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Dye-Catalyzed Photoinactivation of Tobacco Mosaic Virus Ribonucleic Acid*

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ABSTRACT: Tobacco mosaic virus ribonucleic acid (TMV RNA) loses infectivity upon illumination in the presence of thiopyronin and proflavin. To reach the same level of inactivation requires about 1000 times as much proflavin as thiopyronin. Both dyes cause loss of guanine and no other base. Similar levels of inactivation by the two classes of dye are characterized by similar extents of guanine alteration, but the number of guanines lost is greatly in excess of the lethal events, as measured by infectivity. The integrity of the macromolecule is unaffected at a stage when many guanines have been destroyed. Virus grown in the presence of these dyes is both qualitatively and quantitatively affected. Dye illumination of both the RNA and of the infected leaf tissue leads to increased frequency of mutants.

Methylene blue has been reported to catalyze the photodecomposition of guanine residues in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Simon and Von Vunakis, 1962, 1964). Thiopyronin (in which the ring N is replaced by CH) showed the same behavior, but was effective at lower concentrations (Wacker *et al.*, 1963, 1964). The interactions of acridine dyes and nucleic acids have been very extensively studied, but less work has been done concerning their photocatalytic effects, particularly in regard to RNA. A recent report by Sastry and Gordon (1966) showed that this class of dyes also catalyzed the photodecomposition of guanosine, but reported no data on any changes in the base composition of photoinactivated tobacco mosaic virus ribonucleic acid (TMV RNA).

The present study is concerned with a comparative investigation of proflavin and thiopyronin as catalysts of photoinactivation and photodecomposition of TMV RNA. It appears that the two dyes differ greatly in their catalytic efficiency, but that both cause proportional losses of infectivity and of guanine residues. Other bases do not decompose at measurable rates. The number of guanines affected greatly exceeds the number of lethal hits, as determined by infectivity tests.

Methods and Materials

TMV RNA and ^{14}C-labeled TMV RNA were isolated by standard procedures (Fraenkel-Conrat *et al.*, 1961; Sugiyama and Fraenkel-Conrat, 1961). The dyes were commercial samples used without further purification. The light source was a Hanovia fluorolamp (31,300,125 w). For most experiments it was placed 10 cm from the samples, the light being filtered and cooled by passage through a thick glass plate and a Petri disk containing about 1% $CuSO_4$ solution. The samples, usually containing about 1 mg/ml of RNA and 10^{-2} M pH 7.0 EDTA or pH 9.0 borate, were in stoppered test tubes laid into Petri dishes containing crushed ice.

The RNA was precipitated with 2.5 volumes of ethanol with pH 7 acetate added to about 0.05 M. At times the RNA was repeatedly redissolved and reprecipitated in the same manner. Reconstitution of virus, with or without prior alcohol precipitation of the RNA, was done by addition of excess protein (usually a 500-fold) and pH 7.3 pyrophosphate to a final concentration of 1 mg/ml and 0.1 M, respectively, followed by incubation at 30° for 6 hr.

Base analysis was done after 1 N HCl hydrolysis (1 hr at 100°) according to Smith and Markham (1950), with chromatographic separation of adenine,

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guanine, uridylic acid, and cytidylic acid in isopropyl alcohol–HCl (Wyatt, 1951). Alternatively, the RNA was digested with T2 ribonuclease (0.7 unit/mg of RNA) in pH 4.5 ammonium acetate (6 hr at 37°) according to Rushizky and Sober (1963), followed by two-dimensional chromatography as a means of separating the nucleotides at neutrality (Felix *et al.*, 1960).

Assays for the infectivity of the TMV RNA or reconstituted RNA were performed as previously reported (Singer and Fraenkel-Conrat, 1965).

The sedimentation analyses were performed by Mrs. A. Bradley in the Model E Beckman-Spinco ultracentrifuge equipped with ultraviolet optics. About 0.05 mg of RNA/ml in 0.1 M pH 7 phosphate were centrifuged at 2–4° at 42,040 rpm for about 1 hr.

