Ultraviolet Sensitization and Photoreactivation of Tobacco Mosaic Virus Ribonucleic Acid Containing 5-Fluorouracil*

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The incorporation of 5-fluorouracil into the nucleic acid of tobacco mosaic virus sensitizes both the intact virus and the infectious nucleic acid to ultraviolet light at 253.7 mμ. The degree of sensitization depends upon the amount of analog incorporated. Nucleic acid and intact virus preparations, in which 17–20% of the uracil residues have been replaced by 5-fluorouracil, display only slight enhancement in sensitivity. The sensitivity increases with preparations that have 50% of the uracil residues substituted by 5-fluorouracil. Enhanced sensitization to ultraviolet light cannot be attributed to the presence of 5-fluorocytosine in the nucleic acid. The sensitivity of the 50% substituted nucleic acid toward ultraviolet light is wavelength dependent and increases progressively as the wavelength of the irradiation increases from 253.7 mμ to 296.7 mμ. Absorbancy differences between 5-fluorouracil and uracil appear to account for the increased sensitivity of the 5-fluorouracil-containing material toward irradiation in the near ultraviolet.

Extensive evidence has recently accumulated concerning the enhanced ultraviolet and X-ray sensitivity of bacteria (Greer, 1960; Kaplan *et al.*, 1962), bacteriophage (Stahl *et al.*, 1961; Sauerbier, 1961), and mammalian cells (Djordjević and Szybalski, 1960; Delihas *et al.*, 1962; Erikson and Szybalski, 1963) which contain thymine analogs in their DNA. Bacteria (Greer, 1960) and bacteriophage (Stahl *et al.*, 1961; Sauerbier, 1961) also show a concomitant loss in the extent of photoreactivation as the extent of the incorporation of the thymine analog, 5-bromouracil in these cases, into the DNA becomes progressively greater.

Since 5-fluorouracil can be incorporated into the nucleic acid of tobacco mosaic virus to a high degree (Gordon and Staehelin, 1959), we have studied the ultraviolet sensitization and photoreactivation of the analog-substituted nucleic acid in order to ascertain whether sensitization and a loss in photoreactivation occurs in a biological system in which RNA is the carrier of genetic information.

Preliminary reports of the ultraviolet sensitization of TMV[1] containing 5-fluorouracil have appeared (Bećarević *et al.*, 1963; Lozeron and Gordon, 1963).

MATERIALS AND METHODS

Virus Strain.—The U1 (common strain) strain of TMV was used in all experiments.

Preparation of 5-FU-substituted Virus.—5-Fluorouracil and 5-fluorocytosine were gifts of Dr. R. Duschinsky of Hoffmann-La Roche, Nutley, N. J. 5-Fluorouracil-2-^{14}C was obtained from the California Foundation for Biochemical Research, Los Angeles, Calif. Incorporation of 5-FU into the virus was performed by incubating infected Turkish tobacco leaf segments on an aqueous solution of 0.1% 5-FU-2-^{14}C (specific activity 6800 cpm/μmole). The incubation was started 12 hours after infection (Gordon and Staehelin, 1959). An incorporation of approximately 20% was attained under these conditions. A higher incorporation of 50% was obtained by incubating segments on 0.4% 5-FU. Following a 7-day incubation under fluorescent lighting (600 foot-candles), the leaf segments were harvested and the virus was purified by differential centrifugation (Knight, 1962). At the relatively high concentration of 5-FU of 0.4% the virus yields were about 50–70% of the controls in which no analog was present.

Isolation of the Nucleic Acid and Analysis for 5-FU.—The radioactive TMV-RNA, in which 20% of the uracil was replaced by 5-FU-2-^{14}C [TMV-RNA-FU-(20)], was prepared by the heat denaturation procedure (Knight, 1952). Uridylic and 5-fluorouridylic acids were separated from the other 2′,3′-mononucleotides by high-voltage electrophoresis at *p*H 9.2 in 0.10 M ammonium carbonate buffer, and the relative amounts of each nucleotide were determined by optical-density or radioactivity measurements. Analysis of the free bases, uracil and 5-FU, by 2-dimensional paper chromatography (Gordon and Staehelin, 1959) was also performed. Finally, the isotope-dilution technique was used in which a predetermined amount of carrier 5-FU was added to the radioactive TMV-RNA-FU(20) previous to digestion with perchloric acid, and a chromatographic analysis of the free bases was performed. The percentage incorporation determined by the three methods agreed to within 3%.

