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The Behaviour of *Rhizobium* Bacteriophages during and after Exposure to Ultraviolet Radiation

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SUMMARY: After inactivation by ultraviolet radiation, particles of two *Rhizobium* bacteriophages interfered temporarily with the multiplication of active particles of the homologous phage, in liquid cultures of their respective host bacteria. Inactivated particles did not affect the number of plaques produced by active particles in bacterial cultures on agar.

No evidence was found that particles that were inactive singly became active when two or more of them infected the same bacterial cell.

The rate of inactivation approximated closely to that of a first-order reaction. Exposing infected bacteria to visible light increased the residual activities of irradiated phage preparations by amounts equivalent to decreasing the doses of ultraviolet irradiation by a constant factor. Exposing either the irradiated phage preparations or the bacterial cultures separately to visible light had no effect.

Those ultraviolet irradiated phage particles which remained active were so altered that they became relatively unstable.

Much work has been done on the effects of ultraviolet radiation on bacteriophages of *Bacterium coli*, but relatively little on others. The course of inactivation, i.e. of becoming unable to multiply and cause lysis, was found to approximate to that of a first-order reaction, though slight deviations have been recorded (Latarjet & Wahl, 1945; Latarjet & Morenne, 1951). Some inactivated phages have proved able to interfere with multiplication of host bacteria and of active phages (Luria & Delbrück, 1942). Residual activity of some irradiated phage preparations could be increased by increasing their concentration at which they were brought into contact with susceptible bacteria ('multiplicity reactivation' Luria & Dulbecco, 1949) or by exposure of infected host bacteria to visible light ('photo-reactivation' Dulbecco, 1950).

The work described in the present paper was started to see how far these phenomena applied to bacteriophages of strains of *Rhizobium* and was done simultaneously with a comparable work on plant viruses (Bawden & Kleczkowski, 1953). During the work a new phenomenon was encountered; when irradiated phage preparations were stored their residual activity decreased at a much higher rate than did the activity of unirradiated phage preparation stored in the same conditions. A few experiments were made to study some details of this phenomenon.

MATERIAL AND METHODS

Two strains, 317 and Cl₅, of pea and clover nodule bacteria, respectively, and their two homologous bacteriophages, 317 and Cl₅, were used. Each bacteria strain was lysed by its homologous phage but not by the other. The bacterial strains, the bacteriophages, media and the 'poured plate' method of

producing plaques, have been described previously (Kleczkowska, 1945; Kleczkowski & Kleczkowski, 1952). Phage cultures were lysed liquid bacterial cultures passed through a Chamberland L3 filter and stored at 2°.

The source of ultraviolet (u.v.) radiation was a low-pressure mercury discharge lamp made by the Thermal Syndicate Ltd. and fitted with a cylindrical chromium-plated reflector. According to the makers' specifications the intensity of radiation, 99 % of which was of wave-length 2536 Å, was 121 μ W./sq.cm. at a distance of 100 cm.

Undiluted phage cultures were irradiated as layers 0.15 cm. deep in a Petri dish. They were exposed to the lamp at a distance of 20 cm. and rocked during the whole time of exposure. The rocking is assumed to have ensured that all phage particles were equally exposed to the radiation.

The intensity of radiation and its absorption by irradiated materials were not measured. The measurements would be useless because the amount of absorption by phage particles, as distinct from that caused by other constituents of crude bacterial lysates, would still remain unknown.

RESULTS

Interference by inactivated phage with multiplication of active phage and of host bacteria

This problem is considered first because if there were any interference it might need to be allowed for whenever residual activity of an irradiated phage preparation is tested.

The presence of inactivated phage does not interfere with formation of plaques by active phage. This is shown by the fact that adding untreated phage to an irradiated phage preparation, which is then incubated suitably diluted in a culture of host bacteria and plated, has no effect on the number of plaques. Nor is the plaque number affected by adding an irradiated phage preparation to an equal volume of a culture of host bacteria in which untreated phage preparation is then diluted and plated. Thus no active phage particles are destroyed or otherwise made permanently unable to multiply. On the other hand, irradiated phage preparations can stop completely, though temporarily, the multiplication of active phage in liquid cultures of host bacteria and can make the host bacteria unable to multiply and form colonies on agar medium.

