A CRITICAL TEST OF THE RECOMBINATION THEORY OF MULTIPLICITY REACTIVATION[1]

R. DULBECCO

California Institute of Technology, Pasadena, California

Received for publication July 27, 1951

Bacteriophage particles are called active when they are able to give rise to production of active phage following adsorption on their specific host bacterium. This is generally detected by the formation of a plaque when plated on nutrient agar by standard techniques (Adams, 1950). If the particles are exposed to ultraviolet light, a fraction of them loses the ability to give rise to production of active phage. These particles are called inactive. This is a somewhat unfortunate term because the inactive particles are still able to perform functions characteristic of the active ones, in that they adsorb on the host bacteria and kill them. Moreover, the inability to give rise to production of active phage is not necessarily permanent because it can be overcome by at least two means, namely, by multiplicity reactivation (Luria, 1947) and by photoreactivation (Dulbecco, 1950). In multiplicity reactivation, production of active phage takes place in bacteria infected simultaneously with more than one inactive particle, in photoreactivation, it takes place in bacteria infected with one or more inactive particles and subsequently irradiated with visible light.

Luria (1947) formulated an interesting theory of multiplicity reactivation, suggested by analogy with the findings of others on the recombination of genetic markers occurring in bacteria mixedly infected with several active phage particles. Luria assumed that each phage particle consists of a certain number of indispensable genetic units, which are sensitive to ultraviolet light. Each of these units is inactivated independently of the others. When one or more of them are inactivated, the whole particle becomes inactive. However, in bacteria infected with more than one such inactive phage particles, the remaining active units multiply and recombine; and this may lead to the reconstitution of a complete active phage particle under suitable conditions. We call this theory the "recombination theory" of multiplicity reactivation.

Luria and Dulbecco (1949) have presented experiments in support of this theory, based on systematic measurements of the probablility that bacteria infected with more than one inactive phage particle (multicomplexes) yield active phage. We call this probability the survival of multicomplexes. The results were in fair agreement with the prediction of the theory. These experiments, however, had been limited for technical reasons to the determination of survivals generally higher than 10^{-3}, and therefore they did not constitute a critical test of the recombination theory; in fact, the most characteristic features of the theory come to light only at considerably lower survivals, as shown by the following considerations.

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

According to the theory, a multicomplex is able to produce active phage if at least one genetic unit of each kind is active among all the units contributed by the different phage particles in the complex. If the phage is irradiated with a low ultraviolet dose, the complex will generally contain a considerable surplus of active units, so that an additional ultraviolet dose will have a small additional inactivating effect. At high ultraviolet doses no spare units will remain, and an additional ultraviolet dose will have a high inactivating effect. Therefore, the survival curves for the multicomplexes should have a so-called multiple-target character, starting out with a shoulder and ending up with a finite ultimate slope.

The predictions of the theory for the survival curves of multicomplexes can be observed in more detail from the equations given by Luria and Dulbecco (1949). These predictions are:

(1) The ultimate slope at high ultraviolet dose is equal to that of the curve for a single phage particle. This is a very essential result of the theory. It occurs because there is only one surviving genetic unit of each kind left in the multicomplexes surviving at very high doses. This state of affairs is reached only at very much larger doses than in a simple multiple-target model, and the Luria curves, correspondingly, approach their ultimate slope much more gradually than the corresponding curves of the multiple-target theory.

(2) If the number of units in a phage particle is n, and the multiplicity of infection k, the intercept of the asymptote with the axis of ordinates is equal to k^n, an enormously high value.

These characteristics of the Luria curves are illustrated in figure 1 for $n = 10$, 25, and 100, and for average multiplicities $x = 0.5$ and 4. They show that the ultimate slope is approximately reached only at a survival equal to 10^{-7} for $n = 25$; at higher survival neither ultimate slope nor its intercept with the axis of ordinates is determinable. For this reason, the experiments reported by Luria and Dulbecco (1949) do not critically test the theory.

In order to make more critical experiments, our quantitative analysis was pushed a great deal further, both as to accuracy and as to range of ultraviolet dose. This extension was made possible by a technical refinement, namely, the adsorption of phage to bacteria in buffer instead of in a nutrient medium. Exclusion of some of the infecting phage particles due to differences in the timing of adsorption is thus avoided (Dulbecco, 1952). Moreover, an accurate measurement of multiplicity becomes possible by determining the fraction of killed bacteria; thus eliminating the inaccuracies arising in the previous experiments from bacterial growth taking place during the adsorption period.