Detection of 5-Fluorocytosine in the Virus Nucleic Acid.—An isotope-dilution procedure was used. A 0.30-ml sample of carrier 5-fluorocytosine (1.0 mg/ml) was added to an ethanol precipitate of radioactive TMV-RNA-FU(20) (4.0 mg). This mixture was taken to dryness, hydrolyzed with 72% perchloric acid, and diluted with an equal volume of water. An aliquot of the sample was submitted to paper chromatography (Bendich, 1957). The ultraviolet-absorbing spot containing a mixture of cytosine and 5-fluorocytosine was cut out and eluted with water,

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[1] Abbreviations used in this work: 5-FU, 5-fluorouracil; TMV, tobacco mosaic virus; TMV-RNA-N, normal nucleic acid from this virus; TMV-FU(20), tobacco mosaic virus in which 20% of the uracil residues have been replaced by 5-fluorouracil; TMV-RNA-FU(20), the analog-substituted nucleic acid from this virus; TMV-FU(50), tobacco mosaic virus containing 50% 5-fluorouracil substitution; TMV-RNA-FU(50), the analog-substituted nucleic acid from this virus.

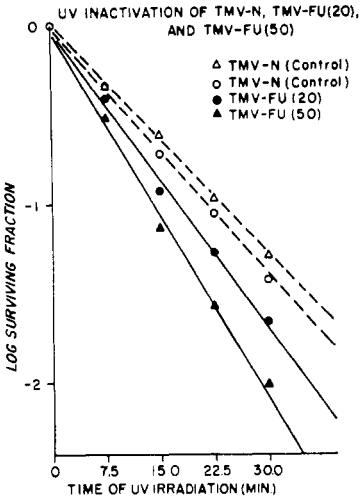

FIG. 1.—Ultraviolet inactivation of TMV-N, TMV-FU(20), and TMV-FU(50) using a filtered Mineralight V-41 lamp source at a distance of 17.0 cm. Lamp output energy was 2.8 ergs/mm² per sec. All irradiations were carried out at 0.10 M sodium phosphate buffer, *p*H 7.0. The same TMV-N control preparation has been used in the two experiments above and the slight variation in photosensitivity for the controls is the result of variation in experimental conditions.

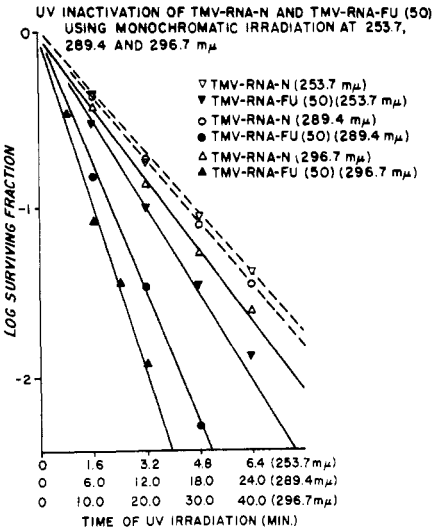

FIG. 2.—Ultraviolet inactivation of TMV-RNA-N and TMV-RNA-FU(50) using monochromatic irradiation at 253.7, 289.4, and 296.7 mμ. Lamp-output intensities were not sufficient to be measured by the ultraviolet dosimeter. All radiations were performed in freshly distilled water.

and the eluate was taken to dryness under nitrogen. The residue was dissolved in 0.05 ml of water and submitted to electrophoresis in 0.05 M ammonium formate buffer, *p*H 2.7. The voltage gradient was 30 v/cm. Under these conditions the movement of 5-fluorocytosine was 5 cm while cytosine moved 16 cm (both toward the cathode).

Irradiations.—Irradiations were carried out with a Mineralight V-41 mercury discharge lamp with a Corning filter No. 9863 (Ultra-Violet Products, Inc., San Gabriel, Calif.) at a wavelength of 253.7 mμ. The approximate energy emitted (ergs/mm² per sec) was calculated by using an ultraviolet dosimeter (Latarjet *et al.*, 1953).

Two ml samples of virus or virus nucleic acid prepared by the phenol method (Gierer and Schramm, 1956) were irradiated with shaking in open Petri dishes 4.5 cm in diameter at 0° in an ice-water bath. TMV and TMV-FU were irradiated at concentrations of 1.75 μg/ml and TMV-RNA and TMV-RNA-FU at concentrations of 40–80 μg/ml made up in either water or 0.05 M sodium phosphate buffer, *p*H 7.0. At specified time intervals appropriate aliquots of the irradiated solutions were withdrawn, diluted with ice-cold 0.10 M phosphate buffer, *p*H 7.0, and assayed. In all experiments normal and fluorouracil-substituted samples were irradiated simultaneously in order to eliminate errors resulting from possible variation in lamp-output energy. In the studies on the wavelength dependence of ultraviolet sensitization, the Bausch and Lomb 50mm × 50mm diffraction grating monochromator with a 200 watt Osram high pressure mercury arc light source and power supply (HBO 200 w/2) was used. The entrance and exit slits were both set at 1.5 mm corresponding to a band width of 10 mμ. The monochromator was positioned vertically so that the cooled sample contained in an open Petri dish could be shaken directly under the irradiation emanating from the exit slit. Irradiation distance was 23.0 cm.

