# THE QUANTITY OF IRRADIATED NON-VIRULENT RABIES VIRUS REQUIRED TO IMMUNIZE MICE AND DOGS

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In a previous paper we pointed out that 1.5 cc. of irradiated tissue culture virus successfully immunizes mice against a subsequent intracerebral or intramuscular test infection (1). It soon developed, however, that extremely large volumes of the irradiated culture virus were needed to immunize dogs. Quantitative studies were undertaken, therefore, to determine the amounts of vaccine necessary for mice and dogs in terms of mouse intracerebral lethal doses.

## *Minimum Volume and Virus Content of Culture Virus Required for Immunization of Mice*

The first experiments dealt with the minimum dose of tissue culture vaccine capable of immunizing mice. In the previous paper (1), we stated that the culture virus usually titred 0.03 cc. of the $10^{-3}$ dilution in 4 to 6 weeks old mice and was therefore said to contain 33,000 mouse intracerebral lethal doses per cc. 1.5 cc., or approximately 50,000 doses, properly irradiated, immunized the mice. More recently we have adopted as a standard for titration 3 weeks old mice rather than those aged 4 weeks or more. In these younger mice the culture virus often titres one dilution higher, 0.03 cc. of the $10^{-4}$ dilution.

Repeated experiments to determine the least amount of irradiated tissue culture virus[1] necessary to immunize mice indicated that 50,000 mouse doses, properly irradiated, gave good protection, whereas less than this amount, although sometimes effective, was not consistently so. The following experiments illustrate the type of result obtained.

*Experiment 1.*—Tissue culture virus, Pasteur strain,[2] prepared as previously described (1), was distributed in three quartz flasks. One was irradiated 20 minutes and then

[1] We are indebted to Dr. George I. Lavin for irradiating the preparations used in these experiments.

[2] This strain was kindly sent by Dr. Pierre Lépine of the Pasteur Institute, Paris, and has been passaged in mice in our laboratory.

tested for virulence, the second 30 minutes and tested, and the third 40 minutes and tested for virulence by inoculating 0.03 cc. intracerebrally into each of five mice. The 30 minute irradiated virus was then injected as a vaccine intraperitoneally into 30 day old Swiss mice in the following manner. Group 1 received 1 cc. in a single injection; group 2, an injection of 0.5 cc. and 2 days later a second injection of 0.5 cc., and group 3 received 0.2 cc. every other day for five injections. Group 4 received 0.5 cc. in a single injection; group 5, 0.25 cc., and 2 days later a second injection of 0.25 cc. Group 6 received 0.1 cc. daily for 3 days and finally, group 7 received no vaccine.

3 weeks after commencing vaccination the mice were tested for immunity by injecting them into the gastrocnemius muscle with 0.01 cc. of virulent rabies virus in serial two-fold dilutions, four mice being employed for each dilution. All mice were observed 4 weeks for signs of rabies.

The virulence of the virus before irradiation (Table I) proved to be 0.03 cc. of the $10^{-4}$ dilution or better. Hence the material is said to contain 330,000 mouse doses per cc. or more. Following irradiation for 20, 30, or 40 minutes, the material injected intracerebrally into mice failed to kill. In the immunity test, the non-vaccinated mice titred 1 to 1,800 according to the method of Reed and Muench (2), whereas the batches of vaccinated mice withstood 23.5 and 47+ times this amount, indicating a strong immunizing potency of the vaccine. In this experiment, 111,000 or more doses of irradiated vaccine given by any of the above methods immunized effectively.

*Experiment 2.*—Tissue culture virus was prepared, irradiated, and tested for virulence as in Experiment 1. 0.5 cc. of the 20 minute irradiated virus was injected as a vaccine intraperitoneally into a batch of 30 day old Swiss mice. The 30 and 40 minute preparations were likewise injected into a second and third batch. A fourth batch received three injections of 0.5 cc. of the 30 minute irradiated vaccine and the fifth batch was left unvaccinated as controls. 3 weeks after commencing vaccination the vaccinated and unvaccinated mice were given a test dose of virulent virus intramuscularly, as described in Experiment 1, and observed 4 weeks for signs of rabies.