Table 1 shows that an irradiated preparation of phage 317 prevented the multiplication of active phage 317 for at least 3 hr., during which the phage concentration of the control increased 30 times. However, this was so only when the irradiated phage preparation was added undiluted to an equal volume of a 24 hr. bacterial culture, i.e. when the number of inactive phage particles in the final mixture was more than 35 times that of the number of bacteria and 500,000 times that of the number of active phage particles. When the concentration of the irradiated phage preparation was decreased to one-quarter, phage multiplication was not completely inhibited and there was no inhibition when the concentration was decreased to one-sixteenth.

After 24 hr. incubation, the concentration of active phage in the culture containing even the most concentrated irradiated phage was almost as high as that of the control. It is obvious, therefore, that the inhibitory effect of the irradiated phage preparation lasted less than 24 hr.

Table 1. *Effect of an ultraviolet-irradiated preparation of phage 317 on multiplication of phage 317*

Materials. Untreated preparation of 317 phage: 5×10^8 plaques/ml. U.v. irradiated preparation of 317 phage: irradiation time was 20 min.; activity fell from 5×10^8 to 50 plaques/ml. U.v. irradiated preparation of Cl_5 phage: irradiation time was 20 min.; activity fell from 18×10^8 to 80 plaques/ml. 24 hr. culture of 317 bacteria: 15×10^6 cells/ml. (haemocytometer count). U.v. irradiated medium: irradiation time was 20 min.

The mixtures: 24 hr. culture of 317 bacteria + an equal volume of:	Phage concentration in the mixtures (in terms of numbers of plaques/ml.)		
	Immediately	After 3 hr.	After 24 hr.
Irradiated 317 phage (undiluted)	530	460	39×10^8 *
Irradiated 317 phage (dil. 1/4†)	600	1,500	—
Irradiated 317 phage (dil. 1/16†)	550	19,000	—
Irradiated Cl_5 (undiluted)	560	7,500	52×10^8
Untreated Cl_5 (undiluted)	500	15,000	54×10^8
Irradiated medium	570	8,000	50×10^8
Untreated medium	550	16,500	53×10^8

One volume of the untreated preparation of 317 phage diluted 1/10⁶ was added to nine volumes of each mixture. Samples of the mixtures were taken for assay of concentration of phage 317 by the plaque count method immediately, after 3 hr. and after 24 hr. incubation at 25°.

* The mean diameter of the plaques was about half that of the control.

† Diluted in untreated medium.

Although after 24 hr. incubation the culture containing irradiated phage produced almost as many plaques as did the control phage culture, the diameter of the plaques (mean = 1.9 mm.) was smaller than that of the control (mean = 3.4 mm.). No explanation of this phenomenon can be offered. It is not caused by a phage mutation, because phage isolated from the small plaques produced plaques of normal size.

Table 2 shows that u.v. inactivated phage Cl_5 also temporarily prevented the multiplication of active phage Cl_5 in a liquid culture of Cl_5 bacteria. Comparison of Tables 1 and 2 shows that the multiplication of active phage was prevented only by irradiated preparations of the homologous phage. Irradiated preparations of the other inhibited only to the same extent as did the irradiated medium.

This apparent specificity probably occurs because each of the two bacterial strains was susceptible to only one of the two phages used. Luria & Delbrück (1942), who used one bacterial host susceptible to two different phages, found that irradiated preparations of one phage could slow the multiplication of either phage in liquid cultures of their common host, whereas irradiated preparations of the other had no effect on multiplication of either. It appears, therefore, that if a u.v. irradiated phage can interfere with the multiplication

of active phage, it does so only in a bacterial strain which is its host and then it interferes with the multiplication of any phage in it.

Luria & Delbrück (1942) found that the u.v. inactivated phage that interfered with phage multiplication also rendered the bacteria unable to multiply, whereas the other inactivated phage had no such effect. Moreover, the ability of the irradiated phage to interfere with phage multiplication could be destroyed by excess of u.v. irradiation, when their ability to make their host

Table 2. *Effect of an ultraviolet irradiated preparation of Cl₅ phage on multiplication of Cl₅ phage*

Materials. Untreated preparation of Cl₅ phage: 18×10^8 plaques/ml. U.v. irradiated preparation of Cl₅ phage: irradiation time was 20 min.; activity fell from 18×10^8 to 80 plaques/ml. U.v. irradiated preparation of 317 phage: irradiation time was 20 min.; activity fell from 5×10^8 to 50 plaques/ml. 24 hr. culture of Cl₅ bacteria: 16×10^6 cells/ml. (haemocytometer count). U.v. irradiated medium: irradiation time was 20 min.

