J. gen. Virol. (1968), 3, 19–24
Printed in Great Britain

Dark Reactivation of Ultraviolet-irradiated Tobacco Necrosis Virus

By A. KLECZKOWSKI
Rothamsted Experimental Station, Harpenden, Hertfordshire

(*Accepted* 22 *November* 1967)

SUMMARY

Some damage caused in tobacco necrosis virus by u.v. radiation could be repaired in darkness in *Chenopodium amaranticolor* (dark reactivation) but not in French bean or in tobacco. By contrast, photoreactivation of the irradiated virus was observed in French bean and in tobacco but not in *Chenopodium*. The kind of damage in u.v.-irradiated virus that is susceptible to repair by photoreactivation appears to be repaired by dark reactivation in *Chenopodium*. In conditions without evidence of any repair, the quantum yield for inactivation of the nucleic acid inside the virus is about $6{\cdot}5 \times 10^{-4}$, and the amount of radiation energy that must be absorbed by the nucleic acid to reduce infectivity to 50 % is about 0·3 J/mg.

INTRODUCTION

The only kind of reactivation of u.v.-irradiated plant viruses known so far is photoreactivation (Bawden & Kleczkowski, 1955, 1959). With bacteria and bacteriophages much work has been done on other kinds of reactivation occurring in darkness, and this paper reports the reactivation in darkness of u.v.-irradiated tobacco necrosis virus, which seems comparable to what is called 'host cell reactivation' of u.v.-irradiated bacteriophages (Ellison, Feiner & Hill, 1960; Sauerbier, 1962; Jansz, Pouwels & Rotterdam, 1963; Rörsch, Edelman & Cohen, 1963).

METHODS

Virus. A purified preparation of tobacco necrosis virus (strain A) was kindly supplied by Dr B. Kassanis, who purified the virus from sap of infected tobacco plants (*Nicotiana tabacum*, var. White Burley) by differential centrifugation.

Irradiation. The source of radiation was a 'Hanovia' low-pressure mercury lamp with a filter to eliminate radiations of wavelengths shorter than 2400 Å, so that most of the radiation was of wavelength 2537 Å. A solution of the virus at a concentration of 0·22 mg./ml. in 0·067 M-phosphate buffer at pH 7·0 was irradiated as a layer 0·16 cm. thick in an open Petri dish so placed that the intensity of the radiation (estimated by the potassium ferrioxalate method of Hatchard & Parker, 1956) was 0·86 mW/cm.^{2}, i.e. $1{\cdot}1 \times 10^{-7}$ einsteins/cm.^{2}/min. The dish was gently rocked during irradiation, which never lasted longer than 75 sec.

Assay of infectivity. Infectivity of irradiated virus preparations was assayed by the local-lesion method using in each experiment three different species of host plant: tobacco (*Nicotiana tabacum*, var. Xanthi-nc.), French bean (*Phaseolus vulgaris*, var.

The Prince) and *Chenopodium amaranticolor*. The plants were aged about 5, $2\frac{1}{2}$ and $4\frac{1}{2}$ weeks, respectively, from the date of sowing. Tobacco plants were stripped to two leaves, French bean plants were in the stage of two primary leaves, and *Chenopodium* plants were stripped to four leaves. Half-leaves of tobacco and French bean and whole leaves of *Chenopodium* were used as 'experimental units', so that there were four 'units' per plant.

In each experiment 24 plants of each species were used, and there were four inocula: an irradiated preparation of the virus and three different dilutions of an unirradiated (control) preparation. For tobacco and *Chenopodium* the inocula were undiluted irradiated preparation and the unirradiated preparation diluted 1/10, 1/40 and 1/160. For French bean all the inocula were further diluted 1/4. The inocula were distributed among the 'experimental units' so that each inoculum occurred once on each plant and, on tobacco and *Chenopodium*, also an equal number of times on each leaf position. Except for these restrictions, experimental units were allotted to the inocula at random.

