EFFECT OF X-RAYS ON NUCLEIC ACID ISOLATED FROM TOBACCO MOSAIC VIRUS

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SUMMARY

Breakage of RNA, X-irradiated after phenol extraction from tobacco mosaic virus, was measured using the ultracentrifuge equipped with u.v. absorption optics. The sedimentation patterns of control solutions of RNA show a homogeneous component corresponding in size to the fraction of monomer in the parent virus. By following the decrease of this RNA component with increasing X-ray dose, reproducible values for the 37 % survival dose, D_0, for breakage of the main chain can be obtained.

The indirect action of radiation was minimized by irradiating RNA in concentrated, oxygen-free, frozen solution. Under these conditions the D_0 for breakage was $21 \cdot 10^5$ R, while D_0 for dried RNA was $4.2 \cdot 10^5$ R, and for RNA in the virus was $4.0 \cdot 10^5$ R (see ref. 1). The anomaly could be due to intramolecular hydrogen bonding[2], since heating the irradiated RNA solutions to 65 or 70° drastically increased breakage leading to an effective D_0 of $4.4 \cdot 10^5$ R.

INTRODUCTION

The radiation sensitive volume of TMV, estimated from X-ray inactivation experiments, is much smaller than the particle size determined by other physical methods and has been shown to correspond to the RNA content of the virus[3,4]. Irradiation of TMV with doses of X-rays much larger than necessary to inactivate the virus does not produce any observable change in size and shape of the virus[5,7]. X-ray diffraction studies[6] indicate that the nucleic acid is embedded in the protein and thus is protected and held in a rigid configuration. When methods for the extraction of intact RNA from TMV became available, LAUFFER, TRKULA AND BUZZELL[7] showed that RNA isolated from irradiated virus was less viscous than that isolated from unirradiated virus. They suggested that both the viscosity decrease and the inactivation of the virus might be caused by radiation-induced breakage of the RNA strand. ENGLANDER, BUZZELL AND LAUFFER[1] developed a centrifuge technique to compare quantitatively the rate of breakage of the RNA isolated from irradiated TMV with the rate of loss of activity of the virus. However, it is possible that the breakage observed was the sum of the irradiation-caused damage plus additional breakage caused by the isolation

Abbreviations: TMV, tobacco mosaic virus; RNA, ribonucleic acid.

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of RNA from the irradiated virus. The centrifuge technique of ENGLANDER *et al*[1]. has been used to study the breakage of RNA isolated before irradiation and to investigate the conditions which affect this breakage.

MATERIALS AND METHODS

Preparation of TMV

The type strain of TMV was isolated from systemically infected Turkish tobacco plants by the SIMMONS resin technique[8], a modification of an earlier low ionic strength method[9] which yields virus preparations containing a large majority of monomers[10]. The important features of this method are the extraction of the virus from the infected leaves at neutral pH in the presence of ion exchange resins and the purification of the virus by alternate low and high speed centrifugations at low ionic strength and neutral pH with the chelating agent, Versene. After purification the virus was stored at 5° in 10^{-3} *M* Versene solution until used. The virus can be stored in this way for long periods of time with no evidence of aggregation[9].

Extraction of RNA

The RNA was extracted from the preparations of TMV by the phenol method of SCHRAMM[11]. The method was modified slightly so that the virus was shaken for shorter periods of time with phenol: 4 min the first time and 1 min the second and third times.

Concentration determination

Concentrations of the various solutions were determined from measurement of of O.D. with a Beckman Model DU spectrophotometer. The RNA concentration was obtained by dividing the O.D. of the solution at 257 mμ by 253 $(g/100\ ml)^{-1}$ (see ref. 12). The concentration of TMV solutions was determined by dividing the O.D. at 265 mμ by the extinction coefficient, 30.6 $(g/100\ ml)^{-1}$ (see ref. 9). The concentrations of several TMV preparations were also checked by refractive index measurements made with a Phoenix differential refractometer. The value of $1.94 \cdot 10^{-4}$ $(g/l)^{-1}$ was used for the specific refractive index increment of TMV in water at a wavelength of 436 mμ (see ref. 9).