The mixtures: 24 hr. culture of Cl ₅ bacteria + an equal volume of:	Phage concentration in the mixtures (in terms of numbers of plaques/ml.)	
	Immediately	After 5 hr.
Irradiated phage Cl ₅	950	810
Irradiated phage 317	890	2500
Untreated phage 317	930	6700
Irradiated medium	860	3000
Untreated medium	870	7000

One volume of the untreated preparation of Cl₅ phage diluted $1/10^5$ was added to nine volumes of each mixture. Samples of the mixtures were taken for assay of concentration of phage Cl₅ by the plaque count method immediately and after 5 hr. incubation at 25°.

bacteria unable to multiply also disappeared. It is probable, therefore, that the interference with both phage and host multiplication are expressions of the same disturbance of host metabolism caused by the u.v. inactivated phage. If so, u.v. inactivated *Rhizobium* phages would be expected to be able to interfere with multiplication of their host bacteria, and Table 3 shows that addition of an irradiated preparation of phage 317 to an equal volume of a 24 hr. culture of 317 bacteria did decrease considerably the number of colonies formed by the bacteria on agar medium. The decrease was probably caused by u.v. inactivated phage and not by the remaining active phage or by some other constituent of the irradiated phage preparation. The number of plaques formed by the irradiated preparation was 50/ml. Thus, if the proportion of active phage particles that formed plaques when the preparation was assayed was $1/m$, the number of active phage particles per ml. of the mixture with the bacterial culture was $25m$. The results of an electron microscopic study by Luria, Williams & Backus (1951) suggest that the value of m is not greater than 10, so that the number of active phage particles per ml. of the mixture with bacterial culture was probably not greater than 250, whereas the approximate number of bacterial cells per ml. of the mixture rendered unable to form colonies was 1×10^6 . Therefore, some constituent of the irradiated preparation other than active phage must have made them unable

to do so. The fact that irradiated medium or irradiated (as well as unirradiated) preparations of a heterologous phage (Cl_6) caused no drop in the number of colonies, shows that the constituent of the irradiated preparation of the homologous phage (317) that caused the drop was probably inactivated phage 317.

Table 3. *Effect of an ultraviolet irradiated preparation of phage 317 on multiplication of its host bacteria*

Materials. All the materials used in this experiment were the same as those used in the experiments shown in Tables 1 and 2.

The mixtures: 24 hr. culture of 317 bacteria + an equal volume of:	Mean numbers of colonies/plate formed by the mixtures used at a dilution of	
	1/10 ⁴	1/10 ⁵
Untreated phage 317	42	4.5
Irradiated phage 317	264	25
Untreated phage Cl_6	380	42
Irradiated phage Cl_6	370	39
Untreated medium	355	36
Irradiated medium	363	39

The mixtures were incubated for 15 min. at room temperature then diluted 1/10⁴ and 1/10⁵ in untreated medium, and 1 ml. of each dilution was plated by mixing with 9 ml. of melted agar medium cooled to 42° and pouring into a Petri dish which was then incubated for 7 days at 25°. There were four replications of each treatment.

However, irradiated medium or irradiated preparations of the heterologous phage can slow bacterial multiplication in liquid cultures if mixed in equal volumes. This agrees with other observations that u.v. irradiation makes media less suitable for bacterial growth, an effect usually attributed to the action of peroxides formed by u.v. irradiation (Coblentz & Fulton, 1924; Bedford, 1927; Proks, 1933; Wyss, Haas, Clark & Stone, 1950). The slight inhibitory effect on phage multiplication given by irradiated medium and irradiated preparations of heterologous phages (Tables 1 and 2) probably resulted from their effect on growth of the host bacteria.

The common feature of the effect of u.v. inactivated phages, on the one hand, and of such inhibitors as ribonuclease or some polysaccharides (Kleczkowski & Kleczkowski, 1952), on the other, is that they interfere with phage multiplication in liquid cultures of host bacteria while having no effect on the numbers of plaques formed on agar. This effect on the former and not the latter, showing an apparent contradiction, has been discussed elsewhere (Kleczkowski & Kleczkowski, 1952).

Tests for 'multiplicity reactivation'

The 'multiplicity reactivation' observed with a few coli bacteriophages (Luria & Dulbecco, 1949) was not observed with the *Rhizobium* bacteriophages used in this work. The numbers of plaques produced by u.v. irradiated preparations of these phages did not increase with the increasing concentration at which they were brought into contact with host bacteria before plating. The phenomenon of 'multiplicity reactivation' does not seem to be very common. It

was observed only in a proportion of a few coli phages tested by Luria & Dulbecco (1949), and it could not be observed with a *Staphylococcus* phage (Price, 1950), or with three different plant viruses (Bawden & Kleczkowski, 1953).

