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# Residual Infectivity and the Extent of Photoreactivation in Three Different Host Plants of u.v.-irradiated Potato Virus X

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SUMMARY

Residual infectivities of u.v.-irradiated preparations of potato virus X were assayed on three different kinds of plants. When the plants were kept in darkness to prevent photoreactivation of the inactivated virus, the residual infectivity appeared the same whether *Chenopodium amaranticolor* or either of two varieties of tobacco was used for assay. When the plants were exposed to daylight, photoreactivation occurred in all three kinds of plants, possibly to a somewhat greater extent in *Chenopodium* than in tobacco plants. Thus, in contrast to the results previously obtained with tobacco necrosis virus, no evidence was obtained for dark reactivation of u.v.-inactivated potato virus X in *Chenopodium*.

In the absence of photoreactivation, the quantum yields for inactivation were about $0{\cdot}8 \times 10^{-3}$ and $1{\cdot}3 \times 10^{-3}$ for the whole virus and for the RNA inside the virus, respectively.

## INTRODUCTION

Kleczkowski (1968*a*) obtained evidence that some damage to tobacco necrosis virus, caused by exposure to ultraviolet radiation (u.v.), was repaired in darkness in *Chenopodium amaranticolor* but not in tobacco (*Nicotiana tabacum*) or French bean (*Phaseolus vulgaris*) plants. The residual infectivity was increased by photoreactivation in tobacco and in French bean, but not in *Chenopodium*, suggesting that the kind of damage repaired in darkness in *Chenopodium* includes all or most of the damage that can be repaired by photoreactivation. The results obtained with tobacco necrosis virus are not generally applicable to all plant viruses, because Werbin *et al.* (1966), who used the same range of host plants to test the residual infectivity of u.v.-irradiated RNA from tobacco mosiac virus, obtained no indication of dark reactivation and observed photoreactivation in all the test plants. Therefore, it seemed desirable that some other plant viruses should be investigated for the phenomenon of dark reactivation and we describe experiments with potato virus X, chosen because it shows more photoreactivation than any other plant virus yet tested (Bawden & Kleczkowski, 1955).

The results give no evidence of dark reactivation in *Chenopodium* of irradiated potato virus X and there was at least as much photoreactivation in *Chenopodium* as in tobacco.

## METHODS

*Potato virus X.* The virus (strain CS 35) from systemically infected leaves of tobacco plants (var. White Burley) was purified by clarifying sap with chloroform, followed by two cycles of differential centrifugation and centrifugation through a sucrose gradient. The preparation was finally dialysed against 0·01 M-borate buffer at pH 7·5, sedimented

at high speed and dissolved in the buffer to give a virus concentration of 1·14 g./l. The solution was divided into samples of 1·5 ml. and stored frozen. For irradiation and inoculating to plants, the solution was appropriately diluted in the buffer.

*Irradiation.* The source of radiation was a 'Hanovia' low-pressure mercury lamp with a filter to eliminate radiation of wavelength shorter than 240 nm., so that most of the radiation was of the wavelength of 253·7 nm. Solutions of the virus were irradiated as layers 0·16 cm. thick in an open Petri dish placed so that the intensity of the radiation (estimated by the potassium oxalate method of Hatchard & Parker, 1956) was $6{\cdot}3 \times 10^{-4}$ joules/cm.$^2$/sec. ($1{\cdot}34 \times 10^{-9}$ einsteins/cm.$^2$/sec.). The dish was gently rocked during irradiation to ensure equal exposure of all virus particles.

The stored virus gave progressively fewer lesions during the course of the work, probably mainly because the assay plants became more resistant to infection with the change of season from winter to spring, but possibly also because the infectivity of the frozen virus preparation decreased during storage. For this reason the concentration at which the virus was irradiated was gradually increased from 0·114 to 1·14 g./l. as the work progressed. However, the proportions of the original infectivity remaining after irradiation were kept at least approximately the same in all experiments, and the times of exposure ($t$ in seconds) were computed from the equation

$$t = \frac{KlV}{I_0\,[1-\exp(-2{\cdot}3\alpha lV)]}, \qquad (1)$$

where $K$ is the number of joules of radiation energy absorbed and scattered by each mg. of the virus, $I_0$ is the intensity of incident radiation in joules/cm.$^2$/sec., $l$ is the thickness of the irradiated layer (in cm.), $\alpha$ is the optical density (at 253·7 nm.) of the virus at a concentration of 1 g./l. and light path of 1 cm., and $V$ is the concentration of the virus in g./l.

