Inactivation of Fixed Rabies Virus, Grown on Embryonated Duck Eggs, by Means of Beta Propiolactone

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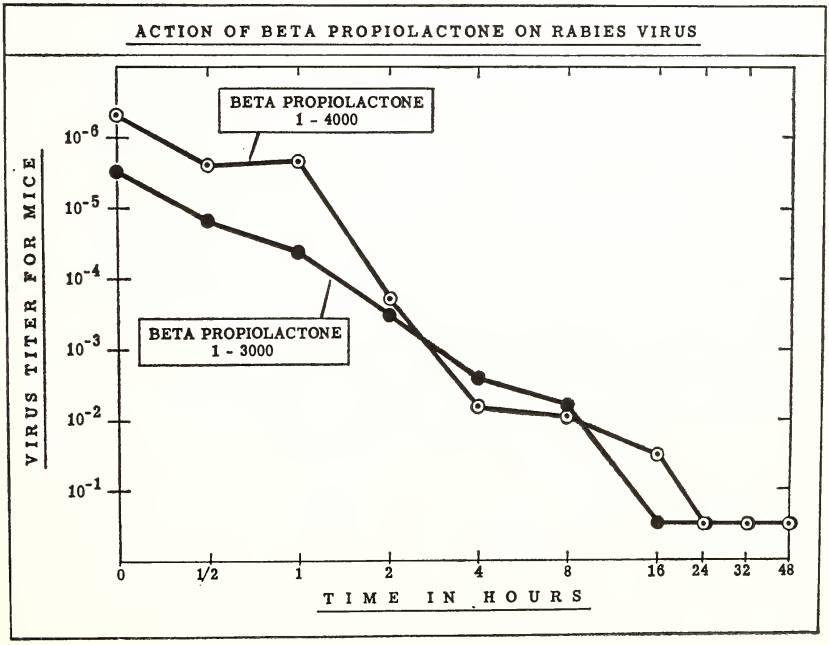
This report concerns details of the inactivation of fixed rabies virus, grown on embryonated duck eggs, through use of beta propiolactone (BPL), also something about the utility of the product prepared in this way. We have previously reported on certain properties of this virus (1, 2) and on vaccine made from it (4), and LoGrippo and Hartman showed good antigenicity of rabies mouse brain vaccine inactivated with BPL (3).

Preparation of the virus

Different laboratory strains of fixed virus have somewhat varying capacities for growing on embryonated duck eggs. We have experimented thus far with six such strains, and a strain supplied by the National Institutes of Health and known as N.I.H. or CVS appears at this time most satisfactory. We are not sure that repeated passage or long time cultivation of a given strain of fixed virus on duck eggs has any merit in production of large batches of virus, and for several reasons have have experimented with first generation duck embryo virus grown either from rabbit brain or mouse brain seed. Attempts have been made with



no very noticeable results to select sub lines of virus (as for obtaining clones) from individual eggs inoculated with limiting dilutions of seed virus. Currently, embryonated duck eggs at 7 days incubation are inoculated with a 10^{-2} dilution of fixed virus and are thereupon re-incubated for 12 additional days. At this time the virus-containing embryos are recovered, and on being proven free of viable bacteria are ground in buffered lactose diluent with a Waring Blender in the cold to make a 10 percent suspension.

Figure 1 shows the increase of virus titer in duck embryos during incubation. It is noted that after day 2 there is a rapid increase in titer up to about 7 days, then a leveling off. In the meantime the embryo grows considerably so that at 12 days post inoculation it may weigh from 12 to 14 grams, at which time the virus is harvested.

