CROSS REACTIVATION OF GENETIC LOCI OF T2 BACTERIOPHAGE AFTER DECAY OF INCORPORATED RADIOACTIVE PHOSPHORUS

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Bacteriophages containing radiophosphorus P^{32} of high specific activity in their desoxyribonucleic acid (DNA) are unstable, i.e., from day to day a progressively decreasing fraction of the initial phage population is still able to form plaques when plated on a sensitive bacterial strain.[1] The cause of death appears to be some direct, short-range effect of the disintegration of a phosphorus atom in the DNA chain, and not the β-radiation attending this radioactive decay.[1, 2] It is the purpose of this communication to show that an appreciable fraction of P^{32} disintegrations destroying the plaque-forming ability of T2 bacteriophage leave the particle still able to donate some but not all of its genetic characters to the progeny of a mixed infection with an undamaged, non-radioactive phage.

Materials and Methods.—Coliphage T2 (wild type), its mutant T2hr_1 (*h* and r_1 unlinked[3]) and its host *Escherichia coli*, strains B(H) and B(H)/2, obtained from Dr. A. D. Hershey, were employed in this study. When assayed on agar plates seeded with a 1:1 mixture of 24 hr cultures of the two indicator strains, T2h^+r^+ make small, turbid and T2hr large, clear plaques. Their recombinants T2hr^+ and T2h^+r make small, clear and large, turbid plaques, respectively. The genotype spectrum of phages issuing from a mixedly infected bacterium plated before burst can be recognized according to the following schema:

If the bacterium yields the allele combination	h,r or h,h^+r	h,r^+ or h,h^+,r^+	h,r,r^+ or h,h^+,r,r^+	h^+,r	h^+,r^+	h^+,r,r^+
then its plaque type will be	large and clear	small and clear	mottled and clear	large and turbid	small and turbid	mottled and turbid

When our stock of T2 wild type was plated on the mixed indicators, a background of no hr, one h^+r and 10 hr^+ plaques were found among 6000 plaques inspected.

P^{32}-unstable T2 phage was grown according to a modification of the technique of Hershey, Kamen, Kennedy and Gest,[1] to be published in detail elsewhere. Radiophosphorus of high specific activity was obtained from the Isotopes Division, Atomic Energy Research Establishment, Ministry of Supply, Harwell, England.

Phage development in infected bacteria was arrested reversibly by quick-freezing and storage in liquid nitrogen at −196°C. in a glycerol-casamino acid medium devised by Fraser.[4] The fraction of infective centers recovered after thawing is near 0.4 and independent of the length of time of storage.[2]

Experimental: Cross Reactivation of Free Phage.—It was discovered by Luria[5] that bacteriophages whose plaque forming ability, i.e., capacity for independent reproduction, has been destroyed by irradiation with ultraviolet light (UV) can, under certain circumstances, still participate in the reproductive processes occurring within the infected cell. One such phenomenon is *cross reactivation:* if a bacterium is mixedly infected with UV-inactivated and with active phage, differing from each other in their genetic properties, then the markers of the inactive phage appear among the progeny issuing from such a cross. Watson[6] later observed that cross reactivation also occurs with phages inactivated by x-rays. Recently, Doermann[7] has found that only part of the genetic material of X-ray inactivated bacteriophage may participate in cross reactivation and that the ability of different genetic loci of such particles to reappear is eliminated separately by the irradiation.

TABLE 1

CROSS REACTIVATION OF P^{32}-INACTIVATED T2*hr* PHAGE WITH ACTIVE T2h^+r^+

TUBE CONTAINS	TITER OF INFECTED BACTERIA PER ML. YIELDING: EITHER *h* OR *r* OR BOTH	h^+ AND r^+
High P^{32}-T2*hr* + bacteria	1×10^5	0
T2h^+r^+ + bacteria	$<10^5$	8.7×10^7
High P^{32}-T2*hr* + T2h^+r^+ + bacteria	1.2×10^6	5.6×10^7