Twelve plants of each species were inoculated in daylight (early in the day in summer) and kept in daylight. The remaining twelve plants were inoculated in a dark room illuminated with a dark green darkroom safelight, and then kept for 24 hr in darkness, after which all plants were kept together in ordinary greenhouse conditions. Lesions on leaves of French bean, tobacco and *Chenopodium* were counted, respectively, 3, 4 and 11 days after inoculation.

Residual infectivity of irradiated preparations were estimated by comparing numbers of lesions formed by them with those formed by three different dilutions of the unirradiated preparations. The latter were plotted against logarithms of virus concentrations, and logarithms of proportions of original infectivity remaining in the irradiated preparations were obtained by graphic interpolation. In most experiments the numbers of lesions formed by the control preparations were roughly proportional to virus concentrations.

Statistical tests. Because the variation within each class of values shown in Table 1 was large by comparison with the differences between the mean values of different classes, a statistical test to assess the significance of the differences was considered necessary, and the *t*-test was used. The test can only be applied under the following conditions: (1) that the values of each class are distributed around their own means with a variance that is the same for all classes, and (2) that the distributions are not very different from the normal. Condition (1) is particularly important for the validity of the *t*-test, and it could not simply be assumed without any evidence because different classes of values were obtained using different species of assay plants, and because the plants were treated differently after inoculation (*D* and *L*).

If condition (1) were fulfilled, the estimate of the variance of the populations of all the classes would be obtained by dividing the sum of squares of deviations of the values of all the classes from the means of their classes (which is 1·2478) by the total number of degrees of freedom (which is 31), thus getting 0·0403, so that the standard deviation would be 0·201.

The assumption that conditions (1) and (2) were fulfilled could not be tested rigorously because of too few values of the variate, but the following considerations show that the assumption cannot be far from being true. If the distributions were normal with the standard deviation of 0·201, the deviations of all the values from the means of their classes, expressed in units of standard deviation (i.e. the deviations divided by 0·201),

would be expected to be evenly distributed among the following five groups: (1) less than $-0{\cdot}84$, (2) between $-0{\cdot}84$ and $-0{\cdot}25$, (3) between $-0{\cdot}25$ and $+0{\cdot}25$, (4) between $+0{\cdot}25$ and $+0{\cdot}84$, and (5) more than $+0{\cdot}84$. The numbers of the values falling into the groups were 8, 5, 9, 8 and 7, respectively, so that the expectation can be considered to be fulfilled.

If the conditions (1) and (2) were fulfilled, the scatter of the estimates of variance obtained separately for each class (i.e. the variance of the estimates of variance) should conform to what would be expected. The estimates of variance are: 0·0792, 0·0554, 0·0337, 0·0143, 0·0098 and 0·0445. When the distribution is normal, the variance of estimates of variance is $v = 2\sigma^2/(n-1)$, when σ^2 is the variance of the population (i.e. 0·0403), and n is the number of values of the variate on which a given estimate of variance is based. The number of values (n) in the classes varied from 5 to 7. When $n = 5$, $v = 0{\cdot}00081$, and when $n = 7$, $v = 0{\cdot}00054$. Therefore, we shall expect the value of v to be between 0·00054 and 0·00081. The value of v computed from the six estimates of variance obtained separately for each class is 0·00068, which agrees with the expectation.

Analysis of variance of the results (after substituting appropriate values for the 5 missing data) showed that variation between the totals of experiments is negligible, so that there is no need to treat the values obtained in the same experiments as 'blocks'. The division of the values into 7 groups obtained in 7 different experiments (as shown in Table 1) can therefore be disregarded.

RESULTS

Residual infectivity of a virus preparation irradiated for 60 sec. was estimated on three different species of host plants which were either kept after inoculation in darkness (*D*) or exposed to daylight (*L*). Table 1 gives the values of logarithms of the reciprocals of proportions of the original infectivity that, according to the different assays, remained in the irradiated virus preparation, and the mean of the values of each class will be used below as an estimate of the extent of inactivation obtained in the specified conditions.