The rate of inactivation and the effect of visible light

Table 4 shows that the rate of inactivation of the 317 phage by u.v. radiation approximates closely to that of a first-order reaction because the value of k , obtained from the equation $p = e^{-kt}$ (where p is the proportion of remaining activity and t the time of exposure), was almost constant when experimental conditions were constant.

Table 4. *The rate of inactivation of phage 317*

Time of exposure to u.v. radiation (min.)	Dilution at which the preparation was plated	Total nos. of plaques formed on 14 plates*	Proportion of remaining activity	k †
0	1/10 ⁸	519	1.00	—
0.283	1/10 ⁸	278	0.54	2.15
1	1/10 ⁷	498	0.096	2.34
2	1/10 ⁸	713	0.014	2.13
4	1/10 ⁴	956	0.00018	2.16

* There were seven separate experiments with the same times of exposure. In each experiment two platings were made with each preparation giving 14 as the total number of platings.

† The value of k is obtained from the equation $p = e^{-kt}$, where p is the proportion of remaining activity and t is the time of exposure (in minutes).

Table 5. *The effect of visible light on the activity of ultraviolet irradiated 317 phage*

Time of irradiation of phage (min.)	Dilution at which the preparation was plated	Plates not exposed to visible light			Plates exposed for 3 hr. to daylight immediately after plating		
		Total nos. of plaques formed on 4 plates	Proportion of remaining activity	k^*	Total nos. of plaques formed on 4 plates	Proportion of remaining activity	k^*
0	1/10 ⁷	165	1.00	—	158	1.00	—
2.25	1/10 ⁵	131	0.0079	2.15	336	0.021	1.72
4.5	1/10 ³	130	0.000079	2.10	765	0.00048	1.70

* Obtained as in Table 4.

Table 5 shows that a smaller value of k is obtained if the agar plates inoculated with mixtures of host bacteria and irradiated phage are exposed for some time to visible light than if they are kept all the time in darkness. In each condition, however, the value of k is constant. The decrease in the value of k arises from the fact that u.v. irradiated phage shows increased residual activity if the infected host bacteria are exposed to visible light. The phenomenon is shown by *Rhizobium* phage in all details as observed by Dulbecco (1950) with a number of coli phages and described as 'photo-reactivation'. The constancy of k under constant conditions of exposure to visible light or in its absence agrees with the so-called 'dose reduction prin-

ciple' of Kelner (1949), according to which exposure to visible light is equivalent to reducing the dose of u.v. radiation by a constant factor. The principle was formulated for u.v. irradiated bacteria and found by Dulbecco (1950) to apply to u.v. irradiated coli phages and by Bawden & Kleczkowski (1953) to some u.v. irradiated plant viruses.

Visible light increases the residual activity of u.v. irradiated phage preparations only if the infected host bacteria are exposed to it. Exposure of the irradiated phage preparations, or of the host bacteria, or of both, separately, i.e. before they are mixed, has no effect. Similarly, the residual activity of u.v. irradiated preparations of some plant viruses is increased by exposing the inoculated host plant to visible light, whereas exposure of irradiated virus preparations *in vitro*, or of the host plant before inoculation, has no such effect (Bawden & Kleczkowski, 1953).

Visible light can counteract lethal and some other effects of u.v. radiation on unicellular (see Kelner, 1951) and on some multicellular (Bawden & Kleczkowski, 1952) organisms by acting on them directly, whereas it can counteract comparable effects of u.v. radiation on bacteriophages and plant viruses only when they have combined with their cellular host organisms. Cellular structure is thus apparently essential for the occurrence of this phenomenon.

The fact that illumination of infected host bacteria cannot increase residual activity of u.v. irradiated phage above a certain limit, so that a proportion of phage particles will remain inactive irrespective of the amount of illumination, led Dulbecco (1950) to conclude that there are two different kinds of injury inflicted by u.v. radiation on phage particles: 'photoreactivable' and 'nonphotoreactivable'. The conclusion that there are at least two and possibly more different kinds of injury indeed seems inescapable. The fact that the value of k is constant when the conditions of illumination are constant means that the process of inflicting each kind of injury is a separate first-order reaction. Conclusions that can be drawn from this are given elsewhere (Bawden & Kleczkowski, 1953).