Sedimentation

A Spinco Model E ultracentrifuge equipped with u.v. absorption optics was used for all centrifugations. Samples were contained in a standard four-degree, sector-shaped cell 12 mm deep. For centrifugation the RNA solutions were diluted to an O.D. at 257 mμ of 1.0, which corresponds to a concentration of 0.04 mg/ml. The nucleic acid was centrifuged at 59,780 rev./min in 0.02 *M*, pH 7.2 phosphate buffer. For TMV the centrifugations were done at a speed of 27,690 rev./min with a virus concentration of 0.3 mg/ml in 0.02 *M* phosphate buffer. All runs were made at about 4°. The rotor temperature, which was controlled by chamber refrigeration, drifted no more than a few tenths of a degree.

Pictures of the absorption pattern, taken every 4 min, were recorded on Kodak "commercial" film. To obtain sufficient film blackening, a 40-sec exposure was required. Films were developed with half strength Kodak DK-50 developer at 20° for exactly 2 min with continuous brushing. Photodensitometric tracings were made

from the films with a modified Spinco Model RA Analytrol adjusted with calibrated neutral density filters to indicate the true O.D. of the film over a wide range. Only in a few cases were small corrections for non-linearity necessary.

Analysis of sedimentation data

The relative amount of homogeneous material in a solution was determined from tracings of the absorption pattern of the solution obtained during centrifugation (Fig. 1). These tracings show the changes, as material is sedimented, in the distribution of the solution density throughout the cell. There is a narrow region in which the tracings show an abrupt change in density, indicating a large number of particles moving at a uniform velocity. These particles can be considered to be a distinct component separate from additional inhomogeneous material. The amount of material in this component was calculated by comparing the change in this narrow region to the total difference in O.D. between the solution before and after the absorbing material is removed by centrifugation. The relative amount of homogeneous material was calculated from sedimentation patterns taken at three different times during the centrifugation. Each value was corrected for radial dilution[13] and the corrected amounts were then averaged.

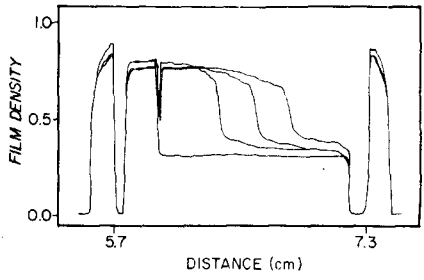

Fig. 1. Superimposed photodensitometric tracings of frames 1, 5, 7 and 9 of a typical preparation of unirradiated RNA.

For RNA solutions the apparent range of sedimentation coefficients calculated from the limits of this narrow region was 29–31 S. At least half of this spread can be attributed to diffusion and to the length of the exposure time; therefore this component can be considered homogeneous[14]. The average S^{w}_{20} value of 30 S for the homogeneous component agrees with that found by BOEDTKER[15] and by GIERER[16]. Both concluded the component consists of intact RNA particles of $2 \cdot 10^6$ molecular weight.

In TMV solutions 60 to 70 % of the material present was in a homogeneous fraction which had an average S^{w}_{20} of 190 S characteristic of TMV monomers[9,17]. The RNA extracted from these virus preparations contained from 50 to 60 % of a homogeneous component; most of the rest of the material moved slower than the intact RNA and only about 10 % moved faster.

Irradiation procedure

The source of radiation was a Westinghouse deep-therapy X-ray machine with tungsten target and glass window. At 200 kV the output, calibrated with a Victoreen Roentgenmeter, Model 70, was 1900 R/min. The average effective wavelength of the radiation was 0.2 Å as determined by absorption in copper[3]. The solutions of RNA were kept frozen during irradiation. A long copper block holding the small sample

cup was placed in a Dewar flask packed with dry ice. The sample temperature was —60° to —65° as measured with a copper-constantan thermocouple. The dry ice was replenished every 1–2 h during irradiation.

Measurements of infectivities

Infectivities of RNA solutions were measured by local lesion assays performed on *Nicotiana glutinosa* plants. Unknown samples were inoculated in parallel with standard samples at several dilutions in a Latin-square pattern to reduce variation resulting from plant or leaf position. Carborundum was dusted onto the leaves and 0.1 ml of the sample in 0.02 *M*, pH 7.2 phosphate buffer was pipetted onto the leaf. A clean spatula was used to spread the liquid over the surface of each leaf. The lesions were counted after five to seven days. Activities of the unknowns were expressed in terms of concentrations of standard sample necessary to produce an equal number of lesions.

