MUTUAL EXCLUSION BETWEEN RELATED PHAGES[1]

R. DULBECCO

California Institute of Technology, Pasadena, California

Received for publication July 27, 1951

The term mutual exclusion is used to designate an extreme form of interference which often occurs when two phage particles attack the same host cell. The cell liberates numerous particles of one of the parental types and not a single particle of the other parental type (Delbrück and Luria, 1942; Dulbrück, 1945). Mutual exclusion is the rule when the two infecting particles are very dissimilar. However, when they are similar and infect the cell simultaneously, both may multiply. Under these conditions genetic recombination between the two parental types may also take place.

Recently French *et al.* (1951) discovered that infection of an *Escherichia coli*, strain B, cell with a particle of the related large phages T2, T4, and T6, and of the unrelated phage T6, within two minutes produces a profound modification in the bacterium. This modification is manifested in the fact that T2 particles superinfecting the cell two minutes or later after the first infection are broken down. In the experiments of Lesley *et al.* (1951) the superinfecting phage was labeled with P32, and its breakdown was tested for by determination of trichloracetic acid soluble label. Up to 50 per cent of the label of the superinfecting phage particles was found in the soluble fraction within a few minutes after its absorption onto bacteria previously infected with another phage of the group mentioned. Although these experiments of Lesley *et al.* (1951) testify only as to the breakdown of the nucleic acid, they lead one to suspect that the entire phage particle is broken down. This would indeed constitute a very potent mechanism of exclusion, but one of very different characteristics from the case where two very dissimilar phages are involved. To distinguish the two, we will call the first one exclusion by "incompatibility" and this new phenomenon, following Lesley *et al.*, exclusion by "stimulation to breakdown." Incompatibility is a general characteristic of dissimilar phage pairs and is independent of timing relationships. Stimulation to breakdown is produced only by the large phages and requires a time differential between the arrival of the stimulating phage and of the one to be broken down.

The discovery of the breakdown of superinfecting phage needs to be supplemented by genetic tests to find out whether the phage whose nucleic acid is broken down is, indeed, unable to contribute genetic markers to the progeny. The present paper presents experiments of this kind, whose results leave no doubt that the breakdown of superinfecting phage is strictly paralleled by exclusion from the progeny of the genetic markers it contains.

We are presenting also some experiments in which the stimulating phage alone or both the stimulating and the superinfecting phage have been inactivated by

[1] Aided by a grant from the National Foundation for Infantile Paralysis.

irradiation with ultraviolet light. These experiments serve to characterize functions which have remained unimpaired in ultraviolet treated phage and their relation to multiplicity reactivation of ultraviolet treated phage.

MATERIALS

The phages, the bacterial strain, the nutrient medium, the buffer, the ultraviolet light equipment, and the irradiation technique were identical with those described in the preceding paper (Dulbecco, 1952).

METHODS

The general plan of the experiment is to infect a bacterial population with T2r$^+$, to superinfect it with T2r after a precisely defined but varied time interval, and to determine the change in the fraction of bacteria giving a mixed yield of r and r$^+$ particles as a function of the time interval between infections. Furthermore, the same experiments are to be done with the sequence of phages reversed. The test for bacteria giving a mixed yield can be made very simply by plating the infected bacteria before lysis and carefully noting the appearance of the plaques at a critical stage of their development. Plaques due to bacteria giving a mixed yield of r and r$^+$ particles have a mottled appearance (Hershey 1946, see appendix for a test of the reliability of this method).

Only the *change* in the fraction of mottle plaques (mixed yielders) with the time interval between infections is significant, not the absolute value of this fraction. The absolute values of this fraction are influenced by the multiplicities of the two infections, and these may vary somewhat from one experiment to the next. The changes of the fraction are well reproducible.

The bacteria were grown in nutrient broth to a concentration of 2×10^8 per ml, centrifuged, washed, and resuspended in buffer at a concentration of 4×10^8 cells per ml.

The primary infection was conducted in a buffered suspension, at a multiplicity (i.e., average number of phage particles adsorbed per bacterium) between 1 and 2. The adsorption rate in this mixture is very high, about 90 per cent per minute. The mixture, in aliquots of 0.5 ml, was distributed into small tubes, which were kept in a water bath at 37 C. During this adsorption period in buffer, stimulation to exclusion does not take place, as will be shown later. After about 2 minutes, when adsorption is practically complete, 0.5 ml of nutrient broth at 37 C is added to each tube. This addition of broth starts the course of events leading to stimulation, and will be referred to as time zero of the experiment. The superinfecting phage is added at various later times, at a multiplicity around 1. In a control tube, broth and the two phages were added simultaneously to the uninfected bacteria.

