Effects of X-irradiated Aqueous Solutions on Vesicular Stomatitis Virus

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Infectivity of vesicular stomatitis virus was inactivated by suspending the virus in aqueous salt solutions that had previously been subjected to X-irradiation. This viral inactivation did not appear to be attributable to hydrogen peroxide or to long-lived free radicals generated by X-irradiation of the salt solutions. Indirect evidence suggested that the deleterious effects on the virus were caused by chemical species resulting from interaction of free water molecules with anions in the solution. Inactivation of infectivity was associated with distortion of the outer layer of the virion, as determined by electron microscopy, and with consequent impairment of adsorption to otherwise susceptible cells.

The inactivation of infectivity of virions suspended in aqueous solutions by X-irradiation or by other ionizing radiation has been attributed to at least two effects. In one, viral infectivity is inactivated by direct hits on the virion (12, 17, 22); in the other, inactivation of viral infectivity is brought about by secondary effects on the virion of short-lived radicals and of new stable chemical substances generated by the action of ionizing radiation on the suspending medium (12, 22, 25). The indirect inactivation by stable toxic products has been thought to be caused by hydrogen peroxide which appears after ionizing irradiation of water (2, 16, 24). The present experiments were designed to test this hypothesis. The model chosen was vesicular stomatitis virus (VSV) infection of L cells, because it has recently been demonstrated (11) that this system is one of the few animal virus-cell systems in which seed inocula can be prepared such that the ratio of physical particles (virions) to infective units is close to unity.

MATERIALS AND METHODS

Medium and diluents. L cells were originally received from W. Earle and were conditioned to grow in medium 199 and 10% horse serum. These cells were used for both growth and titration of the virus. The diluents in the various experiments were water (distilled), 0.15 M NaCl (saline), phosphate-buffered saline (PBS) containing 8.0 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4 (nonhydrated), 0.2 g of KH_2PO_4 , and 1,000 ml of distilled water, or commercial hydrogen peroxide (Fisher Scientific Co., Pittsburgh, Pa.; 30.30%) at 7.4 $\mu\text{g}/\text{ml}$ in water.

Virus. The Indiana serotype of VSV obtained from the American Type Culture Collection was used throughout these studies. It was adapted to grow in

L cells (10), and virus from the 23rd to the 27th passages was used. Stock virus was prepared by inoculating 4-day-old L-cell monolayers at a low multiplicity of infection (usually 5) in order to obtain a good yield of highly infectious VSV (11). The virus was harvested after 20 hr of infection, centrifuged at low speed to remove cellular debris, and then centrifuged at $66,000 \times g$ for 90 min in a type 30 rotor in a Beckman L2-65 ultracentrifuge. The pellet was washed twice in PBS. It was diluted to approximately 10^{10} virions/ml in PBS and was stored in 1-ml amounts at -60°C . Length of storage at this temperature had no effect on titer for the period under investigation (11).

Biological and physical virus quantitation. Plaque titrations were performed in triplicate on 4-day-old monolayers as previously described (11). Physical particles were counted by the sedimentation technique of Sharp (20) as modified by Galasso (10). By this method, the actual number of virions could be determined with a standard error of less than 8% of the mean.

X-irradiation. A Picker therapy unit was used for X-irradiation and was operated at 200 kv and 25 ma, with a 1.26-mm copper filter in addition to the X-ray window. The average dose rate was 260 to 270 r/min. The dosage was measured with a type 250 R Victoreen condenser meter (no. 70, Glasser-Seitz) 1.5 cm from the end of a cone, 3 cm in diameter, that extended 21 cm from the X-ray tube focal spot. The solution to be irradiated was placed in a closed polypropylene tube, 3 cm in length and 3 cm in diameter, which was placed in contact with the cone. The exposure dosage was measured at mid-depth of sample and may have varied by $\pm 11\%$ due to the depth of the sample. The tube was almost completely full, with a minimum of air space remaining, and was always kept in an ice bath during irradiation. After irradiation of the solution, sonically treated stock virus (for 3 min in a model S-102A Raytheon sonic oscillator at 9

kc/sec) was added to a final concentration of 10^8 virions/ml. Sonic oscillation for this period of time has no effect on the infectivity of the virion (11). This mixture was kept at 4 C in the dark for various periods of time. Controls were treated identically using unirradiated solutions. The virus was then diluted with complete medium for titration. In two other experiments, the solutions were irradiated at pH 1.8 and pH 12 and were then readjusted to pH 7 with HCl or NaOH before the virus was suspended.

Hydrogen peroxide determination. The amount of hydrogen peroxide produced during X-irradiation was determined by the starch-iodide test of Savage (19). Concentrations of less than 10^{-3} M can be measured by this technique.

