

# Immunogenicity of Rabies Virus Inactivated by $\beta$ -Propiolactone, Acetyleneimine, and Ionizing Irradiation

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Ionizing radiation,  $\beta$ -propiolactone, and acetyleneimine were compared for their ability as virus-inactivating agents for the preparation of rabies vaccine. Each agent reduced viral infectivity exponentially; ionizing radiation also destroyed viral hemagglutinin. The vaccine prepared by ionizing radiation was equal or superior to that prepared by  $\beta$ -propiolactone in its ability to protect mice from rabies infection. The acetyleneimine-treated vaccine was a less potent immunogen.

An important factor in the preparation of a viral vaccine is that the immunogenicity of the virus be retained at the highest possible level. Achieving this goal, while ensuring the complete inactivation of the virus, quite often presents a technical problem. Commercial inactivated rabies vaccines, of nervous tissue origin or prepared in duck embryos, are inactivated by phenol,  $\beta$ -propiolactone (BPL) or ultraviolet (UV) light. The preparation of a rabies vaccine from virus propagated in tissue culture and inactivated by the various chemical agents mentioned above or UV light has been reported (4, 14). In our laboratory, the BPL-inactivated rabies vaccine has been shown to be twice as potent as vaccine prepared by UV inactivation. We have observed an apparent fragmentation of the rabies virion during BPL treatment, as evidenced by sucrose density gradient centrifugation (H. G. Aaslestad, unpublished data) and a partial loss in hemagglutinin titer (14).

In this paper we compare the inactivation of rabies virus by the chemical agents BPL and acetyleneimine (AEI) and by physical treatment with ionizing radiation. The ability of rabies vaccine inactivated by each of these three agents to protect mice from a lethal rabies virus challenge is also presented.

## MATERIALS AND METHODS

**Virus and cells.** The Pitman Moore strain of rabies virus was propagated in baby hamster kidney

(BHK-21) cell cultures as described previously (12). The medium utilized for roller bottle propagation of the virus was supplemented with 0.1% bovine serum albumin. Rabies virus contained in the tissue culture fluid decanted from infected cells was freed of cellular debris by centrifugation and concentrated 40-fold by ultrafiltration through a Sartorius membrane of 0.01- to 0.02- $\mu$ m porosity (14). The virus concentrate was placed in ampoules in 4-ml portions and frozen at  $-70^{\circ}\text{C}$ . The same preparation of concentrated virus preparation was used in all experiments described in this paper.

**Assay procedures.** Rabies virus infectivity was quantitated by plaque titration on agarose suspensions of BHK-21/13S cells (10) and by intracerebral inoculation of undiluted vaccine into groups of 10 4-week-old mice. Titrations of rabies-specific complement fixation and hemagglutination were carried out in Microtiter plates according to published procedures (5, 12).

Vaccines were evaluated for their protective activity by the National Institutes of Health (NIH) potency test (11). Antigenic values are expressed as the ratio of the immunizing dose of the vaccine under test protecting 50% of mice ( $\text{ED}_{50}$ ) with that of a standard reference vaccine (13), NIH rabies reference vaccine lot number 178.

Protein was determined by the method of Lowry et al. (6).

**Virus inactivation procedures.** Chemical inactivation of concentrated rabies virus with BPL (Test Agar and Co., Inc., Detroit, Mich.) was accomplished by adding the reagent to a final concentration of 0.025% (v/v). After vigorous mixing, the BPL-containing virus suspension was maintained at  $4^{\circ}\text{C}$  for different lengths of time. The reaction was stopped by the addition of sodium thiosulfate (14), and biological assays were carried out immediately after hydrolysis of the BPL. Rabies virus inactivated with

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AEI (Burroughs Wellcome and Co., Dartford, Kent, England) was prepared by adding the reagent to a final concentration of 0.05% (v/v). Inactivation was performed at 37 C. The evaluation of the biological activity of samples from AEI-treated rabies was carried out without further treatment and at the specified time intervals. No adjustment of the pH of the virus concentrate was made in either BPL or AEI treatments.