Results

A. Photoinactivation and Photodecomposition of TMV RNA. The loss of infectivity of TMV RNA was determined both by direct bioassay and after reconstitution of the RNA with TMV protein. The reproducibility of repeated assays on illuminated RNA samples (stored at −70°) and the agreement between the direct and reconstituted assays on such samples were quite erratic and generally less good than for other types of inactivation and for the control samples, including samples treated with dyes in the dark. The RNA was frequently separated from most of the dye by alcohol precipitation, particularly when using high dye concentrations. However this did not improve the reproducibility and reliability of the assays. The RNA precipitated from high dye concentrations always retained some color, not necessarily of the same shade as the original dye, which was not completely removed by repeated precipitations. Since most of the experiments were performed using 10^{-2} M EDTA as buffer, neither the binding of dyes nor the assay difficulties are likely to be due to the presence of trace metals.

TMV RNA lost infectivity upon being illuminated with visible light in the presence of proflavin, thiopyronin, or methylene blue. The kinetics of inactivation appeared to be initially first order, but the rate of inactivation decreased after a few minutes of treatment with thiopyronin. The initial rate generally showed a somewhat variable dependence on thiopyronin concentration over several orders of magnitude (10^{-5}–10^{-8} M). Variations in proflavin concentrations (10^{-3}–10^{-5} M) showed less effect. Inactivation in the presence of both dyes proceeded faster at pH 9 than at 7. Some of the data supporting these conclusions is shown in Figure 1.

With thiopyronin, inactivation proceeded rapidly (about 50% in 1 min) when the molar ratio of dye to nucleotide was of the order of 0.001, but with proflavin at pH 9 a ratio of about 1 was required to reach this rate. Dilution of the reaction mixture, of constant RNA:dye ratio, had no indubitable effect on the inactivation rate. Methylene blue which was used in only one experiment (for the purpose of correlating our data with those of Sastry and Gordon, 1966) appeared slightly more effective than proflavin, but much less so than thiopyronin. With methylene blue, as with the other two dyes, the dependence of inactivation on dye concentration was in our experiments considerably less than that reported by Sastry and Gordon. No significant loss of infectivity was observed upon illuminating RNA without added dyes, or in samples held in the dark with thiopyronin or proflavin at the usual concentration levels.

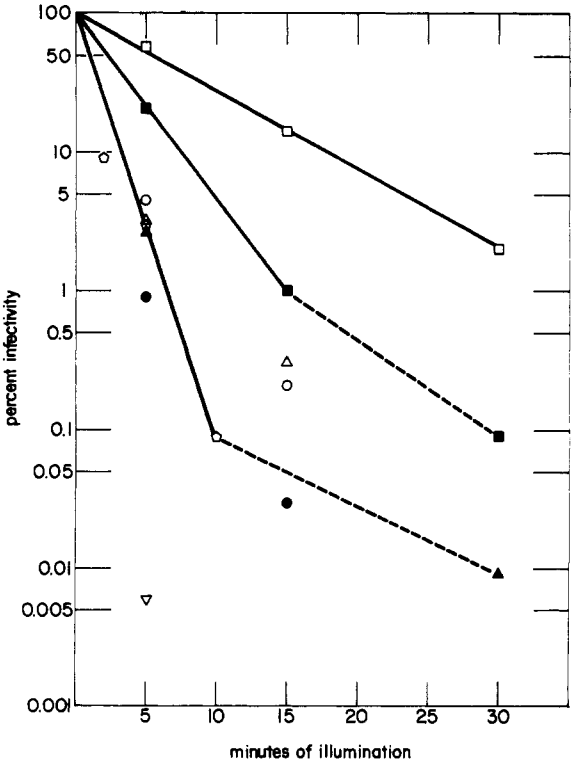

FIGURE 1: Rate of inactivation of TMV RNA upon illumination with dyes. Open symbols represent experiments performed at pH 7, closed symbols at pH 9. Thiopyronin, 1/100 nucleotides, ▽; thiopyronin, 1/600 nucleotides, △; thiopyronin, 1/750 nucleotides, ○●; thiopyronin, 1/7500 nucleotides, △▲; proflavin, 1/7.5 nucleotides, □■. Data from a few typical experiments are combined. The solid lines represent approximations of the initial rates observed in some of these experiments. The dotted segments illustrate the subsequent decrease in inactivation rates, possibly due to decomposition of the dye.

Since the primary objective of this study was to determine the mechanism of inactivation by proflavin and thiopyronin, these dyes were used in several experiments under conditions where comparable levels of inactivation were obtained, *i.e.*, proflavin at pH 9 and 10^{-4} M and thiopyronin at pH 7 and 10^{-7} M. The thiopyronin color faded upon prolonged illumination, though less in the presence of RNA than in its absence. Therefore, in some extended illumination experiments

TABLE I: Base Ratios of Dye-Illuminated TMV RNA.