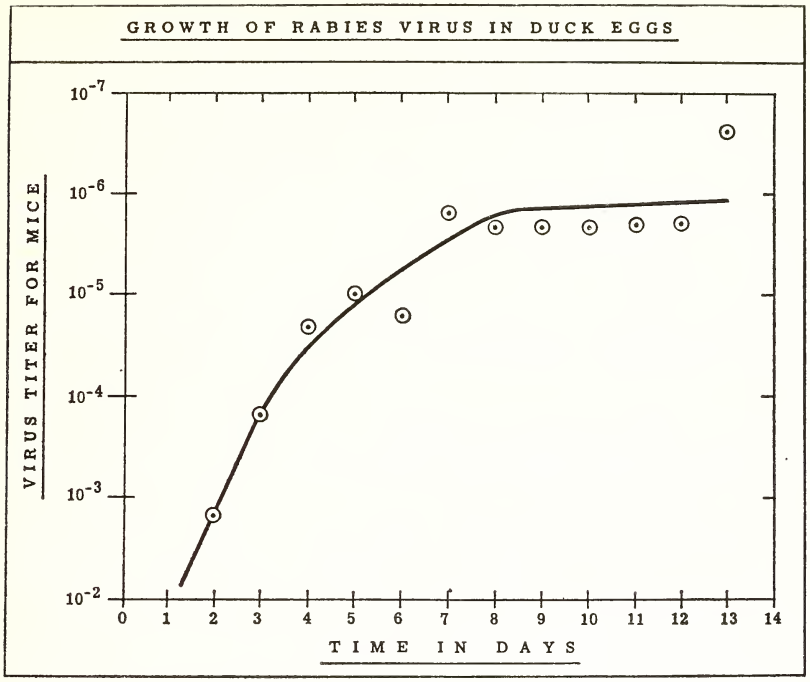
If duck embryo fixed rabies virus is to be kept very long in the fluid state it should remain in the cold, at an alkaline reaction, and be protected from oxygenation with a reducing agent such as cysteine. On the alkaline side this particular agent may also act as an anti-phosphatase as Amos has shown in the case of herpes virus (5). It has appeared that pools of virus having mouse infectivity titers of 10^{-5} or over suffice for preparation of vaccine.

Inactivation

In attempts to inactivate fixed virus in preparing vaccine, we have tried heat, phenol, formalin, ultraviolet light, several nitrogen mustard like compounds, and BPL. In the midst of work with this latter compound LoGrippo and Hartman as mentioned above, published on the probable usefulness of this drug in connection with several virus vaccines, although earlier these authors had published on the use of BPL in other biological preparations. We in turn have subsequently published a brief report of use of BPL in the preparation of several batches of duck embryo rabies vaccine carried through various kinds of animal experimentation and then into man as also cited.

Our experiments have dealt mainly with inactivating duck embryo fixed virus with BPL at ice box temperature or 4° or 5°C although LoGrippo and Hartman preferred 37°C. Action of preferable concentrations of 1:3000 to 1:4000 is usually complete at 20 to 24 hours, however an additional 24 hours standing is advisable for completion of action, and some storage at this temperature is generally necessary.

Figure 2 shows the decrease in mouse infectivity of fixed virus during BPL treatment in the ice box. Since the activity of this drug of course is greater the higher the temperature, haste is essential in sampling the reaction mixture, preparing dilutions for mouse tests, and completing the testing at any point desired. A very great drop in titer of virus after 2 hours is noted, and a further considerable drop after 4 hours. Live virus dwindles off to nil in about 24 hours using BPL 1:4000 and about 16 or 20 hours using BPL 1:3000. Several virus titers placed on the $10^{-0.5}$ dilution level are practically nil since no mice died on the corresponding 10^{-1} dilution shown in Figure 2. A second day's standing in the ice box however is useful for added certainty of complete virucidal action.



Such inactivation curves are intended to indicate general trend and speed of the reaction. Since these preparations of virus are far from being homogeneous, great exactness of measurement of the specific reaction can hardly be approximated. Whether similarities exist or not with the kind of reaction involved in the inactivation of poliovirus by formalin cannot be guessed. It appears the nature of this latter reaction is still a subject of study.

In the use of BPL as indicated, a considerable degree of acidity is generated. Initial suspensions of virus should be well buffered on the alkaline side and it is safest to test the pH from time to time and re-alkalinize the suspension if the pH drops to 6.8 or 6.9, setting it upwards to about pH 7.5. Final pH of 7.2 to 7.4 or a little higher is optimal.

To assure stability of potency of duck embryo rabies vaccine made in this may, we have freeze-dried the product and in this shape it rehydrates readily on addition of sterile water.

