THE ULTRAVIOLET LIGHT AND PHOTOSENSITIZED INACTIVATION OF TOBACCO MOSAIC VIRUS

By GERALD OSTER* AND A. DOUGLAS McLAREN‡

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey, and the Institute of Polymer Research, Polytechnic Institute of Brooklyn, Brooklyn, New York)

(Received for publication, August 5, 1949)

INTRODUCTION

It has been known for some time that viruses are inactivated by ultraviolet radiation. The studies of Hollaender and Duggar (1) and of Price and Gowen (2) have shown that tobacco mosaic virus is inactivated according to the simple exponential law suggestive of a "one-hit" process. Rough calculations made by Uber (3) using the data of Price and Gowen indicate that the quantum yield for the inactivation process is much smaller than that observed for other proteins (4). Since tobacco mosaic virus is obtainable in pure form (Stanley (5)) and since considerable chemical and physical information is available concerning this nucleoprotein (for review, see references 6 and 7), it was felt that a quantitative study of the ultraviolet light inactivation of tobacco mosaic virus might be useful in an understanding of the general problem of the effects of ultraviolet radiation on living systems and on proteins.

Although tobacco mosaic virus is not affected by visible light, it can be rendered inactive if irradiated with visible light in the presence of a dye which is adsorbed on the virus. This photosensitization of the virus by the dye may serve as a model for elucidation of the photosensitized lethal action of visible light on simple biological systems.

In the present work, the quantum yield of ultraviolet light inactivation was determined. Possible physicochemical changes accompanying the inactivation were studied. The ultraviolet destruction of ribosenucleic acid obtained from the virus was also examined. The virus is inactivated by visible light when the dye acriflavine is adsorbed on the virus. The quantum yield of this process was determined and its effectiveness with and without oxygen was studied.

Experimental Methods and Results

Ultraviolet Light Inactivation of the Virus.—Solutions of tobacco mosaic virus purified by differential centrifugation by the method of Stanley (8) were treated with ultraviolet radiation of wave length of 253.7 mμ. The virus samples, between 4 and 5 ml.

* Present address: Davy Faraday Research Laboratory, Royal Institution.

‡ Present address: Carlsberg Laboratory, Copenhagen.

(6.4×10^{-4} gm. virus per ml. in 0.1 molar phosphate buffer at pH 7.0), were placed in quartz vessels and the samples were stirred during the period of irradiation (Fig. 1). An Hanovia mercury Sc-2537 lamp was used with an acetic acid (43 per cent solution) filter which cuts off radiation shorter than 253.7 mμ. Details of this filter system are given elsewhere (9). The samples received no heat from the lamp and remained at room temperature.

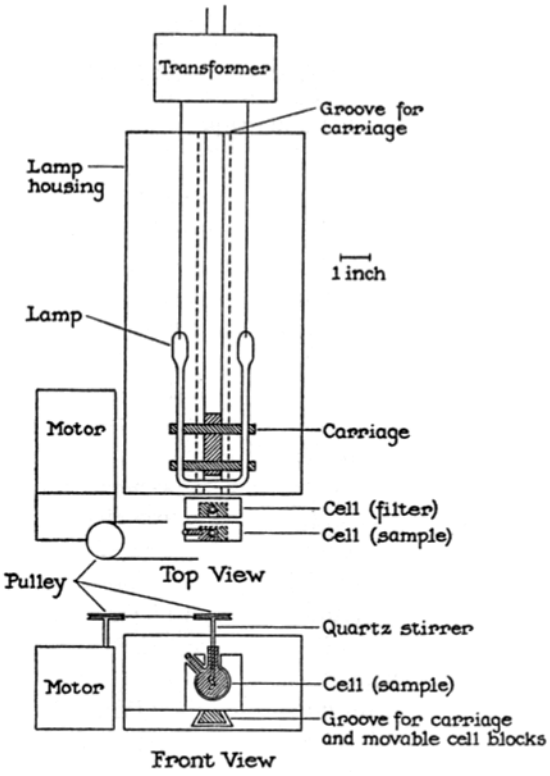

FIG. 1. Irradiation apparatus (see text for explanation).

