THE RELATIONSHIP BETWEEN INACTIVATION OF TOBACCO MOSAIC VIRUS BY X-RAYS AND BREAKAGE OF NUCLEIC ACID*

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SUMMARY

The analytical ultracentrifuge, with u.v. absorption optics, has been used to demonstrate and measure bond breakage in the main chain of RNA strands extracted from X-irradiated TMV.

When the whole virus was irradiated in solution at 0°, the rate of loss of intact RNA strands was about equal to the rate of loss of viral activity. However, the reduction in yield of extracted RNA that accompanied increasing X-ray dose, together with the probability that longer strands would be selectively lost, cast considerable doubt on the significance of this result.

When TMV was irradiated while frozen at about —50° or while in centrifugal pellets with little water present, the correlation between reduction in yield and X-ray dose disappeared. These data indicate a D_0 for strand breakage of $4.0 \cdot 10^5$ R as compared to an average measured D_0 of $1.8 \cdot 10^5$ R for virus inactivation. Thus, breakage proceeds 40 % as rapidly as does inactivation. Also, 40 % of all the ionizations in TMV RNA induced by radiation may be expected to occur among backbone chain electrons. If it is assumed[1] that any ionization within the nucleic acid complement of a virus inactivates the virus, then the present data indicate that ionizations within the backbone chain of TMV RNA break main chain bonds and cleave the strand with an efficiency of close to 100 %. By analogy, then, it is suggested that ionizations elsewhere in the nucleic acid also are effective in breaking bonds.

INTRODUCTION

There is good evidence that absorption of ionizing radiation within the tobacco mosaic virus (TMV) particle inactivates by inducing damage in the nucleic acid (RNA) moiety. EPSTEIN[1] and BUZZELL, TRKULA AND LAUFFER[2] have calculated the X-ray sensitive volume of TMV to be about equal to its RNA volume. GIERER AND SCHRAMM[3] and FRAENKEL-CONRAT, SINGER AND WILLIAMS[4] have demonstrated TMV activity in solutions of practically protein free RNA. GINOZA AND NORMAN[5] have determined the X-ray inactivation rate of infectious RNA to be the same as that of intact TMV. The physical nature of the lethal change remains to be determined.

LAUFFER, TRKULA AND BUZZELL have shown that both X-ray[6] and u.v.[7] irradiation of whole TMV decrease the intrinsic viscosity of subsequently extracted RNA

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strands. They suggested that both the viscosity decrease and inactivation of the virus might be due to a radiation induced breakage of the virus' RNA strands. The present communication reports further investigations of the physical effect on RNA of X-irradiation of TMV. Using the Spinco analytical ultracentrifuge equipped with u.v. absorption optics, we have been able to resolve whole from somewhat degraded RNA, to measure the relative concentrations of the two, and thus to follow the kinetics of an X-ray induced degradation of intact nucleic acid strands.

METHODS AND MATERIALS

The type strain of tobacco mosaic virus was prepared from systematically infected Turkish tobacco plants by the method of BOEDTKER AND SIMMONS[8,9], but with fewer high speed centrifugations.

Virus solutions were sprayed at low pressure in microdrops onto collodion covered grids for electron microscopic examination.

RNA was isolated from the virus by SCHRAMM's phenol method[3] modified by limiting the six extractions to 1 min each as suggested by BAWDEN AND PIRIE[10].

X-radiation with a mean wave length of 0.2 Å, as determined by absorption in copper, was delivered by a 200 kV Westinghouse deep therapy unit. To reduce the effectiveness of radiation induced free radicals of water[11], inactivation experiments were performed at concentrations of 1 % to 2 % of virus, either in 10 % Difco nutrient broth solution or frozen in buffer at about —50°. For the various breakage experiments, TMV was irradiated at 1° and at —50° in buffer and at 1° in wet pellets obtained by centrifuging from neutral, 0.001 *M* Versene solution at 35,000 × *g* for 90 min.

