EXPERIMENTS ON PHOTOREACTIVATION OF BACTERIOPHAGES INACTIVATED WITH ULTRAVIOLET RADIATION[1]

R. DULBECCO

Department of Bacteriology, Indiana University, Bloomington, Indiana[2]

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Kelner (1949), working with conidia of *Streptomyces griseus*, discovered that light belonging to the visible range is capable of reactivating biological material that has been rendered inactive by ultraviolet radiation (UV). Shortly after Kelner's discovery was known, a similar phenomenon in bacteriophages (bacterial viruses) was observed by accident. Plates of nutrient agar containing UV-inactivated phage and sensitive bacteria had been left for several hours on a table illuminated by a fluorescent lamp. After incubation it was noticed that the number of plaques was higher on these plates than on similar plates incubated in darkness. A short report of this phenomenon of "photoreactivation" (PHTR) has already been published (Dulbecco, 1949). The present paper contains the results of a first group of experiments concerning PHTR of seven bacteriophages of the T group active on *Escherichia coli*, strain B.

MATERIALS AND METHODS

Stocks of each phage were prepared by inoculating material from a single plaque into a culture of *E. coli* B in a synthetic medium M9,[3] except for phage T5, of which a stock in Difco nutrient broth was used. In some experiments the phage was purified by two or three steps of differential centrifugation; the phage was resuspended in M/15 phosphate buffer pH 7, with $MgSO_4$ added to a concentration 10^{-3} M. Unless otherwise specified, the experiments described in this paper were performed with phage T2. *Escherichia coli*, strain B, was used throughout. In some experiments bacteria were grown in nutrient broth with aeration and the culture was infected with phage when it was in the logarithmic phase of growth (about 10^8 cells per ml); these bacteria will be referred to as "bacteria in broth." In other experiments bacteria were grown in broth up to a concentration of about 2×10^8 cells per ml, then washed with saline (0.85 per cent NaCl) and resuspended in saline, kept at 37 C for 30 minutes, and then infected; these bacteria will be referred to as "resting bacteria."

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[2] Present address: Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena 4, California.

[3] NH_4Cl, 1.0 g; KH_2PO_4, 3.0 g; Na_2HPO_4, 6.0 g; NaCl, 0.5 g; $MgSO_4$, 0.1 g; distilled water, 1,000 ml; 4 g per liter glucose added after separate sterilization.

Inactivation of the phages was accomplished with a low-pressure mercury discharge lamp (General Electric "germicidal" lamp, 15 watts), giving most of the UV energy in the line 2,537 A. The output of the lamp was kept constant by alimenting it through a "sola" stabilizer and by using it only after it had been burning for at least 20 minutes.

The stocks to be irradiated were diluted in phosphate buffer plus $MgSO_4$ and exposed to the lamp at a 20-inch distance either in an open petri dish with continuous shaking (3 ml of phage in a 10-cm petri dish) or in a quartz cell 2 mm thick with parallel faces. Relative measurements of the incident UV doses were made in some experiments by timing the exposure; in other experiments rela-

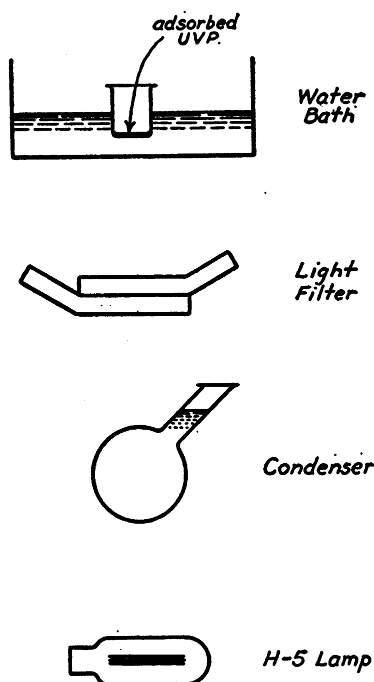

Figure 1. Diagram of the apparatus employed for illumination in liquid.

tive and absolute measurements were conducted with a calibrated Westinghouse SM-200 meter with tantalum photocell WL-775. A dose of UV will be expressed as seconds of exposure to the germicidal lamp. The reactivating light was used in two different ways:

Illumination on the plate. The plates, prepared by the agar layer method (Gratia, 1936; Hershey *et al.*, 1943), were exposed right side up to the light of two parallel fluorescent discharge lamps, 40 watts each, at a distance of 12 inches at room temperature.

Illumination in liquid. The apparatus used is illustrated in figure 1. A mercury discharge lamp, medium pressure (General Electric H-5 lamp, 250 watts) was used as the light source. The light was condensed through a spherical pyrex flask

filled with distilled water and passed through suitable filters (see later section); for white light experiments infrared rays were absorbed by a filter of $CuSO_4 \cdot 5H_2O$ (5 per cent in water, 1 inch thick) and ultraviolet rays shorter than 330 mμ by a Corning glass filter no. 738. A mixture of phage and bacteria was exposed to light in a small beaker (5 ml of mixture in a beaker 4 cm in diameter) kept in a thermostatically regulated water bath and shaken by a reciprocating motion in a horizontal plane to ensure uniform distribution of the material and uniform illumination. Some experiments were done with a 100-watt General Electric H-4 lamp without a condenser.

In the experiments with illumination in liquid the ratio "phage particles: bacteria" was kept very low (about 10^{-3}) to decrease the probability of multiple infection of bacteria and the occurrence of reactivation by multiplicity (Luria, 1947).

EXPERIMENTAL RESULTS

Role of the Bacteria in the PHTR of Inactive Phage

Phage particles inactivated by UV (UVP) can be reactivated by light only if the particles are mixed with sensitive bacteria during illumination. Illumination of UVP alone is without effect, as is shown by the following experiment: Phage T2 was irradiated with the germicidal lamp for 30 seconds (dark survival = 2×10^{-5}) and divided into two equal samples. The first sample was immediately plated and incubated in darkness; the second one was exposed to the light of a fluorescent lamp (80 watts at a 12-inch distance) for 1 hour at room temperature and then divided into two parts, one of which was plated and incubated in darkness, the other under the same light. The sum of plaque counts of two plates for each sample are given in table 1 (I).