Infectivity Tests.—Controls, irradiated virus, and virus nucleic acid samples were assayed on *Nicotiana tabaccum* var. *Xanthi*, n.c. (Fraenkel-Conrat, 1959). Normal and substituted samples were compared directly on opposite sides of the same leaf. Assay concentrations for virus and virus nucleic acid controls were of the order of 0.005 μg/ml and 0.50 μg/ml, respectively.

Photoreactivation Procedure.—The half-leaf assay procedure was used for the photoreactivation measurements. Each of the controls and irradiated solutions was assayed by inoculating six whole leaves chosen randomly from a series of plants, using 0.10 ml of inoculant per leaf. After inoculation the six leaves were immediately cut from the plants, washed with tap water, and cut along the mid-ribs. Opposite half-leaves were placed in separate trays containing cotton saturated with water. This procedure usually required 10 minutes, and was performed under yellow safety lights (Kodak Safelight Filter, Wratten Series 04, Eastman Kodak Co., Rochester, N. Y.). The tray containing one set of half-leaves was kept in the dark for 8 hours before being placed in the light; the other tray was immediately exposed to 1500 foot-candles (measured by a Spectral Professional light meter, Photo Research Corp., Hollywood, Calif.) of artificial light (Ken-Rad fluorescent lights) for 4–5 hours. This procedure was repeated twice for each assay solution, giving a total of 12 half-leaf assays per sample. During the dark and photoreactivation periods, all trays were kept at a temperature of 22–23°. The trays were then transferred to artificial lighting until the lesions were fully developed (about 3 days).

RESULTS

Viability of the Analog-substituted Virus Nucleic Acid.—It has been shown previously (Gordon and Staehelin, 1959; Holoubek, 1963) that the viability of TMV does not change as a result of the incorporation of 5-FU into the nucleic acid. We have again not been able to detect any significant differences in the number of local lesions produced by preparations of TMV-FU and TMV-RNA-FU containing as high as 50% replacement compared to the respective normal preparations when assayed at the same concentration levels.

Attempts to Detect 5-Fluorocytosine in the Virus Nucleic Acid.—Calculations based upon the isotope dilution data (specific activity of 5-fluorocytosine

assumed to be equivalent to that of 5-FU) indicated a recovery of 0.053 μg of 5-fluorocytosine from 2.0 mg of TMV-RNA-FU(20) or approximately 0.40 residue of 5-fluorocytosine per RNA molecule. Thus enhanced ultraviolet sensitization cannot be attributed to the presence of 5-fluorocytosine in the nucleic acid.

Ultraviolet-Inactivation Studies at 253.7 mμ.—The results of these studies are summarized in Figures 1 and 2. The points on the survival curves represent the compilation of data from two separate experiments or a total of 24 half-leaf assays per point.

Experiments carried out with intact virus preparations (Fig. 1) show that the slope ratios increase from 1.2 to approximately 1.5 at 253.7 mμ as the degree of 5-FU substitution increases from 20 to 50%. A similar increase in sensitization as a function of the extent of 5-FU substitution was obtained on comparing the infectious nucleic acids. The increased sensitivity of the analog-containing material is expressed as the survival-curve slope ratio which is defined as the ratio of the first-order rate constant for the inactivation of the analog-containing virus to the first-order rate constant for the normal virus.

In the above studies, the irradiations were performed with the Mineralight V-41 low pressure mercury lamp which, even with the Corning filter 9863, probably emits small amounts of 184.9 mμ radiation. In view of the marked effect of radiation of this wavelength on the photolysis of thymine (Daniels and Grimison, 1963), the ultraviolet sensitization of TMV-RNA-FU(50) was rechecked using monochromatic 253.7-mμ radiation. The same sensitization was obtained indicating that 253.7-mμ radiation is principally responsible for the degree of sensitization observed, rather than contaminating irradiation of other wavelengths.

The Wavelength Dependence of Ultraviolet Sensitivity of TMV-RNA-FU(50).—Irradiations of TMV-RNA-N and TMV-RNA-FU(50) at long wavelengths are illustrated in Figure 2. The slope ratio of 1.5 obtained at 253.7 mμ increases to 2.0 and 2.4 at 289.4 and 296.7 mμ, respectively.