RESULTS

Radiation-breakage of RNA

To prevent thermal denaturation of the RNA and to reduce the indirect effect of radiation, the RNA solutions were kept frozen with dry ice during irradiation. Freezing at dry ice temperatures and subsequent thawing did not affect the homogeneity of the RNA solutions, a finding in agreement with others[15,18]. Nucleic acid was first irradiated at a concentration of 0.04 mg/ml. A comparison of the sedimentation patterns of irradiated with unirradiated RNA showed that there was a decrease in the amount of homogeneous material and a corresponding increase in the amount of material which sedimented slower than the homogeneous fraction. This effect has been interpreted to be the result of breakage of the RNA strands[1]. In Fig. 2, the natural logarithm of the fraction remaining in the homogeneous component (ln survival) is plotted against the X-ray dose. The dose which leaves 37 % of the homogeneous component intact is called D_0 for breakage. The D_0 for breakage of frozen RNA irradiated at a concentration of 0.04 mg/ml was found to be $7.4 \cdot 10^5$ R. This is somewhat greater than the value of $4.0 \cdot 10^5$ R found by ENGLANDER *et al.*[1] for the D_0 of breakage for RNA extracted from irradiated virus.

Further investigation showed that both the presence of oxygen and the concentration of RNA during irradiation had an effect on the amount of breakage. When the concentration of nucleic acid was increased from 0.04 mg/ml to 0.2 mg/ml, the rate of breakage decreased. It was also found that removal of dissolved oxygen from the more dilute RNA solutions by bubbling with purified N_2 gas resulted in an equal decrease in the rate of breakage of the RNA. The least square line for these data indicates a D_0 for breakage of about $18 \cdot 10^5$ R (Fig. 3).

When increased RNA concentration and removal of oxygen were combined, the rate of breakage was further decreased only slightly, suggesting that maximum protection had been achieved. An experiment was also done in which these maximally protected RNA samples were kept at liquid nitrogen temperatures instead of dry ice temperatures during irradiation. This substantial decrease in the temperature of the samples during irradiation did not change the rate of breakage. The results of both experiments are plotted together in Fig. 4. The least squares line indicates a D_0 for breakage of $21 \cdot 10^5$ R.

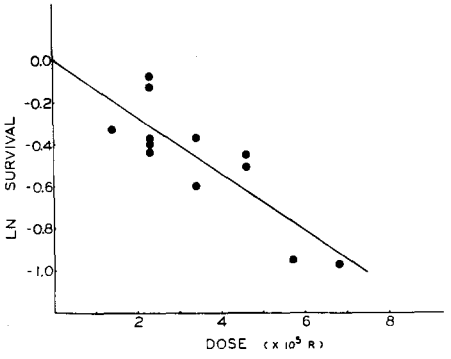

Fig. 2.

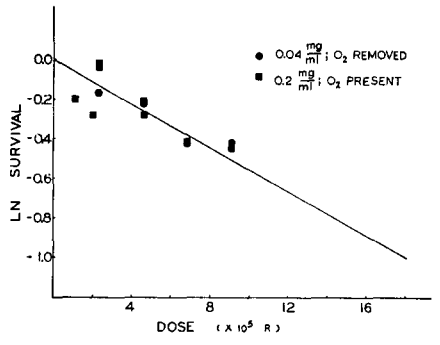

Fig. 3.

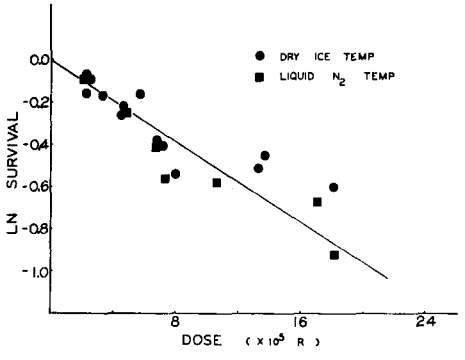

Fig. 4.

Fig. 2. Effect of X-irradiation on RNA in dilute solution.

Fig. 3. Effect of X-irradiation on RNA in concentrated solution or in oxygen free solution.

Fig. 4. Effect of X-irradiation on RNA in concentrated solution with oxygen removed.