Three minutes after superinfection with the second phage, the mixture was diluted into specific anti-T2 serum to inactivate any unadsorbed phage. Four minutes were allowed for serum action, then a further dilution in broth was made and plated before lysis for a count of the fraction of mottled plaques. The plates were inspected for mottling after 8 hours of incubation at 37 C.

EXPERIMENTAL RESULTS

Interactions between active phages. Table 1 and figure 1 show the results of experiments in which the time interval between infections varied between 0 and 12 minutes. The count of mixed yielders drops to 50 per cent of its original value when the interval is one minute, and to 10 per cent when the interval is 4 minutes. Identical results are obtained whether T2r$^+$ is used for the primary infection and T2r for the superinfection, or vice versa.

When broth is not added between the primary infection and the superinfection, the count of mixed yielders drops only slightly. We conclude that the exclusion we observe is dependent on metabolic processes requiring an external energy supply. The small amount of exclusion found in buffer may be due to endogenous metabolism, since the bacteria had not been starved. The physiological state of

TABLE 1

Exclusion between active phages

Multiplicity of T2r$^+$: 1.4; multiplicity of T2r: 0.9

	TIME INTERVAL BETWEEN FIRST AND SECOND INFECTION	PLAQUE COUNTS			FRACTION OF MOTTLED PLAQUES	RELATIVE FRACTION OF MOTTLED PLAQUES
		Mottled	r$^+$	r		
T2r$^+$ first	0	385	223	133	0.51	1.00
	1 min	178	319	137	0.28	0.56
	2 min 25 sec	53	428	120	0.09	0.18
	5 min 40 sec	21	491	132	0.03	0.07
	10 min 40 sec	10	504	139	0.02	0.04
T2r first	0	396	204	233	0.48	1.00
	1 min	147	293	222	0.22	0.47
	2 min 25 sec	66	364	415	0.09	0.16
	5 min 40 sec	23	301	400	0.03	0.07
	10 min 40 sec	22	263	389	0.03	0.07

the bacteria also affects the progress of stimulation: if the bacteria are grown in broth to a concentration of 4×10^8 cells per ml, chilled for 30 minutes, brought back to 37 C, and immediately given a primary infection, exclusion is established more slowly than without chilling.

Primary infection with ultraviolet inactivated phage, superinfection with active phage. The results of an experiment in which T2r$^+$ irradiated with ultraviolet light was used for the primary infection are given in table 2. This ultraviolet dose was so high (380 seconds) that the primary infection, at the moderate multiplicity used, did not contribute appreciably to the plaque count. In calculating the multiplicity of the primary infection, we take into account a slight reduction in the killing ability of the phage suspension as a result of the radiation (see Appendix).

The results in table 2 show that there is no exclusion of the superinfecting active phage if it is given simultaneously with the inactive phage. With a time interval of one minute there is an exclusion of the superinfecting phage in 50 per

cent of the bacteria infected previously with at least one killing particle. For longer intervals exclusion appears to spread more slowly than in the case of