Photoreactivation of Normal and Analog-substituted Nucleic Acid.—Initial studies with TMV-RNA-N and TMV-RNA-FU(20) irradiated with the filtered Mineralight at 253.7 mμ indicated that the extent of photoreactivation was the same in both cases. The results obtained with TMV-RNA-N and TMV-RNA-FU(50) are shown in Figure 3. In the case of the highly substituted RNA there is a slight but experimentally significant decrease in photoreactivation. The points on these curves represent average lesion numbers obtained from two independent experiments or a total of 25 half-leaf assays.

The degree of photoreactivation attained in these studies is somewhat less than that obtained by other workers (Bawden and Kleczkowski, 1959; Rushizky *et al.*, 1960). This is probably a reflection of the physiological condition of the plants. However, attempts to increase the extent of photoreactivation met with failure. Plants kept in the shade or in the light before inoculation or plants grown in the summer or winter months all gave essentially similar results.

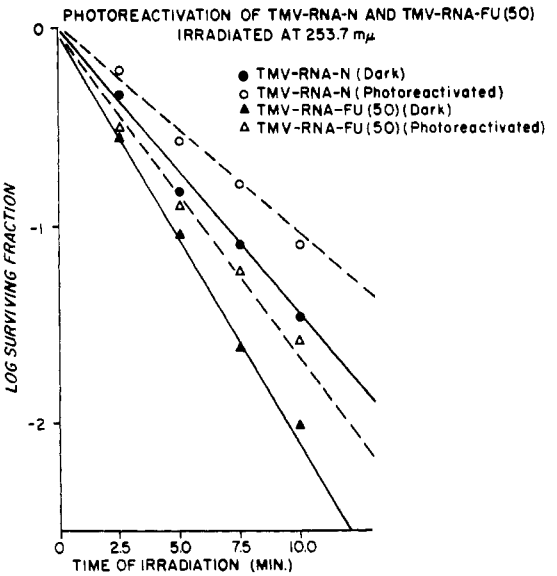

FIG. 3.—Photoreactivation of TMV-RNA-N and TMV-RNA-FU(50) irradiated with a filtered Mineralight V-41 lamp source at a distance of 45 cm. Lamp-output energy was 0.50 ergs/mm^2 per sec. All irradiations were performed in freshly distilled water.

DISCUSSION

The incorporation of 5-fluorouracil into the ribonucleic acid of tobacco mosaic virus sensitized both the intact virus and the infectious nucleic acid to ultraviolet irradiation *in vitro*. The results obtained in the present studies indicate that the inactivation of normal or analog-containing TMV is a first-order process. Ultraviolet-irradiation studies at 253.7 mμ indicate that the degree of sensitization is a function of the amount of analog incorporated. A 20% replacement of 5-FU into the nucleic acid results in a barely detectable sensitization and only at a level of 50% replacement does radiation sensitivity become significant.

The nature of the increased sensitization of the analog-substituted nucleic acid at 253.7 mμ, where the molar extinction coefficients of uracil and 5-fluorouracil are approximately equal, is not known. It is possible that in a manner analogous to 5-bromouracil in DNA (Smith, 1961) sensitization results from the higher quantum efficiency of 5-fluorouracil photodecomposition relative to uracil. Photochemical dehalogenation, postulated as a possible ultraviolet-sensitizing mechanism in 5-bromouracil-substituted bacterial DNA (Wacker *et al.*, 1961), is probably not applicable to 5-fluorouracil-substituted ribonucleic acid. A photolytic removal of the fluorine atom yielding uracil (were such a reaction possible) would merely give rise to a normal biologically active RNA molecule and, consequently, no sensitization toward ultraviolet light would ensue. A more definite explanation of the increased sensitivity of TMV-RNA induced by FU substitution must await more information concerning the photochemistry of the analog and the nature of its photoproducts.