The values of $I_0$ and $l$ are given above and Bawden & Kleczkowski (1959) found the value of $\alpha$ to be about 3·4. Preliminary experiments indicated that a value for $K$ of 0·11 would give a suitable extent of inactivation, and this value was used in all experiments. This means that, taking specific absorbencies (at 253·7 nm.) of the RNA and of the protein of potato virus X as 24 and 0·83, respectively (Bawden & Kleczkowski, 1959), and the RNA content of the virus as 6% (Loring, 1938; Reichmann, 1959*a*), 0·072 joules were absorbed by each mg. of the virus. Then 0·046 joules were absorbed by the RNA contained in 1 mg. of the virus, or 0·77 joules were absorbed by each mg. of the RNA.

*Assays of infectivity.* The infectivities of irradiated preparations were assayed by the local-lesion method using in each experiment three kinds of assay plant: two varieties of tobacco (*Nicotiana tabacum*), namely Xanthi-nc. and White Burley, and *Chenopodium amaranticolor*. Tobacco plants were stripped to two leaves and *Chenopodium* to four leaves. Half-leaves of tobacco 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 kind were used. Twelve plants of each kind were inoculated in daylight (in the morning) and kept in daylight. The remaining 12 plants were inoculated in a dark room illuminated with a yellow darkroom safelight, then kept for 24 hr in darkness, after which all plants were kept together in the greenhouse.

There were five inocula: the irradiated virus preparation used undiluted (concentra-

tion $v$) to inoculate the plants kept for 24 hr in darkness, the irradiated preparation diluted 1/15 used to inoculate plants exposed to daylight, and three solutions of unirradiated virus at concentrations $v/50$, $v/100$ and $v/200$, which were used to inoculate both groups of plants. The inocula were distributed among the experimental units so that each inoculum occurred once on each plant and an equal number of times on each leaf position in each group of 12 plants. Leaves of all plants were dusted with carborundum powder (mesh 600) before inoculation. Lesions on the leaves of tobacco and *Chenopodium* were counted 6 and 10 days, respectively, after inoculation.

The proportion of the original infectivity remaining in an irradiated preparation was estimated from the lesion counts by a statistical method described by Kleczkowski (1968*b*) that estimates both log. $p$ (where $p$ is the proportion) and its variance.

## RESULTS

### *Residual infectivities and photoreactivation in different assay plants*

The values of the logarithms (log. $(1/p)$) of the proportions ($p$) of the original infectivity remaining in the irradiated virus preparations (Table 1) were estimated from results of seven experiments with three different assay plants, which were kept either in darkness for 24 hr after inoculation or in daylight. Considerable variation between the values of log. $(1/p)$ within all six treatments makes a statistical appraisal of the results necessary. The variances of the estimates of values of log. $(1/p)$ were obtained from the variation between numbers of lesions by the method described by Kleczkowski (1968*b*). As differences between the values of the variances are considerable, the conventional analysis of variance cannot be applied to the values of log. $(1/p)$, and so another method of statistical treatment must be sought. Although variances of individual values of log. $(1/p)$ were available, a method of treatment based on the use of weighted means of the values could not be applied because, as can be shown by the method described below, these values differed significantly between experiments, presumably because the extent of inactivation of the virus by u.v. was not quite the same in all the experiments, despite the effort to make it so. Therefore, comparisons between weighted treatment means of values of log. $(1/p)$ could lead to false conclusions, because the means would be biased to different extents in favour of different values of log. $(1/p)$ depending on the magnitudes of their variances. Consequently, ordinary arithmetic means (or totals) of groups of values of log. $(1/p)$ were compared using 'balanced' groups of values, i.e. when a value of a group was missing, either that group was excluded from a comparison with other groups, or the corresponding values of the other groups were excluded from the calculation of their means (or totals). The variances of the means (or totals) were computed from those of the individual values of log. $(1/p)$. The means, the totals and the variances were treated as those of large samples.