Antigenicity and Safety

The potency of this BPL inactivated vaccine, as measured in comparative mouse immunization and challenge tests, has proven to be from about 2 to 4 times the minimal potency prescribed for rabies vaccine used in the U.S.A. The present mouse test, which is a modification of the original Habel test and uses a standard, is of great value in comparing utility of different inactivating agents. There is no need of any special test with vaccine prepared as we have described.

Virus-neutralizing antibody produced in dogs and humans by this vaccine has neutralized all strains of fixed virus with which we have worked in mice. The percentage of dogs producing such antibody compares favorably with that produced by other inactivated vaccine. Table 1 shows virus neutralizing antibody test results in 125 dogs in which no attempt was made to use optimum vaccine dosage concentration for animal use. Approximately 80 percent of dogs getting one dose and 95 percent of dogs getting two doses of duck embryo vaccine, and 95 percent of dogs getting two doses of phenolized vaccine develop antibody. Virus neutralizing antibody tests have been completed in 211 human beings in addition to those previously reported, and a report is contemplated of these as well as tests involving street virus challenge in dogs in which our associate, Dr. R. A. Sauter, has observed good response following use of duck embryo rabies vaccine (6).

TABLE 1

Comparative Results of Rabies Vaccines in 125 Dogs

Vaccine	One 5 cc Dose BPL Killed Vaccine	Two 5 cc Doses BPL Killed Vaccine	One 5 cc Dose Phenolyzed Commercial Vaccine	Two 5 cc Doses Phenolyzed Commercial Vaccine
Total dogs given vaccine	50	40	5	30
Dogs having pre-immune antibody	7	3	0	2
Remaining dogs in which vaccine effects are best measured	43	37	5	28
Number of dogs developing antibody following vaccine	34	35	2	24
Percent of dogs developing antibody	79	94	(40?)	85

It is believed from the work of others as well as ourselves, and a general consideration of the technique that no BPL as such remains in the vaccine. Acidic residue becomes neutralized. Safety of the vaccine has been indicated in some special tests of three batches of vaccine in 36 rats given multiple doses and examined once or twice weekly for one and one half years. Saline was given to a fourth group of 12 rats. During this time there were no early or late local reactions to vaccine or saline. A few rats died of intercurrent respiratory pathology, the deaths in the saline group by happenstance exceeding those in any of the three vaccine groups. Specifically six of twelve rats on saline died, and 3, 1, and 2 rats respectively on three lots of vaccine died during the one and one-half years. These long term tests are in addition to tests conducted with vaccines in general in relation to safety, and the usual safety tests prescribed in the U.S.A. for rabies vaccine to be used in humans.

Discussion

This report is presented to indicate some degree of utility of BPL as a virus inactivating agent in the preparation of duck embryo fixed rabies virus vaccine. The sort of inactivation that is needed in our case is some kind of gentle killing sufficiently moderate to allow inactivated virus to maintain its capacity to act as an antigen. This probably means that the inactivation process with the specific drug must be carried out under conditions which, if the drug were not present, the virulence of the virus would be maintained. In order to know how satisfactorily the fixed virus may have been inactivated, it is necessary first to measure antigenicity with prolonged mouse tests. These may occasionally need repetition for added certainty, and some degree of stability needs to be known. Dog experimentation gives additional results. It is for these reasons along with others that progress in this field is slower than one would think.

It will now be of interest to determine the relative utility of inactivation of virus to form vaccine by combined use of BPL and ultraviolet light compared to either agent used alone in view of the most recent publication on this subject by LoGrippo (7). It may be mentioned that LoGrippo did not attain complete inactivation with any amount of ultraviolet light which he used.

Although of limited interest in comparison to viruses and vaccines now being dealt with over the world, the dissemination of street virus to several species of wild animals in nature making eradication of rabies in many countries next to impossible, it follows that rabies vaccine will likely have to be made and used for many years to come. About the only alternative would be appearance of an effective chemotherapeutic drug or antibiotic not in sight at the present time.

Conclusions

1. Details of the virucidal action of beta propiolactone on fixed rabies virus of duck embryo origin are described.

2. Such completely inactivated virus has proven to be an effective antirabies vaccine showing the degree of potency necessary for general use.

3. Further work is indicated to determine if inactivation by BPL in conjunction with another agent is preferable to use of BPL alone.

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