The results of this test are shown in Table II. The virulence of the virus before irradiation proved to be 0.03 cc. of the $10^{-3}$ dilution. Hence the material is said to contain 33,000 mouse doses per cc. Following irradiation three of the five mice injected with the 20 minute material succumbed to rabies, two of the five injected with the 30 minute, and none injected with the 40 minute irradiated virus. Only this latter preparation was regarded, therefore, as non-virulent. In the immunity test the challenge virus in the non-vaccinated mice titred 1 to 1,280. The mice vaccinated with 16,500 doses withstood three times and those vaccinated with 49,500 doses withstood thirty-one times as much virus, indicating a strong immunity. In passing, it may be noted also that, even though most of the vaccine given in this experiment contained a trace of virulent virus, it was too small in quantity to affect the immunizing potency of the material (1).

TABLE I

*Immunization of Mice with Graded Doses of Irradiated Tissue Culture Pasteur Rabies Virus*

Virulence before irradiation (0.03 cc. in dilutions intracerebrally) $10^{-2}$ – 3/4*:$10^{-3}$ – 2/4:$10^{-4}$ – 2/4.
" following " (0.03 cc. undiluted " ) 20 minutes – 0/5:30 minutes – 0/5.

*Immunity of Vaccinated Mice*

| Dose of vaccine | Mortality of mice given test virus intramuscularly (0.01 cc.) in dilutions | | | | | | | Titre† of virus in mice | Difference in titre of virus in vaccinated and non-vaccinated mice |
|---|---|---|---|---|---|---|---|---|---|
| | 1/80 | 1/160 | 1/320 | 1/640 | 1/1,280 | 1/2,560 | 1/5,120 | | |
| Group 1: 1 cc., 1 dose (330,000 M.D.‡) | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | — | — | <80 | 47+ |
| " 2: 0.5 cc., 2 doses " | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | — | — | <80 | 47+ |
| " 3: 0.2 cc., 5 " " | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | — | — | <80 | 47+ |
| " 4: 0.5 cc., 1 dose (165,000 M.D.) | 2/4 | 0/4 | 0/4 | 0/4 | 0/4 | — | — | 80 | 23.5 |
| " 5: 0.25 cc., 2 doses " | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | — | — | 80 | 23.5 |
| " 6: 0.1 cc., 3 " (111,000 " ) | 0/4 | 1/4 | 0/4 | 0/4 | 0/4 | — | — | <80 | 47+ |
| " 7: No vaccine | — | 2/4 | 4/4 | 3/4 | 3/4 | 3/4 | 0/4 | 1,800 | |

* 3/4 = 3 of 4 mice injected died of rabies. † Estimated by method of Reed and Muench (2).
‡ Mouse intracerebral lethal doses in vaccine prior to irradiation. — = material not tested.

TABLE II

*Immunization of Mice with Graded Doses of Irradiated Tissue Culture Pasteur Rabies Virus*

Virulence before irradiation (0.03 cc. in dilutions intracerebrally) $10^{-2}$ – 3/3*:$10^{-3}$ – 3/3:$10^{-4}$ – 0/3.
" following " (0.03 cc. undiluted " ) 20 minutes – 3/5:30 minutes – 2/5:40 minutes – 0/5.

*Immunity of Vaccinated Mice*

| Dose of vaccine | Mortality of mice given test virus intramuscularly (0.01 cc.) in dilutions | | | | | | | | Titre† of virus in mice | Difference in titre of virus in vaccinated and non-vaccinated mice |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1,280 | 1/2,560 | 1/5,120 | | |
| Group 1: 0.5 cc., 1 dose (16,500 M.D.‡). Irradiated 20 min. | 4/4 | 2/4 | 3/4 | 2/4 | 1/4 | 2/4 | — | — | 320 | 4.0 |
| " 2: Same dose. Irradiated 30 min. | 4/4 | 3/4 | 3/4 | 3/4 | 2/4 | 0/4 | — | — | 416 | 3.1 |
| " 3: Same dose. Irradiated 40 min. | 3/3 | 3/4 | 2/4 | 3/4 | 2/4 | 1/4 | —* | — | 415 | 3.1 |
| " 4: 0.5 cc., 3 doses (49,500 M.D.). Irradiated 30 min. | 0/4 | 1/4 | 0/4 | 0/4 | 0/4 | 0/4 | — | — | <40 | 31.2 |
| " 5: No vaccine | — | — | 3/4 | 3/4 | 2/4 | 3/4 | 1/4 | 1/4 | 1,280 | |

Footnotes the same as in Table I.

TABLE III

*Immunization of Mice with Graded Doses of Concentrated Irradiated Tissue Culture Pasteur Rabies Virus*

Virulence before irradiation (0.03 cc. in dilutions intracerebrally) $10^{-2}$ – 2/2*:$10^{-3}$ – 2/2:$10^{-4}$ – 1/2:$10^{-5}$ – 0/2.
" following " (0.03 cc. undiluted " ) 45 minutes – 0/4.