In another similar experiment the UVP was first spread on the surface of a nutrient agar plate and then exposed to the light; after illumination sensitive bacteria were spread on the same plate in darkness. In this condition also PHTR was not produced.

These experiments clearly indicate that illumination of UVP in the absence of bacteria has no reactivating effect; they do not show, however, whether PHTR occurs only for adsorbed phage or also for nonadsorbed phage in the presence of bacteria. This point was investigated by mixing UVP with bacteria in nutrient broth without added NaCl (under these conditions the adsorption is slight), illuminating the mixture, and testing for reactivation of the nonadsorbed phage particles. A sample of phage irradiated with the germicidal lamp for 30 seconds was mixed with a culture of bacteria in broth without added NaCl, containing 10^9 cells per ml. The mixture was exposed to the light of an H-4 lamp at a 6-inch distance for 10 minutes at 28 C, then centrifuged; samples from the supernatant were plated and incubated both in darkness and in the light. The plaque counts (two plates for each sample) are given in table 1 (II), together with an assay of the irradiated phage diluted in broth by a factor equal to the one used in the experiment. The result of this experiment clearly indicates that the unadsorbed phage particles are not reactivated by light.

Illumination of bacteria alone followed by the addition of UVP does not produce any PHTR. Bacteria spread on the surface of several nutrient-agar plates were exposed to the light of a fluorescent lamp (80 watts, 12-inch distance) for 4 hours at room temperature; then UVP were spread on the same plates in darkness, and the plates were incubated in darkness. Control plates, spread with bacteria at the same time, were kept in darkness and received UVP at the same time as the illuminated plates. Equal numbers of plaques were found in all plates whether the bacteria had been preilluminated or not, showing that preillumination of bacteria does not cause PHTR of UVP added later. In another experiment a suspension of resting bacteria was illuminated with a light of 365-mμ wave length at 37 C for a period long enough to give a very high PHTR in adsorbed UVP, and the UVP was added at the very moment at which the light was turned off; no measurable PHTR was observed.

TABLE 1

Effect of light on inactivated phage T2 alone and on unadsorbed inactivated phage T2r mixed with sensitive bacteria

EXPERIMENT	TREATMENT	PLAQUE COUNT 0.1 ML
I. Illumination of UVP alone	1. UVP not illuminated and plated with B. Plates incubated in darkness.	17
	2. UVP illuminated alone and plated with B. Plates incubated in darkness.	6
	3. UVP illuminated alone and plated with B. Plates incubated under light.	609
II. Effect of light on unadsorbed phage in presence of bacteria	1. UVP alone.	72
	2. UVP mixed with B in saltless broth; illuminated 10 minutes; centrifuged; supernatant plated with B; plates incubated in darkness.	86
	3. Same as II, 2, but with plates incubated under light.	>1,000

If bacteria killed by heating to 60 C for 20 minutes are substituted for living bacteria, no PHTR takes place. Actually the plaque count decreases, probably because of an irreversible adsorption of phage by the dead bacteria without the release of new phage.

Illumination of bacteria prior to infection does not diminish the photoreactivability of UVP added later, as shown by the following experiment: Bacteria were spread on the surface of nutrient agar plates and exposed to the light of a fluorescent lamp (80 watts, 12-inch distance) for 4 hours at room temperature; then UVP was spread on the plates, which were afterwards incubated under the same light. After incubation the plates showed the same number of plaques as control plates containing nonpreilluminated bacteria and UVP, incubated under the same light.

From these experiments with phage T2 one may conclude that PHTR occurs only for UVP adsorbed on sensitive bacteria and that illumination either of UVP or of bacteria before infection has no detectable effect.

To test how soon after phage adsorption PHTR can occur, UVP and bacteria were mixed on several plates, and the plates were immediately exposed to the light of an H-4 lamp at an 8-inch distance at 28 C. The exposure was continued for 10, 20, 30, or 50 seconds. The plaque count was found to increase even after 10 seconds, showing that no measurable delay exists between adsorption and the beginning of PHTR and that PHTR has no measurable latent period.

Action of Bacterial Extracts on PHTR

Some attempts were made to obtain PHTR by illuminating mixtures of UVP with cell-free bacterial extracts. Bacteria were grown in nutrient broth to a concentration of about 5×10^8 cells per ml and harvested in a Sharples centrifuge. Two extraction procedures were used: (a) the thick bacterial suspension was frozen at -30 C, the frozen paste was then ground with carborundum powder and extracted with phosphate buffer (pH 7.5) for about 10 minutes, and the extract was clarified by centrifugation; (b) the bacteria were broken in a sonic vibrator after the bacterial paste was diluted with an equal volume of phosphate buffer, and the extract was clarified in the centrifuge. In both cases the supernatant was a thick, yellowish liquid, which showed a high degree of enzymatic activity (methylene blue reduction, tryptophanase). Both extracts still contained a few living cells, which could be eliminated either by filtration or by repeated freezing at -30 C.

UVP was mixed into various dilutions of the extracts, and the mixtures were kept either in light or darkness and assayed for active phages at different times. Only extracts still containing living cells gave some PHTR. Removal of almost all living cells eliminated PHTR.

PHTR as a Function of the Dose of the Inactivating UV Light

For several phages of the T group (T2, T4, T5, T6) the curve obtained by plotting the logarithm of the active fraction against the UV dose approaches a straight line (Latarjet and Wahl, 1945), at least for high values of the dose, whereas an inflection with downward concavity, of dubious origin, may appear for low doses. Three other phages (T1, T3, T7) show, on the contrary, an inflection with upward concavity of unknown origin.

If the inactivated phages are adsorbed on bacteria and exposed to light of high intensity for a sufficient length of time, the active fraction increases and reaches a maximum (see later section). After this maximum is reached, the curve showing the logarithm of the active fraction against the UV dose has for each phage the same shape as the curve obtained in darkness, but for a given UV dose the slope of the curve obtained after PHTR is lower than the slope of the curve in darkness.

The fact that both curves in the light and in darkness tend to be straight lines with different slopes for high UV doses is an indication that absorption of

UV light in the phage has a probability, a, of producing a photoreactivable inactivation and a probability, b, of producing a nonphotoreactivable inactivation; $a + b$, the probability of producing any inactivating damage, is proportional to the cross section of the phage for UV. Assuming $a + b = 1$, a is the photoreactivable sector of the cross section, b the nonphotoreactivable sector; b is measured by the ratio of the slope of the curve after maximum PHTR to the slope of the curve in darkness, both measured in the straight parts.