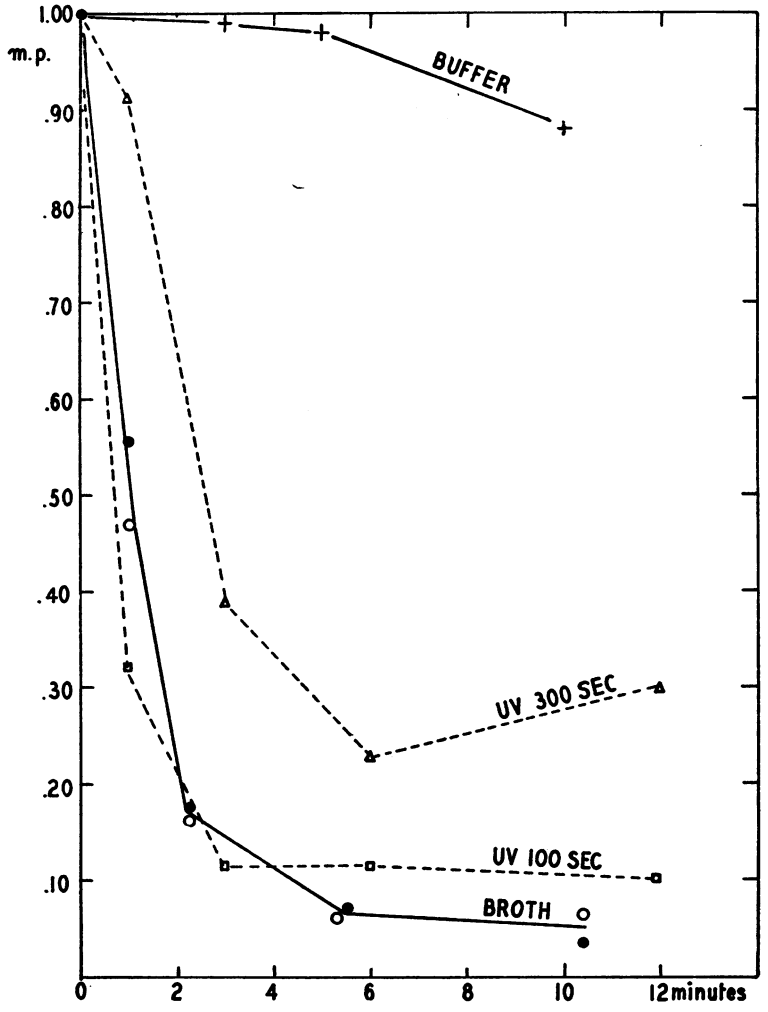

Figure. 1. The relative fraction of mottled plaques as a function of the time interval between infections. The relative fractions of mottled plaques (m.p.) are plotted versus the time intervals given in minutes. Solid lines refer to experiments performed with active phage, dashed lines to experiments performed with irradiated phage: the ultraviolet dose is given near the curves. Solid circles refer to an experiment in which the sensitizing phage was T2r$^+$, open circles to an experiment in which the sensitizing phage was T2r.

stimulation by active phage. We conclude that T2 heavily irradiated with ultraviolet light is very nearly as potent as active phage in stimulating the exclusion of superinfecting phage.

Both primary and superinfecting phage inactivated by irradiation with ultraviolet light. When both primary and superinfecting phage have been inactivated

by an appreciable dose of ultraviolet light, phage production can come about only through multiplicity reactivation, either by cooperation between the particles of

TABLE 2

Exclusion of active T2r by ultraviolet light-irradiated T2r+

T2r+ was irradiated with 380 seconds of ultraviolet light. Multiplicity of infection of T2r+ (total) = 1.6; multiplicity of infection with killing particles = 0.95. Bacteria infected with T2r that did not receive a killing T2r+ particle: 192 plaques (calculated).

TIME INTERVAL BETWEEN FIRST INJECTION (T2r+ IRRAD.) AND SECOND INFECTION (T2r ACT.)	COUNTS OF r PLAQUES	r PLAQUES FROM BACTERIA RECEIVING AT LEAST ONE KILLING T2r+	r PLAQUES, FRACTION OF THE NUMBER OBSERVED AT TIME ZERO
No T2r+	536		
0	521	329	1.00
1 min	379	187	0.56
2 min 20 sec	358	166	0.50
5 min	316	124	0.37
10 min	303	111	0.33

TABLE 3

Exclusion between two irradiated phages

Dose of irradiation: 100 seconds in the first experiment, 300 in the second one. Multiplicity of infection: first experiment T2r+ = 3, T2r = 2; second experiment: T2r+ = 2.5, T2r = 1.7. First phage: T2r+ in both experiments.

	TIME INTERVAL BETWEEN FIRST AND SECOND INFECTION	PLAQUE COUNTS			FRACTION OF MOTTLED PLAQUES	RELATIVE FRACTION OF MOTTLED PLAQUES	RELATIVE FRACTION OF MOTTLED PLAQUES DUE TO MULTIPLICITY REACTIVATION BETWEEN PARTICLES OF FIRST AND SECOND INFECTION
		Mottled	r+	r			
	min.						
Ultraviolet light irrad. 100 sec	0	81	43	31	0.52	1.00	1.00
	1	17	77	13	0.16	0.32	0.30
	3	6	91	3	0.06	0.12	0.20
	6	6	90	7	0.06	0.12	0.26
	12	4	78	4	0.05	0.10	0.00
Ultraviolet light irrad. 300 sec	0	145	50	99	0.49	1.00	1.00
	1	40	31	16	0.46	0.93	0.23
	3	11	32	14	0.19	0.39	0.12
	6	6	32	14	0.12	0.23	0.09
	12	4	16	5	0.16	0.32	0.00

the primary infection, or by cooperation involving both the particles of the primary infection and those of the superinfection. The efficiency of the second of these two types of multiplicity reactivation decreases as the time interval between the two infections increases until we reach as a base level the plaque count produced by the primary infection alone (see table 3). This drop-off of the multiplicity reac-

tivation produced by interaction between the two phages occurs at a similar rate as does the exclusion phenomenon described in the preceding experiments. The experiments show, moreover, that they are mainly the mixed yielders which disappear from the plaque count when this part of the multiplicity reactivation disappears. We learn from this experiment two lessons about multiplicity reactivation: (1) multiplicity reactivation fails under precisely those conditions under which mixed growth fails, and (2) under conditions which permit multiplicity reactivation to occur, the genetic markers of both parents enter into the progeny in 50 per cent of the cases, i.e., in at least one-half of these cases *both* parents are reactivated.