At a distance of 3.5 cm. from the lamp, a fused quartz cell of 1.0 cm. depth and 2.6 cm. inside diameter was mounted in a 4.2 cm. square block drilled to fit and also mounted on a sliding rail. The quartz cell was equipped with two arms, each 0.6 cm. inside diameter, at an angle of 45° with each other. A fused quartz stirrer driven at 150 R.P.M. was employed. Between the lamp and the two-armed cell was placed, in a similar block, another quartz cell which contained the acetic acid.

The virus solution was irradiated for a certain length of time, the contents of the cell emptied, the next sample was irradiated for a shorter time, the cell emptied, and so forth. The intensity of light striking the contents of the cell was determined by a uranyl oxalate actinometer (10). With this actinometer the error in intensity at 253.7

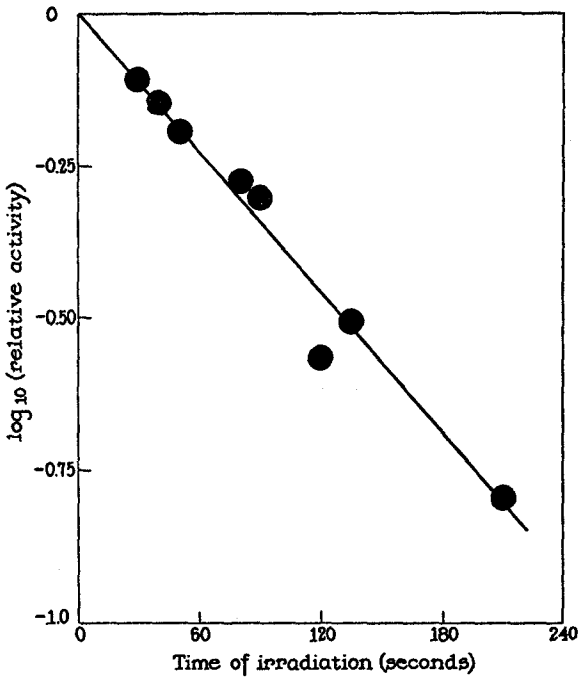

FIG. 2. Biological activity of tobacco mosaic virus as a function of time of irradiation (253.7 mμ).

TABLE I

Biological Activity of Ultraviolet Light-Treated Tobacco Mosaic Virus

Time of treatment	Relative activity
sec.	*per cent*
0	(100)
30	78
40	71
50	64
81	53
90	50
120	27
135	31
210	16

mμ is not greater than 2 per cent of the absolute value (9). The virus concentration employed corresponds, at 253.7 mμ, to a 96.8 per cent absorbing system. This wave length is nearly that for the maximum in the ultraviolet absorption spectra for the virus due primarily to the nucleic acid which it contains (6, 11).

The biological activity of the virus was determined on *Nicotiana glutinosa* by the

local lesion method of Holmes and others (12). The untreated virus was diluted to a virus concentration of 6×10^{-6} gm. per ml., and was applied on either half of the leaves and compared with the irradiated virus which had been diluted to give a comparable number of lesions on the other half. In Table I are given the mean values of three sets of determinations corresponding to nearly 20,000 lesions counted. Fig. 2 shows the logarithm (base ten) of the relative activity as a function of the time of treatment. The results follow the simple exponential decay curve.

The distance between the mercury lamp and the cell containing the virus was increased so that the intensity of the light on the cell was halved. Then the inactivation of the virus proceeded in twice the times given in Table I; *i.e.*, the inactivation depended on the dosage but not the intensity.

The quantum yield Φ for this process, that is the number of virus particles which are inactivated for each quantum of light absorbed, is defined as:

$$\Phi = \frac{\text{No. of moles virus particles inactivated per ml.}}{\text{No. of einsteins absorbed per ml.}} = \frac{cf}{MEF} \tag{1}$$

c = concentration in gm. virus per ml.
f = fraction of molecules inactivated during time of exposure.
M = molecular weight of the virus.
E = einsteins falling on 1 ml. of the solution during exposure.
F = fraction of light absorbed by the virus at the given wave length, corrected for the average amount of light absorbed by the active virus.

