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Inactivation of Infectivity of RNA of Tobacco Mosaic Virus during Ultraviolet-Irradiation of the Whole Virus at Two Wavelengths

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SUMMARY

Loss of infectivity by tobacco mosaic virus exposed to ultraviolet radiation appeared to result entirely from changes in the RNA of the virus. Any changes that may have occurred in the virus protein did not seem to contribute to loss of infectivity of the virus, but there was an interaction between the protein and the RNA because free RNA was equally sensitive to inactivation by absorbed radiation energy of any wavelength (i.e. the extent of inactivation depended entirely on the number of absorbed quanta irrespective of the wavelength), while inside the virus the RNA was about 24 times more sensitive to inactivation at 230 mμ than at 280 mμ. Inside the virus the RNA seemed to be largely protected by the protein from damage by radiation of 280 mμ and 254 mμ, but not of 230 mμ to which it was about as sensitive as when free.

Ultraviolet irradiation at any wavelength caused at least two kinds of damage to free RNA, one of which was photoreversible, but the photoreversible damage did not occur in the RNA irradiated when inside the virus.

INTRODUCTION

Action spectra for the inactivation of tobacco necrosis virus and of its free RNA by ultraviolent radiation (u.v.) are identical, and parallel the absorption spectrum of the RNA, i.e. inactivation of infectivity depends entirely on the amount of radiation energy absorbed by the RNA whether or not it is inside the virus and irrespective of the wavelength within the range of 230 to 290 mμ. Sensitivity of the RNA to absorbed radiation is therefore the same in each circumstance, as is also the amount of photoreactivation (Kassanis & Kleczkowski, 1965). By contrast, although free RNA from tobacco mosaic virus (TMV) behaves like free RNA from tobacco necrosis virus (Rushizky, Knight & McLaren, 1960), intact TMV behaves quite differently from intact tobacco necrosis virus. The action spectrum for inactivation of TMV, first obtained by Hollaender & Duggar (1936) and confirmed by several workers, does not parallel the absorption spectrum of the RNA. The curve of the action spectrum rises rather slowly as the wavelength decreases from 300 to 250 mμ, and then rapidly as the wavelength decreases below 250 mμ. In other words, susceptibility of a dilute solution of TMV to inactivation by a given amount of incident radiation energy increases

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rapidly as the wavelength decreases below 250 mμ. There is also evidence that at least at wavelengths longer than 250 mμ the virus protein and RNA interact so that the RNA is partially protected from damage by u.v. The RNA must absorb more radiation energy to be inactivated to a given extent when in the virus than when free (Siegel, Wildman & Ginoza, 1956; McLaren & Takahashi, 1957). Also, some of the infectivity lost when free RNA is irradiated at 254 mμ can be regained by photoreactivation, whereas none can be regained when the intact virus is irradiated (Bawden & Kleczkowski, 1959). Thus, inside the virus the RNA seems to be completely protected from photoreversible damage. Protection of the RNA by the protein, complete against photoreversible and partial against non-photoreversible damage by u.v., may result from some details of structure in the combination of RNA with the protein, or perhaps from a partial transfer of energy from RNA to protein.

A possible reason for the rapid increase in susceptibility of TMV to inactivation as the wavelength decreases below 250 mμ may be that the RNA inside the virus becomes more sensitive to absorbed radiation, perhaps because the protein protects the RNA less. However, it may be that the RNA is about equally sensitive at all wavelengths but, as suggested by McLaren & Moring-Claesson (1961), at a short wavelength (e.g. 230 mμ at which about 90% of all absorbed energy is absorbed by virus protein) infectivity is lost because not only the RNA, but also the protein is damaged. They suggest that 'the host plant can not extract active RNA from a coat of protein which has become denatured by u.v. light, and therefore the quantum yield is higher than would be expected if the RNA was equally available to the plant after irradiation at any wavelength'. A similar explanation was suggested by Siegel & Norman (1958).

We thought it of basic importance to determine whether irradiation causes changes in virus protein contributing to the loss of infectivity of the virus, particularly at wavelengths shorter than 250 mμ. Our approach was based on the following reasoning. If the loss of infectivity during irradiation of the virus only partly reflects damage to the RNA and is partly caused by damage to the protein (so that some virus particles are noninfective because the RNA is damaged and some because the host plant cannot release undamaged RNA from damaged protein coat), the RNA isolated by the phenol method from irradiated virus should be relatively more infective than the irradiated intact virus. This was tested after irradiating the virus at wavelengths of 230 mμ and 280 mμ, i.e. near the two extremes of the known action spectrum. Published results show that the relative infectivities are probably the same after irradiation at 254 mμ (Siegel & Norman, 1958; Bawden & Kleczkowski, 1959; Goddard *et al.* 1966).

