

# Inactivation of Thirty Viruses by Gamma Radiation<sup>1</sup>

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Decimal reduction values (*D* value) for 30 viruses were determined. The weighted *D* values of the viruses suspended in Eagle's minimum essential medium ranged from 0.39 to 0.53 Mrads. It was necessary to increase the radiation dose by a factor of >3 to inactivate virus suspended in Eagle's minimum essential medium as compared to the same virus suspended in distilled water. The destruction rate curves were of a first-order reaction.

Radioinactivation of viruses has received sporadic attention during the last 25 years. The use of attenuated viral vaccines overshadowed the concept of utilizing gamma radiation in killed vaccine preparations. Recently, renewed interest has been generated in the use of gamma radiation as a method for inactivating viruses and other pathogens in water, sewage, and in foods. The side reactions and infections produced by some attenuated vaccines have stimulated this revival of interest.

The use by various investigators of differing units of measurement to describe the radiation dosage has discouraged comparisons of early viral inactivation studies and those more recently published. Various data, however, indicate that viruses are more resistant to gamma radiation than are other microorganisms and that viral inactivation demonstrates a "one-hit" or first-order reaction rate curve when the suspending medium contains free radical scavengers or is in a frozen state.

Gamma radiation inactivation studies of some animal viruses include polyoma virus (1, 5, 11); Rous sarcoma virus (3); vaccinia virus (4, 8, 9, 12, 14); Newcastle disease virus (22, 23); influenza viruses A and B (16-18; Sullivan et al., *Bacteriol. Proc.*, p. 155, 1968); mumps virus (18); rabies virus (21); echoviruses 1, 7, and 11 (2; Sullivan et al., *Bacteriol. Proc.*, p. 155, 1968); poliovirus (2, 8); coxsackieviruses A9 and B2 (Sullivan et al., *Bacteriol. Proc.*, p. 155, 1968); reovirus 1, simian virus 40 (Sullivan et al., *Bacteriol. Proc.*, p. 155, 1968); rubella virus (10); St. Louis encephalitis virus (8); Western equine encephalitis virus (8); Venezuelan encephalitis

virus (19); measles virus (2); hoof and mouth disease virus (15); smallpox virus (17); and herpes simplex virus (17). In these studies, the method of reporting the response of the virus to the radiation dose and the media used to suspend the virus under investigation varied extensively.

The present study was undertaken on 30 selected viruses believed to be of public health significance in environmental studies. The viruses were suspended in Eagle's minimum essential medium (MEM) containing 2% fetal bovine serum, which was used as a radical scavenger, and in distilled water.

## MATERIALS AND METHODS

**Viruses.** The viruses selected for radioresistance studies were poliovirus I Mahoney, poliovirus I Lotshaw, poliovirus II Y-SK, poliovirus II Lansing, poliovirus III Leon, poliovirus III Nadler, coxsackievirus A9 CME 456, coxsackievirus A-11 Belgium YR 169, coxsackievirus B1 Conn. 5, coxsackievirus B2 Ohio 1, coxsackievirus B3 PF, coxsackievirus B4 JVB, coxsackievirus B5 Faulkner, echovirus 4 SEC, echovirus 5 Noyce, echovirus 6 D'Amori, echovirus 7 Wallace, echovirus 9 Hill, echovirus 11 Gregory, echovirus 12 Travis, echovirus 18 SEC, simian virus 40 DBS, reovirus 1 Lang, adenovirus 2 NIAID, adenovirus 3 Meacham, adenovirus 5 NIAID, adenovirus 12 NIAID, herpes simplex virus HF, Newcastle disease virus B1, and influenza virus A NWS. All of the viruses were passaged at least twice in primary kidney cell cultures from African green monkeys (*Cercopithecus aethiops*) by using Liebovitz's L-15 medium supplemented with 2% fetal bovine serum and 0.07% NaHCO<sub>3</sub> (13). Cell sheets showing advanced cytopathic effect were frozen and thawed three times and the virus was harvested. The harvest was clarified by centrifugation for 15 min at 1,060 X g at 4°C. The titers of the harvested viruses were determined by using a plaque-forming unit (PFU) assay system. The virus material was dispensed into borosilicate glass ampoules; the ampoules were flame-

<sup>1</sup> Part of the research reported here was done by Robert Sullivan in partial fulfillment of the requirements for the Ph.D. degree from the Department of Civil and Environmental Engineering, University of Cincinnati, Cincinnati, Ohio.

sealed and stored at  $-60^{\circ}\text{C}$ . These procedures provided a pool of known titer for each strain of virus used throughout the investigation.