In this study $c = 6.4 \times 10^{-4}$ gm. virus per ml. In 1 minute at a wave length of 253.7 mμ the fraction of virus inactivated (see Fig. 2) is equal to 0.409 (the remaining activity is 59.1 per cent). During 1 minute 2.37×10^{-7} einsteins fell on a milliliter of solution, as shown from actinometer measurements. The molecular weight of the active virus is now known to be 41 millions (7, 13) (Uber (3) took $M = 17$ millions, an earlier estimate now known to be incorrect.) At this wave length the optical density D is, for a solution of 6.4×10^{-4} gm. virus per ml., 1.50 corresponding to 3.2 per cent transmission or an opacity of 0.968.

Because of the large size of the virus particles, however, much of this opacity is due to light-scattering (14). Tobacco mosaic virus is known to contain about 6 per cent ribose nucleic acid (15). The nucleic acid accounts for nearly one-third of the optical density of the virus at 253.7 mμ. Other ultraviolet-absorbing constituents in the virus are, from the work of Knight (16), tryptophane (2.1 per cent), tyrosine (3.8 per cent), and phenylalanine (8.4 per cent). At 253.7 mμ these substances have molar coefficients (D/k where k is in moles per liter) of 3600 (in HCl), 400, and 200 respectively (data from Loofbourow (17)). Taking into account the concentrations of these ultraviolet-absorbing substances in the virus, we calculate that the optical density of the virus at

253.7 mμ should be about 50 per cent of that observed, indicating that the other 50 per cent of the optical density is due to light-scattering by the virus particles. A direct calculation of the light-scattering of the particles at this wave length is difficult because the particles are greater in length than the wave length of the light (18). However, with the use of light-scattering formulae (Equation 10 of reference 18) and using the index of refraction for visible light of the virus solution (14), nearly 80 per cent of the optical density of the scattered light may be accounted for (compare reference 19). It is difficult to correct for secondary scattering; *i.e.*, light scattered by the particles which is then scattered and absorbed by the particles. We shall assume that the scattered light leaves the solution without being reabsorbed by the particles. Pertinent data for a solution of the virus of concentration 6.4×10^{-4} gm. per ml. are as follows:—

	Density	Transmitted	Opacity
		per cent	*per cent*
Observed	1.50	3.2	96.8
Calculated from absorbing constituents	0.75	17.8	82.8

Since we are concerned with light absorbed by active virus, we must correct the absorption by the average value of the activity during the duration of the time considered. For a period of irradiation of 1 minute, this is seen graphically (Fig. 2) to be equal to 78 per cent. In summary, then, the fraction of light absorbed F equals opacity $\times$ fraction due to absorbing constituents $\times$ fraction absorbed by active virus (average value during 1 minute) $= 0.968 \times 0.85 \times 0.78 = 0.64$. Thus the quantum yield is

$$\Phi = \frac{cf}{MEF} = \frac{6.4 \times 10^{-4} \times 0.409}{4.1 \times 10^{7} \times 2.37 \times 10^{-7} \times 0.64} = 4.3 \times 10^{-5}$$

Stanley (20) has performed qualitative experiments on the ultraviolet light inactivation of tobacco mosaic virus and found no measurable changes in certain chemical and serological properties of the virus. The increase in turbidity observed by Stanley (a full mercury arc was employed) was probably due to heat denaturation since in the present work it was found that irradiation of the solutions kept at room temperature produces inactivation without an increase in turbidity. Irradiation at 37°C. resulted in an increase in turbidity, however, although heating the normal virus at this temperature for the same length of time does not give an increase in turbidity.

In order to observe any possible chemical or physical modification of the virus, several tests were made on virus which had been subjected to ultraviolet light irradiation. It was found that a sample irradiated for as long as 30

minutes and having less than 2 per cent activity remaining showed no change in viscosity. Viscometric measurements give an indication of the shape of the virus (21), but here the intrinsic viscosity did not change under irradiation. This is in contrast to sonic irradiation which breaks down the rod-like virus particles (13). Other physical properties of the virus which remained unchanged were its moving boundary in the ultracentrifuge, its optical turbidity, its isoelectric point, its ultraviolet absorption spectra, and its appearance in the electron microscope. The irradiated sample was sedimented in the ultracentrifuge but no nucleic acid was found in the supernatant fluid. It was found, however, that the irradiated virus was more sensitive to heat denaturation than the untreated virus, but this change in property was not studied in any detail. This effect has also been found for proteins (22).