When the assay plants were kept in darkness, the extent of inactivation appeared almost the same according to the assays on French bean and on tobacco plants, but it was much smaller according to the assays on *Chenopodium*, the difference being highly significant ($P < 0{\cdot}001$). We can therefore assume that some damage caused in the virus by u.v. was repaired in darkness in *Chenopodium*, but not in the other two species of host plants. The phenomenon will be referred to as 'dark reactivation'.

When French bean and tobacco plants were used the extent of inactivation appeared smaller when the plants were kept in light (*L*) than in darkness (*D*), the differences being highly significant ($P < 0{\cdot}005$). This was not so when *Chenopodium* plants were used. We can conclude therefore that some damage caused in the virus by u.v. was repaired by photoreactivation in French bean and in tobacco, but not in *Chenopodium*.

The difference between the results obtained in darkness and in light was slightly greater when tobacco was used than when French bean was used, but the difference between the differences was too small to be significant ($P = 0{\cdot}3$) and thus not sufficient to support the possibility of a difference in the extent of photoreactivation in the two species of plants.

When *Chenopodium* plants were used, the extent of inactivation of the irradiated virus appeared greater when the plants were kept in light than in darkness. Exposure of *Chenopodium* to daylight may not only therefore not have resulted in photoreactivation of the virus, but may have interfered with the process of dark reactivation. However, the difference between the results was too small to be significant ($P = 0{\cdot}12$).

The extent of inactivation of the virus, assayed in French bean or tobacco plants when they were exposed to daylight, was greater than when assayed in *Chenopodium* kept in darkness, and the difference between the result with French bean in light and *Chenopodium* in darkness was highly significant ($P = 0{\cdot}005$). More damage caused in the virus by u.v. appeared to be repaired by dark reactivation in *Chenopodium* than by photoreactivation in French bean, and possibly also in tobacco.

Table 1. *Logarithms of the reciprocals (log. (1/p)) of proportions (p) of the original infectivity remaining in an irradiated virus preparation*

			Experiment no.							
Class	Assay plant		1	2	3	4	5	6	7	Mean
I	French bean	*D*	2·0	2·6	2·1	2·8	2·4	2·2	2·35	2·35 ± 0·075
II		*L*	—	1·9	1·85	1·7	2·25	1·95	2·3	1·99 ± 0·08
III	Tobacco	*D*	2·5	2·2	2·5	2·35	2·3	2·2	2·7	2·39 ± 0·075
IV		*L*	—	1·9	—	1·95	1·9	1·65	1·9	1·86 ± 0·09
V	*Chenopodium*	*D*	1·65	1·75	1·7	1·6	1·45	1·7	1·6	1·64 ± 0·075
VI		*L*	—	1·75	2·0	—	2·0	1·9	1·5	1·83 ± 0·09

D, Inoculated plants kept in darkness; *L*, inoculated plants exposed to daylight.

As the residual infectivity of the same irradiated virus preparation varied when assayed in the different conditions shown in Table 1, the quantum yield computed from the extents of inactivation will also vary. Assuming that no dark reactivation at all occurred in French bean or in tobacco plants, the quantum yield computed from the extent of inactivation assayed on these plants when kept in darkness would be concerned with all kinds of damage causing loss of infectivity, whereas the quantum yields from the extents of inactivation assayed on these plants when exposed to daylight, or on *Chenopodium* when kept in darkness, would be concerned with the kind of damage that could not be reversed by photoreactivation or by dark reactivation, respectively.