U.v. absorption spectra of nucleic acid solutions were measured in the Beckman DU spectrophotometer. Maxima and minima were found at 257–258 mμ and 230–231 mμ, respectively. RNA concentration was found by dividing O.D. at 257 mμ by the factor 25.3 (mg/ml)$^{-1}$ (ref. 12). Virus concentrations were determined with a differential refractometer by use of the factor 0.0017 (mg/100 ml)$^{-1}$ (ref. 13). Relative nucleic acid yields were calculated by assuming the virus to be 6 % by weight RNA.

For the analytical centrifugations, conditions were so controlled that the spinning rotor reached a temperature of about 4° when full speed was attained. The rotor temperature drifted no more than several tenths of a degree during the runs. Samples were run at an O.D. at 257 mμ of 1.00 to 1.25, corresponding to concentrations of 0.004 % to 0.005 % for nucleic acid, which was centrifuged at 59,780 rev./min, and to 0.04 % for TMV, which was centrifuged at 25,980 rev./min. Pictures of the absorption profile of the spinning cell were obtained on Kodak "commercial" film (Fig. 1). A picture was taken during acceleration (frame 1), at speed (frame 2), and every 4 min thereafter (frames 3–10). Exposure time was 40 sec. Films were developed in half strength Kodak DK 50 developer at 20° for 2 min with continuous brushing. Photodensitometric tracings were made with a suitably adapted Beckman analytrol adjusted by use of calibrated neutral density filters to read true density of the films.

Viscosities were measured at 4.0° with a Cannon-Manning semi-micro viscometer having an average velocity gradient of 300 sec^{-1}.

Optical activity of RNA solutions was measured with a Rudolph model 80 precision polarimeter at wavelengths 546 mμ and 589 mμ.

Viral activity was assayed by the local lesion method using *Nicotiana glutinosa*. Unknowns were inoculated in parallel with standard samples at several dilutions on the plan of a Latin square so that each sample was inoculated once on each plant and once on each leaf position. Activities of unknowns were expressed in terms of concentration of standard sample necessary to produce an equal number of lesions.

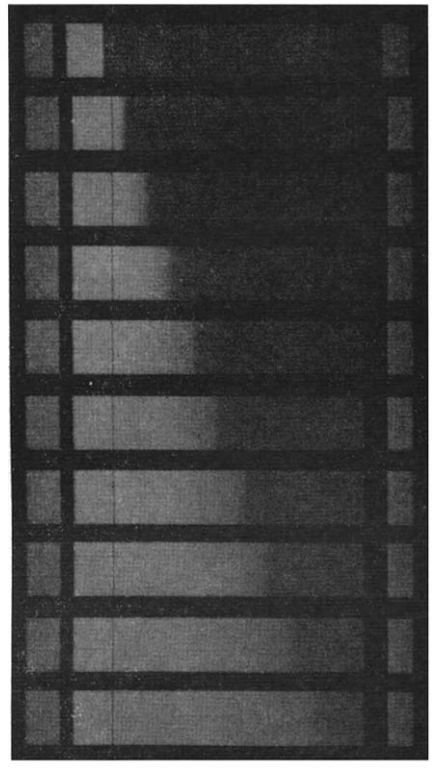

Fig. 1. Positive print of pictures obtained with the u.v. absorption optics of the analytical ultracentrifuge showing the sedimentation pattern of unirradiated RNA.

The inactivation dose (D_0) was estimated from a straight line plot of logarithm of activity remaining *versus* the X-ray dose.

The solvent employed throughout was 0.02 *M* phosphate buffer at pH 7.2 unless otherwise specified.