The survival of the multicomplexes has been determined, both in darkness and after exposure to white light under conditions allowing maximum photo reactivation. The reason for superimposing photoreactivation upon multiplicity reactivation was the idea that ultraviolet damage is of a variety of types and that this variety might be reduced by eliminating the photoreactivable part (Dulbecco, 1950). Multiplicity reactivation might be simpler if studied after this elimination.

As to the effect of photoreactivation, the recombination theory predicts that

the survival curves after photoreactivation should be related to those before photoreactivation by a simple dose reduction factor, representing the ratio of the cross sections of each unit before and after photoreactivation. The ultimate slope of the survival curves after photoreactivation should be the same for multicomplexes as for monocomplexes.

The results of our experiments clearly contradict the recombination theory of multiplicity reactivation. In this paper only the negative conclusions will be discussed, reserving the formulation of a new theory to a later paper.

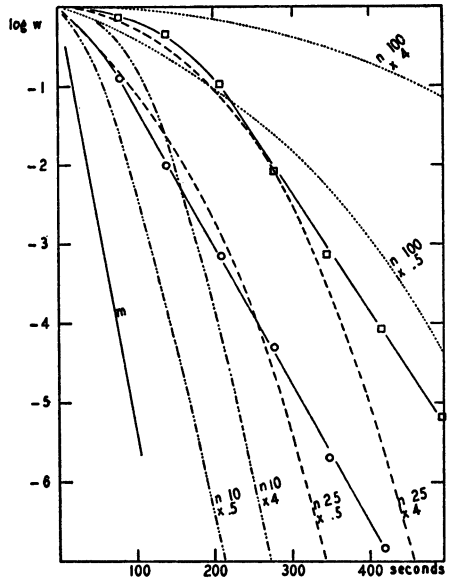

Figure. 1. Theoretical and experimental survival curves for monocomplexes and multicomplexes. The log w is plotted versus the ultraviolet dose given in seconds of irradiation as described in the text. m = survival curve for monocomplexes. Dotted lines: theoretical curves for $n = 100$; dashed lines: theoretical curves for $n = 25$; broken lines: theoretical curves for $n = 10$. Number of units (n) and average multiplicity of infection (x) used in the calculation are indicated near to each line. Solid lines: experimental curves obtained at multiplicity 4 (squares) and 0.5 (circles). Multicomplexes of multiplicity greater than 10 have been neglected in the calculation of the theoretical curves.

MATERIALS AND METHODS

Phages T2r+ and T2r, and *Escherichia coli*, strain B, were used in these experiments. For most experiments, lysates of the phages, produced in a synthetic medium containing glucose and ammonium chloride, were used; some control experiments were run with phage preparations purified by two steps of differential centrifugation.

Ultraviolet irradiation was given with a GE "germicidal" lamp, emitting 80 per cent of its energy in the wave length of 2537 A. The lamp was fed through a "sola" voltage stabilizer. The samples to be irradiated were made up in 3 ml buffer and were irradiated in a 10 cm Pyrex watch glass at a distance of 80 cm

from the center of the lamp. Exposure times lasted between 70 seconds (survival without reactivation 10^{-4}) and 700 seconds.

The buffer was an M/15 phosphate buffer, pH 7, to which had been added 10^{-3} M $MgSO_4$ and 10^{-1} M NaCl.

The bacteria were grown in Difco nutrient broth to a concentration of 10^8 cells per ml, washed, and resuspended in buffer at the original concentration. In some experiments the bacteria were resuspended in one-tenth of the original volume, thus raising their concentration to 10^9 cells per ml.

The irradiated phage was added to this bacterial suspension in buffer, and the mixture was kept in darkness at 37 C for 12 minutes to allow time for adsorption. One sample of the mixture was then plated in darkness on nutrient agar plates and another sample was plated after exposure to the photoreactivating light emitted by a GE mercury discharge lamp A-H5 (Dulbecco, 1950). During the photoreactivating exposure the sample was held in a water bath at 37 C. The time of exposure was adequate to ensure a maximum photoreactivation.

The multiplicity of infection was determined in each experiment by measuring the fraction of surviving bacteria in a suitable control tube in which the phages were in excess by a factor of about 1.5, and assuming a Poisson distribution of the phage among the bacteria. The errors introduced into this calculation by deviations from the Poisson distribution of the phage among the bacteria are negligible (Dulbecco, 1949). In the other tubes containing different amounts of phage and a constant amount of bacteria, the multiplicity was calculated on the basis of this calibration value. In basing our estimate of the multiplicity of infection on the killing ability of the phage, we are making the tacit assumption that phage which has been damaged by ultraviolet light to the point of being unable to kill a bacterium also will not participate in multiplicity reactivation.