**Radiation source.** A cobalt-60 well source located at General Electric Missile and Space Division, Evendale, Ohio, was used in this study. The chamber consisted of 36 pencil sources arranged in concentric tiered groupings of 6, 12, and 18. The irradiation rig, cylindrical in shape, was placed in the center of the tiers. The tube holders had inner and outer positions, which put the tubes, at any given height, farther from or closer to the source. Dosimetry was calibrated by cobalt glass dosimeters. During irradiation, the rig holding the samples was rotated at appropriate periods through a series of 26 steps so that one complete revolution occurred during the exposure time of 3 hr.

**Viral assay.** A PFU assay system was used (20). This system consisted of (i) the diluent, which was Eagle's MEM with nonessential amino acids, 2% fetal bovine serum, pH 7.0; (ii) African green monkey kidney primary cell cultures in 6-oz (ca. 180

ml) prescription bottles (45-cm<sup>2</sup> cell sheets); and (iii) an overlay of 0.95% Ionagar no. 2 (Oxoid), Eagle's medium as above, 2% fetal bovine serum, 0.5%  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.0015% neutral red, 0.19%  $\text{NaHCO}_3$ , and 1% (sterile) cow's milk. The Eagle's medium was in Hanks balanced salt solution and did not contain phenol red (6, 7). The material to be assayed was pipetted onto the cell sheet and incubated for 1 hr at  $36^{\circ}\text{C}$ . The overlay agar medium was added after the adsorption period and was allowed to solidify at room temperature. The bottles were incubated agar side up at  $36^{\circ}\text{C}$ , and the cell sheets were examined daily for plaques, which were marked and recorded as they appeared.

The virus to be assayed was diluted in Eagle's MEM or distilled water to approximately 10,000 PFU/ml, 1.2 ml of the suspension was dispensed into 13 by 53 mm borosilicate glass tubes, and the tubes were flame-sealed. Five tubes containing the virus material to be tested were placed in a position on the rig so that, when lowered into a cobalt-60 gamma energy source,

TABLE 1. Summary of radioresistance for viruses suspended in Eagle's minimum essential medium plus 2% fetal bovine serum (preliminary study)

Virus	No. of observations	Weighted <i>D</i> value (Mrad)	99% Confidence limits	No. of runs	<i>D</i> value for runs that could not be pooled
Adenovirus					
2	27	0.41	0.38-0.44	2	
3	19	0.49	0.42-0.61	2	
5	42	0.44	0.39-0.50	3	
12	55	0.46	0.42-0.52	4	
Coxsackievirus					
A-9	27	0.42	0.37-0.47	2	
A-11	60	0.48	0.44-0.52	3	
B-1	25	0.41	0.35-0.49	2	
B-2	46	0.44	0.41-0.48	2	
B-3	40			3	0.35, 0.43, 0.43
B-4	26	0.50	0.46-0.55	2	
B-5	14	0.41	0.35-0.51	1	
Echovirus					
4	19	0.46	0.42-0.51	2	
5	29	0.49	0.43-0.55	2	
6	32	0.51	0.41-0.68	2	
7	64			4	0.38, 0.55, 0.49, 0.57
9	19	0.50	0.42-0.60	1	
11	49			4	0.42, 0.47, 0.50, 0.48
12	72	0.50	0.46-0.55	5	
18	17	0.44	0.37-0.55	1	
Poliovirus					
I-MAH	22	0.49	0.43-0.58	2	
I-Lotshaw	17	0.53	0.44-0.65	1	
II-Y-SK	28			2	0.54, 0.41
II-Lansing	49			4	0.50, 0.50, 0.54, 0.41
III-Leon	41			3	0.41, 0.54, 0.44
III-Nadler	15	0.48	0.44-0.54	1	
Herpes simplex virus	33	0.43	0.39-0.47	3	
Newcastle disease virus	88	0.52	0.49-0.56	6	
Reovirus I	15	0.42	0.41-0.43	1	
Simian virus 40	74	0.45	0.41-0.51	4	
Influenza virus A	42	0.49	0.43-0.56	3	