The relative sensitivity of the analog-substituted nucleic acid as compared to the normal nucleic acid increases with increasing wavelengths in the region between 253.7 and 296.7 mμ. Since the absorption spectrum of 5-fluorouracil compared to uracil is shifted to the red (Duschinsky *et al.*, 1957; Berens and Shugar, 1963), an attempt was made to determine whether absorbancy differences at these long wavelengths might account for this long-wavelength dependency of ultraviolet sensitivity. The quantum yields for the ultraviolet inactivation of the infectious nucleic acid are constant at wavelengths between 230 and 280 mμ (Rushizky *et al.*, 1960). If absorbancy differences between uracil and 5-fluorouracil account for the wavelength dependence of ultraviolet sensitivity at various wavelengths, the ratios of ultraviolet sensitivity

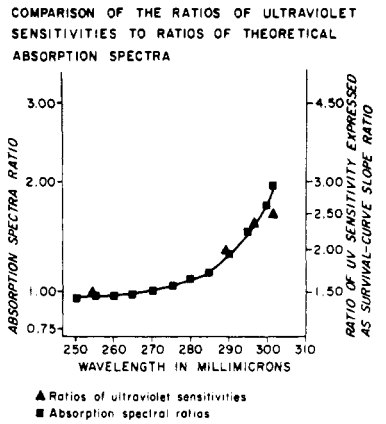

FIG. 4.—Comparison of the ratios of ultraviolet sensitivities expressed as survival-curve slope ratios to ratios of the theoretical absorption spectra of TMV-RNA-FU(50) to TMV-RNA-N at various wavelengths. ▲, Experimentally determined ratio of ultraviolet sensitivity of TMV-RNA-FU(50)/TMV-RNA-N; ■, calculated ratios of the corresponding theoretical absorption spectra. See discussion for calculations.

and ratios of the absorption spectra of the analog-substituted nucleic acid to normal nucleic acid should be similar functions of wavelength (Boyce and Setlow, 1963). The latter ratios, however, cannot be obtained experimentally since the shift in the absorption spectrum of the nucleic acid resulting from uracil replacement by 5-fluorouracil, even at the 50% level of substitution, is not of sufficient magnitude to be determined accurately. Thus analysis of data in these studies was performed in a manner similar to that used for the determination of the effect of absorbancy differences between 5-bromouracil and thymine on the sensitivities of 5-bromouracil-substituted *E. coli* and phage DNA to ultraviolet light of long wavelengths (Boyce and Setlow, 1963; Setlow and Boyce, 1963). The theoretical absorption spectra were calculated from the sum of the products of the molar extinction coefficients (as the ribonucleosides at *p*H 7.2) and the molar ratios of the bases. The spectral ratio is thus

$$\frac{1.18\epsilon_A + 1.0\epsilon_G + 0.73\epsilon_C + 0.5\epsilon_U + 0.5\epsilon_{FU}}{1.18\epsilon_A + 1.0\epsilon_G + 0.73\epsilon_C + 1.0\epsilon_U}$$

where ϵ_A, ϵ_G, ϵ_C, ϵ_U, and ϵ_{FU} are the molar extinction coefficients of the ribonucleosides of adenine, guanine, cytosine, uracil, and 5-fluorouracil, respectively, and the base ratios are taken from literature values (Gordon and Staehelin, 1959). The calculated ratios are plotted in Figure 4. The molar extinction coefficients of the ribonucleosides in the region of long wavelengths are so low that the calculations could be subject to error. However, within the limitations of the theoretical treatment, fairly good agreement is found between the observed ratios of ultraviolet sensitivities and the calculated spectral ratios (Fig. 4). It is thus concluded that absorbancy differences can account for, at least semiquantitatively, the increased sensitivity of the 5-fluorouracil-containing nucleic acid toward ultraviolet light of long wavelengths.

In the T1, P22H5 phage (Sauerbier, 1961), and T2 phage bacterial-host systems (Stahl *et al.*, 1961), a concomitant loss in photoreactivation is observed as the extent of 5-bromouracil incorporation into DNA increases. In these systems the loss in photoreversibility has been attributed to the inability of a host system to repair the photochemical damage induced in 5-bromouracil in order to yield a biologically active unit. It would be reasonable to expect that the same phenomenon would be observed in the case of the 5-fluorouracil-substituted TMV-RNA; however, no differences in the degree of photoreactivation were observed between the normal and 5-fluorouracil-substituted infectious nucleic acids irradiated at 253.7 mμ at the 20% level of substitution. At the 50% level of 5-fluorouracil substitution, only a slight but experimentally significant decrease in photoreactivation was found (Fig. 3). This is in direct contrast to photoreactivation studies on 5-bromouracil-substituted $\overline{T2}u^+$ phage in which no photoreactivation was detected (Stahl *et al.*, 1961). Thus, it would appear that a definite conclusion regarding the photoreversibility of irradiation damage to 5-fluorouracil cannot be made at present.

Further photoreactivation studies are currently being performed in which normal and 50% substituted 5-fluorouracil preparations are irradiated at long ultraviolet wavelengths (290–300 mμ). It is hoped similar studies can be carried out on nucleic acid preparations with levels of 5-fluorouracil substitution higher than the 50% replacement presently available.

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