METHODS

Tobacco mosaic virus (TMV), type strain, was purified from sap of infected tobacco leaves by several alternate precipitations by one third saturation with ammonium sulphate and by adjusting the pH value to 3·4, followed by differential centrifugation.

Irradiations were made with a Hilger and Watts large-aperture quartz-prism monochromator, using a 250 W xenon discharge lamp as a source of radiation. The width of the entry slit was 1·5 mm. and that of the exit slit 1·11 mm. and 2·0 mm. with the wavelengths of 280 mμ and 230 mμ, respectively. One ml. samples of a 0·05% solution of TMV in 0·066M-phosphate buffer of pH 7·0 were irradiated in a quartz cuvette of square cross-section with sides 1 cm. long. The cuvette was positioned so

that the whole beam of radiation, which was also square in cross-section, fell on the irradiated fluid. Light from the visible part of the spectrum was used for positioning the cell. A quartz stirrer, driven by an electric motor, ensured that all virus particles were equally exposed to radiation.

Intensities of radiation were measured actinometrically by the sensitive potassium oxalate method of Hatchard & Parker (1956). The actinometric solution was placed in the same quartz cuvette used for irradiating the virus, and irradiated in the same way.

RNA was isolated by the phenol method in a cold room at 4°. One ml. of water was added to 1 ml. of a 0·05% solution of irradiated or unirradiated TMV in 0·066 M-phosphate buffer at pH 7·0, and mixed with 2 ml. of water saturated phenol (at 4°). The mixture was shaken vigorously by hand for 1 min. and centrifuged for 10 min. at 10,000 rev./min. The water phase (top) was transferred to a small separation funnel and extracted three times with 20 ml. of diethyl ether, which was removed after each extraction with a pipette. Finally, the solution was placed in a vacuum to remove most of the dissolved ether, and the volume was adjusted to 5 ml. with 0·033 M-phosphate buffer at pH 7·0 The solution and/or its dilutions in the buffer were kept in an ice bath until inoculated to test plants.

Infectivity was assayed by the local-lesion method, using leaves of *Nicotiana tabacum* var. Xanthi-nc. Infectivities of irradiated whole virus and of the RNA extracted from it were assayed separately by comparison with their own controls. No comparison was intended or needed of infectivities of the whole TMV and of RNA extracted from it. For estimating the infectivity of the whole irradiated TMV, a sample of 0·025 ml. was withdrawn from 1 ml. of irradiated solution before this was subjected to the procedure for isolating the RNA. The sample was added to 2·5 ml. of 0·066 M-phosphate buffer at pH 7·0, thus diluting it about 1/100.

For reasons discussed below, the doses of irradiation given were such that the residual infectivity was about 25% of the original. Four inocula were used in each assay; one was an irradiated preparation and the others were three different dilutions of an unirradiated (control) preparation. The irradiated solution of the virus was diluted 1/100 and the unirradiated solution 1/100, 1/200 and 1/400. The RNA preparation from irradiated virus, obtained as described above, was used undiluted and the preparation from unirradiated virus was undiluted, diluted 1/2 and 1/4. Leaves of plants to be inoculated with the RNA were dusted with carborundum powder (grade 600); the powder was washed off after inoculation. With these conditions the numbers of lesions were usually proportional to the concentrations of the virus and of the RNA, and the use of three different dilutions of the control made it possible to see whether this was so in every individual assay.

Twelve plants, all stripped to two leaves, were used in each assay, and each of the four inocula was inoculated (by rubbing with the forefinger dipped in the inoculum) to 12 half-leaves so that each inoculum occurred once on each plant and 6 times on each leaf position. Except for these two restrictions, the positions of half-leaves were allocated to each inoculum at random. The proportions of original infectivity remaining in irradiated preparations were estimated by a statistical method (Kleczkowski, in preparation) that gives an estimate of log p (where p is the proportion), and also the variance of the estimate.