Electron microscopy. Treated and control virions were prepared for electron microscopic examination in the same manner as the particle count preparations (10). However, the collodion film pseudoreplicas were floated off the agar receiving surface into a 1% phosphotungstic acid (PTA) solution at pH 2, instead of into water as previously described. After allowing the stain to act for 1 min, the film was placed on a 400-mesh copper grid previously covered with Formvar. The grids were then examined with an RCA EMU 3 H electron microscope.

RESULTS

Effect of irradiated aqueous solutions on VSV. VSV was suspended in cold (4 C) previously X-irradiated (30,000 r) water, in saline (0.15 M), or in PBS (pH 7.2) to a concentration of 10^8 virions/ml, and was stored in the dark at 4 C within 30-min postirradiation. Controls were treated in the same manner, but unirradiated solutions were used. Samples of virus were removed at different time intervals and were diluted further, in complete medium, for plaque titration. This dilution also presumably served to terminate the reaction of virus with inactivating compounds generated by irradiation. The fraction of surviving infectivity (N/N_0) was plotted against the time of virus exposure to the preirradiated solution at 4 C. As shown in Fig. 1, preirradiated water had little effect on the virus but both preirradiated (30,000 r) saline and preirradiated PBS induced significant inactivation of VSV, 66% and 84%, respectively, at 3 hr.

The negative results obtained with irradiated water suggested that formation of hydrogen peroxide in the irradiated solutions had little to do with the viral inactivation. To rule out the effect of hydrogen peroxide formed by the interaction of short-lived radicals in irradiated water, VSV was treated with commercial hydrogen peroxide (7.4 μ g/ml) in water. No significant effect was apparent (Fig. 1). The amount of hydrogen peroxide generated in irradiated water was 1.6 μ g/ml, in saline was 1.4 μ g/ml, and in PBS was 0.9 μ g/ml. The addition of phosphate salts to irradiated water or the addition of 10 μ g/ml of hydrogen peroxide per ml either to un-

FIG. 1. Inactivation rates of purified VSV suspended in previously X-irradiated aqueous solutions (dose 30,000 r) and commercial hydrogen peroxide. Symbols: $\square$, X-irradiated PBS sterilized either by autoclaving or by filtration; Δ , X-irradiated saline; $\circ$, X-irradiated water (addition of phosphate salt to X-irradiated water or addition of 10 μ g of hydrogen peroxide per ml to either unirradiated PBS or to saline gave similar results); $\bullet$, commercial hydrogen peroxide (7.4 μ g/ml in water).

irradiated PBS or to saline had no effect on viral infectivity (Fig. 1). Therefore, the hydrogen peroxide generated by prior irradiation of the aqueous solutions does not appear to be the inactivator of the VSV.

To determine whether autoclaving would alter the constituents of PBS and thereby enhance the inactivating effects, the same experiment was performed with membrane-filtered (Millipore Corp., Bedford, Mass.) PBS. Inactivation obtained with irradiated PBS sterilized in this manner was reduced, but the degree of reduction was not consistent in several experiments. Cahn (8) has shown that detergents used to stabilize the filters are washed off into the material being filtered and can be toxic. Therefore, the experiments were repeated with filters which had been washed with hot water to remove the detergents. The inactivation rates obtained with irradiated PBS which had been previously sterilized with these washed filters were reproducible and were the same as those obtained with irradiated PBS which had been sterilized by autoclaving (Fig. 1). It was concluded that autoclaving does not alter the constituents of PBS making it more susceptible to change by X-irradiation, and that detergents and proteins (16) in the filters do show some protective effect on the virus, possibly by quenching of the toxic product. It has been demonstrated that as little as 0.1 g of peptone per liter protects virus from indirect irradiation effects (16). Therefore, very small amounts of detergents or protein could have caused the inconsistent results.

It was not possible to examine the relationship of phosphate concentration to inactivation since phosphate salt solutions in concentrations greater than 0.07 M phosphate readily crystallized when subjected to X-irradiation.

To ascertain the role of long-lived radicals which may be generated by irradiation, electron spin resonance (ESR) studies were carried out under the direction of R. Livingston of the Oak Ridge National Laboratories. (ESR detects any chemical species with unpaired spin in irradiated solutions.) No such radicals could be detected, above the estimated sensitivity of the instrument (10^{14} radicals), in PBS examined within 30 min of irradiation. It was concluded within the limits of the sensitivity of the ESR studies, that long-lived radicals were not involved. However, the interaction of short-lived free radicals appeared to be involved, as was demonstrated by an experiment (Fig. 2) in which PBS was irradiated in the frozen state (in dry ice). This was done to immobilize the short-lived radicals produced by X-irradiation. No inactivation was observed when VSV was suspended in melted solutions which had been irradiated in the frozen state.