The photoreactivable sector, a, varies between 1 (complete photoreactivability) and 0 (no photoreactivability) and can therefore be used as an index of the photoreactivability. Values of a for different phages are given in table 2.

Influence on PHTR of the Interval of Time between Infection and Exposure to Light

In the experiments reported in the present and following sections the influence of various experimental conditions on PHTR was analyzed. A quantitative determination of PHTR was made by measuring either the "active fraction" or the "amount of PHTR" in an UVP sample after a given exposure to light. The active fraction is the ratio of the number of active particles after PHTR to the total number of adsorbed particles and is equal to the sum of the fraction active in the darkness plus the fraction reactivated by light; the amount of PHTR is the reactivated fraction.

TABLE 2

Photoreactivability of the phages of the T group

PHAGE	T1	T2	T3	T4	T5	T6	T7
Photoreactivable sector of cross section (a)	0.68	0.56	0.39	0.20	0.20	0.44	0.35

The influence on PHTR of the interval of time between infection and exposure to light was determined for UVP adsorbed on bacteria in broth and on resting bacteria (see "Material and Methods"). Bacteria and UVP were mixed in darkness; samples of the mixture were kept in darkness for various intervals of time and then exposed to light for a period long enough to produce maximum PHTR. After illumination, samples were plated and incubated in darkness, and the active fraction was determined. In this procedure the bacteria infected with irradiated phage particles had to be exposed to light much longer than the latent period between infection and liberation of phage adsorbed on bacteria in broth. When bacteria in broth were used, therefore, the mixtures were plated before the end of the latent period and illumination was continued by exposing the plates; when resting bacteria were used, illumination could be continued indefinitely in liquid, since no phage liberation takes place under these conditions.

Experiments with bacteria in broth. The experiments were performed with phage T2 at 28 C. The amount of PHTR decreased rapidly as the time interval between infection and the beginning of exposure to light increased; after about 20 minutes only a small amount of PHTR was produced, as is shown in table 3.

This decrease in PHTR might be caused by a gradual decrease in the amount of PHTR per time unit as the time interval between infection and illumination increases, by a limitation of the time interval after infection in which PHTR can occur, or by both. The amount of PHTR per time unit was determined in experiments in which exposure to light was started at various times after infection. The results, shown in figure 2, indicate that the amount of PHTR per time unit remained practically constant for about 15 minutes. The decline in maximum PHTR must be due, therefore, to a limitation of the time within which PHTR can occur after infection, the useful time interval ending between 20 and 30 minutes after infection under the experimental conditions; after this time very little or no PHTR can take place.

Experiments with resting bacteria. As is shown in table 4, the maximum amount of PHTR obtainable in phage T2r irradiated with the germicidal lamp for 18 seconds remains fairly constant for at least 70 minutes after infection at 37 C;

TABLE 3

The effect of the time interval between infection and exposure to light (bacteria in broth)

Phage T2r, irradiated for 20 seconds with the germicidal lamp, was mixed with bacteria and adsorption was allowed to continued for 2 minutes, after which it was interrupted by serum anti-T2. Exposure to light (H-4 lamp, 12-inch distance) was begun at various times and was continued for 100 minutes at 28 C. Amount of PHTR is lower than in experiment reported in table 4, because in the present experiment a lower light intensity was used, and the time in which the light could be utilized for reactivation was limited, since bacteria in broth were used.

TIME INTERVAL BETWEEN INFECTION AND EXPOSURE TO LIGHT	ACTIVE FRACTION
min	
0	5.3×10^{-3}
10	1.4×10^{-3}
20	3.5×10^{-4}
30	5.0×10^{-4}
Active fraction in darkness	3.0×10^{-4}

longer intervals have not been tested. The amount of PHTR per time unit is not influenced by the time interval between infection and illumination.

The differences between experiments with bacteria in broth and with resting bacteria indicate that under the experimental conditions the system "UVP-metabolizing bacteria" undergoes a gradual change that in its late phases prevents PHTR, a change absent in the system "UVP–resting bacteria."

Kinetics of PHTR

PHTR as a function of the time of exposure to the reactivating light. The following experiments employed inactive phage T2r and resting bacteria, with illumination in liquid. Inactive phage diluted in phosphate buffer was mixed with bacteria at time 0 at 37 C in darkness, and 10 minutes were allowed for complete adsorption. At the eleventh minute a sample was plated in darkness; at the twelfth minute the mixture was exposed to light, and samples were taken at

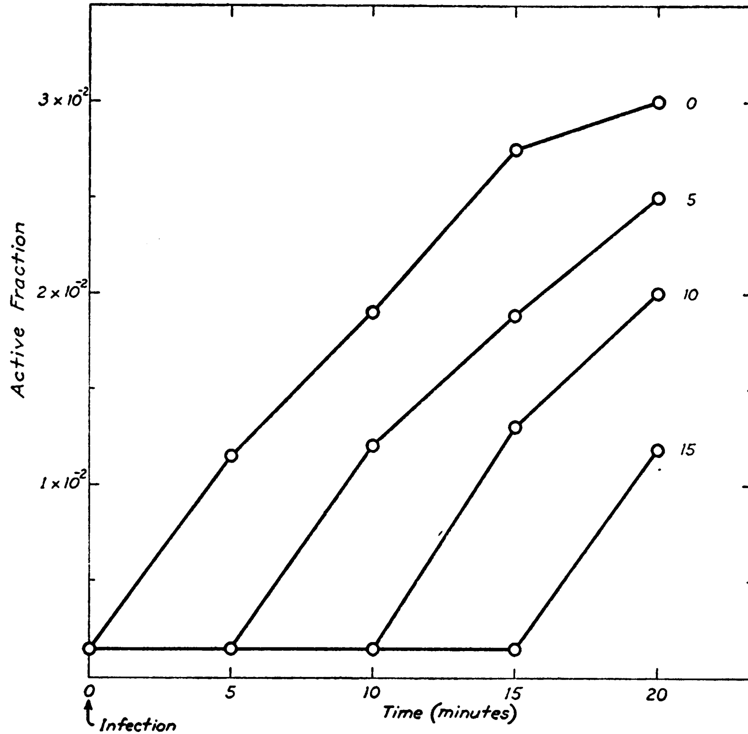

Figure 2. The fraction of active particles as a function of the time of illumination (in minutes) and of the interval between infection and exposure to light. Each curve gives the active fraction as a function of the time of illumination (in minutes) for a different interval between infection and exposure to light; the interval is indicated (in minutes) at the right end of each curve. Phage T2r was irradiated for 20 seconds with the germicidal lamp, adsorbed on bacteria in broth, and exposed to light in broth at 28 C.