Physical inactivation of rabies virus with ionizing radiation was achieved with gamma radiation from a Cobalt-60 well source at the National Bureau of Standards, Washington, D.C. Rabies virus was kept frozen with dry ice during irradiation to reduce free radical effects. The Cobalt-60 source emitted gamma radiation at a rate of  $1.8 \times 10^6$  r/hr; doses of radiation were increased by lengthening the time of exposure. A description of the irradiation rig and handling procedures has been published (8). Irradiated rabies virus was kept at -70 C until assayed for biological activity.

**RESULTS**

The rabies virus concentrate employed in the following inactivation studies had an infectivity titer of  $3 \times 10^9$  plaque-forming units (PFU) per ml. Its rabies-specific complement-fixation titer was 380 units per ml, and it agglutinated goose red blood cells to a titer of 760 units per ml. The protein concentration in concentrated rabies virus preparation was 36.8 mg/ml. The majority of the protein in the rabies concentrate was bovine serum albumin, as determined by analysis on a diethylaminoethyl cellulose chromatographic column (Aaslestad, unpublished data).

Figures 1-3 summarize the inactivation and degradation kinetics determined for viral infectivity, complement fixation, and hemagglutination. When BPL was used as the inactivating agent, infectivity was exponentially reduced 100,000-fold during the first 6 hr, whereas complement-fixing activity remained similar to that of the untreated vaccine (Fig. 1). BPL treatment reduced the hemagglutination titer to one-half of the control value after 4 hr of treatment; however, no further loss in hemagglutinin titer was detected during the subsequent 20 hr of treatment. Complement fixation by the rabies virus concentrate remained at 100% of the control value, even after exposure to BPL for 24 hr.

Data obtained for AEI-inactivated rabies virus are shown in Fig. 2. Loss of infectivity followed an exponential pattern with respect to time of contact with the inactivating agent. Only 2 hr of exposure to AEI was required to achieve inactivation equivalent to that found for BPL-treated virus at 6 hr. No loss of either complement fixation or hemagglutination titer

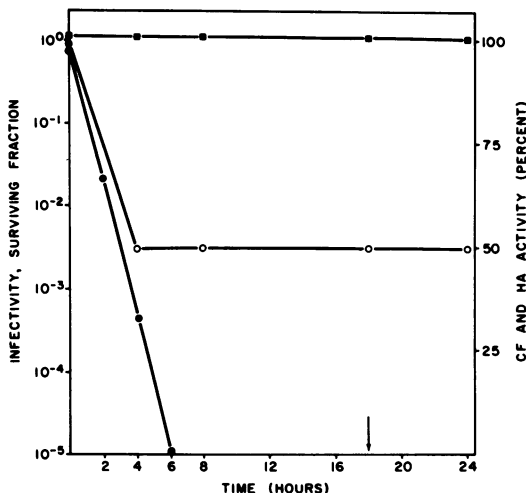


FIG. 1. Inactivation of rabies virus by  $\beta$ -propiolactone (BPL). Concentrated rabies virus was treated with BPL during the periods indicated. Viral complement fixation (■), viral hemagglutination (○), and infectivity, as measured by plaque formation (●), were compared with those of a control sample which had not been exposed to BPL. Experimental details are given in the text. The arrow denotes the point at which vaccine was assayed for antigenic value.

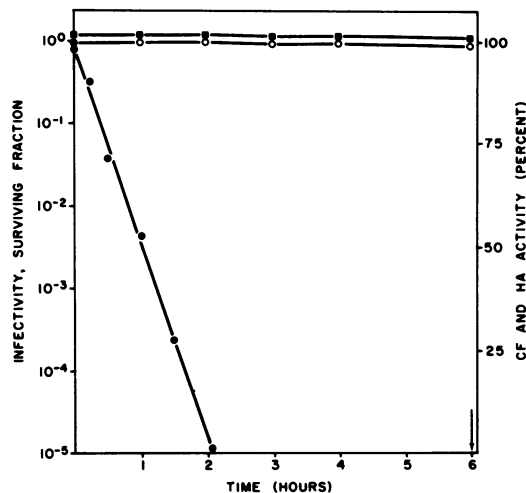


FIG. 2. Inactivation of rabies virus by acetyleneimine. See legend to Fig. 1 for definition of symbols and experimental design.

was observed during 6 hr of treatment with AEI.