The quantum yields computed below are for inactivation of virus nucleic acid inside the virus. It was found previously that inactivation of infectivity of tobacco necrosis virus (strain A) was entirely a function of the amount of radiation energy absorbed by the nucleic acid irrespective of whether it was inside the virus or free (Kassanis & Kleczkowski, 1965), so that radiation energy absorbed by virus protein is irrelevant. The quantum yields were computed on the assumption that the rate of inactivation assayed in each of the conditions followed first-order kinetics, at least approximately. Results of a few experiments made to test the assumption did not suggest any serious deviation from first order kinetics. The quantum yields (Φ) were therefore computed from the formula

$$\Phi = \frac{[2{\cdot}3M \log (1/p)]}{D}, \qquad (1)$$

where p is the proportion of the original infectivity of the virus preparation, according to a given assay, still remaining after irradiation; M is the number of moles of virus nucleic acid under each cm.^{2} of the irradiated area; and D is the number of einsteins absorbed by the nucleic acid.

To compute the values of M and D, the molecular weight of the virus was taken as 9×10^6 daltons, the content of RNA as 18 %, specific optical density of the virus (i.e. the optical density at a concentration of 1 g./l. for a light path of 1 cm.) as 5·3 and of the RNA as 25, both at the wave-length of 2537 Å (Kassanis & Kleczkowski, 1965). As the amount of virus under each cm.^{2} of irradiated area was 0·035 mg., it can be computed from all the above data that 30 % of the applied radiation energy was absorbed by the RNA. The amount of applied radiation energy during 60 sec. irradiation was $1{\cdot}1 \times 10^{-7}$ einsteins per cm.^{2}, so that the amount absorbed by RNA was $D = 0{\cdot}33 \times 10^{-7}$ einsteins (or 0·0155 J). The number of moles of the virus, and therefore of the moles of RNA, under each cm.^{2} of irradiated area was $3{\cdot}9 \times 10^{-12}$. Substituting the values of M and D in equation (1), we get

$$\Phi = 2{\cdot}7 \times 10^{-4} \times \log (1/p). \qquad (2)$$

If we take the mean value of log $(1/p)$ obtained by the assay on tobacco plants kept in darkness, i.e. 2·39, we get $\Phi = 6{\cdot}4 \times 10^{-4}$. It can also be computed from the above data that the amount of energy that must be absorbed by each mg. of virus RNA to reduce infectivity to 50 % of the original, according to the assay on tobacco plants kept in darkness, is 0·31 J. Both values are close to those previously obtained (Kassanis & Kleczkowski, 1965). Quantum yields according to assays other than those on tobacco plants kept in darkness can be obtained by substituting the corresponding mean values of log $(1/p)$ shown in Table 1 in equation (2).

DISCUSSION

The fact that the results of assays of the extent of inactivation of a preparation of tobacco necrosis virus by u.v.-irradiation depended on the conditions of the assay was interpreted as evidence that in some conditions some damage caused in virus particles by u.v. was repaired. Whether the interpretation is correct may be doubtful, but until evidence to the contrary is obtained, it can be accepted as a working hypothesis. Similar phenomena occurring with u.v.-irradiated bacteriophages, which have been much more extensively investigated, are at present generally accepted as evidence of repair.

The results can be interpreted to mean either that dark reactivation of u.v.-irradiated virus occurred in *Chenopodium* and not in French bean and tobacco plants, or that it occurred in all three plant species, but to a greater extent in *Chenopodium* than in the other two species. No evidence is, however, at present available for any dark reactivation in French bean or in tobacco.

The results obtained with tobacco necrosis virus do not seem to be generally applicable to plant viruses even when the same set of species of assay plants is used. Residual infectivity of an u.v.-irradiated preparation of RNA isolated from tobacco mosaic virus was assayed by Werbin *et al.* (1966) on the same three species of host plants as used in the present work, and no evidence for any dark reactivation was obtained; but photoreactivation occurred in all three species. It is possible that the

kind of damage in u.v.-irradiated tobacco necrosis virus that can be repaired by dark reactivation in *Chenopodium* does not occur in free nucleic acid of tobacco mosaic virus. Alternatively, the molecules of the free nucleic acid that cannot start infection because of damage by u.v. are decomposed by plant enzymes before any damage can be repaired by dark reactivation.