TABLE 4

The effect of the time interval between infection and exposure to light (resting bacteria)

Phage T2r, irradiated for 18 seconds with the germicidal lamp, was adsorbed onto resting bacteria suspended in saline. Exposure to light (H-5 lamp with condenser, wave length 365 mμ) began at various times. Illumination was carried out in liquid.

TIME INTERVAL BETWEEN INFECTION AND EXPOSURE TO LIGHT	ACTIVE FRACTION
min	
0	5×10^{-2}
10	5×10^{-2}
30	6×10^{-2}
50	5.4×10^{-2}
70	6×10^{-2}
Active fraction in darkness	10^{-3}

various time intervals thereafter and plated in darkness; all plates were incubated in darkness. The experiments lasted 140 minutes at most; control experi

ments showed that resting bacteria that have adsorbed active phage do not liberate any phage in this time interval. The active fraction always increased with the time of illumination, the increase becoming less and less with increasing time, so that a maximum was reached as is shown in figure 3. The time at which the maximum was reached depended on the light intensity, a longer time being required when the intensity was lower; when the light intensity was varied in such a way that the maximum was reached in a period between 20 and 140 minutes, approximately the same maximum was reached in all cases, as is shown in figure 3.

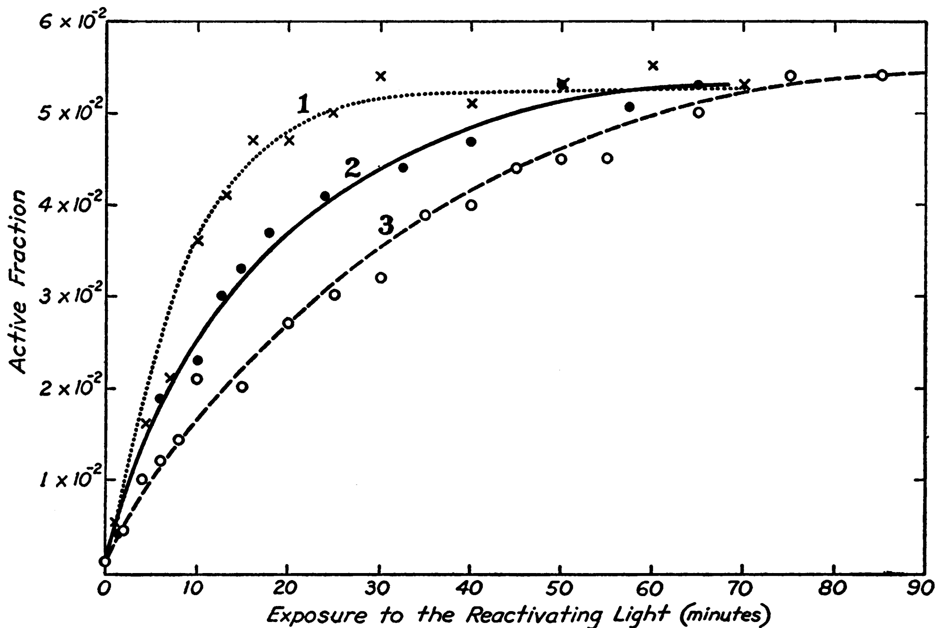

Figure 3. The fraction of active particles as a function of the time of illumination and of the light intensity. The active fraction is plotted against the time of illumination (in minutes). Phage T2r was irradiated for 20 seconds with the germicidal lamp, adsorbed on resting bacteria, and illuminated in liquid at 37 C. Curve 1 was obtained with a light of intensity 10 (in arbitrary units), curve 2 with a light of intensity 2.9, and curve 3 with a light of intensity 0.6.

The amount of PHTR (defined in previous section) observed in a sample of UVP after a given time of illumination ($p(t)$), divided by the amount of PHTR obtained in the same sample when PHTR has reached the maximum value ($p(\infty)$), will be indicated at the "relative amount of PHTR"; it can vary between zero and one.

By subtracting the relative amount of PHTR from unity, one obtains the fraction of photoreactivable particles that are still inactive after a time, t, of illumination $\left(= 1 - \frac{p(t)}{p(\infty)}\right)$. The logarithm of this quantity plotted against the time of illumination always gave a straight line for different intensities of

the reactivating light and for different doses of the inactivating UV. A curve of this type is reproduced in figure 4. The linearity of the experimental curves was found to be statistically significant by comparing, with the χ^2 test, the experimental data for the active fractions with data calculated on this assumption, as is shown in table 5. This result shows that PHTR is a one-hit phenomenon; a

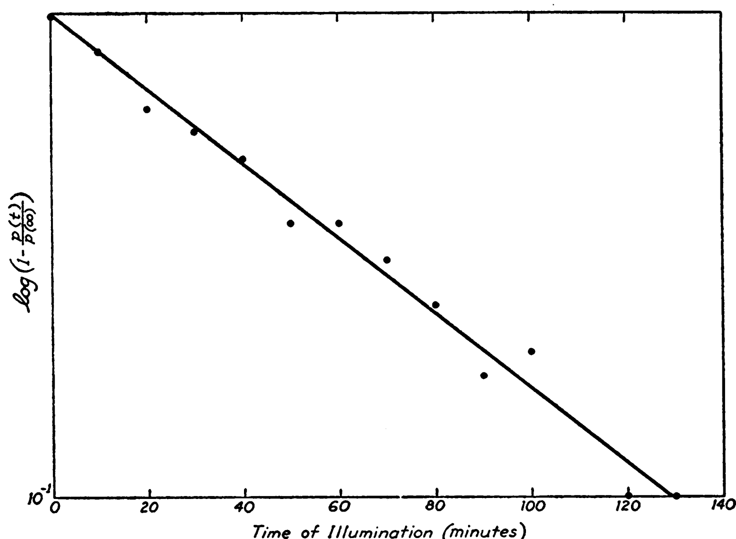

Figure 4. The logarithm of the fraction of photoreactivable particles that has not been reactivated after a given time of illumination $\left(1 - \frac{p(t)}{p(\infty)}\right)$ plotted against the time of illumination (in minutes). Phage T2r was irradiated for 20 seconds with the germicidal lamp, adsorbed on resting bacteria, and illuminated in liquid at 37 C.