T2 bacteriophage, inactivated by decay of P^{32} incorporated in its DNA is likewise capable of being cross reactivated. To demonstrate this reactivation, a stock of radioactive T2*hr* was prepared and decay of the free phage was permitted to progress to 0.0005 of its original titer. Bacteria were then mixedly infected at a multiplicity of two each with this inactivated stock of T2*hr* and a non-radioactive stock of T2h^+r^+ and plated before burst on mixed bacterial indicators B + B/2. The different plaque types formed by the infected bacteria were scored separately, according to the schema outlined above, to determine the titer of infective centers liberating any of the alleles of the inactivated T2*hr* stock. In two control tubes bacteria were infected under otherwise identical conditions with either only the inactivated T2*hr* or only the active T2h^+r^+ stock.

The result of this experiment is presented in table 1, where the number of bacteria yielding in mixed infection with T2h^+r^+ one or both of the marker loci of the inactive T2*hr* have been listed and compared with the number of bacteria yielding T2*hr* in the absence of the non-radioactive T2h^+r^+. It

is seen that the number of bacteria yielding some of the loci of the P^{32}-inactivated parent in the cross is more than ten times greater than that of bacteria infected with survivors of the radioactive decay. This tenfold excess, then, is the manifestation of cross reactivation.

Cross Reactivation of Adsorbed Phage.—It has been shown[2] that if a bacterium is singly infected with P^{32}-unstable phage, the inactivation due to radioactive decay of the infective center proceeds, during the early stages of the latent period, at nearly the same rate as the loss of titer of the free phage. We have now studied cross reactivation of P^{32}-unstable T2 whose radioactive decay had taken place only after adsorption to a bacterium also infected with stable phage. The advantage of this method over cross reactivation after P^{32} decay of free phage is that aliquots of the same population of infected cells may be examined at different stages of decay. Decay of adsorbed phage can, furthermore, proceed in a dilute suspension, eliminating irradiation effects on the phage found upon storage of undiluted highly radioactive lysates.

A suspension of bacteria in broth, containing $M/100$ KCN, was infected at a multiplicity of one each with a non-radioactive stock of T2h^+r^+ and with a one-day old stock of T2*hr*, containing P^{32} at a specific activity of 120 millicuries/mg. and referred to hereafter as "high-P^{32}-T2*hr*." The presence of KCN permits adsorption but arrests further phage development.[8] After elimination of unadsorbed phage by centrifugation, the infected bacteria were frozen and stored at −196°C. Aliquots were then thawed from day to day, plated on the mixed indicators B + B/2 before and after burst, and the different plaque types scored separately.

The results of this experiment are presented in figure 1. The logarithm of titer has been plotted against $1-e^{-\lambda t}$, λ being the fractional rate of decay of P^{32} per day, $t-1$ the number of days elapsed between freezing and thawing, and hence $1-e^{-\lambda t}$ being the fraction of P^{32} atoms having decayed by the t-th day. (Such a plot gives a straight line P^{32} mortality curve of free phage if the loss of plaque forming ability is due to the decay of a single phosphorus atom.[1]) It is seen that on the day on which the cross was made, there were 10^7 bacteria per ml. yielding only the *hr* parent, 10^7 per ml yielding only the h^+r^+ parent, and 10^7 per ml. yielding both parents and their recombinants together. In addition, we find two classes of infected bacteria in which only one of the marker loci of the high-P^{32}-T2*hr* parent appear, in addition to those of the stable parent T2h^+r^+: 3×10^6 bacteria per ml. yield h,h^+,r^+ and 10^5 bacteria per ml. yield h^+,r,r^+. The over-all yield after burst contains 55% h^+r^+, 31% *hr* and about 7% each of the two recombinants h^+r and hr^+. (The fraction of recombinants is rather low for two unlinked markers like *h* and r_1, due to the fact that at the multiplicity of infection employed here only 0.46 of all infected bacteria were infected with both parents.)