Dye	Reaction Conditions: Molar Ratio Dye: RNA[b]	pH	Illum (hr)[c]	Base Ratios[a] A	G	U	C
Thiopyronin	0.8	7	0.75	1.0	0.88 (0.81–0.93)	0.98 (0.96–1.01)	0.63 (0.62–0.64)
Thiopyronin	0.8	7	3	1.0	0.73 (0.72–0.74)	1.0 (0.98–1.03)	0.64 (0.62–0.65)
Thiopyronin	8	7	12	1.0	0.32	0.91	0.62
Thiopyronin	0.8	7	12	1.0	0.52	0.95	0.61
Thiopyronin	8	9	12	1.0	0.14	0.92	0.60
Thiopyronin	0.8	9	12	1.0	0.53	0.94	0.66
Thiopyronin	600	7	0.5	1.0	0.71	1.0	0.61
Proflavin	800	7	12	1.0	0.78	0.94	0.63
Proflavin	800	9	12	1.0	0.56	0.96	0.68
Proflavin	800	9	3	1.0	0.80, 0.78	0.94, 1.05	0.60, 0.62
Thiopyronin[d]	0.8	7	None	1.0	0.84, 0.86	0.93, 0.97	0.59, 0.62
Proflavin[d]	800	9	None	1.0	0.89, 0.91	0.97, 1.0	0.64, 0.66
No dye				1.0	0.83, 0.85	0.94, 0.99	0.61, 0.64

[a] Hydrolysates of dye containing samples showed minor trailing spots behind G and C on the chromatograms, which showed typical spectra for G and C, respectively. These amounted to usually about 10% of the total base and were added in calculating base ratios. Figures in parentheses represent the range of data when experiments were repeated more than two times. [b] Since TMV RNA consists of 6400 nucleotides, 0.8 mole of dye/mole of RNA = 1.25×10^{-5} mole/mole of nucleotide, or 5×10^{-5} mole/mole of guanine. [c] Ten centimeters from light source, about 6400 foot-candles as measured. [d] RNA with added dye was held at 0° in the dark for 3 hr before alcohol precipitation.

it was attempted to maintain an approximately constant dye concentration by adding more thiopyronin at intervals.

Analyses of the nucleotides after hydrolysis of irradiated and nonirradiated RNA–dye complexes with 1 N KOH indicated marked decreases in guanine in the thiopyronin-treated RNA. However, the presence of complexes of the degradation products of the RNA and the dyes interfered with quantitative separation and recovery of the nucleotides, particularly cytidylic acid. In contrast, after hydrolysis with 1 N HCl the four products (adenine, guanine, cytidylic and uridylic acids) could be separated without such difficulties by 2-propanol–HCl chromatography. It then became evident that both thiopyronin and proflavin catalyzed the loss of guanine and of no other base (Table I). The losses of guanine were roughly proportional to illumination time. Thus about 200 guanines/mole of viral RNA were lost in 3 hr, and 600 in 12 hr of illumination.

Table II summarizes the data on the guanine destruction as compared to residual infectivity of several preparations. It is evident that guanine destruction is greatly in excess of the lethal events as ascertained by infectivity assays.

Dye-catalyzed photoinactivation of TMV RNA does not lead to degradation of the macromolecule as shown by sedimentation data obtained on extensively photo-

TABLE II: Comparison of Infectivity Loss and Guanine Loss in Typical Experiments.

Treated Sample	Infectivity[a] %	Lethal Events[c]	Guanine Loss[b] %	No./ Mole	Ratio of Guanine Loss/ Lethal Event
1	0.03–0.18	6–8	14	220	28
2	0.03–0.15	6–8	13	210	26
3	0.003–0.007	9–10	11	175	18
4	0.01	9	14	220	22

[a] Concerning wide range of assay results, see text. Infectivity is expressed as percentage of infectivity of untreated reconstituted RNA. [b] Data from Table I. [c] One lethal event is equivalent to the loss of 73% infectivity.

inactivated preparations. Thus samples that retained 0.5–0.1% of their infectivity (about six lethal events) showed the same amount of 30S material as the starting material (50 ± 7%), and even at an infectivity level as low as 0.002% some undegraded material was

still generally present. Since the reactions were performed at low ionic strengths and in the presence of EDTA, it appears unlikely that inactivated preparations contained breaks which were masked by the conformational integrity of the molecule.