TABLE 5

A comparison between observed and calculated active fractions after different times of illumination

EXPERIMENT NO.	UV DOSE IN SECONDS	DEGREES OF FREEDOM	χ^2	P
312	10	10	12.1	>0.20
313	30	11	10.6	>0.40
315	20	12	14.3	>0.20
319	20	11	19.8	0.05

photoreactivable particle is reactivated by one quantum only, independently of the UV dose.

The dependence of the amount of PHTR on the time of illumination is expressed by the equation

$$p(t) = (1 - e^{-ft})\,F(D)$$

in which t is the time of illumination, $F(D)$ the photoreactivable fraction, which is a function of the dose, D, of UV. Value f is the probability per time unit that

a particle is photoreactivated and can be called the PHTR rate; it is proportional to the slope of the line giving $\log\left(1 - \frac{p(t)}{p(\infty)}\right)$ versus time of illumination. Value f may depend on several variables, such as dose of UV, intensity of the reactivating light, temperature, and metabolic condition of the bacteria during PHTR. This dependence will be examined in the next sections.

Dependence of PHTR rate on dose of UV and intensity of the reactivating light. PHTR rate (f) was determined for UVP inactivated with different UV doses, adsorbed on resting bacteria, and illuminated in liquid at 37 C with light of constant intensity; it was found to be approximately constant for doses of UV between 10 and 30 seconds. The results, however, are not yet definite on this point, and a decrease of f by a factor 1.2 when the UV dose increases from 10 to 30 seconds cannot be excluded. This result shows that the probability for an adsorbed quantum to reactivate a photoreactivable phage particle is practically independent of the inactivating UV dose.

Value f was also determined for different intensities of the reactivating light on UVP inactivated with the same UV dose, adsorbed on resting bacteria, and illuminated in liquid at constant temperature. The intensity was varied either by changing the distance of the sample from an H-4 lamp—assuming the intensity to be inversely proportional to the square of the distance—or by lowering the intensity of a monochromatic light by filters and measuring with a thermopile the relative intensities. Value f was found to increase almost linearly with light intensity for low intensities but for high intensities to tend to a maximum as is shown in figure 5. The highest value of the PHTR rate observed in these experiments was about 1.4×10^{-3} sec^{-1} and corresponds to a half-time of about 8 minutes.

For low light intensities, f being a linear function of the intensity, the probability of PHTR occurring in a bacterium-phage complex is a linear function of the dose of the reactivating light (equal to intensity $\times$ time), whereas for high intensities the same dose has less effect. For low intensities and relatively short exposures the dependence of amount of PHTR on light dose is also approximately linear.

Action Spectrum of PHTR

Seven wave lengths were tested for photoreactivating activity. The corresponding monochromatic lights were obtained in the following ways (see Bowen, 1946):

(1) Group of lines near 313 mμ of the mercury arc (with a small amount of 334 mμ). Light: mercury lamp H-4 without glass envelope. Filter: 3 cm $NiSO_4 \cdot 7H_2O$, 350 g + $CoSO_4 \cdot 7H_2O$, 10 g made up to a liter with water; 1 cm potassium hydrogen phthalate, 5 g in 1,000 ml water.

(2) Group of lines 365 mμ of the mercury arc. Mercury lamp H-5 (General Electric); Corning glass filter combination nos. 738, 5860.

(3) Group of lines 404 mμ of the mercury arc. Lamp H-5. Filter: 2 cm $Cu(NO_3)_2 \cdot 6H_2O$, 200 g in 100 ml water. Iodine 0.75 g in 100 ml carbon tetrachloride.

(4) Group of lines 434 mμ of the mercury arc. Lamp H-5. Filter: 2 cm $CuSO_4 \cdot 5H_2O$, 25 g + 300 ml ammonium hydroxide (d = 0.88), made up to 1 liter with water; 1 cm $NaNO_2$, 75 g in 100 ml water.

(5) Band around 500 mμ (between 480 and 520 mμ, center 500 mμ). Projection lamp with ribbon filament. Filter: Wratten no. 47, Wratten no. 58, 2 cm $CuSO_4$, 5 per cent.

(6) Line 546 mμ of the mercury arc. Mercury discharge lamp H-5. Corning glass filter combination, nos. 3484, 4303, 5120.

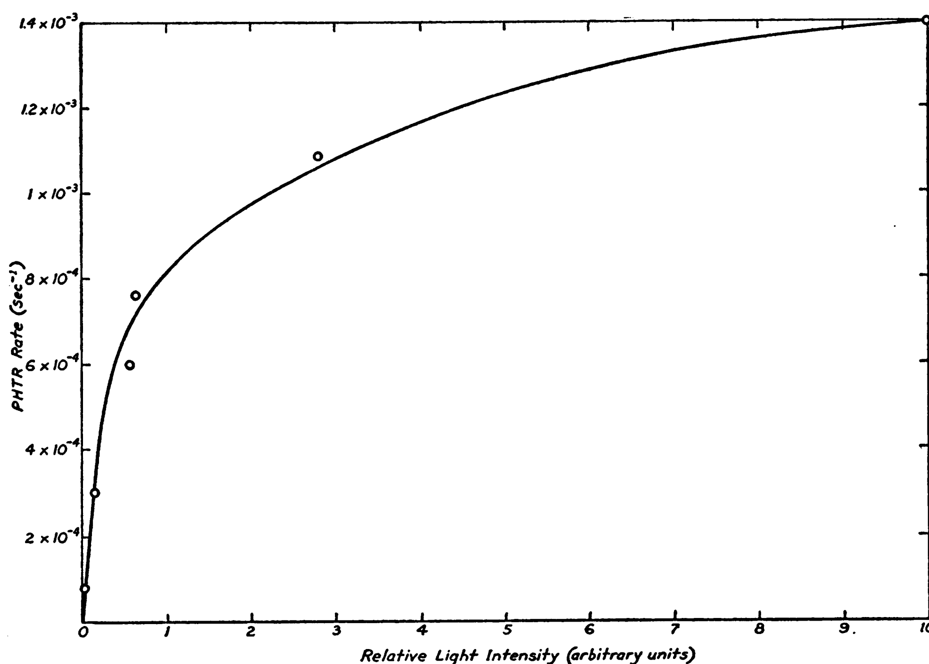

Figure 5. The PHTR rate as a function of the intensity of the reactivating light. The PHTR rate is expressed in sec^{-1} and the light intensity in arbitrary units. Phage T2r was irradiated for 20 seconds with the germicidal lamp, adsorbed on resting bacteria, and illuminated in liquid at 37 C.