Analysis of the ultracentrifuge data

A set of pictures obtained from unirradiated RNA is reproduced in Fig. 1. The two outer areas provide reference lines at known distances from the rotor axis; the inner oblong represents the cell itself. From left to right can be distinguished the air space above the meniscus, the meniscus as a sharp line, a non-absorbing region of solvent from which all the molecules have migrated, a region of increasing absorption indicating particles moving within a range of velocities, an abrupt increase in absorption indicating a major fraction of particles moving with uniform velocity, and a uniform region extending to the bottom of the cell. Superimposed densitometric

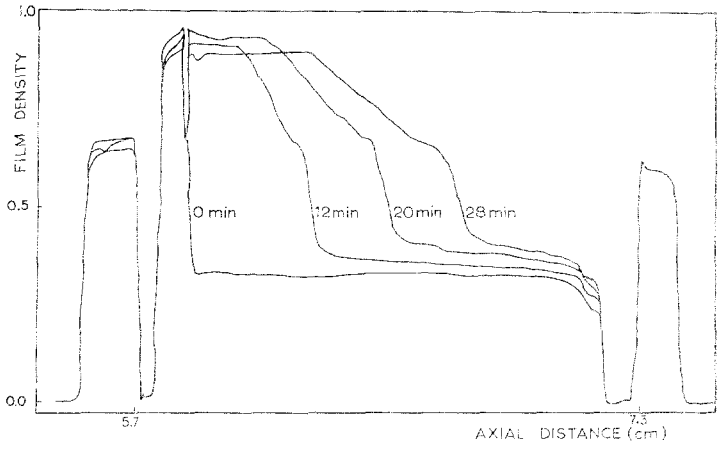

Fig. 2. Superimposed photodensitometric tracings of frames 1, 5, 7 and 9 of Fig. 1.

traces of frames 1, 5, 7 and 9 are presented in Fig. 2. The leading component moves with a sedimentation coefficient corrected to water at 20°, S^{w}_{20}, of 31.

Traces were analyzed to determine the relative amounts of RNA sedimenting as the leading component and as the hetero-dispersed, slower component. With the conditions of film treatment previously described and the RNA concentration here employed, the trace height was approximately proportional to concentration. Corrections were made when necessary for non-linearity at the very top and bottom of the traces. Correction was also made for the dilution experienced by all sedimenting components[14]. Traces 1, 5, 7 and 9 (Fig. 2) were used in the analysis of each centrifuge run. From frames 5, 7 and 9 was taken the average trace height for the region of the cell containing buffer only. The difference between this height and the height of trace 1 was taken to indicate undiluted, total RNA concentration in the cell. The corrected heights corresponding to the leading component and to the trailing component were expressed in terms of percent of the total. The use of this measure renders the analysis independent of dilution errors in previous handling of the samples and of differences in film development among the various runs. Estimations from the three traces of the fraction of the total in each component were averaged.

RESULTS

Data concerning unirradiated virus and RNA extracted from it are presented in Table IA. A number of points are noteworthy.

Half of the parent virus sedimented with a leading component with S^{w}_{20} of 190. This is the full length monomer[15] and the slower material can be only broken rods which normally occur in TMV preparations. The sedimentation pattern for RNA from unirradiated virus approximates that of the virus. Thus the RNA moving with the leading component is derived from full length virus rods while the more slowly sedimenting strands are derived from broken rods. It appears probable on the basis of hydrodynamic data that the entire RNA complement of the virus sediments in one piece as the leading component. The S^{w}_{20} of this component is 31. Intrinsic viscosity characteristic of the component is estimated to be about 200 ml/mg when allowance

TABLE IA

UNIRRADIATED SAMPLES

Sample	*Extraction yield (%)*	*Absorption maximum/minimum*	*Fraction in main component (%*
Parent TMV I			51
TMV I, frozen and thawed			49
RNA from TMV I	95	2.56	46
	79	2.36	45
	86	2.15	44
RNA from TMV II pellet	100	2.44	75