RESULTS

Comparison of relative infectivities of irradiated TMV and of RNA isolated from it

To assay infectivity of free RNA from irradiated TMV, the infectivity of all the yield of RNA extracted by the phenol method from irradiated virus was compared with infectivity of all the yield of RNA from unirradiated virus. (The quantities of irradiated virus were too small for any accurate physical estimation of concentrations of RNA in the extracts.) This introduced an error because of a variation between yields obtained on different occasions from similar materials, in addition to the error of estimating infectivity. Therefore, as already stated, a statistical method was used to estimate log p from results of each individual experiment, and a mean of several estimates was needed to make the standard error of the mean reasonably small. The same statistical method was used to obtain the values of log p for irradiated TMV.

Table 1. *Estimated logarithms of proportions (p) of original infectivity remaining in the whole irradiated TMV and in preparations of RNA isolated from the irradiated TMV*

Irradiation at 280 mμ. The dose of supplied radiant energy was $1{\cdot}7 \times 10^{-6}$ einsteins (0·74 J)			Irradiation at 230 mμ. The dose of supplied radiant energy was $3{\cdot}1 \times 10^{-7}$ einsteins (0·16 J)		
	Log p			Log p	
Expt no	TMV	RNA	Expt no	TMV	RNA
1	−0·60	−0·49	7	−0·62	.
2	−0·47	−0·43	8	−0·66	.
3	−0·63	−0·51	9	−0·50	−0·39
4	−0·61	−0·25	10	−0·68	−0·87
5	−0·67	−0·58	11	−0·59	−0·48
6	−0·50	−0·92	12	.	−0·59
			13	.	−1·11
			14	.	−0·38
			15	−0·59	−0·31
Means	−0·58	−0·53	Means	−0·61	−0·59
	±0·03	±0·11		±0·03	±0·10
p	0·26	0·30	p	0·25	0·26

The virus was inactivated only to about 25% of the original infectivity for three reasons. First, a more extensive inactivation would have required inconveniently long periods of exposure. Supplying only 0·16 J of radiation energy at a wavelength of 230 mμ to bring infectivity to about 25% of the original required 6 hr exposure. Secondly, with the amount of virus that could be irradiated, more inactivation would have made lesions caused by the RNA too few to estimate the residual infectivity. Thirdly, it was desirable to avoid radiation damaging the virus protein extensively in a way that might affect the yield of RNA extracted by phenol, especially because the yield from still-infective and from inactivated particles might be affected differently. This was especially relevant with irradiation at 230 mμ at which the virus protein absorbed about 10 times more radiation energy than the RNA. Inactivation to about 25% of the original infectivity was considered to be a safe maximum for estimation of residual infectivity.

Estimates of log p obtained with free RNA from irradiated TMV were much more

dispersed than those obtained with irradiated whole TMV (Table 1). However, the mean values of variances (not shown in the table) of estimates of log p, computed from the variation in the numbers of lesions obtained in individual experiments, were similar. The estimations of infectivities of the inocula, therefore, were about equally precise with TMV and with RNA. The values of the variance obtained from results of all experiments could therefore be pooled, giving the value of 0·007 based on several hundreds of degrees of freedom.

The values of the variances of estimates of log p obtained with the whole irradiated virus, computed from the actual dispersion of the values of log p (as shown in Table 1), were 0·006 and 0·004 for inactivation at 280 mμ and 230 mμ, respectively, both with 5 degrees of freedom (D.F.). These values did not differ significantly (by the variance ratio rest) from 0·007 (see above), and so we concluded that the estimation of infectivity of the inocula was the only source of error in getting the values of log p for the whole irradiated virus. Therefore the variance of 0·007 was used to obtain the standard error of the mean values of log p for irradiated TMV (Table 1).

The values of the variances of estimates of log p obtained with free RNA from irradiated TMV, computed from the actual dispersion of the values of log p, were 0·049 (with 5 D.F.) and 0·087 (with 6 D.F.) for inactivation at 280 mμ and 230 mμ respectively. Both values being significantly greater than 0·007 (see above), we concluded that in addition to the error of assay of infectivity of RNA there was another source of error contributing to the dispersion of the estimated values of log p. This doubtless came from a variation in yields of RNA extracted from the virus. However, the two values of the variance (i.e. 0·049 and 0·087) did not differ significantly from each other, and so could be pooled, giving the value of 0·07 (with 11 D.F.) which was used to compute the standard errors of the mean values of log p obtained with free RNA (Table 1).

The means of the values of log p obtained with TMV and with free RNA at any one of the two wavelengths were close to each other, so we concluded that the residual infectivities of irradiated TMV and of free RNA isolated from it did not differ appreciably. Consequently, loss of infectivity when TMV was irradiated resulted entirely from changes in its RNA.