Implication of H radicals. It was then hypothesized that the effect could be due to the reaction of the H radicals or the e_{aq}^- (hydrated electrons) which are produced during radiolysis of water with anionic species in solution (1). This reaction could produce stable reactive chemical products

which would then cause the inactivation of virus. To determine which of the two radical species (H or e_{aq}^-) was involved, the pH of PBS was lowered to 1.8 prior to irradiation with 30,000 r. Under these conditions, the H radical formation is known to increase coincidentally with the removal of e_{aq}^- by the following reaction (18):

The *G* value (the yield of each product expressed in molecules formed per 100 electron volts of energy absorbed) for the H radical at pH 1.8 was $G_H = 3.65$, as compared to *G* values at neutral pH of $G_H = 0.5 \pm 0.1$ and $G_{e_{aq}^-} = 2.7$. On the other hand, at pH 12, the $G_{e_{aq}^-}$ was again 2.7, whereas the H radical disappears (1). We found (Fig. 2) a marked increase in viral inactivation when PBS was irradiated at pH 1.8, as compared with the inactivation of PBS irradiated at neutral pH. In sharp contrast, PBS irradiated at pH 12 had no effect on viral infectivity. This finding indicated that the H radical is indirectly responsible; if e_{aq}^- were involved, the effect would have decreased at pH 1.8 and increased at pH 12.

Dose responses. We also performed a series of experiments to determine the effects of radiation dose, viral concentration, and kinetics of inactivation. An increase in the radiation dose to 45,000 r resulted in a viral inactivation curve similar in shape to that of 30,000 r (Fig. 2), but steeper and more pronounced (92% inactivation) at 3 hr. Figure 3 shows that the degree of inactivation of VSV was greater when lower concentrations of virus were suspended in PBS previously irradiated with 30,000 r. These inactivation data suggest a concentration and time dependency. The dose-response curve of viral inactivation with constant viral concentration (10^8 virions/ml) plotted against increasing irradiation indicated a cumulative inactivation response (Fig. 4). Extrapolat-

FIG. 2. Inactivation rates of purified VSV suspended in previously X-irradiated PBS. Symbols: $\square$, X-irradiated (30,000 r) PBS sterilized either by autoclaving or filtration; $\circ$, X-irradiated (30,000 r) frozen PBS; $\bullet$, X-irradiated (30,000 r) PBS at pH 12; $\times$, X-irradiated (30,000 r) PBS at pH 1.8; $\triangle$, X-irradiated (45,000 r) PBS at pH 7.0.

FIG. 3. Variation in the degree of inactivation of VSV suspended at different concentrations in PBS previously irradiated with 30,000 r.

FIG. 4. Radiation dose response of VSV inactivation. Symbols: ○, dose response for 0.5-hr exposure of virus to X-irradiated PBS; ●, dose response for 1-hr exposure of virus to X-irradiated PBS.

ing the linear portions of the curves back to zero dose shows that, to inactivate one virion, more than two interactions between the virion and the chemical radiation products are required (3). This is in agreement with calculations of G_H values after irradiation of solutions at different pH values. Assuming a sevenfold increase in the G_H of irradiated PBS at pH 1.8 over that of PBS at a neutral pH, an approximately twofold or greater increase in viral inactivation should occur. The observed data fall within the limits of this estimate.

Effect of irradiated PBS on viral adsorption. It seemed likely that the toxic products generated by irradiation might inactivate the virus by acting on the surface of the virion. To test this hypothesis, experiments were undertaken to examine the effect of irradiated solutions on viral adsorption. Since no biological assay is available to quantitate inactivated virus, adsorption studies were conducted by counting physical particles with the aid of the electron microscope (10).

Virus exposed to previously irradiated PBS (30,000 r) for 3 hr at 4°C and control virus suspended in unirradiated PBS were diluted in complete medium. These virus suspensions were then used to resuspend pellets of L cells, yielding a virus to cell ratio of 15. The suspensions were divided among a number of tubes which were placed on a roller drum (30 rev/min) at room temperature. The tubes were removed periodically

and were subjected to low-speed centrifugation for 10 min to remove the cell-virus complexes. Unadsorbed virus particles in the supernatant fluid were counted in the electron microscope (Fig. 5). We observed a lag period as well as a decrease in adsorption for the virus exposed to irradiated PBS, compared to the control virus. Per cent adsorption is based on the number of particles added to the cells and the number of particles recovered in the supernatant fluid. Both samples reached a peak after 1 hr. Maximal adsorption by this technique was 73% for the control and only 42% for the treated virus.

Effect of irradiated PBS on virus morphology. The decreased adsorption of virus exposed to X-irradiated PBS could be caused by an effect on cellular attachment sites on the surface of the virion. Therefore, the treated particles were examined by electron microscopy to determine whether any alteration could be found in the outer layer of the virus.