TABLE 2. *Radioresistance of nine selected viruses suspended in Eagle's minimum essential medium plus 2% fetal bovine serum*

Virus	No. of observations	Weighted <i>D</i> value (Mrad)	99% Confidence limits	No. of runs	<i>D</i> value for runs that could not be pooled
Adenovirus 2	103			4	0.46, 0.43, 0.45, 0.38
Coxsackievirus					
A-9	117	0.46	0.44-0.48	4	
B-2	106	0.45	0.42-0.48	4	
Echovirus 11	67	0.43	0.38-0.49	4	
Herpes simplex virus	87			3	0.39, 0.46, 0.42
Influenza virus A	85	0.46	0.44-0.49	3	
Poliovirus III-Leon	104			4	0.48, 0.45, 0.40, 0.38
Reovirus I	99	0.44	0.41-0.49	4	
Simian virus 40	49	0.39	0.36-0.43	2	

they received a predetermined dose of irradiation during a period of slightly over 3 hr. The levels of irradiation used in the screening study were 0.51, 1.09, 1.44, and 1.95 Mrad. Temperature was maintained at  $0.5 \pm 0.2$  C during irradiation. A 1-ml amount from each of the five tubes at each dose level was assayed for surviving virus.

**Calculation of *D* values.** *D* values were calculated by the following formula. A linear model was assumed, and the parameters  $\beta_0$  and  $\beta_1$  were estimated for each run. The model is as follows:  $Y = \beta_0 + \beta_1 X + \epsilon$  where,  $Y = \log_{10}$  plaque count,  $\beta_0, \beta_1 = \text{true but unknown regression coefficients}$ ,  $X = \text{concentration in Mrad}$ ,  $\epsilon = \text{experimental error}$ .

The proportion sum of squares due to linear regression ( $R^2$ ) was  $\geq 0.9$  for most runs, and the model used to obtain an estimate of virus radioresistance seems to be an adequate representation of the data. The value of 1 over the estimate of slope ( $\beta_1$ ) is the *D* value in Mrad.

In the study of nine selected viruses, the levels of radiation were increased in an attempt to obtain a broader range of values for the determination of the slope of the destruction rate curves. The levels used were 0.25, 0.49, 0.98, 1.26, 1.50, 1.75, 1.95, and 2.20 Mrad.

## RESULTS

Data from the screening tests of 30 viruses were statistically analyzed by using the linear regression model, and the *D* values were computed from the inverse slope of the line (Mrad versus  $\log_{10}$  PFU/ml). Data from replicate runs were compared to determine whether the slopes from the runs could be pooled. Table 1 shows the result of these homogeneity of regression line tests for the 30 strains in the preliminary study. A weighted estimate of *D* is given for those runs that could be pooled, and individual estimates are presented for tests that were significant. A 99% confidence interval is also given for the pooled results. This means that the true but unknown value is expected to fall between the upper and lower limits 99 times in 100. The total observations for each strain are listed in Table 1.

Nine viruses were selected for more intensive study by using eight doses of radiation per determination. An attempt was made to select viruses representative of the picornavirus, reovirus, papovavirus, adenovirus, herpesvirus, and myxovirus groups. They were also chosen for plaque-forming characteristics, consistently high titer, and ease of handling in the laboratory.

Gamma-radiation destruction-rate studies at  $0.5 \pm 0.2$  C were performed on these representative viruses. Viral populations of approximately 10,000 PFU/ml were suspended in modified Eagle's MEM for the determination of gamma-radiation destruction-rate curves. Experiments were run for each viral population, and the data derived from these experiments were statistically analyzed to determine the 99% confidence limits of the *D* values for these viruses.