Quantum yields for inactivation at 230 mμ and 280 mμ

Although we concluded that inactivation of TMV by u.v. resulted from changes in its RNA, we did not know whether the energy received by the RNA was only that absorbed directly by it, or whether some absorbed by protein was transferred to the RNA. (This was discussed by McLaren & Shugar, 1964). The quantum yields for inactivation of RNA inside the virus are computed below on the assumption that the energy received by RNA was the energy directly absorbed by it. The content of RNA in TMV is taken as 5 %, and the molecular weight of the RNA as 2×10^6. The specific absorbence of TMV was found to be 9·8 at 230 mμ and 2·6 at 280 mμ, and the specific absorbence of free RNA was 10·0 at 230 mμ and 11·5 at 280 mμ. It follows that the amount of radiation energy scattered and absorbed by 1 ml. of 0·05 % solution of TMV, containing $1{\cdot}25 \times 10^{-11}$ moles of RNA, was about $3{\cdot}1 \times 10^{-7}$ einsteins at 230 mμ (when log $p = -0{\cdot}61$) and about $1{\cdot}62 \times 10^{-6}$ einsteins at 280 mμ (when log $p = -0{\cdot}58$), of which $(0{\cdot}5/9{\cdot}8) \times 3{\cdot}1 \times 10^{-7} = 0{\cdot}16 \times 10^{-7}$ and $(0{\cdot}575/2{\cdot}6) \times 1{\cdot}62 \times 10^{-6} = 0{\cdot}36 \times 10^{-6}$ einsteins respectively, were absorbed by the RNA.

Thus, the quantum yield for inactivation of RNA at 230 mμ is (McLaren & Takahashi, 1957)

$$\phi_{230} = \frac{2{\cdot}3 \times 1{\cdot}25 \times 10^{-11} \times 0{\cdot}61}{0{\cdot}16 \times 10^{-7}} = 110 \times 10^{-5},$$

and at 280 mμ it is

$$\phi_{280} \quad \frac{2{\cdot}3 \times 1{\cdot}25 \times 10^{-11} \times 0{\cdot}58}{0{\cdot}36 \times 10^{-6}} = 4{\cdot}6 \times 10^{-5}.$$

The RNA inside the virus seems to have been about 24 times more sensitive to inactivation at 230 mμ than at 280 mμ. These yields were only about half those reported previously (McLaren & Moring-Claesson, 1961), but the ratio is almost the same, namely 22.

Because of the relatively large quantum yield at 230 mμ, it seemed possible that the RNA inside intact virus irradiated at this wavelength may not have been protected from the photoreversible kind of damage, i.e. that some of the lost infectivity may have been regained by photoreactivation. To test whether this was so, a 0·05% solution of TMV was irradiated at 230 mμ with a dose of $2{\cdot}8 \times 10^{-6}$ einsteins (1·23 J). Two lots of 12 plants (each plant stripped to two leaves) were used to assay infectivity of the irradiated virus. The unirradiated solution of the virus was inoculated at dilutions 1/50, 1/100 and 1/200 and the irradiated solution at 1/10. One lot of plants was inoculated and kept in daylight, and the other was inoculated in a dark room illuminated with a yellow light and then kept for 24 hr in darkness. The values of log$p$ were $-1{\cdot}04$ and $-1{\cdot}10$, respectively, i.e. the remaining infectivities were 9% and 8% of the original. Thus, there was no photoreactivation.

DISCUSSION

If the RNA inside the virus were equally sensitive to absorbed u.v. at any wavelength, and if the greater sensitivity of TMV to inactivation at 230 mμ than at 280 mμ resulted from some change in virus protein produced by 230 mμ but not by 280 mμ, the quantum yield for inactivation of RNA inside the virus at 230 mμ would be the same as at 280 mμ, i.e. $4{\cdot}6 \times 10^{-5}$. Therefore, log p for RNA that has absorbed $0{\cdot}16 \times 10^{-7}$ einsteins of energy at 230 mμ while inside the virus, and has then been isolated, would be expected to be

$$\log p = -0{\cdot}4343 \times 4{\cdot}6 \times 10^{-5} \times 0{\cdot}16 \times 10^{-7} / 1{\cdot}25 \times 10^{-11} = -0{\cdot}0256$$

(i.e. $p = 0{\cdot}94$), which differs greatly from $-0{\cdot}59$ ($p = 0{\cdot}26$), which is the mean of seven actually estimated values. The assumption that the RNA inside the virus is equally sensitive to absorbed u.v. of any wavelength must therefore be rejected.