Relative instability of active phage in irradiated preparations

Tables 6 and 7 show that when u.v. irradiated and unirradiated phage preparations are incubated in identical conditions, the former lose their activity at a proportionally higher rate than the latter. The more a preparation is irradiated the higher is the relative rate of the decrease of its remaining activity (Table 6). The difference between irradiated and control preparations is noticeable when the incubation temperature is near 0°, but it becomes more obvious as the temperature increases, i.e. when both irradiated and control preparations become less stable (Table 7).

It is concluded that irradiated phage particles that still remain active are less stable than those in control preparations. Their relative instability does not result from the presence in the medium of any materials that are produced by u.v. radiation and are harmful to phage particles, because unirradiated phage preparations diluted in irradiated preparations are as stable as their

controls. The instability is, therefore, a property of the irradiated phage particle and it is a result of either a change in the structure of the particle caused by u.v. radiation, or a selective effect of the radiation. The latter possibility seems unlikely, because it necessitates the assumption that the

Table 6. *Effect of incubation at 34° of preparations of phage 317 ultraviolet irradiated for various lengths of time*

Time of irradiation (min.)	Dilution at which the preparation was plated	Numbers of plaques formed on 4 plates by samples plated		Drop in activity to
		Immediately after irradiation	After 3 hr. incubation at 34° following irradiation	
0	1/10 ⁷	305	295	97 %
1·75	1/10 ⁸	84	60	71 %
3·5	1/10 ⁴	225	74	33 %
5·0	1/10 ²	980	257	26 %

Table 7. *Effect of incubating an ultraviolet irradiated preparation of 317 phage at different temperatures*

	Untreated phage		Phage irradiated for 4 min.	
	Nos. of plaques on 4 plates	Remaining proportion of original activity (%)	Nos. of plaques on 4 plates	Remaining proportion of original activity (%)
A sample plated				
Immediately	350	100	660	100
After 24 hr. incubation at:				
2°	385	110	520	80
20°	370	105	390	60
34°	290	80	45	7

Samples of unirradiated phage preparation were plated at a dilution of 1/10⁷ and those of the irradiated preparation at 1/10⁸.

more stable particles are more easily inactivated by u.v. radiation than the less stable ones. Moreover, it would have to be assumed that phage particles differ in susceptibility to u.v. radiation, and this means that the value of *k* should decrease as the residual activity of irradiated phage preparations decreases to the values reached in the experiments shown in Tables 6 and 7, whereas in fact the value of *k* remained approximately constant (Tables 4 and 5). It seems, therefore, more likely that u.v. radiation can so alter a phage particle that, although still active, it is rendered unstable.

The relative instability of particles that remain active after exposure to u.v. radiation is a phenomenon that does not seem to be limited to bacteriophages, for it has also been observed in preliminary experiments with trypsin (Kleczkowski, to be published). It was not observed with a few u.v. irradiated plant viruses that were tested (Bawden & Kleczkowski, 1953), but the failure

may have resulted from failure to adjust experimental conditions to the much greater stability of the viruses used.

Alper (1952) has just reported that inactivation of a phage by X-rays still goes on after the irradiation of its preparation has ended. Two different causes of this could be distinguished. In the first place X-rays alter phage particles making them susceptible to the inactivating effect of hydrogen peroxide, and, secondly, X-rays produce hydrogen peroxide in the medium. Diluting irradiated phage in unirradiated medium can, therefore, prevent its further quick inactivation.

Instability of residual activity of u.v. irradiated preparations of the *Rhizobium* bacteriophages is not likely to be caused by a similar combination of two causes, for diluting the preparations 1/1000 in unirradiated medium did not make them more stable. On the contrary, they even became less stable, suggesting the presence of some protective material in the crude bacterial lysate.

When residual activity of u.v. irradiated phage preparations falls as a result of ageing, the ability of the preparations to respond to exposure of infected host bacteria to visible light seems to remain unchanged. The ratio of the number of plaques formed with exposure to visible light to that formed without exposure does not seem to alter appreciably. It is concluded that irradiated phage particles that still are fully active and those active only if the infected host is exposed to visible light, both lose their activities at the same relative rate. The ability of an u.v. irradiated phage particle to be active only if the infected host is exposed to visible light may be a transition stage between fully active and fully inactive states, but there is no evidence for this.

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