(7) Group 576–579 mμ of the mercury arc. Lamp H-5. Filter: 1 cm mixture of $CuCl_2 \cdot 2H_2O$, 10 g in 10 ml water and $CaCl_2$, 3 M, 90 ml; 2 cm $K_2Cr_2O_7$, 15 g in 200 ml water.

The efficiency of the different lights was determined in the following way: For each light the range of intensity was first determined, in which the PHTR rate is approximately proportional to the light intensity; the intensity of the most effective wave lengths was reduced by filters until it fell into this range. The rate of PHTR was then determined for each wave length, and the relative intensities were measured with a thermopile. The time of illumination was short, so that the amount of PHTR was very nearly proportional to the dose of reactivating light (see previous section).

The dose of light of each wave length necessary to give a standard amount of

PHTR in a given time was calculated from these data, and the reciprocal of this dose (given in arbitrary units) was taken as a measure of the activity of that wave length. In figure 6 the activity of the wave lengths tested is plotted against the wave length.

The activity of a given light may be underestimated, since it is known that light of the wave lengths used in PHTR may damage the bacteria (Hollaender, 1943) or the phages (Wahl and Latarjet, 1947). The killing action of the seven wave lengths on active phage adsorbed on bacteria was therefore determined, and it was found that with the light intensity and the time of illumination used in the PHTR experiments an appreciable killing activity was only evident for wave length 313 mμ. To correct for this killing activity, the amount of PHTR

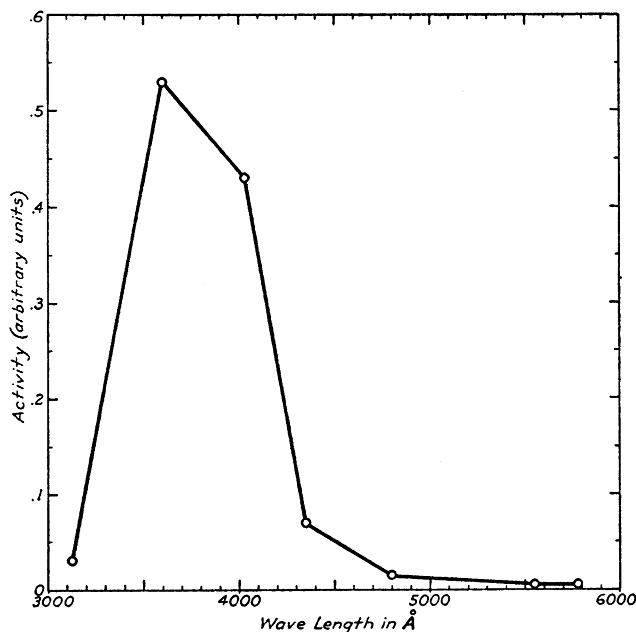

Figure 6. The action spectrum of PHTR. The activity of each wave length, given in arbitrary units, is plotted against the wave length. Phage T2r was irradiated for 20 seconds with the germicidal lamp, adsorbed on resting bacteria, and illuminated in liquid at 37 C.

obtained after a given exposure to this light was increased by a factor equal to the decrease in titer of active phage adsorbed on B exposed to the same light for the same length of time in equal experimental conditions. The curve of PHTR as a function of the time of exposure to 313 mμ light, obtained in this way, was almost linear and was used in calculating the activity of the light.

The activity of the seven wave lengths tested gives only the general shape of the action spectrum. It consists of a band covering the range from about 300 mμ on the side of the short wave lengths to about 500 mμ on the side of the long ones, with a maximum around 365 mμ. The greatest photoreactivating activity occurs therefore in the near ultraviolet.

The action spectrum of PHTR is related to the absorption spectrum of the

pigment that absorbs the reactivating light (see Loofbourow, 1948, for discussion of this relation); we tried, therefore, to obtain on this basis some information about the photosensitive pigment. The action spectrum is not detailed enough to give a specific indication; it shows, however, that the pigment is not contained in the unmodified phage, since the absorption spectrum of purified