Effect of hydrogen peroxide on RNA

The conditions that reduce the breakage of RNA, that is, removal of oxygen and increase of concentration, are standardly used to eliminate indirect action which results from the irradiation of water. It appeared anomalous that the radiation breakage of extracted RNA should in all cases be less rapid than breakage of RNA in the intact virus, which presumably is protected from indirect action[19]. A possible way that indirect action could affect the RNA in the whole virus is by the formation of organic peroxide groups on the protein[20]. These organic peroxide groups might then break the RNA later during the extraction procedure. For this reason the effect of hydrogen peroxide on RNA was studied. Storage of RNA with 2400 μmoles/l of hydrogen peroxide in the frozen state for several hours did not cause a drastic reduction of the amount of homogeneous RNA (Table I). A possible explanation is suggested by the reports that minute traces of stabilizer present in commercial peroxide reduce its effectiveness[21]. This has been questioned by CONWAY AND BUTLER[22], who showed that trace amounts of metallic-reducing catalysts are necessary for the activity of hydrogen peroxide on deoxyribonucleic acid. The virus from which the RNA was prepared was suspended in Versene, which would remove these catalysts from both commercial and irradiation-produced peroxides[23].

It is possible to measure the amount of peroxides produced in irradiated solution by using the oxidation of KI and determining liberated iodine (I^-_3) by optical absorption at 353 mμ (see ref. 24). The amount of peroxides produced when water was irradiated under various conditions was measured in this way. Distilled water was

TABLE I

EFFECT OF PEROXIDE ON RNA

Treatment	*Homogeneous material (%)*
Control	69
2400 μmoles/l H_2O_2	50
Control	68
X-rayed water, 45 μmoles/l H_2O_2	59

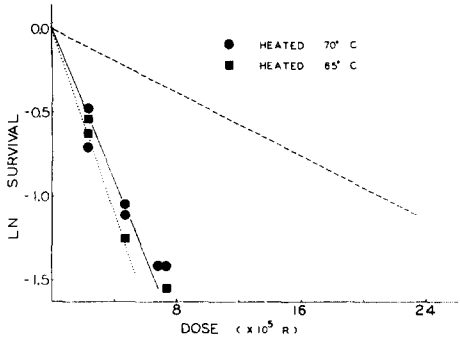

Fig. 5. Breakage of RNA by combined action of irradiation and heat. Dashed line, breakage by irradiation alone; dotted line, breakage of RNA irradiated in intact virus[1].

frozen with dry ice and irradiated for 12 h. The KI test showed, in agreement with data in the literature[25], that 45 μmoles of H_2O_2/l were present in this irradiated water after thawing, an amount about equal to the steady state achieved in another experiment in 1 h of irradiation at 0°. RNA added to this irradiated water showed only a small amount of breakage (Table I). Thus hydrogen peroxide was not effective. Organic peroxides might be more effective. However, subsequent results, which resolved the anomaly of the relatively slow radiation breakage of extracted RNA, indicated that further research on the effect of peroxides was unnecessary.

Heating and other post-treatments of irradiated RNA

Heating irradiated nucleic acids is known to increase the damage[26]. For this reason the following heating experiments were carried out on RNA after irradiation. After thawing, a portion of the irradiated sample was removed, diluted and centrifuged immediately; the remainder was used for heat treatment. For heating at 40° a 5-ml test tube containing 0.8 ml of buffer was placed in a water bath for several minutes to reach temperature equilibrium. The RNA was then added and the sample was heated with shaking for 1 or 2 min. The tubes contained approx. 4 times as much buffer as RNA. The heat treatment was stopped by plunging the tube into an ice bath. For the higher temperatures, test tubes containing 1-ml samples of diluted RNA solution were taken from an ice water bath and placed in the high temperature bath for 5 min. The heating was stopped by returning the samples to the ice water bath. The samples were centrifuged without further dilution. The results are shown in Table II. From Table II it can be seen that heating decreased the amount of material in the homogeneous component of the irradiated sample, but had little effect on unirradiated samples. Data for concentrated samples of RNA, irradiated with oxygen removed and heated at 65 or 70° after irradiation, are plotted in Fig. 5. The dashed line shows the breakage before heating. The solid line represents the least squares fit of the results from the heated samples and indicates a reduction in D_0 from 21 to $4.4 \cdot 10^5$ R. Thus, the breakage obtained after heating was very similar to that observed by ENGLANDER *et al.*[1] for the breakage of RNA extracted from irradiated TMV shown by the dotted line. Heating at 40° was done on RNA preparations irradiated under various conditions; therefore calculation of D_0 is not in all cases meaningful. Heating at 40° of a few samples of RNA irradiated at high concentrations with oxygen removed, yielded a value for D_0 of about $10 \cdot 10^5$ R.