TABLE IB

IRRADIATED SAMPLES

Sample	*Dose ($R \cdot 10^5$)*	*Extraction yield (%)*	*Absorption maximum/minimum*	*Fraction in main component (%)*
RNA from TMV I	0.39	72	2.28	37
irradiated in solution	1.2	66	2.22	22
	1.6	72	2.08	19
	2.1	59	2.02	15
	3.5	49	2.14	10
RNA from TMV I	0.78	81	2.20	30
irradiated frozen	1.8	80	2.16	36
	2.7	85	2.29	23
	3.7	76	2.07	24
	5.5	69	2.09	16
RNA from TMV II	1.0	88	2.14	52
irradiated in pellets	1.9	85	2.20	40
	2.8	84	2.03	37

is made for the viscosity contribution of the broken strands. Taking the partial specific volume as 0.57[16] and using the equation of MANDELKERN AND FLORY[17] for a random coil, one obtains a mol. wt. of $2 \cdot 10^6$ if the effective hydrodynamic unit is assumed to be spherical. These data and calculations agree with those recently published by BOEDTKER[18] for heat extracted TMV RNA and by GIERER[19–21] for phenol extracted TMV RNA. Both authors have concluded that the leading component corresponds to the whole RNA complement of TMV.

Data characterizing RNA extracted from irradiated virus are summarized in Table IB. In Fig. 3 is given a representative series of photodensitometric traces showing comparable sedimentation patterns for RNA extracted from virus exposed to different doses of X-rays. These data show that when parent virus is subjected to increasing doses of X-irradiation more and more of the RNA appears in the trailing component at the expense of the leading component. This is thought to reflect breakage of nucleic acid strands.

Fig. 4 is a semi-log plot of the relative fraction of RNA remaining in the leading component after various doses of irradiation. The plot indicates that RNA breakage

proceeds as a first order, single event reaction. The 37 % dose, D_0, for breakage of RNA extracted from TMV which has been X-irradiated in solution at 0° is $1.8 \cdot 10^5$ R. Several determinations of the D_0 for inactivation of the parent TMV irradiated under the same conditions (except for inclusion of 10 % broth in the virus solution) gave the values $1.6 \cdot 10^5$ R, $2.0 \cdot 10^5$ R and $2.4 \cdot 10^5$ R, so that apparent RNA breakage and

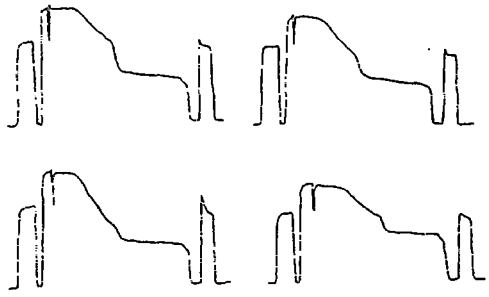

Fig. 3. Tracings after 20 min of sedimentation (frame 7) for RNA extracted from virus receiving various radiation treatments: upper left unirradiated; upper right $0.39 \cdot 10^5$ R in solution; lower left $1.56 \cdot 10^5$ R in solution; lower right $1.75 \cdot 10^5$ R, frozen at —50°.

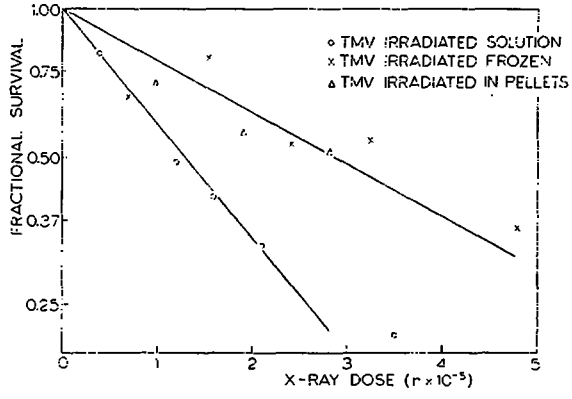

Fig. 4. Semi-log plot of fraction of intact RNA to survive breakage after various X-ray doses.

loss of viral activity proceed at essentially the same rate. However, the persistent decrease in the yield of extracted RNA with increasing X-ray dose makes the significance of this result questionable. A selective loss of longer strands could obscure the true breakage rate. When the virus was irradiated while frozen at about —50° or in a wet pellet the radiation induced reduction in yield was essentially eliminated (Table IB). These data are plotted in Fig. 4 and indicate a D_0 for RNA breakage of $4.0 \cdot 10^5$ R. The D_0 for inactivation of frozen virus was measured at $1.2 \cdot 10^5$ R and $1.6 \cdot 10^5$ R in two experiments.