Electron micrographs of untreated virus stained with PTA at neutral and acid pH values revealed typical VSV particles, measuring 69.1 by 176 nm. These dimensions are in excellent agreement with the detailed studies of Bradish et al. (7), Howatson and Whitmore (14), Simpson and Hauser (23), and Bergold and Munz (5).

VSV which had been suspended for 3 hr in PBS previously X-irradiated with 30,000 r showed an average reduction in particle width of greater than 10 nm and in particle length of about 4 nm (57.5 by 172.1 nm). These values represent the

FIG. 5. Adsorption rate of VSV suspended in X-irradiated (30,000 r) PBS (●) was determined by physical particle count of unadsorbed virions in the supernatant fluid of cell-virus suspensions. This was compared to a similar experiment with virus suspended in unirradiated PBS (○).

average of measurement of more than 100 particles from electron micrographs projected on a screen at a final magnification of $238,000\times$. These particles were stained with PTA at an acid pH to provide greater contrast in obtaining ac-

curate overall particle dimensions (Fig. 6 and 7). Normal virus particles are shown in Fig. 6; Fig. 7 shows particles treated for 3 hr with irradiated PBS.

Electron micrographs were also used to deter-

FIG. 6. Normal untreated VSV particles stained with PTA at acid pH. $\times 95,250$.

FIG. 7. VSV particles treated for 3 hr with X-irradiated PBS, demonstrating the "eroded" virions from which the outer layer and surface projections have been removed. $\times 95,250$.

mine the per cent of virions affected. When treated with X-irradiated PBS (30,000 r) for 3 hr, 73% of the particles lost their outer layer; when particles were suspended in X-irradiated saline for the same period of time, 42% were similarly denatured. Particles with minor alterations in dimension were considered unaffected. These figures are in excellent agreement with the biological inactivation rates, which indicated an infectivity [plaque-forming units (PFU)] drop of 84% for particles treated with irradiated PBS.

DISCUSSION

Previously irradiated aqueous salt solutions appear to inactivate various viruses (2, 12, 24, 25). The present experiments were undertaken to determine the nature of this effect. Since it has been shown that the ratio of the number of virions to the infectivity titer of animal viruses is usually greater than 10 (21) and that these noninfectious particles may affect experimental results (6), we chose a model in which this ratio approaches unity (11). To avoid the formation of incomplete or inhibitory forms which may be produced by VSV (9, 13, 15), the virus to cell ratio was carefully controlled; the virus suspension used in these experiments had an average titer of 1.3 virions per PFU.

When aqueous solutions were exposed to X-irradiation and then used as diluent for the virus, the virus became inactivated in increasing proportion with the increase in dissolved ionic species. It was easily shown that this effect was not a consequence of the hydrogen peroxide generated by irradiation, as was reported by Alper (2) and Johnson (16). Nor did this effect seem to be the result of long-lived radicals, since no such radical species could be detected by ESR studies. Prior irradiation of PBS at low and high pH values seemed to indicate that viral inactivation was caused by the indirect involvement of the H radicals produced during the radiolysis of water. This was demonstrated by prior irradiation of PBS at low and high pH values. When the pH was lowered, increasing the H radical concentration, viral inactivation was increased. On the other hand, when the pH was raised, decreasing the H radical concentration, virus was not inactivated. The H radical was able to interact with anionic species in irradiated solutions, resulting in reactive toxic chemical products (1) which are stable and which can, in turn, presumably react with the virion producing inactivation. The nature of these stable reactive chemical products is not known but their formation appears to be dependent on the anionic constituents of the solutions. The dependence of this type of inactivation on the

anionic species in solution has also been reported by Augenstine (4) and may account for the greater inactivation of VSV by irradiated PBS as compared to saline solution.

It appeared that the toxic product formed can cause an irregular erosion of the surface of the virion, as observed by electron micrographs, probably by an oxidative reaction. This may explain the reduced ability of the virions to adsorb onto susceptible cells, which results in loss of infectivity.

The inactivation curves with PBS irradiated at 30,000 r began to depart from linearity just below 50% survival, sooner than those of PBS irradiated at 45,000 r or at pH 1.8. Since it has been shown that minute amounts of protein, sulfhydryl compounds (16), or detergents can inhibit the inactivation, it is possible that a sufficient amount of debris, originating from the eroded virion, is present in the 30,000-r material to interact with the toxic products, which act as a quencher, and cause the early deviation. Although the other two curves (of PBS irradiated at 45,000 r or at pH 1.8) may be steeper because the initial ratio of toxic chemical radiation products to virions was greater than that of the 30,000-r material, the involvement of debris was less critical. Thus, it is believed that the actual rate of viral inactivation is the rate observed during the first 0.5 to 1 hr. The radiation dose-dependency plotted from this time period demonstrates a multiple interaction between virions and toxic chemical radiation products.

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