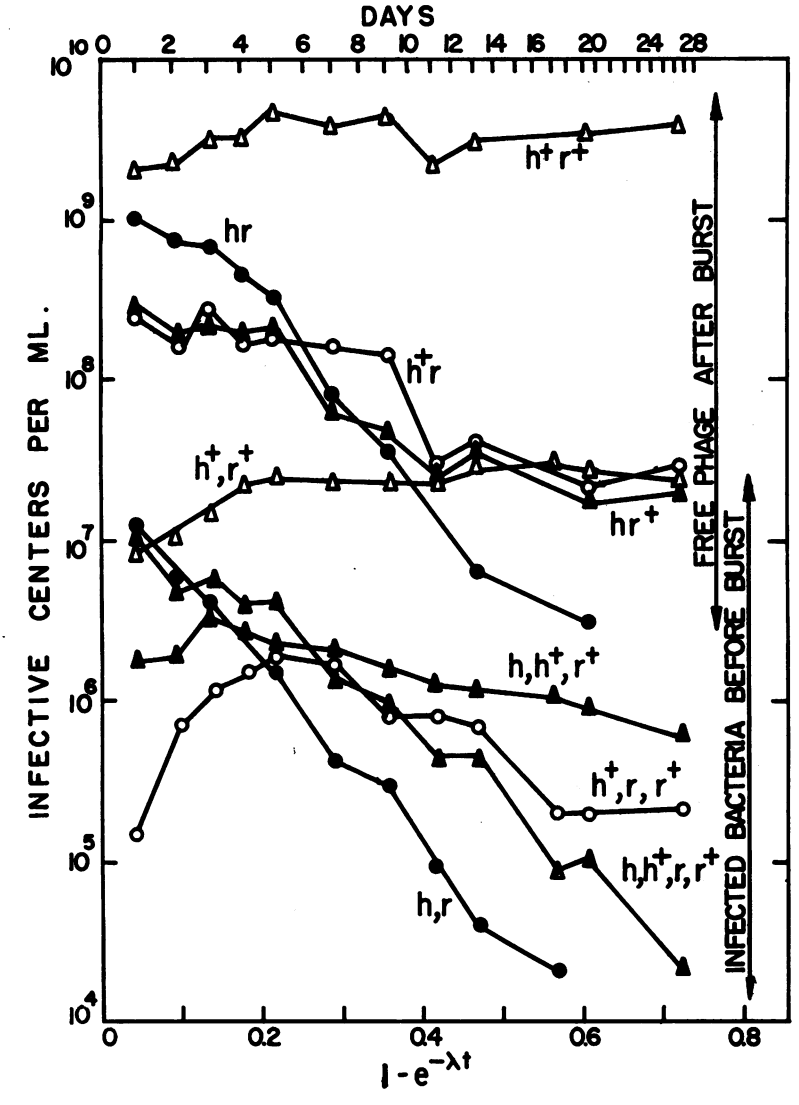

FIGURE 1

DECAY OF HIGH-P^{32}-T2*hr* IN MIXED INFECTION WITH T2h^+r^+

SYMBOL	PLAQUE TYPE	ALLELES LIBERATED BY INFECTED BACTERIUM PLATED BEFORE BURST	PHAGE TYPE PLATED AFTER BURST
●	Large, clear	*h,r*	*hr*
▲	Small, clear	h,h^+,r^+	hr^+
○	Large, turbid or mottled, turbid	h^+,r,r^+	h^+r
△	Small, turbid	h^+,r^+	h^+r^+
◬	Mottled, clear	h,h^+,r,r^+	...

As radioactive decay of the adsorbed high P^{32}-T2*hr* proceeds in the frozen state, the five different classes of infected cells are seen to behave in the following way:

(1) Those yielding only *hr* disappear rapidly at a rate equal to the inactivation of free high P^{32}-T2*hr*, the latter having been measured in a control not shown here.[2]

(2) Those yielding only h^+r^+ increase during the first five days, after which they remain constant at about twice their initial titer.

(3) Those yielding both alleles of both loci h,h^+ and r,r^+ decrease at about 0.7 the rate of the inactivation of high P^{32}-T2*hr*.

(4) Those yielding only the *h* locus of the radioactive parent in addition to h^+ and r^+ decrease at about 0.2 the rate of inactivation of high P^{32}-T2*hr*.

