	254	1.32
	70	265	202	1.31
	73	2,131	1,574	1.35
	83	1,520	1,097	1.39
	Σ	4,540	3,323	1.37

bacteria yielding active phage is greater than the number of bacteria infected with the residual active phage. Experiments with T2 and T6 gave results similar to those with T4. This indicates that XRP, like UVP, can be reactivated by multiple infection.

In order to compare results with those obtained with UVP, we should know how many bacteria contain two or more inactive particles that can be re-

activated by multiple infection. Evidence is not yet available, however, as to whether the XRP particles that have lost the ability to kill bacteria can be reactivated. We have decided to consider only the "killing" particles as reactivable, since we feel that quantitative analysis based on the alternative assumption, i.e., that all particles are reactivable, is more likely to be misleading. In the following discussion the "multiplicity" of XRP will therefore refer to the average number of adsorbed particles with killing ability.

It is seen in table 7 that the probability that a multiple-infected bacterium would liberate active phage increases with increasing multiplicity up to a multiplicity of approximately 2. Beyond this point the probability rapidly falls off. This is in marked contrast to the situation with UVP, with which the probability increases up to a multiplicity of 10 or 20. We believe that this difference is related to the fact that when XRP is used, in addition to the inactive particles that can kill, there are also adsorbed a number of particles that cannot kill.

TABLE 7

Probability of reactivation of XRP T4 as a function of the multiplicity of infection

Experiment no. 26. XRP T4, 6 hits per particle. Each mixture contained a constant amount of bacteria and various amounts of irradiated phage.

MIXTURE NO.	PHAGE INPUT*	MULTIPLICITY* OF INFECTION	(a) BACTERIA WITH RESIDUAL ACTIVE PARTICLES	(b) BACTERIA WITH TWO OR MORE INACTIVE PARTICLES	(c) BACTERIA THAT LIBERATE ACTIVE PHAGE	(d) EXCESS (c) − (a)	RATIO (d)/(b)
1	2.54×10^8	3.6	9.20×10^5	3.6×10^7	9.5×10^5	3×10^4	0.00083
2	1.27×10^8	1.8	4.60×10^5	2.9×10^7	9.0×10^5	4.4×10^5	0.015
3	6.30×10^7	0.9	2.30×10^5	1.4×10^7	4.0×10^5	1.7×10^5	0.012
4	3.15×10^7	0.45	1.15×10^5	5×10^6	1.6×10^5	4.5×10^4	0.009

* Only particles able to kill bacteria are included in these calculations. For every killing particle there are 6 nonkilling ones.

For an irradiated sample with 6 hits, there are six particles that cannot kill for one that can. We have shown earlier that those particles, though unable to kill by single particle action, can, if adsorbed in large numbers, suppress active phage production. It is, therefore, probable that our failure to detect increased reactivation at high multiplicities (table 7) is due to the suppression of active phage production by the large excess of particles unable to kill.

At low multiplicities the number of adsorbed particles unable to kill is too small to influence the amount of detectable reactivation. We can, therefore, use reactivation results employing low multiplicities for a comparison with reactivation of UVP. The comparison can be made on the basis of the probability of reactivation, which is the ratio between the number of bacteria that liberate active phage and the number of multiple-infected bacteria. The fraction of bacteria containing 2 or more "killing" particles is: $1 - (x + 1)e^{-x}$, where x is the multiplicity of infection. For all phages so far tested, T2, T4, and T6, the probability of reactivation, for equal percentage of active survivors and equal multiplicity, is much lower for XRP than for UVP. For example, when the aver-

age multiplicity is 1, the probability that a multiple-infected bacterium liberates active phage is 0.3 for UVP T4 with 2×10^{-2} survival. With XRP T4 with 2×10^{-2} survival, this probability is only 0.01. Since reactivation of XRP is very slight and experimentally difficult to ascertain, confirmation of the phenomenon was desired by other methods.

Luria (1947) reported cross reactivation of UVP T2 by mixed infection with active T4 or T6. Cross reactivation of XRP T2 by mixed infection with active T6 was tested and found to occur. Table 8 shows the results of two typical experiments, which indicate that active T6 increases the number of bacteria liberating T2.

TABLE 8

Cross reactivation of XRP T2 by active T6

Bacteria were mixedly infected with XRP T2 (6 hits) and active T6. The multiplicity of XRP T2 was 0.1, that of active T6 was 1.8. Platings were done on B/6 before burst.