Although u.v.-irradiated tobacco necrosis virus is susceptible to photoreactivation (it is photoreactivated in French bean and in tobacco), none occurred in *Chenopodium* in spite of the fact that this plant seems to have a photoreactivating system as it photoreactivated u.v.-irradiated RNA isolated from tobacco mosaic virus (Werbin *et al.* 1966) and u.v.-irradiated red clover vein mosaic virus (Varma, 1967). This suggests that the kind of damage, caused by u.v. in tobacco necrosis virus that is susceptible to repair by photoreactivation is also repaired in this plant in darkness.

As the test plants were kept in darkness only for 24 hr to prevent photoreactivation, in *Chenopodium* the irradiated virus may remain photoreactivable for more than 24 hr, and so what appears to be a result of dark reactivation may have been a result of delayed photoreactivation. The possibility would still exist in principle even if the plants were kept in darkness for more than 24 hr. However, in such plants as tobacco an irradiated virus remains photoreactivable only for about $1\frac{1}{2}$ hr (Bawden & Kleczkowski, 1955). The sequence of events in *Chenopodium* would therefore have to be at least about 20 times slower than in tobacco to allow for the possibility of delayed photoreactivation, whereas the time of appearance of lesions was only about 3 times longer. Moreover, photoreactivation in *Chenopodium* of u.v.-irradiated free nucleic acid of tobacco mosaic virus (Werbin *et al.* 1966) and of u.v.-irradiated red clover vein mosaic virus (Varma, 1967) was prevented by the usual prodecure, when batches of inoculated plants were kept in darkness for 6 and 24 hr respectively.

REFERENCES

BAWDEN, F. C. & KLECZKOWSKI, A. (1955). Studies on the ability of light to counteract the inactivating action of ultraviolet radiation on plant viruses. *J. gen. Microbiol.* **13**, 370.

BAWDEN, F. C. & KLECZKOWSKI, A. (1959). Photoreactivation of nucleic acid from tobacco mosaic virus. *Nature, Lond.* **183**, 503.

ELLISON, S. A., FEINER, R. R. & HILL, R. F. (1960). A host effect on bacteriophage survival after ultraviolet irradiation. *Virology* **11**, 294.

HATCHARD, C. G. & PARKER, C. A. (1956). A new sensitive chemical actinometer. II. Potassium ferrioxalate as a standard chemical actinometer. *Proc. R. Soc.* A **235**, 518.

JANSZ, H. S., POUWELS, P. H. & ROTTERDAM, C. VAN (1963). Sensitivity to ultraviolet light of single- and double-stranded DNA. *Biochim. biophys. Acta* **76**, 655.

KASSANIS, B. & KLECZKOWSKI, A. (1965). Inactivation of a strain of tobacco necrosis virus and of the RNA isolated from it, by ultraviolet radiation of different wave-lengths. *Photochem. Photobiol.* **4**, 209.

RÖRSCH, A., EDELMAN, A. & COHEN, J. A. (1963). The gene-controlled radiation sensitivity in *Escherichia coli*. *Biochim. biophys. Acta* **68**, 263.

SAUERBIER, W. (1962). The bacterial mechanism reactivating UV-irradiated phage in the dark (host-cell reactivation). *Z. Vererb. Lehre* **93**, 220.

VARMA, A. (1967). Studies on red clover vein mosaic virus and some associated viruses. *A thesis for the degree of Doctor of Philosophy of the University of London.*

WERBIN, H., HIDALGO-SALVATIERRA, O., SEEAR, J. & MCLAREN, A. D. (1966). Comparative photoreactivation of ultraviolet light-inactivated tobacco mosaic virus ribonucleic acid on *Chenopodium*, Pinto bean, and tobacco plants. *Virology* **28**, 202.

(*Received* 16 *September* 1967)