The data of table 3, obtained from two experiments differing in ultraviolet dose, show clearly the loss of mixed yielders with increasing time interval between first and second infection: however, they also show several apparent discrepancies which should be mentioned. These arise because of the complicated effects produced by multiplicity reactivation.

In the case of exclusion between active phage (table 1) we have seen that the loss of mottled plaques is paralleled by an increase of plaques of the primary infection, so that the sum of these two classes remains approximately constant; plaques of the superinfecting type also remain approximately constant in number. With both phages inactive (table 3), the sum of mottled and primary infecting type plaques no longer is constant but decreases as the interval between infections grows longer. This occurs because the mottled plaques arise only from multiplicity reactivation, and exclusion of the superinfecting particle generally results in complete loss of the given bacterium as a phage yielder. The effect is much more prominent at the higher ultraviolet doses, where multiplicity reactivation is more important.

Moreover, at the higher ultraviolet dose, plaques of the primary infection type actually decrease in number with time, as do those of the superinfecting type at both doses. This indicates that in some bacteria the sensitizing phage is reactivated by the superinfecting phage, which, however, does not appear in the yield. This fraction decreases with increasing time interval between infections, as does the fraction of mottled plaques. At low ultraviolet dose this phenomenon is not apparent because the superinfecting phage contributes little to the reactivation of the first.

DISCUSSION AND SUMMARY

Bacteria infected with phage T2r$^+$ are stimulated to exclude superinfecting phage T2r arriving after a time interval, and vice versa. The superinfecting phage is excluded in 50 per cent of the bacteria if the time interval between infections is one minute. This time scale is similar to the one established by Lesley *et al.* (1951) for the disruption of the nucleic acid of superinfecting phage.

T2 irradiated with ultraviolet light for 380 seconds leads to this stimulation to exclude superinfecting phage at nearly the same rate as does active phage. When the active phage is added simultaneously to the bacteria, the inactive phage does not exclude it.

If both the primary and the superinfecting phage have been inactivated by irradiation with ultraviolet light, the ability of the superinfecting phage to cooperate with the primary phage in multiplicity reactivation drops off at the same rate at which the stimulation to exclusion is manifested. Under conditions which permit multiplicity reactivation to occur, the genetic markers of both parents enter into the progeny, i.e., both parents are reactivated in at least 50 per cent of the cases.

The stimulation to exclusion is dependent on processes requiring an external energy supply. It is practically absent in resting bacteria in buffer.

Exclusion of a phage from multiplication in a bacterium infected a few minutes earlier with a similar active or ultraviolet killed phage has been noted repeatedly (Luria and Delbrück, 1942; Luria, 1945; Delbrück and Bailey, 1946; Luria and Dulbecco, 1949). Each of the earlier observations fits well into the body of facts here reported.

It seems very likely that the stimulation to breakdown discovered by Lesley *et al.* (1951) and the stimulation to exclusion described in the present paper represent two aspects of the same function of phage, a function which is highly resistant to ultraviolet light. The principal characteristic of this function consists, perhaps, in the rapid mobilization of a powerful enzyme system attacking nucleic acids. It is tempting to view the destruction of the chromatinic bodies of infected bacteria (Luria and Human, 1950) as a third aspect of the same function. This destruction, too, takes place within the first minutes and occurs with ultraviolet phage as with active phage.

If mixed infections are made in broth, a certain amount of exclusion must be expected to take place, particularly if the adsorption rate is not very high and the adsorption time not very short.

A detailed study of the metabolic requirements for this stimulation could be undertaken, since the exclusion mechanism provides a convenient quantitative test of the progress of stimulation. Such a study would supplement the method developed by Benzer (1951) for the study of intracellular phage growth. Benzer uses the increase in radiation resistance of infective centers during the first half of the latent period as an index of the progress of infection. This method is relatively insensitive during the first 3 minutes after infection where the one here proposed will have the highest sensitivity.

REFERENCES

BENZER, S. 1951 *In press.*

DELBRÜCK, M. 1945 Interference between bacterial viruses. III. The mutual exclusion effect and the depressor effect. J. Bact., **50**, 151–170.