Inactivation of the virus by ultraviolet light irradiation was found to be independent of whether oxygen or oxygen-free nitrogen (prepared as described below) was bubbled through the system for 2 hours before and during the period of irradiation.

Degradation of Virus Nucleic Acid

Ribose nucleic acid is released from the virus on heating. Samples of nucleic acid made in this way and purified by the method of Cohen and Stanley (23) were found to have an intrinsic viscosity of 13, *i.e.* for a rod, an axial ratio of 10. This corresponds to "Nucleate C" of Cohen and Stanley and has, according to their measurements, a molecular weight of 15,000. This would correspond to about 12 tetranucleotides per nucleic acid molecule.

On irradiation of an 0.3 per cent solution of nucleic acid for 21 hours in a coil Sc-2537 resonance lamp with an acetic acid filter, the intrinsic viscosity decreased from the initial value to practically zero, suggesting that the molecules had broken down to much smaller molecules. This solution has no appreciable transmission and, therefore, only material in the outer surface of the vessel was irradiated; but due to the long exposure, convection currents and Brownian movement would be expected to allow all the molecules to be irradiated. It was necessary to use such a concentrated solution in order to make viscosity measurements.

It is known that degradation of ribose nucleic acid is accompanied by an increase in absorption at 260 mμ (24, 25). We found a 10 per cent increase in absorption (on suitable dilution) with the above solution of virus nucleic acid when it had been irradiated. A dilute solution of the virus nucleic acid (8×10^{-5} gm. per ml.; $F = 0.60$ at 253.7 mμ) was irradiated for 3 minutes at 253.7 mμ in the apparatus described in the previous section. The maximum in the ultraviolet spectra increased by 10 per cent in optical density. A further indication that the nucleic acid was degraded by the ultraviolet irradiation is that after irradiation, the nucleic acid failed to give the typical metachromatic staining with safranin (25).

Photosensitized Inactivation of the Virus

Tobacco mosaic virus particles are negatively charged at pH values above the isoelectric point of the virus and will, therefore, adsorb basic dyes. The basic dye chosen was acriflavine since its binding with the virus has been studied in detail (25). The spectra for this dye are given in Fig. 3 (filled circles). There are maxima at 450 mμ and 260 mμ; the former corresponding to that for the chromophoric group and the latter to that for the benzene rings. The dye

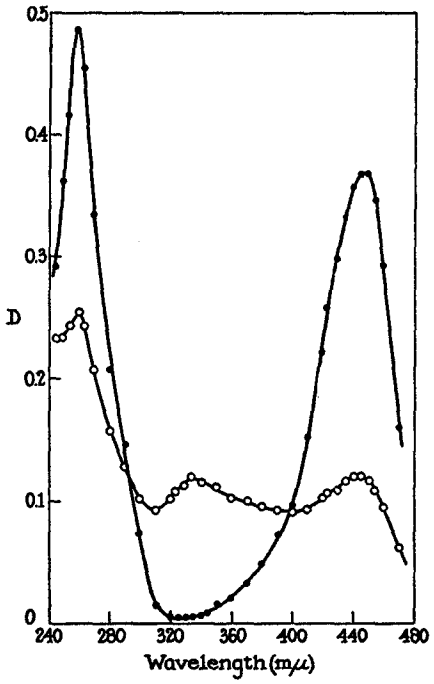

FIG. 3. Absorption spectra of acriflavine (8.3×10^{-6} molar) untreated (filled circles) and irradiated with blue light for 45 minutes (open circles).

and the dye-virus systems were irradiated with the 436 mμ line of mercury by placing in front of a mercury arc a blue glass filter transmitting this wave length. The lamp used was a water-cooled H-6 (General Electric) high pressure lamp. To further eliminate possible heating of the sample, a glass water bath was placed between the lamp and the filter. The sample was placed beyond the filter 8 inches from the lamp. The number of einsteins falling on a milliliter of solution per hour was 8.0×10^{-5} as determined by a uranyl oxalate actinometer.