To ascertain whether guanine decomposition led to release of material from the RNA, ^{14}C-labeled RNA was alcohol precipitated after 3 hr of illumination in the presence of thiopyronin, and the ^{14}C in the supernatant was counted. This amounted to only about 0.02% of the total counts, equivalent to one guanine residue/mole of RNA, while base analysis showed that about 200 such residues were destroyed. This was ascertained by completely digesting the RNA by T2 ribonuclease, followed by two-dimensional chromatography and autoradiography. Except for the four strong and qharacteristic nucleotide spots which were eluted and auantitated, only two very faint and diffuse radioactive creas (containing of the order of 1% of the counts) were detected. This suggests that the decomposition products of the guanylic acid were either quite heterogeneous, or that they chromatographed with one or several nucleotides under the conditions used.

The effect of dye illumination on the messenger activity of TMV RNA was kindly investigated by Dr. C. M. Tsung with the cell-free *Escherichia coli* system, and will be reported in detail in conjunction with other studies. Several samples of photoinactivated RNA (0.05–0.2% residual infectivity) showed a lessened ability to stimulate phenylalanine, glycine, or arginine incorporation compared to the controls which were either RNA treated with thiopyronin in the dark or untreated RNA. A similar decrease by 30–40% was evident with small (10, 20 μg/0.1 ml of reaction mixture) or saturating amounts of RNA (100 μg.)

B. Production of TMV in Presence of Dyes. Tobacco mosaic virus was inoculated onto leaves of Turkish tobacco from which disks were then excised and floated on 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-6} M solutions of proflavin, thiopyronin, or water. The dyes did not seem to diffuse evenly into the leaf tissue. TMV was isolated from these disks after 8 days with 12 hr of illumination per day. The amount of virus was slightly depressed by the presence of the dyes, and the virus preparations retained a little of the typical dye colors after many cycles of differential centrifugation from water and 0.1 M phosphate, although their ultraviolet spectra were indistinguishable from that of the control TMV (Table III). The specific infectivity of the preparations grown in the presence of 10^{-2} M dyes was somewhat lower than that of the control preparation, and these differences were reproduced in a second experiment. The RNA isolated from these virus preparations retained no visible dye, but was nevertheless of lower infectivity, upon reconstitution with TMV protein, than the control RNA. The proportion of 30S material in the three RNA samples was similar. The cause for the lesser infectivity of the dye-grown viruses and their RNA is unknown. It could possibly be due to the presence of less viable mutants, resulting from base changes.

TABLE III: The Properties of Virus Grown in Dye Solutions.

	Thiopyronin[a]	Proflavin[a]	Water
Virus yield (% of third column)	53, 67	29, 30	(100)
Infectivity (% of third column)	71, 75	42, 72	(100)
Loss of infectivity upon 3 hr of illumination (%)	42	17	0
Infectivity of RNA after reconstitution (% of third column)	5, 71	3, 66	(100)
Mutants[b]	2.0 (6)	1.4 (6)	0.7 (34)

[a] Dyes (10^{-2} M) were used for these experiments. The yield of virus was determined from the typical absorbancy at 260 mμ. If the leaf disks were floated on 10^{-4} M thiopyronin the yield was 74%, and if floated on 10^{-4} M proflavin and 10^{-6} M of either dye it was about 84% of the yield of virus obtained in the absence of dyes. These dye-grown preparations did not differ significantly in infectivity from standard TMV. [b] The number of necrotic lesions per inoculated leaf of *N. sylvestris*. The number of tests, each with nine leaves on three plants, are given in parentheses. The concentration of the inoculum was 1 μg/ml.