EXPERIMENTAL RESULTS

In all of the experiments reported in this paper the ultraviolet doses are so large that the contributions to the plaque counts made by free phage particles or by bacteria infected with only one phage particle (monocomplexes) are entirely negligible compared to the contributions made by multiple infected bacteria, or multicomplexes. We express our results, therefore, as the fraction w (Luria and Dulbecco, 1949):

$$w = \frac{\text{multicomplexes yielding active phage}}{\text{total number of multicomplexes}}.$$

The total number of multicomplexes has been calculated from the multiplicity of infection by assuming a Poisson distribution of the phage particles among the bacteria. The deviation from a Poisson distribution of the phage particles among the bacteria does not affect this calculation, provided the multiplicity is not too low or too high (Dulbecco, 1949). High values of multiplicity have not been used to avoid complication due to lysis from without, which occurs easily in bacteria in buffer (Benzer and Weigle, personal communication). In the range of multiplicity used, lysis from without is negligible, and the accuracy of

the determination of the multiplicity has been confirmed by experiments of mixed infection with T2r+ and T2r. The fraction of mixed bursts, detected by mottled plaques (Hershey, 1946), was in agreement with the calculated multiplicity of the two phages, except at very low multiplicities around 0.05 where the fraction of mixed bursts was somewhat higher than calculated, at most by a

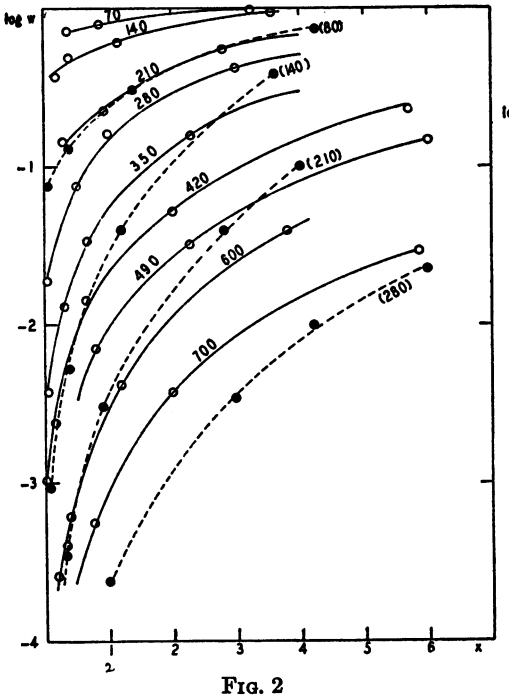

FIG. 2

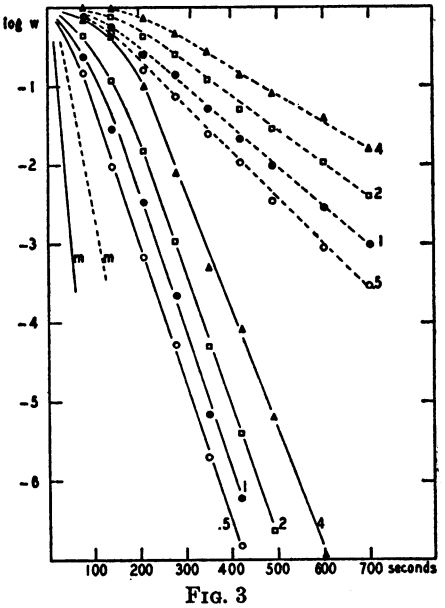

FIG. 3

Figure 2. Survival curves for monocomplexes and multicomplexes at different average multiplicity of infection, in darkness and after maximum photoreactivation. The log w is plotted versus the ultraviolet dose given in seconds of irradiation as described in the text. Solid lines refer to curves obtained in darkness; dashed lines to curves obtained after maximum photoreactivation. The average multiplicity of infection is indicated near each curve.

Figure 3. Curves of log w as a function of the average multiplicity of infection, at different ultraviolet doses. The log w is plotted versus the multiplicity (x). Open circles and solid lines refer to data obtained with maximum photoreactivation, solid circles and dashed lines refer to data obtained in darkness. The ultraviolet dose, in seconds of irradiation as described in the text, is indicated near each curve; figures in parentheses refer to experiments performed in darkness.

factor two. This discrepancy may be due to the inhomogeneity in the length of the bacteria, which, at low multiplicity, leads to a distortion of the distribution of the phages, increasing the multicomplexes and decreasing the monocomplexes.