The following reasoning was used. Let $m_1, m_2, \ldots, m_n$ be the means of $n$ groups of values of log $(1/p)$, let the variances of the means be $v_1, v_2, \ldots, v_n$, respectively, and let $v_s$ and $v_l$ be the smallest and the largest of them. We also compute the variance of the $n$ values of $m_i$ from their dispersion, i.e. the value of

$$s^2 = \frac{1}{n-1} \sum_{i=1}^{n} (m_i - \bar{m})^2,$$

where $\bar{m}$ is the mean of the $n$ values of $m_i$. The value of $s^2$ is compared with those of

Table 1. *Logarithms of the reciprocals (log.(1/p)) of the proportions (p) of the original infectivity remaining in irradiated virus preparations (numbers without parentheses) and their variances (numbers in parentheses)*

| Treatment no. | Assay plant | | Experiment no. 1 | 2 | 3 | 4 | 5 | 6 | 7 | Mean of all experiments | Mean of Expts 1, 2, 4, 6, 7 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Xanthi | *D** | 1·77 (0·0079) | 2·04 (0·0093) | 2·19 (0·0130) | 1·65 (0·0071) | 2·23 (0·0071) | 1·78 (0·0039) | 1·71 (0·0045) | 1·910 (0·00108) | 1·790 (0·00131) |
| 2 | | *L* | 0·97 (0·0066) | 1·17 (0·0094) | — | 0·99 (0·0097) | — | 0·95 (0·0063) | 0·71 (0·0057) | | 0·958 (0·00151) |
| 3 | White Burley | *D* | 2·16 (0·0076) | 1·98 (0·0069) | 2·04 (0·0130) | 1·77 (0·0099) | 1·91 (0·0059) | 1·63 (0·0024) | 1·54 (0·0030) | 1·861 (0·00099) | 1·816 (0·00119) |
| 4 | | *L* | 1·10 (0·0029) | 0·91 (0·0071) | 1·20 (0·0253) | 0·67 (0·0068) | 0·95 (0·0099) | 0·88 (0·0045) | 0·63 (0·0058) | | 0·838 (0·00108) |
| 5 | *Chenopodium* | *D* | 1·91 (0·0136) | 2·12 (0·0091) | 2·13 (0·0170) | 1·65 (0·0062) | 2·01 (0·0058) | 1·48 (0·0036) | 1·52 (0·0067) | 1·831 (0·00127) | 1·736 (0·00157) |
| 6 | | *L* | 1·03 (0·0139) | 1·02 (0·0271) | 1·15 (0·0313) | 0·34 (0·0088) | 0·74 (0·0082) | 0·51 (0·0039) | 0·60 (0·0048) | | 0·700 (0·00234) |

* *D*, inoculated plants kept in darkness; *L*, inoculated plants exposed to daylight. The numbers of degrees of freedom, on which the variances are based, are 32 when the assay plants are Xanthi and White Burley, and 30 when the assay plants are *Chenopodium*.

$v_s$ and $v_l$ by the variance-ratio test with $n-1$ and $\infty$ degrees of freedom. If $s^2$ does not differ significantly from $v_s$, the values of the means are not considered to differ from each other significantly. The opposite conclusion is not drawn from $s^2$ being significantly greater than $v_s$. When, however, $s^2$ is significantly greater than $v_l$, the values of the means are considered to be dispersed more than could have occurred by chance, and so some means differ significantly from others. Again, the opposite conclusion is not drawn from $s^2$ not being significantly greater than $v_l$.

When the assay plants were kept in darkness, estimates of the values of log. $(1/p)$ were obtained with all plants in all the seven experiments, and so the means of all the values obtained on different plants could be compared. The variance of the means, computed from their dispersion, was 0·0016 (with 2 D.F.), which was not significantly greater than 0·00099, the smallest variance of those of the three means ($R = 1{\cdot}62$, $\therefore\ P = 0{\cdot}2$). We conclude, therefore, that there was no evidence for the extent of inactivation being different when assayed on the three different plants in the dark, and so there was no evidence for dark reactivation in any of the plants.

The values of log. $(1/p)$ were always very much smaller when assay plants were exposed to daylight than when they were kept in darkness. A proportion of inactivated virus was therefore photoreactivated in all three kinds of plants. To compare the extent of photoreactivation in different plants, the the mean values of log. $(1/p)$ were used from the experiments (nos 1, 2, 4, 6 and 7) in which the values of the logarithms both for plants kept in darkness and exposed to daylight were obtained with all three kinds of plants. The difference between the means of the values of log. $(1/p)$ obtained on a given kind of plant when kept in darkness and when exposed to daylight was used as a measure of the extent of photoreactivation, the variances of the differences being the sums of the variances of the means. The differences were: 0·832, 0·978 and 1·036 with the variances 0·00282, 0·00227 and 0·00391 for Xanthi, White Burley and *Chenopodium*, respectively. The variance of the differences, computed from their dispersion, was $s^2 = 0{\cdot}011$ (with 2 D.F.). Its excess over 0·00227, the smallest of the variances of the three differences, was highly significant ($P < 0{\cdot}001$), but its excess over 0·003, which is the mean of the three variances, was only just significant ($P < 0{\cdot}05$), and its excess over 0·00391, which is the greatest of the three variances, was not significant ($P > 0{\cdot}05$). The extent of photoreactivation in *Chenopodium* was therefore probably greater than that in the other two kinds of plants, but the evidence for this is not conclusive.