It is possible that the observed breakage of RNA extracted from irradiated

TABLE II

EFFECT OF HEATING ON IRRADIATED RNA

Dose ($R \times 10^6$)	*Conditions of irradiation*	*Heat treatment (time and temp)*	*Homogeneous material (%)*	*Surviving fraction*
Control	All samples at dry ice temperature and		45	
Control	with RNA concentration 0.2 mg/ml		45	1.00
Control		1 min, 40°	46	
1.1			37	0.82
1.1		1 min, 40°	26	0.58
2.0			34	0.75
2.0		1 min, 40°	19	0.42
Control			64	
Control		1 min, 60°	69	
Control		1 min, 60°	68	
Control			63	1.00
1.1			72	1.1
1.1		1 min, 40°	61	0.97
1.7			65	1.05
1.7		1 min, 40°	59	0.94
Control			56	
Control	Oxygen removed		55	1.00
2.3			52	0.93
2.3		2 min, 40°	41	0.73
5.7			48	0.86
5.7		2 min, 40°	36	0.64
13.7			36	0.64
13.7		2 min, 40°	17	0.30
Control	All samples with RNA concentration		61	
Control	0.2 mg/ml and O_2 removed		62	
Control		5 min, 65°	61	
Control			63	1.00
Control		5 min, 70°	63	
Control		5 min, 65°	63	
Control			66	
Control		5 min, 70°	64	
2.3	Liquid N_2 temperature		58	0.92
2.3		5 min, 70°	30	0.48
2.3		5 min, 65°	32	0.51
4.6			49	0.78
4.6		5 min, 70°	22	0.35
7.4			36	0.57
7.4		5 min ,70°	15	0.24
7.4		5 min, 65°	13	0.20
6.8	Dry ice temperature		42	0.67
6.8		5 min, 70°	15	0.24
Control			54	
Control			57	1.00
Control		5 min, 70°	50	
Control		5 min, 65°	48	
2.3	Dry ice temperature		51	0.98
2.3		5 min, 70°	32	0.61
2.3		5 min, 65°	31	0.60
4.6			46	0.89
4.6		5 min, 70°	17	0.33
4.6		5 min, 65°	15	0.29

virus might be the cumulative effect of radiation and the development of additional breakage by the extraction process. To test the effect of the extraction process, samples of irradiated RNA were treated in the same manner as the virus is treated to isolate the RNA. This process will be called "re-extraction". The results are shown in Table III. Re-extraction did not affect unirradiated RNA but increased the breakage of irradiated RNA. Actual contact with phenol must be necessary because, if solutions of irradiated RNA were shaken with ether and then bubbled with N_2 gas, the final steps in the extraction process, no increase in RNA breakage was observed. The breakage is not quite as great as breakage of the RNA extracted from the irradiated virus.

TABLE III

EFFECT OF POST-TREATMENTS ON IRRADIATED RNA

Dose ($R \times 10^5$)	*Post-treatment*	*Homogeneous material* (%)	*Slower sedimenting material* (%)
Control		62	17
Control	Re-extraction	63	12
3.4		48	41
3.4	Re-extraction	37	55
6.8		42	35
6.8	Re-extraction	35	42
Control		56	22
Control	Shaken with ether	62	12
8.0		33	48
8.0	Shaken with ether	32	45

Irradiation of dried RNA

Irradiation of dried RNA was also studied. Shortly after extraction, 0.2 ml of the RNA preparation with concentration adjusted to 0.2 mg/ml in 0.02 *M* phosphate buffer was placed on a cover slip and dried in the frozen state. After drying, the samples were sealed between two sheets of parafilm by pressure. The dried RNA samples were cooled during irradiation to a temperature slightly below 25°. After

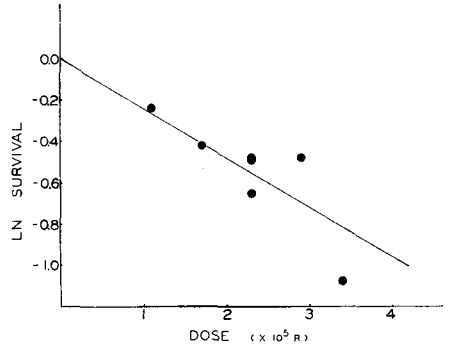

Fig. 6. Breakage of RNA irradiated in dry state.