The absorption spectra of the dye after 45 minutes' irradiation at 436 mμ are shown in Fig. 3 (open circles). Although the incident light corresponds to the region of absorption for the chromophoric group, the benzene rings are

also destroyed by the visible light and a new absorption peak is produced in the spectra at 335 mμ. The yellow fluorescence of the dye is also reduced on irradiation. In Fig. 4 (filled circles) is shown the decrease in absorption of the dye at 436 mμ as a function of time of irradiation. The optical density of the dye is proportional to its concentration and the decrease with time follows a first order reaction. In determining the quantum yield for the destruction of the dye, it is necessary to take into account the change in transmission of the solution, since as the color is lost there are less quanta absorbed by the solution. Then the quantum yield is given by the expression

$$\Phi = \frac{cf}{MEF'(1 - e^{-kt})}$$

where k is the rate of decrease of optical density. From Fig. 4 (filled circles) k equals 1 reciprocal hour (60 minutes to reduce the optical density to 1/eth of its value) so $1\text{-}e^{-kt} = 0.66$. For an optical density of 0.38 ($c/M = 8.3 \times 10^{-9}$) the fraction, F', absorbed in 1 cm. cell thickness is 0.24, so

$$\Phi = \frac{8.3 \times 10^{-9} \times 0.33}{8.0 \times 10^{-5} \times 0.24 \times 0.66} = 2.2 \times 10^{-4}$$

In the calculation of the quantum yield for the dye we did not take into account the fraction of the light which strikes the system and is transformed into innocuous yellow light by fluorescence. Taking a fluorescein solution of sufficient dilution (1.8×10^{-5} gm. per liter with a small amount of ammonium hydroxide) to give the same optical density as the acriflavine solution used, we find that at 436 mμ the acriflavine solution is one-half as fluorescent as is the fluorescein solution. Since at this dilution the quantum efficiency of fluorescence of fluorescein is nearly unity (28), then the effiiciency of acriflavine is one-half; *i.e.*, half the blue light which strikes the solution is wasted as yellow fluorescent light which is not appreciably absorbed by the dye. The quantum yield calculation above must be corrected for this effect, that is, for the dye $\Phi = 4.4 \times 10^{-4}$.

In the presence of virus, the dye is not decomposed as rapidly as it is in the absence of the virus. In Fig. 4 (open circles) is shown the decrease in absorption at 436 mμ of the dye in the presence of the virus with the scattering due to the virus (6.4×10^{-4} gm. per ml.) subtracted. The destruction of the dye is approximately linear with time of irradiation.

The amount of dye adsorbed on the virus particles was determined by sedimenting the virus from the dye solution and measuring the loss of dye in the supernatant fluid (25). In the present case, a 10 ml. mixture of 8.30×10^{-6} molar dye and 6.4×10^{-4} gm. virus per ml. in distilled water gave in the supernatant fluid 5.80×10^{-6} molar dye; *i.e.*, 3.91×10^{-6} moles of dye were bound

to 1 gm. of virus. Taking a molecular weight of 41 millions for the virus particles, then there are 160 dye molecules adsorbed on each virus particle.

The inactivation of tobacco mosaic virus in the presence of the dye as a function of time of irradiation with blue light is shown in Fig. 5. The relative activities were determined by the half-leaf method described earlier. It will be seen that the inactivation is a first order reaction with a rate constant nearly that for the destruction of the dye in the absence of the virus (Fig. 4, filled

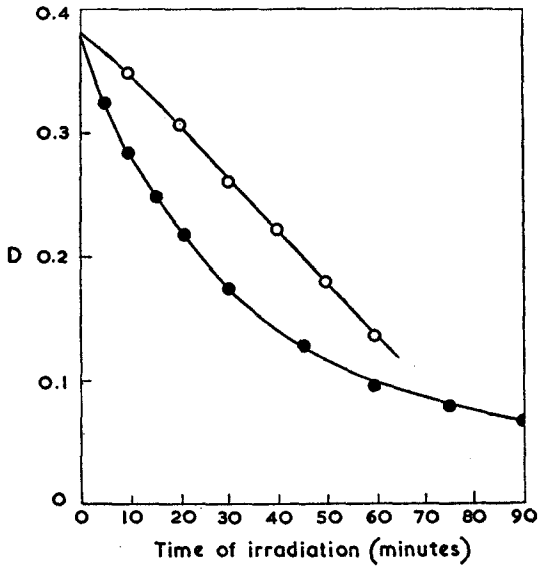

FIG. 4. Optical density at 436 mμ for acriflavine as a function of time of irradiation with blue light; dye alone (filled circles) and dye in the presence of the virus (open circles).

circles). No appreciable amount of inactivation of the virus takes place when a solution of virus in the absence of the dye is irradiated with blue light. The inactivation of the virus in the presence of the dye was considerably reduced when salt is added. Thus for the same dye and virus concentrations as above but in the presence of 0.1 molar NaCl, the relative activity after 1 hour irradiation was 88 per cent compared with 20 per cent when no salt was present. The intensity of fluorescence of the dye in the untreated sample was not influenced by the presence of the salt.