*Quantum yield for inactivation*

To compute the quantum yield for inactivation of potato virus X by u.v.-radiation at the wavelength of 253·7 nm. in the absence of photoreactivation, we shall make use of the fact that when the assay plants were kept in darkness, the extent of inactivation was not affected by the kind of assay plant. We can therefore use the weighted mean of the mean values of log. $(1/p)$ obtained with the three kinds of plant. The weighted mean is $1{\cdot}87 \pm 0{\cdot}019$, so that the 95 % confidence interval (fiducial limits) for the value of log. $(1/p)$ is between 1·83 and 1·91.

If inactivation of the virus by u.v. is assumed to proceed according to first order kinetics, the quantum yield ($\Phi$) can be obtained from the equation

$$\Phi = \frac{2{\cdot}3 \log(1/p)}{E}. \qquad (2)$$

To obtain the quantum yield for inactivation of the virus or of the RNA inside the virus, $E$ of equation (2) must be substituted by the number of einsteins absorbed per mole of the virus or of the RNA, respectively. The amounts of absorbed energy were 0·072 joules = $15{\cdot}3 \times 10^{-8}$ einsteins per mg. of virus and 0·046 joules = $9{\cdot}8 \times 10^{-8}$ einsteins by the RNA inside each mg. of the virus (see Methods). If the weight of a particle of the virus is taken as $35 \times 10^6$ daltons (Reichmann, 1959*b*), 1 mg. of the virus is equivalent to $2{\cdot}9 \times 10^{-11}$ moles of the virus, and therefore also of the RNA. Thus, the amounts of absorbed energy were $5{\cdot}3 \times 10^3$ and $3{\cdot}4 \times 10^3$ einsteins per mole of the virus and of the RNA, respectively. Substituting in equation (2) these values for $E$, and 1·87 for log. $(1/p)$, we get $0{\cdot}8 \times 10^{-3}$ and $1{\cdot}3 \times 10^{-3}$ as quantum yields for inactivation of the virus and of the RNA inside the virus, respectively.

It can also be computed from the above data that 0·012 joules of radiation energy must be absorbed by each mg. of the virus (0·12 joules by each mg. of virus RNA) to halve the infectivity of the virus. This measure of the energy requirement for inactivation is independent of the weight of the virus particle and so may be preferable.

## DISCUSSION

Kleczkowski (1968*a*) interpreted his results with u.v.-irradiated tobacco necrosis virus as evidence of dark reactivation in *Chenopodium*, but discussed the possibility that the reactivation may have been photoreactivation occurring after the assay plants had been kept in darkness for 24 hr, the delay being possibly because all the relevant processes proceed much more slowly in *Chenopodium* than in the other assay plants. Potato virus X also takes much longer to produce visible lesions in *Chenopodium* than in tobacco, but irradiated potato virus X was photoreactivated in *Chenopodium* during a few hours exposure to daylight and not photoreactivated in plants kept for 24 hr in darkness after inoculation. There is thus no evidence that the extended period of lesion development in *Chenopodium* delays photoreactivation, supporting the conclusion that the results with u.v.-irradiated tobacco necrosis virus demonstrated dark reactivation in *Chenopodium*.

Thus, u.v.-inactivated tobacco necrosis virus and potato virus X seem to differ basically in their behaviour when inoculated to *Chenopodium* plants: some infectivity of tobacco necrosis virus, but not of potato virus X, is restored in the dark, whereas some infectivity of potato virus X, but not of tobacco necrosis virus, is restored by photoreactivation. Irradiated potato virus X behaves similarly to RNA from tobacco mosaic virus (TMV-RNA) (Werbin *et al.* 1966).

The difference in behaviour between potato virus X and tobacco necrosis virus in *Chenopodium* in the light may not be a difference between the extents to which they are photoreactivated, but may be a consequence of the extent to which the damaged viruses are reactivated in the dark. The damage in tobacco necrosis virus that is reversed by photoreactivation seems also to be reversible by dark reactivation, so dark reactivation will obscure the effect of photoreactivation, whereas damage in potato virus X, or in TMV-RNA, is not reversed in the dark, so the effect of photo reactivation is not obscured. The basic question, therefore, is why should the mechanism responsible for dark reactivation act on u.v.-inactivated tobacco necrosis virus, but not on potato virus X or TMV-RNA? Two hypotheses will be considered.