The survival curves for multicomplexes are given in figure 2. These were obtained in two steps. In each experiment the ultraviolet dose was kept constant, and the average multiplicity was varied from sample to sample; figure 3 shows the results of a series of such experiments. For drawing the survival curves of

multicomplexes for a given fixed multiplicity, the appropriate values of w were taken by interpolation from these primary plots. Since the average multiplicity of infection had been determined in each experiment with considerable accuracy, the interpolated values of w are reliable. This is confirmed by the fact that repeated determinations of w gave very similar results. In figure 2 the survival curves are given for multicomplexes of average multiplicity 0.5, 1, 2, 4, both in darkness and after maximum photoreactivation. The actual multiplicities from which these data were derived varied between 0.03 and 6.

These survival curves represent populations of multicomplexes of different individual multiplicity. Survival curves were obtained also for two uniform classes of complexes, the 2- and 3-complexes, after maximum photoreactivation, by using the following limits of the curves of log w versus the average multiplicity x:

$$\lim_{x=0} \log w = \log f(2)$$

$$\lim_{x=0} \frac{d \log w}{dx} = \frac{1}{3} \frac{f(3) - f(2)}{f(2)}$$

where f (2) and f (3) are the probabilities that a 2- and a 3-complex, respectively, yield active phage. These two limits are easily derived from the definition of w given in Luria and Dulbecco (1949). The first limit is the intercept of a curve of log w versus x with the axis of ordinates, the second one is its slope in the same point; both can be determined graphically. These determinations are not very precise, however, because the values of w in the proximity of $x = 0$ are less accurate. Several points of the survival curves for 2- and 3-complexes determined in this way are given in figure 4. In this calculation the inhomogeneity of bacterial lengths has not been taken into account; it would decrease slightly the values of all points; the curve for 2-complexes would be shifted without change in slope; the curve for 3-complexes would slightly increase its slope.

The survival curves of the mixed population (figure 2) have the following characteristics:

1. The curves are of the multiple target type for every multiplicity, both in the presence and in the absence of photoreactivation—that is, all the curves have a shoulder at low ultraviolet dose.

2. The ultimate slopes of the curves are very much smaller than the corresponding ultimate slopes of the survival curves for monocomplexes.

3. The curves obtained at different multiplicities tend to slightly different ultimate slopes. The difference is barely noticeable in the absence of photoreactivation, but appreciable in the presence of photoreactivation.

4. The ultimate slopes after maximum photoreactivation are considerably smaller than without photoreactivation, at any given multiplicity.

5. The two sets of curves are not compatible with the simple assumption that photoreactivation is equivalent to a reduction of the ultraviolet dose by a constant factor.

The intercepts of the asymptotes with the axis of ordinates and the ultimate slopes of the curves are given in table 1.

The survival curves for homogeneous populations of 2- and 3-complexes are also of the multiple-target type; they have two interesting features: (1) the final

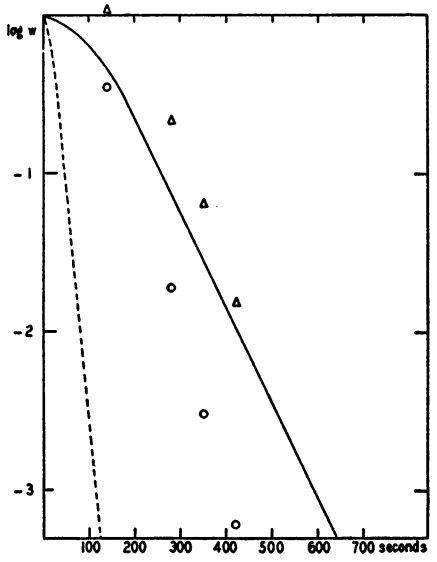

Figure 4. Points of survival curves for 2 and 3-complexes after maximum photoreactivation. The log w is plotted versus the ultraviolet dose given in seconds of irradiation as described in the text. Circles refer to 2-complexes, triangles refer to 3-complexes. Dashed line: survival curve for monocomplexes after maximum photoreactivation. Solid line: survival curve for mixed multicomplexes of average multiplicity 0.5 after maximum photoreactivation.