EXPERIMENT NO.	COUNT ON B/6 WITH T6 ALONE	COUNT ON B/6 WITH XRP T2 ALONE	COUNT ON B/6 WITH XRP T2 PLUS ACTIVE T6
30	0	1.7×10^6	3.1×10^6
32	0	1.0×10^6	1.9×10^6

TABLE 9

Cross reactivation between XRP T2r$^+$ and UVP T2r

Bacteria were mixedly infected with XRP T2r$^+$ (6 hits) and UVP T2r (5×10^{-3} survival). The multiplicity of the inactive phages was 0.05, so that almost all of the mixed infections resulted from one particle of XRP and one particle of UVP. Plating was done before burst and the number of mixed bursts ("mottled plaques") counted.

EXPERIMENT NO.	MAXIMUM NUMBER OF MIXED BURSTS EXPECTED FROM BACTERIA WITH RESIDUAL ACTIVE T2r$^+$	NUMBER OF MIXED BURSTS ("MOTTLED PLAQUES") FOUND
35	6.3×10^2	6.3×10^2
36	1.1×10^3	4.6×10^3
37	9.6×10^2	2.8×10^3

Dr. A. H. Sturtevant suggested (personal communication to S. E. Luria) that cross reactivation might occur between XRP and UVP. To facilitate detection of such cross reactivation, genetic markers were employed, and bacteria were mixedly infected with XRP T2r$^+$ and UVP T2r. In this way, the UVP alone gives only r progeny, the XRP alone gives only r$^+$ progeny; bacteria in which cross reactivation occurs between XRP and UVP may give a mixed burst containing T2r$^+$ and T2r. The bacteria giving mixed yields of r and r$^+$ are detected by plating before burst and determining the number of "mottled plaques" (Hershey, 1946; Dulbecco, 1949). The results of a series of such experiments are tabulated in table 9 and demonstrate beyond doubt the existence of cross reactivation, since in all cases the number of mixed bursts was much greater than was expected from residual active phage.

Experiments employing the genetic markers r and r$^+$ were also used to show

that XRP can contribute genetic characters in mixed infection with active phage. Bacteria were infected with one particle of active T2r$^+$ and one particle of XRP T2r. Not only were all the mixedly infected bacteria able to liberate active T2r$^+$, but in addition some of the bacteria produced mixed yields containing T2r$^+$ and T2r. As table 10 indicates, the number of these bacteria greatly exceeds the number expected from the residual active T2r; this shows the ability of the XRP particles to participate in genetic recombination.

In summary, our experiments show that XRP particles can take part in reactivation leading to the production of active phage particles. The probability of reactivation for particles with a given survival is lower for XRP than for UVP. This difference may be due to the fact that X-rays produce in the phage particles some type of damage that cannot be reactivated. An alternative explanation is that each adsorption of X-rays results in damage to a larger portion of the phage particle. As yet we cannot rule out either of these possibilities, though the much lower photoreactivability of XRP suggests that these two inactivating

TABLE 10

Reactivation of XRP T2r by active T2r$^+$

Bacteria were mixedly infected with XRP T2 (3 hits) and active T2r$^+$. The multiplicity of infection of both phages was 0.05, so that almost all the mixed infections resulted from one particle of each type. Plating was done before burst and the number of mixed bursts ("mottled plaques") counted.

EXPERIMENT NO.	NUMBER OF MIXED BURSTS EXPECTED FROM RESIDUAL ACTIVE T2r	NUMBER OF MIXED BURSTS FOUND
41	5.8×10^4	2.1×10^5
42	4.2×10^4	1.7×10^5
43	5.6×10^4	1.4×10^5
44	7.2×10^4	2.0×10^5

agents may differentially damage different portions of the phage. Experiments testing the effect of X-rays on specific genetic determinants should be useful in answering this question.

DISCUSSION

By means of X-ray irradiation we have been able to obtain phage particles that have lost some of their specific properties, such as the abilities to kill the host bacterium or to exclude another phage type. Some of these properties are lost at different rates, which fact suggests that they depend on the integrity of different parts of the phage particle. It is probable that these parts function at different stages during phage-host interaction, so that phage growth will be blocked at different stages depending on which part is damaged.