(5) Those yielding only the *r* locus of the radioactive parent in addition to h^+ and r^+ increase during the first five to eight days by a factor of 10, reaching a maximum titer of 2×10^6/ml. After this point they disappear again at about 0.4 the rate of inactivation of high P^{32}-T2*hr*.

The reliability of our scoring of the genotype spectrum of phages liberated from an infected bacterium has been tested by a number of single burst experiments[3, 9] carried out at different stages of the decay. The actual composition of the phage yield of individual cells can be determined in this way, and it was found that plaque type analysis before burst gives a reasonably accurate picture of the distribution of the infected bacteria into the five classes discussed above. An exception was the class yielding h,h^+,r^+ whose frequency seemed to have been somewhat overestimated by analysis before burst at early stages of the decay.

The titer of the four-phage genotypes liberated after burst and their average burst size, defined here as the ratio of the titer of a genotype in the yield after burst to the titer of all those infected bacteria which yield this genotype, appear to be affected by the radioactive decay in this manner:

(1) The titer of *hr* decreases at almost the same rate as high P^{32}-T2*hr*, the average burst size decreasing from 50 to 25.

(2) The titer of h^+r^+ rises by a factor of two and remains constant thereafter, the average burst size having increased from 100 to 200.

(3) The titer of the recombinant hr^+ decreases at about 0.4 the rate of high P^{32}-T2*hr*, the burst size remaining nearly constant between 20 to 30.

(4) The titer of the recombinant h^+r decreases at about 0.4 the rate of high P^{32}-T2*hr* the average burst size increasing from 30 to 100.

Survival of Individual Loci.—These observations demonstrate some quantitative aspects of cross reactivation of P^{32}-inactivated T2. The fact that the class of bacteria yielding both alleles of both loci decreases at only 0.7 the rate of free high-P^{32}-T2*hr* means that 0.3 of those "lethal" P^{32} hits which destroy the ability of the particle to propagate itself independently leave it still able to donate both *h* and r_1 to the progeny of a mixed infection. The

appearance of the class yielding the alleles h,h^+ and r^+ but not r must be due to the fact that some "lethal" hits in the high-P^{32} DNA still leave the h locus free to participate in the cross while excluding the r_1 locus. Since this class disappears at only 0.2 the rate of inactivation of high-P^{32}-T2*hr*, 0.8 of the "lethal" hits still permit the participation of the h locus. Similarly, the waxing and waning of the class yielding r,r^+,h^+ but not h reflects the possibility of survival of the r_1 locus with exclusion of h. The disappearance of this class at 0.4 the rate of high-P^{32}-T2*hr* death means that 0.6 of the lethal hits still permit the participation of the r_1 locus. Finally, the increase of the class yielding only h^+r^+ indicates that once both h and r loci have been prevented by P^{32} decay from participation in a mixed infected bacterium originally belonging to the class yielding both alleles and both loci, viable phages bearing the alleles of only the stable parent may still issue from the cell.

From these numerical relations it appears that the elimination of h and r_1 loci by P^{32} decay are independent events since the probability of 0.7 that a lethal hit destroys the ability of the particle to donate both loci is near the sum of the probabilities of eliminating either one, i.e., 0.2 + 0.4. Secondly, the higher the maximum titer to which the two classes of bacteria yielding only one of the alleles of the high-P^{32}-T2*hr* rise during decay, the smaller can be the probability that a lethal hit eliminates both h and r_1 at the same time. A calculation shows that the actual maxima observed require this probability to be small.

The interpretation of the average burst sizes of the four genotypes after burst is complicated by the fact that more than one class of infected bacteria contributes to the yield of each type. Nevertheless, on the first day of the experiment, when little decay has as yet taken place, most of the phages which possess one or both of the alleles of the high-P^{32}-T2*hr* parent originate from bacteria still infected with active T2*hr*, whereas at late stages of the radioactive decay, the vast majority of such genotypes issue from cells in which the loci of the high-P^{32}-T2*hr* have survived only through cross reactivation. Hence the fact that the average burst sizes of these genotypes remain fairly constant indicates that a locus, once reactivated in this fashion, is not at a very serious disadvantage in the competition of intracellular growth between the two parental phages.