DELBRÜCK, M., AND BAILEY, W. T., JR. 1946 Induced mutations in bacterial viruses. Cold Spring Harbor Symp. Quant. Biol., **11**, 33–37.

DELBRÜCK, M., AND LURIA, S. E. 1942 Interference between bacterial viruses. Arch. Biochem., **1**, 111–141.

DULBECCO, R. 1952 A critical test of the recombination theory of multiplicity reactivation. J. Bact., **63**, 199–207.

FRENCH, R. C., LESLEY, S. M., GRAHAM, A. F., AND VAN ROOYEN, C. E. 1951 Studies on the relationship between virus and host cell. III. The breakdown of P^{32} labelled

T2r+ bacteriophage adsorbed to *E. coli* previously infected by other coliphages of the T group. Can. J. Med. Sci., **29**, 144.

HERSHEY, A. D. 1946 Mutation of bacteriophage with respect to type of plaque. Genetics, **31**, 620–640.

LURIA, S. E. 1945 Mutations of bacterial viruses affecting their host range. Genetics, **30**, 84–99.

LURIA, S. E., AND DELBRÜCK, M. 1942 Interference between inactivated bacterial virus and active virus of the same strain and of a different strain. Arch. Biochem., **1**, 207–218.

LURIA, S. E., AND DULBECCO, R. 1949 Genetic recombinations leading to production of active bacteriophage from ultraviolet inactivated bacteriophage particles. Genetics, **34**, 93–125.

LURIA, S. E., AND HUMAN, M. L. 1950 Chromatin staining of bacteria during bacteriophage infection. J. Bact., **59**, 551–560.

APPENDIX

The identification of mottled plaques with mixed yielders. If a bacterium yields r+ particles and r particles in comparable numbers, it will give rise to an unmistakable mottled plaque. The likelihood of overlooking a mixed yielder is appreciable only if one of the types is very much in the minority. To estimate this error bacteria were mixedly infected with active T2r+ and inactive T2r (irradiated for 60 seconds with ultraviolet light). Under these conditions most of the mixedly infected bacteria yield a large number of r+ particles and a small number of r particles. The infected bacteria were plated, incubated, and scored for plaque type. After scoring, the plaques were picked up and their content examined by the technique described by Anderson (1948).

TABLE 4

Scoring of plaques as mottled or nonmottled as a function of their content of r particles

FRACTION OF r PARTICLES	MOTTLED	NONMOTTLED
<0.005	—	137
0.005–0.01	—	14
0.01–0.05	—	9
0.05–0.10	2	2
0.10–0.20	2	—
0.20–0.30	3	—
0.30–0.40	0	—
0.40–0.50	4	—
0.50–0.60	2	—
>0.60	1	—

The results, given in table 4, show that plaques containing more than 10 per cent r particles are invariably scored as mottled, those containing less than 5 per cent r particles invariably as nonmottled, those containing 5 to 10 per cent r particles are variably scored. Thus the error due to misclassification can become appreciable only if the bacteria yield the two types in very unequal proportion.

The killing ability of ultraviolet phage. A suspension of bacteria in buffer was

distributed into a number of tubes and each tube infected with the same amount of ultraviolet phage, the only difference between the tubes being the ultraviolet dose given previously to the phage. After 12 minutes' incubation at 37 C, suitably diluted samples from each tube were plated for colony count.

TABLE 5

Fraction of phage particles irradiated with different ultraviolet doses able to kill the bacteria

ULTRAVIOLET DOSE (SECONDS)	BACTERIAL TITER	BACTERIAL SURVIVAL	MULTIPLICITY*	MULTIPLICITY, FRACTION OF THE MAXIMUM AT TIME ZERO
No phage	8.0×10^7			
0	3.6×10^6	0.045	3.1	1.0
100	5.9×10^6	0.074	2.6	0.84
200	7.2×10^6	0.090	2.4	0.77
400	1.4×10^7	0.175	1.74	0.56
800	2.5×10^7	0.31	1.17	0.38
1200	3.4×10^7	0.42	0.87	0.28

* Calculated from the equation: multiplicity = $-\log_e$ survival.

The results, given in table 5, show that the titer of phage particles able to kill bacteria decreases slowly with increasing ultraviolet dose. The resistance of the killing function of phage T2 is about forty times greater than the resistance of the ability to multiply.

The adsorption rate decreases even more slowly than the killing titer. The time allowed for adsorption in the experiments on the reduction in killing ability was adequate to ensure almost 100 per cent adsorption.

REFERENCES

ANDERSON, T. F. 1948 The inheritance of requirement for adsorption cofactors in the bacterial virus T4. J. Bact., **55**, 651–657.