Figure 3 depicts the effect of increasing amounts of ionizing radiation on rabies infectivity, complement fixation, and hemagglutination activity, as compared to a nonirradiated control. Virus infectivity was reduced exponentially as a function of dosage. After treat-

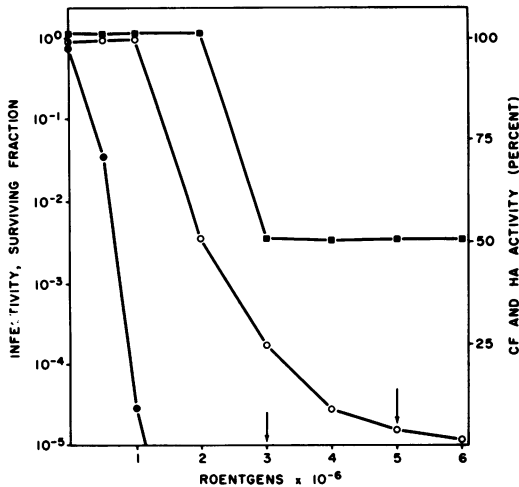


FIG. 3. Inactivation of rabies virus by ionizing radiation. See legend to Fig. 1 for definition of symbols and experimental design.

ment with  $2 \times 10^6$  r, the residual infectivity was less than 100 PFU/ml. While no virus infectivity was detected by plaque assay at a radiation level of  $3 \times 10^6$  r, one-half of the complement-fixing titer and one-fourth of the hemagglutinin titer remained. A high level of complement-fixing activity was detected in virus concentrates receiving as much as  $6 \times 10^6$  r, whereas the hemagglutination titer gradually dropped below 1% of the control.

The immunogenic potency of the rabies virus vaccines inactivated by each of the three methods described above was determined next. It was assumed that exposure of rabies virus to the chemical agents BPL and AEI three times longer than required to inactivate five logs of titratable virus would be adequate for complete inactivation. The ability of AEI-prepared rabies vaccine to protect mice was, therefore, determined after treatment for 6 hr. Rabies virus was exposed to BPL for 18 hr at 4 C, with an additional 2-hr treatment at 37 C to prepare the BPL-rabies vaccine. Rabies vaccine prepared with ionizing radiation was tested at three radiation dose levels:  $3 \times 10^6$ ,  $5 \times 10^6$ , and  $7 \times 10^6$  r. Each of these vaccine preparations was innocuous for 21-day-old mice that were inoculated intracerebrally in an undiluted form.

Table 1 gives the antigenic values determined for the 40-fold concentrated rabies vaccine inactivated by each of three different ways. Irradiation with gamma rays to a level of  $5 \times 10^6$  r resulted in a vaccine preparation

with a maximum antigenic value of 92. The antigenic values of duplicate BPL-inactivated vaccines were 55 and 61. The antigenic value of the AEI-inactivated vaccine was found to be only 19.

## DISCUSSION

Both physical and chemical methods have been used in efforts to inactivate rabies virus. In addition to rendering the virus noninfectious, the inactivation method should have the least adverse effect on the vaccine's immunogenicity. Furthermore, the inactivating method should not introduce a toxic substance into the vaccine, in the form of either residual reagent or altered vaccine components which may be toxic to the recipient.