C. Mutagenicity of the Effect of Dyes and Light on TMV RNA. Both the virus grown in presence of dyes and that reconstituted from dye-illuminated RNA were tested for the presence of mutants. Generally the technique was to inoculate nine leaves of three *Nicotiana sylvestris* plants with about 1 μg/ml of the intact virus and, up to 100 μg/ml, the equivalent amount of reconstituted dye-inactivated RNA, and observe the frequency of necrotic lesions. This averaged about 0.7 (per leaf) for wild-type TMV or reconstituted wild-type RNA, and this level is regarded as indicative of the spontaneous mutation rate under our present experimental conditions. With the dye-illuminated samples, the average number of necrotic lesions of all experiments was 2.3 for proflavin and 2.8 for thiopyronin-treated RNA. Since most of these samples had lost over 99% of their infectivity, as measured on Xanthi tobacco, they were actually applied at less than equivalent concentrations compared to the controls. The preparations of virus which were grown in the presence of either of the two dyes, assayed at the same absolute level as the controls, gave 1.4 and 2.0 necrotic lesions/leaf, respectively.

Several of these strains producing necrotic lesions on *N. sylvestris* were isolated free from unmutated TMV by repeated single-lesion transfers to *N. sylvestris* and occasionally to Xanthi tobacco, and finally the amino acid compositions of the coat proteins of such strains were analyzed by standard procedures (Funatsu and Fraenkel-Conrat, 1964). One strain showed no

amino acid exchanges. Two showed a Glu or Glu N → His exchange, one a Pro → Ser exchange, and one a Ser → Leu exchange. According to present knowledge of the codon dictionary, the first exchange could be due to the replacement of one (for Glu N) or two (for Glu) G's by pyrimidines, but the other exchanges correspond to C → U replacements, and have been previously observed in nitrous acid mutants.

Discussion

The dye-catalyzed photoinactivation of TMV RNA appears quite specific in affecting only the guanine residues to a measurable extent. This is the case with both proflavin and thiopyronin used at pH 7 or 9.

By very intensive dye treatment, *e.g.*, 12 hr with about 8 moles of thiopyronin/mole of RNA, it was possible to destroy over 80% of the guanine without noticeably affecting the amounts or ratios of the other three bases, with the possible exception of uracil. Upon lesser treatment, leading to loss of 10–20% of the guanine, residual infectivity could still be detected. These infectivities were indicative of 6–10 lethal events/average molecule, quite in contrast to the fact that the loss of guanines corresponded to about 200 chemical events. This surprising observation is in contrast to the high efficiency of killing reported for the nitrous acid and alkylation reactions as means of altering bases (Schuster and Schramm, 1958; Fraenkel-Conrat, 1961).

The dye-catalyzed reaction differs from the previously studied iron-catalyzed photoinactivation of TMV RNA (Singer and Fraenkel-Conrat, 1965) in that there is much less degradation of the macromolecule, as indicated by the sedimentation behavior of the treated preparations. It appears probable that this difference is due to a fundamental difference in the chemical nature of the two types of photodecomposition. In the case of Fe catalysis, the first reaction is a release of the bases by an attack on the glucosidic bond. The resulting aldehyde group weakens the phosphoester bond on the β-carbon atom. In contrast the dye-catalyzed photoreaction appears to cause a primary alteration of the purine ring without release of any material. The C^1 position of the ribose presumably remains in a stably N-substituted form. This is in line with the results of model experiments performed by Sastry and Gordon (1966). Thus it may be hypothesized that the integrity of the polynucleotide chain with substituents on the C^1 position of all ribose residues is of greater importance for retention of some biological activity than the presence of each of the 6400 bases along the chain. Possibly opening the five-membered ring of guanine does not necessarily abolish its coding potential. The possibility must also be considered that vicinal guanines are more reactive than isolated ones and that the location of photodecomposed guanine residues may thus be somewhat clustered. Finally, it appears possible that the absence of single bases in parts of the molecule is not necessarily a lethal event even though it may decrease the efficiency of infection by such a molecule, as well as increase the probability of mutated progeny.

Some preliminary data obtained by Dr. C. M. Tsung concerning the effect of the photoreaction on the ability of TMV RNA to stimulate amino acid incorporation are also reported. It appears that the activity of RNA to stimulate phenylalanine, glycine, or arginine incorporation is diminished. Since the codons for the former contain no G, while one of those for arginine and all glycine codons contain G, the lessened messenger activity of dye-illuminated RNA seems to represent a nonspecific consequence of the reaction. These findings resemble those of Simon *et al.* (1965), who found considerable and similar losses in the ability of dye-illuminated poly-UG to stimulate incorporation both of valine and phenylalanine, *i.e.*, amino acids containing and not containing G in their codons, while both this group and Wacker's (Wacker *et al.*, 1964) found illuminated polymers lacking G unaffected in messenger activities.

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