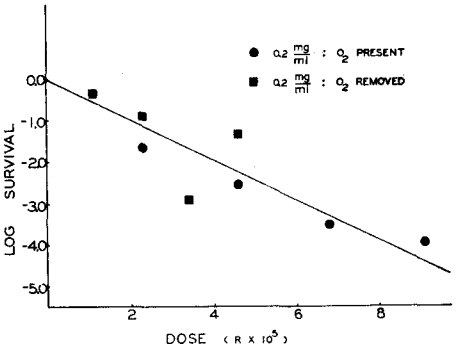

Fig. 7. Inactivation of infectious RNA.

irradiation, the samples were allowed to redissolve slowly in buffer at about 5° for 1 h: the final concentration was 0.04 mg/ml in 0.02 *M* phosphate buffer. Drying has negligible effect on the homogeneity of the RNA. The results of the X-irradiation of dried RNA, shown in Fig. 6, indicate a D_0 for breakage of $4.2 \cdot 10^5$ R. Therefore, radiation breaks dried RNA apparently as fast as the RNA in the virus. Radiation breakage of dried RNA presumably would be almost completely caused by direct effect.

Biological inactivation of RNA

The biological activity of infectious RNA from TMV is also destroyed by X-rays[18]. RNA was irradiated at a concentration of 0.2 mg/ml with and without treatment to remove oxygen. The results are plotted together in Fig. 6 because they are only slightly different for these two conditions. The D_0 for inactivation was found to be about $1 \cdot 10^5$ R. It is substantially lower than the D_0 for the breakage of RNA under any of the conditions used; however, it is nearer the D_0, $1.8 \cdot 10^5$ R, for inactivation of the parent virus[1]. GINOZA AND NORMAN[18] found that D_0 for inactivation of RNA was $3 \cdot 10^5$ R for long wave length X-rays[19].

GIERER AND SCHRAMM[26] investigated the infectivity of nucleic acid solutions prepared by the phenol method and concluded that the infectivity could not be the result of TMV contamination. It has been shown that the infectivity of various RNA batches prepared in this laboratory by the phenol method was not caused by residual virus[28], but such tests were not performed on the individual RNA preparations used in these inactivation studies.

DISCUSSION

Treatment with heat or phenol after irradiation develops breakage not previously observed. Such mild after-treatment has little effect on unirradiated RNA. DOTY *et al.*[2] have proposed that as many as 60 % of the bases of the single stranded RNA molecule are hydrogen bonded to form a system of closed loops. It is possible that these bonds are strong enough to hold two segments of a loop together even though radiation had produced a break somewhere in the main chain. DOTY found that the hydrogen bonding could be eliminated by heating. Thus the development of breaks by heating irradiated RNA might result from allowing the broken parts to separate by elimination of intramolecular hydrogen bonds. Phenol also could break the hydrogen bonds.

The data of DOTY *et al.*[2] indicate that some hydrogen bonds are broken even at 40° and that practically all are eliminated at 65°. From the results of our experiments it can be estimated that heating irradiated RNA at 40° reduced the D_0 for breakage from $21 \cdot 10^5$ R to about $10 \cdot 10^5$ R and that heating at 65 or 70° reduced the D_0 to $4.4 \cdot 10^5$ R, a value in good agreement with the D_0 of $4.0 \cdot 10^5$ found[1] for the breakage of RNA irradiated in the intact virus. These data are therefore in good agreement with the hydrogen bonded loop hypothesis. The rate of breakage of dried RNA is also similar to that observed for irradiated RNA heated at 65 or 70°. In this case an unfolding of the RNA molecule might occur because of the sharp reduction in dielectric constant when the RNA goes from a water medium to an air medium.

Breakage of RNA extracted from irradiated virus is closely similar in extent to breakage of irradiated free RNA after heating. In addition it has been found that

breakage cannot be comparably increased by heating the RNA extracted from irradiated virus[29]. This can be explained by the assumption that when RNA is held in a rigid configuration in the intact virus it is unable to hydrogen bond to itself in the form of closed loops and therefore all breaks are observed on extraction. It is concluded that RNA breakage in the whole virus is not an artifact of the extraction procedure, but an accurate indication of the direct radiation action.

The D_0 found for the inactivation of infectious RNA was $1 \cdot 10^5$ R which is slightly lower than the D_0 of $1.8 \cdot 10^5$ R found by ENGLANDER *et al.*[1] for the inactivation of TMV. It is not unreasonable to expect that some products of indirect action might have an effect on the isolated RNA. While there was no observed difference between the inactivation rate in the presence or absence of oxygen, production by indirect action of free radicals not affected by oxygen concentration could result in some extra damage.

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