In 1951, a mouse brain suspension of rabies virus was successfully inactivated by the ionization induced by high-energy electrons (13). The resultant vaccine was considered superior to the phenol-prepared antigen then in use. Cobalt-60 radiation on the rabies virus concentrate in the experiments reported here resulted in a rapid exponential inactivation of infectivity (Fig. 3). Since one primary ionization is needed to break the single-stranded viral ribonucleic acid, inactivation with a minimum loss of immunogenicity was anticipated. The data in Table 1 clearly show the potent immunizing character of this vaccine. The gradual reduction of hemagglutinin titer during radiation (Fig. 3) is secondary to virus inactivation. It is probably due to the oxidation of specific sulfhydryl functional groups to disulfhydryl groups, since Kuwert et al. (5) reported that the sulfhydryl groups are essential for rabies

TABLE 1. Protective activity of rabies vaccines prepared by inactivation with  $\beta$ -propiolactone (BPL), acetyleneimine (AEI), and gamma irradiation

Inactivating agent	ED <sub>50</sub> <sup>a</sup> (log)	Antigenic value <sup>b</sup>
BPL	3.30	55
	3.34	61
AEI	2.82	19
Cobalt-60		
$3 \times 10^6$ r	3.27	52
$5 \times 10^6$ r	3.52	92
$7 \times 10^6$ r	3.39	68
NIH reference 178	1.56	1

<sup>a</sup> The ED<sub>50</sub> is that dilution of vaccine which protected 50% of the mice against rabies challenge (CVS strain, 21 LD<sub>50</sub>).

<sup>b</sup> The antigenic value is the ratio of ED<sub>50</sub> of the test vaccine to that of the reference vaccine.

hemagglutination. The inclusion of a suitable sulfhydryl compound might spare the loss of viral hemagglutinin. It has been reported that histidine can selectively protect viral antigenicity over viral infectivity of myxoviruses during gamma radiation (7), and Gruber (3) has shown that such is the case when arbovirus hemagglutinin and complement-fixation titers are determined during inactivation of virus with gamma radiation. Determination of whether higher antigenic values than those reported in Table 1 can be obtained in the presence of sulfhydryl agents or histidine must await additional experiments.

The slight loss of rabies complement-fixing activity at high radiation levels and the complete resistance of this antigen to treatment by BPL or AEI (Fig. 1-3) may have little significance for the immunogenicity of the rabies virion. The titers determined represent virion antigen, as well as soluble antigen present in infected cell tissue culture fluids (9). Since as much as 50% of the complement-fixing titer may be due to soluble antigen and since complement-fixing titers have been observed to increase slightly after BPL treatment (Aaslesstad, *unpublished observations*), care must be taken in interpreting these data.

The inactivation kinetics observed when either BPL or AEI was used (Fig. 1 and 2) to inactivate rabies virus were both exponential in nature, suggesting one-hit inactivation. The different reagent concentrations and treatment temperatures used preclude a direct comparison of these two agents. However, inactivation in each case is considered to be the result of alkylation of imidazole functional groups in the viral ribonucleic acid (2).

The BPL inactivation kinetics of rabies infectivity (Fig. 1) are similar to those previously reported (14), although less destruction of viral hemagglutinin was observed than in earlier experiments (5, 14). AEI served as an effective inactivating reagent for rabies virus, confirming the work of Crick and Brown (1). The significance of the full retention of rabies hemagglutinin during AEI treatment is not understood in light of the poor antigenic value of the AEI vaccine, unless the hemagglutinin antigen is assumed to be of little or no importance in immunogenicity (Table 1). From inspection of the data shown in Fig. 1-3, one might have anticipated that the AEI vaccine would serve as the most native antigen and therefore score the highest antigenic value.

The data summarized in Table 1 point to the use of ionizing radiation as an alternative

choice to BPL for the inactivation of rabies virus. The variation in antigenic value among the gamma-irradiated samples may reflect experimental error inherent in the mouse titration employed in the assay. (Arko, R. J., T. J. Wiktor, and R. K. Sikes, *Bull. World Health Organ., in press*). The fact that during radiation inactivation the vaccine may be kept frozen and no subsequent treatment is required to remove undesirable chemicals is an advantage over chemical inactivation. The ability of gamma radiation to penetrate is far greater than that of ultraviolet radiation, thus permitting large volumes of vaccine to be treated. In addition, excessive gamma radiation, threefold or more over the amount required for the destruction of viral infectivity, does not result in significant loss of antigenic potency. A disadvantage of this method is the need for expensive and not commonly available equipment.

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