By examining the rates at which the different properties are lost, it should be possible to draw a tentative sequence of the various events in phage growth. For instance, if a certain type of inactive particle can accomplish steps a, b, and c, another type can go through steps a and c, and still another can accomplish only

step c, we may conclude that the order of these events in reproduction is c, a, b. This type of analysis, utilizing XRP and UVP, permits identification of the following two stages of phage growth:

Adsorption. The distinctness of this stage is shown by finding a phage, the nonkilling XRP, that can adsorb but cannot progress further in reproduction. The adsorption stage must involve a rather deep change in the bacterial cell, since nonkilling XRP can cause both lysis from without and lysis inhibition. In turn, the latter processes are ascribed to an early step in phage-host interaction. As expected, UVP can also lyse from without and inhibit lysis.

Lysis from without occurs under three main conditions: (1) infection of a bacterium by a great excess (100 or more) of phage particles; (2) infection by several phage particles in the absence of nutrients or in the presence of metabolic "inhibitors" (Cohen, 1949); (3) infection by several particles of nonkilling XRP in nutrient medium. A possible clue to its understanding comes from the fact that UVP does not cause lysis from without at low multiplicities in the presence of nutrients. This suggests that lysis from without is the result of an inability to progress to further steps in reproduction. Under this hypothesis, in the absence of nutrients the phage cannot go beyond the adsorption stage and tends instead to cause lysis from without. Supporting this viewpoint is the observation that lysis from without can occur without breakdown of the bacterial "nuclei" (see below).

Invasion. This stage is illustrated by UVP and killing XRP. These particles possess the ability to kill bacteria by single infection but are unable to reproduce. In cells infected with UVP the "chromatinic bodies" disintegrate, suggesting that the UVP kill bacteria by destroying their nuclear apparatus (Luria and Human, 1950). Similar changes have been observed with killing XRP. This stage is further characterized by a blocking of DNA synthesis (Cohen, 1948), inability to form adaptive enzymes (Monod and Wollman, 1947; Luria and Gunsalus 1950), and suppression of increase in respiratory rate (Cohen, 1949).

Mutual exclusion is apparently established in this stage, since we find that the ability to exclude is destroyed by X-rays at the same rate as the ability to kill. In agreement with this idea is the observation (Delbrück, personal communication) that when cells are infected in buffer, that is, in the absence of a nutrient source, mutual exclusion is not established by giving one phage type a head start, presumably due to failure of the process to go beyond the "adsorption" stage.

Such a complete reorganization of the bacterial cell suggests a deep intromission of the infecting phage in the bacterial affairs. We therefore call this stage "invasion" and characterize it as the stage following adsorption during which the infecting phage particle destroys the nuclear apparatus of the host bacterium. Electron micrographs of cells infected with killing particles, compared with others of nonkilling particles, should be of interest in deciding whether after adsorption the former type disappears from the bacterial surface, while the latter does not.

Our picture admittedly presents many gaps. For instance, the stage at which multiplicity reactivation occurs is still unknown. Since all UVP particles can

kill and reactivate excellently by multiple infection, it seems plausible to believe that this reactivation occurs either during or following invasion. Photoreactivation, on the other hand, can occur by exposure to light of bacteria infected in buffer, where invasion presumably does not take place, and may take place both before and after invasion.

Further progress in this type of analysis will be dependent on the availability of additional types of inactive phage. It may be possible through the use of chemical inactivating agents acting in more specific ways to subdivide reproduction into finer steps. For instance, we might be able to obtain phage that can adsorb but not inhibit lysis nor lyse bacteria from without; or we might obtain phage that can kill but not exclude another phage. In any case, the present success with UVP and XRP offers strong incentive for further work with inactive phage.

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SUMMARY

The inactivation by X-rays of bacteriophages of the T group suspended in broth has been investigated, and the properties of the inactive viruses have been studied. Inactivation is a simple exponential function of the dose, indicating a one-hit inactivating mechanism. All the inactive particles are able to adsorb on sensitive bacteria, but only a fraction of them retain the ability to kill bacteria or to exclude another phage type. The ability to inhibit lysis or to lyse bacteria from without is not lost when the ability to kill bacteria is destroyed. Multiplicity reactivation and photoreactivation of X-ray-inactivated phage are observed in slight amounts. The results have been used to distinguish two stages in the early period of phage reproduction, the first stage being called "adsorption," the second "invasion."

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