TABLE 1

Dependence of the slope and the target number of the survival curves for multicomplexes on the multiplicity, in darkness and after maximum photoreactivation

		AVERAGE MULTIPLICITY OF INFECTION				
		0.5	1	2	4	Monocomplexes
Darkness	Slopes	2.5	2.5	2.4	2.1	9.7
	Target number	5–6	14–18	60–70	150–180	3.5
Maximum photoreactivation	Slope	0.8	0.7	0.6	0.5	4.2
	Target number	2.5	2.5	3.2	4.0	1.6

slope of the curve for 2-complexes is intermediate between that for monocomplexes and that for mixed multicomplexes of low average multiplicity; (2) at high ultraviolet dose, the survival of 3-complexes is 10 to 20 times higher than that of 2-complexes; the ratio increases with the ultraviolet dose.

These survival curves for 2- and 3-complexes suggest how the curves for mixed multicomplexes are to be interpreted. Complexes of increasing multiplicity have

survival curves characterized by decreasing slope and probably increasing target number; summed together in a mixed population, they give rise to a resultant curve which tends to the final slope of the class of complexes of highest multiplicity in the population. For this reason the final slopes of the survival curves for mixed multicomplexes do not change very much with multiplicity and differ considerably from the final slopes of the survival curves for monocomplexes, at all multiplicities. The slopes of the survival curves for homogeneous populations of complexes of multiplicity 1, 2, 3 decrease much more regularly than is apparent from the survival curves for mixed multicomplexes.

DISCUSSION

We want now to compare our results with the predictions of the recombination theory. Figure 1 contains both the theoretical curves and the experimental curves for the multiplicities 0.5 and 4. It is apparent immediately that the theoretical curves are not appropriate for describing the data since they contradict all the predictions of the theory. (1) The ultimate slope is not equal to that of the curves for a single particle, but is actually about one-fifth of that slope: this asymptotic slope is reached very quickly. (2) The intercepts with the axis of ordinates are below 200 for each of the experimental curves, although exceedingly high for the theoretical curves. (3) The curves obtained after maximum photoreactivation are similar in general shape to those obtained in darkness but are not related to them by a constant dose reduction. Their ultimate slope is only a fraction (between one-fifth and one-eighth) of the corresponding ultimate slopes for monocomplexes after photoreactivation.

For survival greater than about 10^{-3} the data do not differ widely from the theoretical curves. When the recombination theory was proposed, only data obtained at low dose were available, which seemed to give at least rough quantitative support to the theory. It now appears that this agreement was fortuitous. The present critical comparison between theory and experiments has brought to light important discrepancies which seem to invalidate the theory.

Nevertheless, since the discrepancies occur principally at high ultraviolet dose, one must consider the possibility that the recombination theory describes the basic mechanism of multiplicity reactivation and that the discrepancies arise from other complicating effects at high ultraviolet doses. One would have to assume, however, that the influence of such complicating effects is to increase the efficiency of the reactivation mechanism. Moreover, the discrepancies affect all characteristics predicted by the theory, and do so in an unmistakable way.

We conclude that the simple recombination theory of multiplicity reactivation does not give a satisfactory interpretation of the phenomenon.

According to the present experiments, the principal phenomenon in multiplicity reactivation is the reduction of the ultimate slope of the survival curves of multicomplexes, as compared with monocomplexes. This may be interpreted as a reduction of the cross section of the phage particles for ultraviolet light under conditions of multiple infection. Such a reduction may come about by a repair of the ultraviolet damage in multicomplexes, which does not take place in mono-

complexes; or it may come about by a mechanism which would make certain parts of the phage particle dispensable in multicomplexes. However, any substitutive mechanism in which a damaged part or activity in one of the infecting particles is simply replaced by a similar undamaged part or activity in another particle could not be reconciled with the present data since it would lead to predictions similar to those derived from the recombination theory.

SUMMARY

Precise survival curves for phage production by bacteria multiple infected with phage T2 inactivated by ultraviolet light have been determined for a wide range of ultraviolet doses, with and without photoreactivation. The results are not compatible with the recombination theory, according to which multiplicity reactivation is due to a recombination of undamaged units from several parental particles.

REFERENCES

ADAMS, M. H. 1950 Methods of study of bacterial viruses in "Methods in Medical Research," Vol. 2, The Year Book Publishers, Chicago.

DULBECCO, R. 1949 On the reliability of the Poisson distribution as a distribution of the number of phage particles infecting individual bacteria in a population. Genetics, **34**, 122–125.

DULBECCO, R. 1950 Experiments on photoreactivation of bacteriophages inactivated with ultraviolet radiation. J. Bact., **59**, 329–347.

DULBECCO, R. 1952 Mutual exclusion between related phages. J. Bact., **63**, 209–217.

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

LURIA, S. E. 1947 Reactivation of irradiated bacteriophage by transfer of self-reproducing units. Proc. Natl. Acad. Sci., **33**, 253–264.

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