Discussion.—In order to account for the phenomenon of multiplicity reactivation, Luria[5, 10] proposed a theory which envisions the elimination of radiation-induced lethal genetic factors through recombination. In the hope of supporting this theory, Luria and Dulbecco observed in unpublished cross-reactivation experiments that UV irradiation may eliminate separately genetic markers of T2 phage. A low yield of the surviving marker loci, however, did not permit them to distinguish between (a) direct genetic damage with subsequent reactivation by recombination or (b) physiologi-

cal, i.e. non-genetic, damage and reactivation with subsequent random loss of part of the genetic material. Dulbecco[11] has shown more recently that the details of Luria's theory lead to consequences which do not fit all the observed facts of multiplicity reactivation of UV-inactivated phage. The nature of cross reactivation of x-ray inactivated phage found by Doermann[7] and of P^{32}-inactivated phage reported here indicates, however, that a process of reactivation by genetic recombination might well exist. For the appearance and slow decay of the two classes of infected bacteria producing in relatively high yield only one of the marker loci of high-P^{32}-T2*hr* suggests that in these cells only a part of the genetic apparatus and not the entire P^{32}-inactivated phage was "reactivated." Hence the surviving loci would appear in infective progeny through recombination with phage deriving from the active partner of the cross.

If we now consider the fate of a locus which has not survived radioactive decay, two possibilities suggest themselves for its elimination. On one hand, the "lethal" P^{32} disintegrations may have actually destroyed the locus itself or a section of the linkage group of which this locus forms a part, in which case the fraction of all "lethal" P^{32} disintegrations eliminating a particular locus, such as the factor 0.4 for r_1, represents a fraction of the total phage DNA "sensitive" for this locus. On the other hand, the locus itself may have remained intact after P^{32} decay and the fraction of all disintegrations eliminating the locus represent the probability that it is not separated by recombination from a randomly located P^{32} damage. The operational distinction between these alternatives resides in whether or not the probability of elimination of a locus depends on the conditions of cross reactivation, such as multiplicity of infection of the active partner, latent period, and other factors influencing the recombination process.

A theory of recombination in bacteriophage has recently been proposed in which the intracellular or vegetative phages are thought to engage in genetic exchange through random matings in a "mating pool."[12] Some multiplication precedes mating, indeed the beginning of mating appears to await the "filling" of the mating pool by a sufficient number of replicates of the parental vegetative phages. If cross reactivation is considered in terms of this theory, it seems as if the surviving loci of the P^{32}-inactivated phage commence their multiplication already before recombination with the undamaged partner, for the relatively high burst size of the "reactivated" loci indicates that multiplication of their alleles in the active parent did not begin much sooner than their own. In an experiment not presented here in detail we found, furthermore, that among the first phages matured intracellularly at the conclusion of the eclipse period, the "reactivated" loci are already present in approximately their final proportion. If loci surviving the P^{32} decay, however, commence their multiplication before recombination with the active partner, then it would appear that

the elimination of loci is mainly due to their destruction and not their failure to be recombined away from damaged sites. For if an eliminated locus such as r_1 had undergone any appreciable multiplication in a mixedly infected bacterium, it seems unlikely that there could be a probability as high as 0.4 that not one of the r_1 duplicates succeeded in any of the numerous matings to be recombined into an entirely healthy vegetative phage. Experiments on the cross reactivation of linked markers[7] should assist in the understanding of these questions.

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*EFFECT OF X-RAYS ON CHROMATID ABERRATIONS IN AIR AND IN NITROGEN**

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Since the discovery by Thoday and Read[1] that the frequency of x-ray-induced chromosomal aberrations is drastically lowered by exposure during radiation in atmospheres of reduced oxygen tension, a considerable volume of work has been devoted to the study of the mechanisms involved. To account for the chromosomal effects observed, two hypotheses have been advanced. Giles and coworkers[2–5] have considered that the action of oxygen is such as to govern the frequency of primary breaks (differential breakage hypothesis), this in turn determining the frequency of those aberrations arising from the illegitimate reunion of these broken ends. Their case