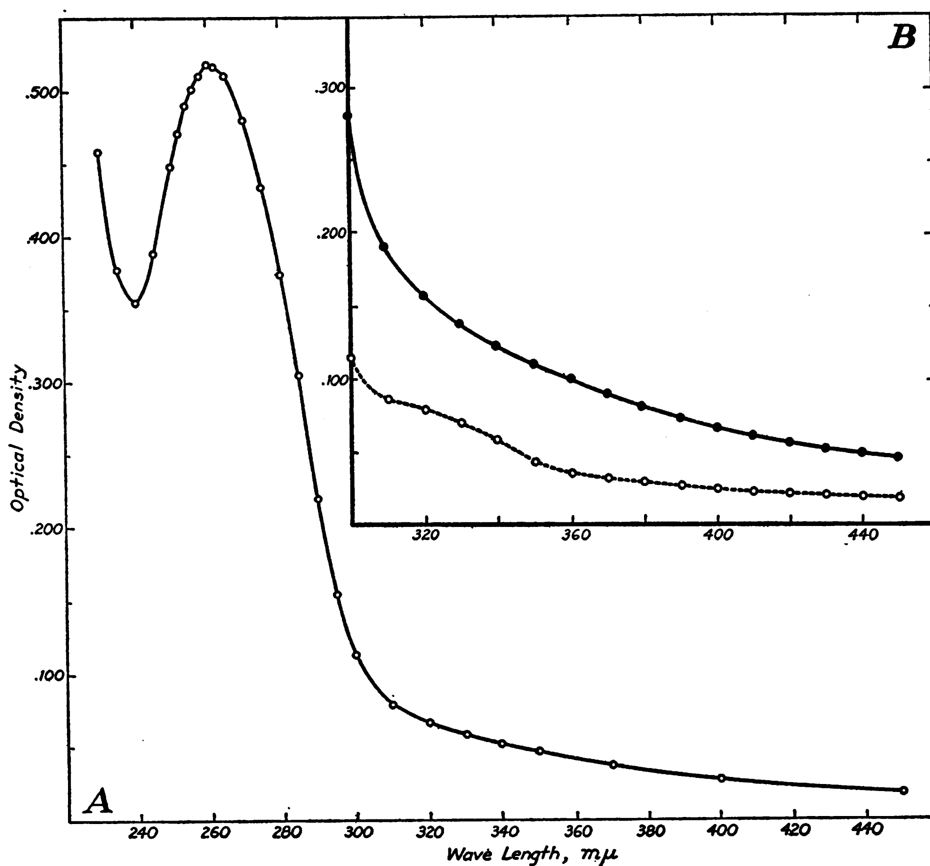

Figure 7. Absorption spectra of purified phage T2. The optical density is plotted against the wave length. The spectra were obtained with a Beckman quartz spectrophotometer. The phage was suspended in phosphate buffer (M/15, pH 7) plus $MgSO_4$ 10^{-3} M. *A*. Active phage, concentration 5.5×10^{10} infecting units per ml. *B*. Upper curve: absorption spectrum of active phage for wave lengths longer than 320 mμ, concentration 2.3×10^{11} infecting units per ml; lower curve: absorption spectrum of the same phage after 2 hours' irradiation with the germicidal lamp at a distance of 12 inches.

phage has no band comparable to the action spectrum of PHTR. This is shown by figure 7*A*, which reproduces the absorption spectrum of purified phage T2. For wave lengths longer than 320 mμ the absorption closely follows Rayleigh's law of scattering and is, therefore, due to scattering of the light. This is shown more convincingly by plotting the logarithm of the optical density at different wave lengths against the logarithm of the wave length; according to Rayleigh's

law one should obtain a straight line with slope 4 (see Oster, 1948, 323, formula 6). As shown in figure 8*A*, the curve, obtained from the same data used for figure 7*A*, is a straight line in the range of wave lengths 320 to 450 mμ; the slope of the curve is 3.7, instead of 4, owing to the size of the phage particles, which is larger than required for the strict application of Rayleigh's law (La Mer, 1948).

The pigment might be formed in the phage after UV irradiation. To check this point a suspension of purified phage T2 containing 2.3×10^{11} particles per ml was irradiated in an open shallow container with the germicidal lamp at a distance of 12 inches for variable lengths of time up to 4 hours, and the absorption

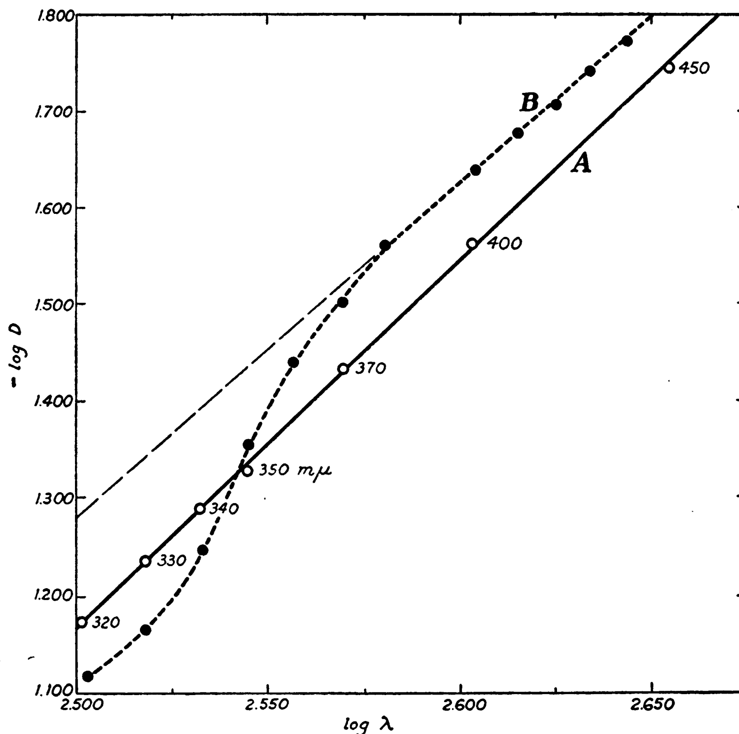

Figure 8. The logarithm of the optical density, *D*, of purified phage T2 versus the logarithm of the wave length for the range 320 to 450 mμ. *A*. Active phage (the same data as in figure 7*A*). *B*. Phage after long UV irradiation (same data as in figure 7*B*). Dashed line shows the curve expected for pure scattering.

spectrum was determined at regular intervals. The absorption spectrum was found to undergo complex changes as the irradiation proceeded; we shall limit our attention to the modifications occurring in the range of wave lengths longer than 320 mμ. A general decrease in absorption in this region was observed, and after about one hour of irradiation a faint band was noticed, which became more evident during the next hour (figure 7*B*). This band has maximum absorption around 330 mμ and extends to about 380 mμ on the side of longer wave lengths (figure 8*B*); its limit on the side of shorter wave lengths cannot be determined because of overlapping with the general phage absorption.

The maximum absorption of this band is located at a shorter wave length than the maximum of the action spectrum of PHTR; this difference, however, is not such as to exclude the band from belonging to the photosensitive pigment, because the location of the band may be shifted toward longer wave lengths if the pigment is bound with some bacterial constituent after adsorption of the inactive phage on bacteria.

Influence of the Metabolic Conditions of Bacteria in PHTR of Phages

PHTR is not appreciably different whether the phage is adsorbed by bacteria in broth or in a synthetic medium; the rate of PHTR is somewhat lower with resting bacteria than with bacteria suspended in nutrient media.

The influence of oxygen on PHTR was determined. Resting bacteria and UVP were placed in separate compartments of a Thunberg tube, nitrogen was bubbled for about 20 minutes through the bacterial suspension, the tube was then evacuated by a pump, and air was replaced with nitrogen, the operation being re-

TABLE 6

PHTR rate and Q_{10} at different temperatures

The Q_{10} was determined from the ratio of Q_d of the rates at two successive temperatures, using the formula: $Q_{10} = (Q_d)^{10/d}$, where d is the temperature interval between two observations. Phage T2r irradiated for 20 seconds with the germicidal lamp, illuminated in liquid.