Table 2 shows results for nine selected virus strains. These data were analyzed in the same manner as those in the preliminary study, and the results are similar to those reported in Table 1. Weighted *D* values ranged from 0.39 to 0.46 Mrad. The highest single run was for poliovirus III where *D* was calculated as 0.48 Mrad. A typical destruction rate curve is shown in Fig. 1.

Five virus strains, one run each, were tested in a water substrate. These data are shown in Table 3 and are based on only three concentrations. The results are not extensive but tend to indicate a significantly lower resistance of these five strains in water as compared to the same five in Eagle's MEM plus 2% fetal bovine serum substrate. The *D* values ranged from 0.10 to 0.14 Mrad.

## DISCUSSION

Gamma radiation destruction rate kinetics on 30 viruses suspended in Eagle's MEM containing 2% fetal bovine serum showed a first-order curve configuration. The amount of gamma radiation necessary to reduce the number of viral PFU/m

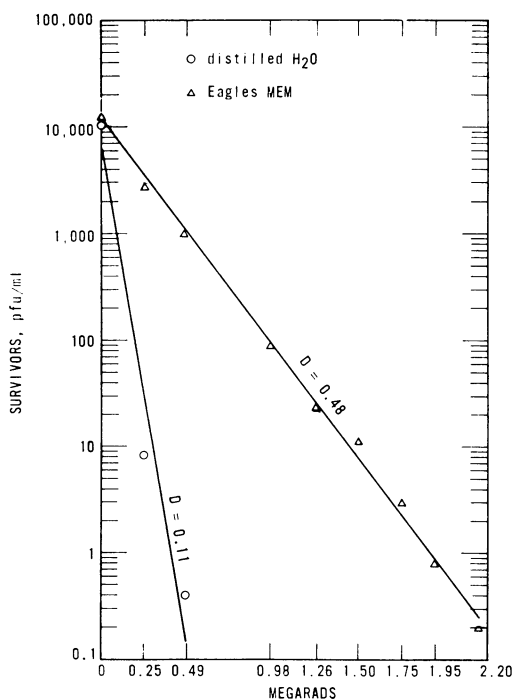


FIG. 1. Typical radioresistance curve of poliovirus III in distilled water and Eagle's MEM.

TABLE 3. Radioresistance of five selected viruses in water

Virus	No. of observations <sup>a</sup>	D value (Mrad)	99% Confidence limits
Coxsackievirus A-9	9	0.12	0.08-0.21
Coxsackievirus B-2	11	0.14	0.10-0.21
Echovirus 11	8	0.14	0.11-0.21
Influenza virus A	11	0.10	0.06-0.25
Poliovirus III Leon	11	0.11	0.07-0.24

<sup>a</sup> Only one run was performed for each virus, and radiation exposures were for only three dosages including zero.

by 90% (weighted *D* value) ranged from 0.39 to 0.53 Mrad.

Nine of these 30 viruses were selected for further study. These nine viruses were from the picornavirus, reovirus, myxovirus, papovavirus, and herpesvirus groups. Their weighted *D* values ranged from 0.39 to 0.46 Mrad when they were irradiated in the Eagle's MEM with 2% fetal bovine serum. The values agree with published *D* values for other viruses in the liquid state. Viruses in the frozen state, however, have been reported to require more radiant energy for inactivation and had higher *D* values (8).

The *D* values determined for five of these viruses were significantly affected by the suspending medium. At least a threefold difference was noted when the *D* values of the same virus suspended in distilled water and in Eagle's MEM were compared. The differing *D* values obtained with the variation in suspending medium indicated that if radiation is used to sterilize viruses suspended in menstua other than those reported, it will be necessary to investigate the rate of viral inactivation in the suspending medium utilized.