The extent to which the protein protects the RNA from inactivation by u.v. can be obtained by comparing the quantum yields for inactivation of RNA inside the virus with those previously obtained for inactivation of free RNA. Kleczkowski (1963) found quantum yields for inactivation of free RNA without photoreactivation of about 8×10^{-4} at 280 mμ and about 10^{-3} at 230 mμ. The quantum yields with photoreactivation are half the above values, because the 'dose reduction' factor is about 0·5 (Bawden & Kleczkowski, 1959; Rushizky *et al.* 1960). The above values agree with the results previously obtained by McLaren & Takahashi (1957) and by Bawden

& Kleczkowski (1959) in spite of differences in concentration of RNA when irradiated, but differ from those obtained by Rushizky *et al.* (1960) who obtained values about 3 times greater and concluded that quantum yield depends on the concentration at which RNA is irradiated. There is, therefore, a disagreement here that should be resolved in future by a special investigation. In the meantime we shall use the smaller values of quantum yields, as they are based on the majority of the results to date.

As RNA irradiated inside the virus does not undergo photoreversible changes, quantum yields for inactivation of free RNA with photoreactivation (i.e. when photoreversible changes have been reversed) seem relevant for comparing the susceptibility of free RNA with that inside the virus to inactivation by u.v. At 280 mμ the quantum yield for free RNA (i.e. 4×10^{-4}) is about 10 times greater than that for RNA inside the virus. The extent of protection is therefore considerable. However, at 230 mμ the quantum yield for RNA inside the virus is twice that of free RNA with photoreactivation (i.e. 5×10^{-4}), and is about the same as the quantum yield for free RNA without photoreactivation. As no photoreactivation could be demonstrated with TMV inactivated at any wavelength, including 230 mμ, the susceptibility of RNA to non-photoreversible damage seems actually twice as great when RNA is inside the virus as when it is free. Therefore the RNA inside the virus seems not to be protected from damage by irradiation at 230 mμ. On the contrary, the union with the protein seems to increase the susceptibility of the RNA to inactivation at 230 mμ, but this may be questionable because the quantum yields that are compared may not be exactly correct. If it is true, a possible explanation could be that some energy absorbed by the virus protein during irradiation at 230 mμ is transferred to RNA, whereas this does not happen during irradiation at wavelengths longer than 250 mμ. On the contrary, the protective effect of the protein at the longer wavelengths may, at least partly, result from some energy absorbed by the RNA being transferred to the protein.

The binding of protein subunits to RNA that has recently been found to occur when TMV is irradiated with u.v. (Goddard *et al.* 1966) seems not to be relevant to the mechanism of inactivation of TMV; it if were, the RNA freed from irradiated virus by phenol, which breaks the binding, would be expected to be relatively more infective than the irradiated virus, whereas the relative infectivities were the same after irradiation at any of the tested wavelengths. That they are the same after irradiation at 230 mμ is especially significant because damage to protein at this wavelength was previously suspected to contribute to loss of infectivity of TMV.

Some other recent work, however, is relevant to the observations that irradiation causes at least two kinds of damage in free RNA, one photoreversible and the other not, whereas irradiated virus exhibits only the latter. Tao, Gordon & Nester (1966) report that RNA irradiated in H_2O and D_2O was photoreactivated to the same extent, although the rate of inactivation was greater in H_2O. This suggests that hydration of a 5,6-pyrimidine double bond is involved in inactivation (see McLaren & Shugar (1964) for a discussion of pyrimidine photochemistry). However, intact TMV was inactivated at the same rate in H_2O and D_2O, suggesting that the double bonds of RNA are not hydrated in the intact virus.

Still another kind of damage may occur in free RNA, because Evans, Savige & McLaren (1966) found that HCN, at a concentration that eliminates hydration in polyuridylic acid, had no effect on the photoreversible fraction of damage in irradiated RNA. It seems most unlikely that plants in daylight could reverse RNA-H_2O,

RNA-D_2O and RNA-HCN addition compounds with equal ease, and a third kind of damage seems implicated. Neither pyrimidine dimer formation (Tao *et al.* 1966) nor hydrate formation (Evans *et al.* 1966) seem to be among initial photochemical changes within the u.v. dose range of biological significance.

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