TEMPERATURE (C)	PHTR RATE (SEC^{-1})	Q_{10}
3	1.25×10^{-5}	
11	6.2×10^{-5}	7.5
16	1.3×10^{-4}	4.2
24	2.3×10^{-4}	2.1
30	3.9×10^{-4}	2.4
37	5.6×10^{-4}	1.7

peated 4 times. Phage and bacteria were mixed and the tube was exposed to light. The control consisted of an open tube from which oxygen had not been removed. The same amount of PHTR was observed in both tubes. Oxygen is therefore not necessary for PHTR, at least not for the initial photochemical reaction. We also found that cyanide in 10^{-2} M concentration does not affect PHTR.

The Effect of Temperature on PHTR

PHTR can occur at temperatures too low to allow growth of active phage (+1 C). To obtain a measurable amount of PHTR at this temperature, one must mix UVP and bacteria in the dark at 37 C to allow enough adsorption, then chill the mixture to 1 C and expose it to light.

Determinations of PHTR rate with constant illumination were made at 3, 11, 16, 24, and 37 C, using phage T2 irradiated for 20 minutes with the germicidal lamp. The Q_{10} was determined for each interval, and the results are reproduced in table 6.

The behavior of the Q_{10} is similar to that found for complex bacterial activities and for enzymatic reactions (Rahn, 1932) and is an indication that the physiological state of the bacterium conditions the probability of the photoreactivating event.

PHTR of Phage Inactivated with X-Rays

It was previously reported (1949) that no PHTR had been detected for phage inactivated by X-rays. This statement is valid only if X-ray irradiation is performed on phage in synthetic medium. With phage T2 inactivated by X-rays in nutrient broth a slight amount of PHTR can be observed. This amount is probably reduced by the poor adsorption of phage inactivated by X-rays, as discovered by Watson (1948). After correction for the limited adsorption (data kindly supplied by Watson), the PHTR of X-ray-inactivated phage is still considerably less than that for phage inactivated to the same extent by UV.

SUMMARY AND DISCUSSION

In the following brief discussion we shall try to arrange the results of our experiments in an order that will bring out their theoretical implications, and we shall present a working hypothesis for the mechanism of PHTR.

(1) The damage caused by UV in bacteriophage consists of two kinds: photoreactivable and nonphotoreactivable damage. These two kinds of damage occur with comparable cross sections; they may reflect the presence of two kinds of UV-absorbing constituents in each phage particle. Further information should be obtainable by determining the action spectrum for the two types of inactivation.

(2) Only phage particles adsorbed on bacteria undergo PHTR; PHTR can occur within a few seconds after adsorption, indicating that PHTR is due to reactions occurring in the early phase of the interaction between inactive phage and bacterium; perhaps surface reactions are involved, and this may account for the failure to reproduce PHTR with bacterial extracts. PHTR does not require the presence of external metabolic substrates or of oxygen and is not inhibited by cyanide, but is influenced by the physiological condition of the bacteria after infection.

(3) The photosensitive pigment has an action spectrum with a maximum near 365 mμ. Normal phage does not have an absorption band corresponding to this action spectrum. UV-treated phage shows an absorption band with a maximum near 330 mμ. Perhaps this is the photosensitive pigment created by UV irradiation in the phage. The shift from 330 to 365 mμ could be due to the binding of the pigment with bacterial constituents upon adsorption of a phage particle on the bacterium. The alternative possibility is that the photosensitive pigment exists in the bacterium prior to infection. Further studies of the absorption band of UV-irradiated phage and of the action spectrum of PHTR are needed.

(4) At low intensities of illumination the probability of PHTR occurring in a bacterium-phage complex is proportional to the dose of light. From this we might conclude that the individual light quanta absorbed by the bacterium-

phage complex do not co-operate to produce PHTR but that each quantum individually has a chance to accomplish PHTR and that this chance is independent of any other quanta absorbed by the complex. We would thus be led to conjecture that PHTR is due to a primary photochemical reaction.

(5) The picture is complicated by the finding that at high intensities the rate of PHTR ceases to be proportional to the intensity of illumination, reaching a maximum value, and by the finding of a complex temperature dependence. These findings require the participation of dark reactions in the mechanism of PHTR.

(6) The probability of PHTR per time unit (PHTR rate) is practically independent of the dose of UV used for inactivation. This finding shows that photoreactivation is an all-or-none phenomenon, and it may indicate that photoreactivable inactivation is always due to one injury, elimination of which restitutes activity.

To explain all the known features of PHTR, we propose the following working hypothesis: The photoreactivable inactivation is due to formation of molecules of an inhibitor in the phage; although many of these molecules may be formed in one phage particle, just one molecule is the inactivating one in each case, for example, by blocking a reaction necessary for phage growth in a small area of the contact surface between phage and bacterium. Restoration of phage activity requires the permanent removal of the inactivating inhibitor molecule. Dissociation does not occur by thermal activation; but absorption of a light quantum of a given wave length produces a transient and reversible dissociation. During the time the inhibitor is dissociated it can be captured by a receptor and destroyed with dark reaction. This makes the removal permanent and constitutes reactivation.

PHTR therefore requires a system made by phage, inhibitor, pigment, and receptor. The inhibitor belongs to the phage, the receptor to the bacterium being perhaps of enzymatic nature; the pigment may belong to either one and may be identified either with the inhibitor or with the receptor. The system is completed after adsorption of the inactive phage on bacteria.

The probability of PHTR (PHTR rate) for low light intensity is proportional to the time integral in which the inhibitor is dissociated and therefore to the number of quanta absorbed (dose of the reactivating light). For high intensities the activation times due to absorption of different quanta overlap somewhat, so that equal doses become less efficient. When the light intensity is so high that the inhibitor is dissociated without interruption during illumination, the probability of PHTR reaches a maximum value. The probability of PHTR is also proportional to the probability that the dissociated inhibitor will be captured and destroyed by the receptor in the time unit and therefore depends on temperature, which influences the dark reactions, and on some physiological conditions of the bacteria, which may affect the efficiency or the amount of the receptor.

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