*First Hypothesis.* The kind of damage in tobacco necrosis virus that is susceptible

to dark reactivation does not occur in potato virus X or TMV-RNA. Such a specific kind of damage could be conditioned by some structural feature of the nucleic acid of tobacco necrosis virus. The fact that irradiated nucleic acids isolated from different viruses differ considerably in the extent to which they can be photoreactivated—for example, TMV-RNA and RNA from tobacco necrosis virus (Kassanis & Kleczkowski, 1965)—seems to support this possibility. Alternatively, the damage occurring in tobacco necrosis virus might be specified by an interaction between the RNA and the protein of the virus. This is possible, but seems unlikely because the RNA of tobacco necrosis virus is inactivated by u.v. at the same rate whether it is free or inside the virus (Kassanis & Kleczkowski, 1965).

However, if we assume that the kind of damage reversible by dark reactivation occurs only in the RNA of tobacco necrosis virus, a problem arises concerning photoreactivation, because damage in tobacco necrosis virus reversible by photoreactivation also seems reversible by dark reactivation. If this kind of damage does not occur in potato virus X or in TMV-RNA, the photoreactivable kind of damage that does occur in them must be of another kind. The question then arises why this kind of damage does not occur in the RNA of tobacco necrosis virus. If the dark-reactivable damage to tobacco necrosis virus is conditioned by a structural feature of the virus, then this same structural feature may make the virus insusceptible to damage of the kind found in potato virus X, that is repaired only in the light.

*Second hypothesis.* The same damage occurs in the RNA of all three viruses, but the hypothetical enzyme responsible for dark reactivation acts on only some species of RNA, and only the RNA of tobacco necrosis virus of viruses yet tested is affected. The advantage of this hypothesis is that no further hypotheses are needed to explain the facts considered above. Experimental data are not yet sufficient for us to accept or reject either hypothesis.

Another result of this work that calls for discussion concerns the magnitude of the quantum yield for inactivation by u.v. of the RNA inside potato virus X, namely about $1{\cdot}3 \times 10^{-3}$ (in the absence of photoreactivation). This value is quite close to those known for inactivation of free nucleic acids of other viruses when irradiated in similar conditions, i.e. in buffered solutions at a pH near 7: about $10^{-3}$ for TMV-RNA (Kleczkowski, 1963) and about $0{\cdot}7 \times 10^{-3}$ for the RNA of tobacco necrosis virus (Kassanis & Kleczkowski, 1965). When the nucleic acid is inside the virus, it may be as susceptible to inactivation by u.v. as when free, for example the RNA of tobacco necrosis virus (Kassanis & Kleczkowski, 1965), or it may be protected by the virus protein from inactivation by u.v., so that the quantum yield for inactivation of the RNA is decreased; for example, the RNA of tobacco mosaic virus (Kleczkowski & McLaren, 1967). As the quantum yield for inactivation of the RNA inside potato virus X is comparable to those of free RNA of other viruses, it does not seem likely that it is protected from inactivation by the virus protein.

## 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). Some properties of decomposition products of potato virus X. *Virology* **7**, 375.

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.

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

KLECZKOWSKI, A. (1963). The inactivation of ribonucleic acid from tobacco mosaic virus by ultraviolet radiation at different wave-lengths. *Photochem. Photobiol.* **2**, 497.

KLECZKOWSKI, A. (1968*a*). Dark reactivation of ultraviolet-irradiated tobacco necrosis virus. *J. gen. Virol.* **3**, 19.

KLECZKOWSKI, A. (1968*b*). Experimental design and statistical methods of assay. In *Methods in Virology*, vol. 4, pp. 615–730. Ed. by K. Maramorosch and H. Koprowski. New York and London, Academic Press.

KLECZKOWSKI, A. & MCLAREN, A. D. (1967). Inactivation of infectivity of RNA of tobacco mosaic virus during ultraviolet irradiation of the whole virus at two wave-lengths. *J. gen. Virol.* **1**, 441.

LORING, H. S. (1938). Properties of the latent mosaic virus protein. *J. biol. Chem.* **126**, 455.

REICHMANN, M. E. (1959*a*). Potato X virus. II. Preparation and properties of purified, non-aggregated virus from tobacco. *Can. J. Chem.* **37**, 4.

REICHMANN, M. E. (1959*b*). Potato X virus. III. Light scattering studies. *Can. J. Chem.* **37**, 384.

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.

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