DISCUSSION

Evaluation of the method

The difficulties associated with the analytical ultracentrifugation of low concentration solutes have received considerable attention. The question is whether sedimenting solute particles can create a density gradient in the cell sufficient to withstand

the mixing tendencies of mechanical shock and local temperature fluctuation encountered during the run. SCHUMAKER AND SCHACHMAN[22] and SHOOTER AND BUTLER[23] have been able to perform evidently convection free centrifugations of desoxyribonucleic acid preparations at concentrations as low as 0.02 mg/ml. With the conditions of centrifugation previously described we have found a rather high degree of reproducibility for the measurements we have made. No run of this project showed disturbances in the principle boundary indicative of significant convection. The possibility of convection below and/or above the boundary, however, cannot be excluded. Indeed the JOHNSTON-OGSTON effect[24] might be expected to evoke convection above the boundary. The low concentrations used make it seem unlikely that the error in concentration measurement deriving from this effect[25] would here be of significant size.

Electron micrographs of virus stock I showed that about 50 % by wt. of the virus particles were the uniform, 3000 Å monomer. The ultracentrifugal analysis estimated this value at 50 %. Three runs of unirradiated RNA (stock I, three independent extractions) gave values of 45 %, 46 % and 47 % in the leading component. Eight runs of a somewhat different sample (five independent extractions) gave values between 38 % and 41 % in the forward boundary. Thus the method can measure reproducibly the fraction of intact RNA in the solutions of interest in this study.

Breakage of bonds in TMV RNA

X-irradiation of the whole virus decreased the sedimentation coefficient of some of the extracted RNA previously in the leading edge. Intrinsic viscosity was concurrently decreased. These observations constitute good evidence for scission of RNA strands. The alternative possibility of an extensive change in shape and hydration is ruled out also by the observation that no change in specific optical rotation of the RNA occurred even after ten times the D_0 dose.

In the RNA extraction procedure, the aqueous TMV solution is shaken with phenol and then centrifuged to bring down the phenol rich phase and denatured virus protein. RNA strands incompletely separated from their denatured protein matrices during the shaking operation might well be lost in centrifugation and it seems likely that longer strands would be selectively removed. When TMV was irradiated in solution, extraction losses of RNA increased with increasing X-ray dose (Table IB). Therefore the very interesting result, that RNA breakage and virus inactivation proceeded at nearly the same rate, is suspect. It seems probable that the decrease in fraction of whole strands observed in the ultracentrifugal analysis was due both to breakage and to selective loss during extraction.

That the decrease in RNA yield was due to chemical attack on the protein by X-ray induced free radicals of water was indicated by the experiments involving the irradiation of TMV in the frozen state and in centrifuged pellets with very little water present. Here the yield was largely independent of dose and the ultracentrifugal analysis should measure the true rate of RNA breakage. The data indicate a D_0 for RNA breakage of $4.0 \cdot 10^5$ R as compared to an average value of $1.8 \cdot 10^5$ R for virus inactivation. The rate of main chain breakage, then, is about 40 % of the rate of virus inactivation. From the reported base ratios in TMV RNA[26–28] and the known primary structure of RNA, the fraction of electrons that are associated with atoms in the main backbone chain can be calculated. The number is close to 40 % inde-

pendent of whether only bonding electrons are considered or whether all the electrons are included.

If it is assumed, following EPSTEIN[1], that any ionization within the RNA moiety inactivates the virus, then the present data indicate that ionizations associated with main chain atoms lead to breaks in main chain bonds with an efficiency of about 100 %, when the RNA is irradiated while enclosed in its protein matrix. Since ionizations break main chain bonds with high efficiency, it is likely that ionizations elsewhere in the nucleic acid also are effective in breaking bonds and this, therefore, becomes a plausible explanation for X-ray inactivation.

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