#### ACKNOWLEDGMENT

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#### LITERATURE CITED

1. Basilico, C., and G. DiMayorca. 1965. Radiation target size of the lytic and the transforming ability of polyoma virus. *Proc. Nat. Acad. Sci. U.S.A.* 54:125-127.
2. Benyesh, M., E. C. Pollard, E. M. Opton, F. L. Black, W. D. Bellamy, and J. L. Melnick. 1958. Size and structure of ECHO, poliomyelitis and measles virus determined by ionizing radiations and ultrafiltration. *Virology* 5:256-274.
3. Bryan, W. R., E. Lorenz, and J. B. Moloney. 1950. Studies on the effects *in vitro* of roentgen radiation on the biological activity of an agent of chicken tumor 1 (*Rous sarcoma*). *J. Cancer Inst.* 10:1215-1237.
4. Decker, C., J. Guir, and A. Kirm. 1969. Infectivity and capacity for DNA replication of vaccinia virus irradiated by  $\gamma$ -rays. *J. Gen. Virol.* 4:221-227.
5. Defendi, V., F. Jensen, and G. Sauer. 1967. Analysis of some viral functions related to neoplastic transformation, p. 645-663. *In* J. S. Colter and W. Paranchych (ed.), *The molecular biology of viruses*. Academic Press Inc., New York.
6. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science* 130:432-437.
7. Hanks, J. H., and R. E. Wallace. 1949. Relation of oxygen and temperature in the preservation of tissues by refrigeration. *Proc. Soc. Exp. Biol. Med.* 71:196-200.
8. Jordan, R. T., and L. L. Kempe. 1956. Inactivation of some animal viruses with gamma radiation from cobalt-60. *Proc. Soc. Exp. Biol. Med.* 91:212-215.
9. Kaplan, C. 1960. The antigenicity of gamma irradiated vaccinia virus. *J. Hyg.* 58:391-398.
10. Kenny, M. T., K. L. Albright, J. B. Emery, and J. O. Bittle. 1969. Inactivation of rubella virus by gamma radiation. *J. Virol.* 4:807-810.
11. Latarjet, R., R. Cramer, and L. Montagnier. 1967. Inactivation by u.v., x, and gamma radiation of the infecting and transforming capacities of polyoma virus. *Virology* 33:104-111.
12. Lea, D. E., and M. H. Salaman. 1942. The inactivation of vaccinia virus by radiations. *Brit. J. Exp. Pathol.* 23:27-37.
13. Leibovitz, A. 1963. The growth and maintenance of tissue-cell cultures in free gas exchange with the atmosphere. *Amer. J. Hyg.* 78:173-180.
14. McCrea, J. F. 1960. Ionizing radiation and its effects on animal viruses. *Ann. N.Y. Acad. Sci.* 83:692-705.
15. Polatnick, J., and H. L. Bachrach. 1968. Ionizing irradiation of foot-and-mouth disease virus and its ribonucleic acid. *Arch. Gesamte Virusforsch.* 23:96-104.
16. Polley, J. R. 1961. Factors influencing inactivation of infectivity and hemagglutinin in influenza virus by gamma radiation. *Can. J. Microbiol.* 7:535-541.
17. Polley, J. R. 1961. Preparation of soluble antigens with gamma radiation. *Can. J. Microbiol.* 7:135-139.

18. Polley, J. R. 1962. The use of gamma radiation for the preparation of virus vaccines. *Can. J. Microbiol.* 8:455-459.
19. Reitman, M., and H. R. Tribble, Jr. 1967. Inactivation of Venezuelan equine encephalomyelitis virus by  $\gamma$ -radiation. *Appl. Microbiol.* 15:1456-1459.
20. Sullivan, R., and R. B. Read, Jr. 1968. Method for recovery of viruses from milk and milk products. *J. Dairy Sci.* 51: 1748-1751.
21. Traub, F. B., U. Friedemann, A. Brasch, and W. Huber. 1951. High intensity electrons as a tool for preparation of vaccines. 1. Preparation of rabies vaccine. *J. Immunol.* 67: 379-384.
22. Wilson, D. E., and E. C. Pollard. 1958. Radiation studies on the infective property of Newcastle disease virus. *Rad. Res.* 8:131-141.
23. Woese, C., and E. C. Pollard. 1964. The effect of ionizing radiations on various properties of Newcastle disease virus. *Arch. Biochem. Biophys.* 50:354-357.