The destruction of the dye and the photosensitized inactivation of the virus are considerably influenced by the presence of oxygen, in complete contrast with the case of ultraviolet light inactivation. In Table II are shown the re-

sults on the photosensitized inactivation of the virus when bubbling air, oxygen, and nitrogen through the solutions. The gases were bubbled for 2 hours prior to and during the irradiation. The nitrogen was bubbled through Fieser's

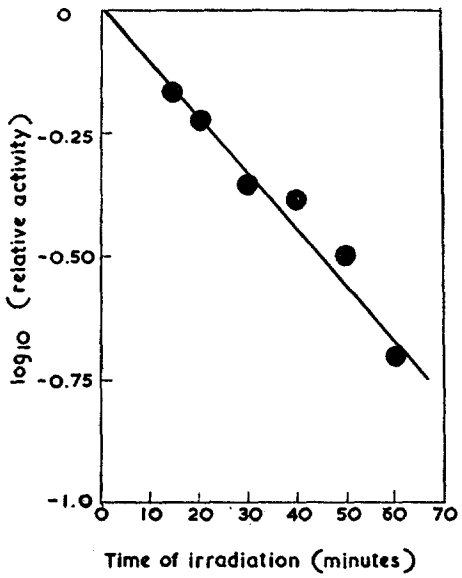

FIG. 5. Biological activity of tobacco mosaic virus in the presence of acriflavine as a function of time of irradiation with blue light.

TABLE II

Effect of Gases on Irradiation for 40 Minutes with Blue Light

	Control (unirradiated)	Nitrogen	Air	Oxygen
Density of dye at 436 mμ.....	0.380	0.237	0.208	0.102
Relative activity of virus, *per cent*.....	(100)	50	42	Less than 1

solution and through lead acetate solution to remove traces of oxygen and hydrogen sulfide respectively (29).

DISCUSSION

Only light which is absorbed is effective in photochemical reactions; therefore, one must distinguish between decrease in transmission due to light-scattering and that due to consumptive absorption. For proteins which are small compared with the wave length of light, at 260 mμ the optical density due to scattering equals $3.3 \times 10^{-5}\ cM$ (Equation 10 of reference 18. Index of refrac-

tion increment for proteins about equal to 0.18). This is not an important factor for low molecular weight proteins (*e.g.*, a 1 per cent solution of molecules of molecular weight 35,000 gives an optical density due to scattering of 0.011), but for particles of virus dimensions the scattering constitutes an important part of the opacity of the system. For particles of sizes comparable with the wave length of light no simple formula for the scattering may be given, and it is simpler to subtract the density of the known absorbing constituents from the observed opacity of the system. With an exponential decline of active virus with time of irradiation at constant intensity, and with a first power dependence of rate constant on intensity, one may say that although every quantum absorbed does not cause inactivation, when inactivation does occur the primary process involves one quantum (22). In another form

$$-\frac{dV}{dt} = \Phi I_{\text{abs.}} \frac{V}{V_0} \tag{3}$$

where V/V_0 gives the fraction of the light absorbed at any time, assuming the molecular extinction coefficients of active and inactive virus to be identical (as observed for tobacco mosaic virus). $I_{\text{abs.}}$ is the intensity of light absorbed. Upon integration there results

$$\ln \frac{V_0}{V} = \frac{\Phi I_{\text{abs.}}}{V_0} t \tag{4}$$

For any given initial molar concentration V_0 we have

$$\frac{\Phi I_{\text{abs.}}}{V_0} = k_I \text{ (a constant)} \tag{5}$$

which shows that k_I is a pseudo first order constant which depends on V_0 (since $I_{\text{abs.}}$ is a function of V_0). For any initial virus concentration we have the well known formula

$$V = V_0 e^{-k_I t} \tag{6}$$

The quantum yield calculated by either Equation 1 or 4 is, of course, the same.

The question arises as to the meaning of the quantum yield 4.3×10^{-5} for the virus. Two mechanisms suggest themselves: (*a*) any highly, thermally excited portions of the molecule, subject to a Boltzmann distribution, may be sites of photochemical rupture; (*b*) localized sensitive groups are involved. The latter mechanism is more amenable to calculation. Considering this possibility we note that only one quantum out of 23,300 is effective. There are 13,000 aromatic residues per virus particle. Must some particular linkages be hit with a quantum yield around 0.5 or may any group be hit with a yield of 4.3×10^{-5}? It is pertinent to note that the quantum yields for rupture of the peptide

bonds (9), the disulfide bond (26), and a pyrimidine (27) are in the neighborhood of 0.01 to 0.02. Since these values are much nearer 0.5 than 4.3×10^{-5}, it seems probable that 25 to 50 linkages are sensitive.

With regard to the degradation of the nucleic acid, we can make a rough estimation of the quantum yield in the following way. Assuming that a 10 per cent increase in light absorption by the dilute solution also means a breakdown to tetranucleotides, as apparently it does in the concentrated solution (see experimental), we calculate for a 3 minute exposure

$$\Phi = \frac{8 \times 10^{-5}}{\frac{15{,}000}{12} \times 3 \times 2.37 \times 10^{-7} \times 0.6} = 0.1$$

There are some reasons favoring a preponderance of depolymerization over oxidative degradation during irradiation of the free nucleic acid. (In the virus neither process takes place.) First, the viscosity, absorption, and staining properties of the irradiated nucleic acid are similar to those found after ribonuclease activity (25), a reaction typically considered as a depolymerization. Second, the above calculation assumes that the breakdown of purine and pyrimidine rings is slower than the depolymerization. This is probably correct, since the quantum yield for destruction of an ethoxy methyl amino pyrimidine was found to be only 0.018 (27). The approximate calculation above simply serves to show that the quantum efficiency for destruction of nucleic acid as well as for protein linkages is much higher than for the intact virus. The nucleic acid–protein framework of the virus apparently serves to hold together free radicals formed by quanta and thus allows for a high probability of recombination without net chemical change and with an over-all low quantum efficiency. The high stability of the virus may be contrasted with irradiation of enzymes which show relatively higher quantum yields accompanied by physical destruction of the molecules (22).

The mechanism of photosensitized inactivation is different from that of ultraviolet light inactivation. The former process requires the presence of a fluorescent molecule and is dependent on oxygen concentration (30), while the latter is a direct absorption mechanism proceeding at a rate independent of oxygen (22). Our data show that only adsorbed dye is effective, since, (*a*) the virus partially inhibits the destruction of the dye (Fig. 4, open circles) and (*b*) inactivation of the virus is slower if the dye is eluted by salt. In addition, if the fluorescent molecules were not attached to the virus particles they would lose their fluorescent energy to the solvent, since the lifetime of the dye is considerably less than the rate of diffusion of the dye to the virus.

The photosensitized inactivation of the virus proceeds under either oxygen or nitrogen; however, the effect is enhanced by oxygen. It is probably significant that the molar concentration of dissolved oxygen under an atmosphere of

pure oxygen or air is nearly that of the dye. This indicates that two mechanisms are operating, the one involving oxygen being the more efficient. The oxygen-dependent mechanism is perhaps related to similar effects observed with ionizing radiation (31).

SUMMARY

The quantum yield for the inactivation of tobacco mosaic virus has been determined at 253.7 mμ and found to be 4.3×10^{-5}. The possible significance of the observed one-hit process of inactivation has been discussed in terms of the kinetics and the rupture of model substances including nucleic acid. The ultraviolet light inactivation, which proceeds independent of oxygen, occurs without change in physicochemical properties, with the possible exception of an enhanced sensitivity to thermal denaturation.

The photosensitized inactivation of virus by acriflavine has been found to proceed parallel with the destruction of the dye. The action was found to be dependent upon adsorbed dye, and the inactivation is enhanced by the presence of oxygen.

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