*Immunity of Vaccinated Mice*

| Dose of vaccine | Mortality of mice given test virus intramuscularly (0.01 cc.) in dilutions | | | | | Titre† of virus in mice | Difference in titre of virus in vaccinated and non-vaccinated mice |
|---|---|---|---|---|---|---|---|
| | 1/80 | 1/160 | 1/320 | 1/640 | 1/1,280 | | |
| Group 1: 1.5 cc., 1 dose (49,500 M.D.‡) | 0/4 | 0/4 | 0/4 | 0/4 | — | <80 | 6.3+ |
| " 2: 0.15 cc., 1 dose (49,500 M.D.) concentrated 10 times | 0/4 | 0/4 | 0/4 | 0/4 | — | <80 | 6.3+ |
| " 3: 0.5 cc., 1 dose (46,600 M.D.) | 3/4 | 2/4 | 1/4 | 0/4 | — | 160 | 3.2 |
| " 4: 0.5 cc., 1 dose (166,000 M.D.) concentrated 10 times | — | 0/2 | 0/3 | 0/3 | — | <160 | 3.2+ |
| " 5: 0.01 cc., 1 dose (3,300 M.D.) concentrated 10 times | 3/4 | 3/4 | 2/4 | 0/3 | — | 320 | 1.6 |
| " 6: No vaccine | — | 3/4 | 3/4 | 1/4 | 0/3 | 400 | |

Footnotes the same as in Table I.

Protocols with further data are shown (Table III), supporting the conclusion that approximately 50,000 irradiated doses of culture virus are required to immunize mice.

### *Minimum Volume and Virus Content of Culture Virus Required for Immunization of Dogs*

The findings with mice led to a rough assay of the amount of vaccine required to immunize dogs. The following experiment shows that beagle dogs of the sort used in previous work with vaccines (3), weighing 10 kilos, or about 500 times as much as 20 gm. mice, were not immunized by 75 times but were immunized to some extent by 500 times the mouse dose of irradiated culture vaccine.

*Experiment 3.*—Tissue culture virus was prepared, tested, irradiated 40 minutes, and tested again for virulence as in the previous tests. Four beagle dogs, 4 to 6 months old and weighing 14 to 16 pounds, each received 450 cc. of the irradiated vaccine intraperitoneally and another batch of four similar dogs each received 75 cc. Five additional dogs were set aside as controls. 3 weeks later, each received 0.25 cc. of virulent virus diluted 1 to 200 (about one lethal dose (3)) into the neck muscles of the right and left sides. They were observed for signs of rabies for 2½ months.

The culture virus before irradiation titred $10^{-3}$ and hence contained 33,000 mouse doses per cc. Following irradiation the vaccine was not virulent for the mice. All five non-vaccinated dogs succumbed to the test virus on the 12th, 13th, 14th, 23rd, and 34th days and those receiving 75 cc. on the 15th, 16th, 19th, and 25th days respectively, whereas of the four receiving 450 cc., one died of rabies on the 14th and one on the 63rd days, and the other two remained well.

In view of the fact that dogs such as those used in our tests (3) needed as much as 500 cc. of irradiated vaccine as prepared to become immune to the test virus, and hence of a possible relation between weight of animal and amount of vaccine required, attempts were made to secure a virus preparation with a greater number of mouse doses per cc. Efforts to increase the titre of the culture virus have thus far been unsuccessful. Concentration procedures, on the other hand, were encouraging as far as they were carried out.

*Experiment 4.*—Tissue culture virus was centrifuged at 1,000 R.P.M. for 5 minutes, the supernatant drawn off, tested for virulence, irradiated 45 minutes with the mercury vapor lamp, and tested again for virulence. 116 cc. of this relatively clear material, free of obvious tissue fragments, were evaporated to dryness at low temperature in reduced atmospheric pressure and then resuspended in distilled water to 11.6 cc., or one-tenth of its original volume.

Sixteen mice were injected intraperitoneally with 1.5 cc. of the unconcentrated and sixteen mice with 0.15 cc. of the concentrated vaccine. Moreover, sixteen were injected with 0.5 cc. of the unconcentrated and sixteen with 0.5 cc. of the concentrated vaccine. Sixteen mice were given 0.01 cc. of the concentrated vaccine and sixteen mice remained unvaccinated. 3 weeks later, all mice were tested for immunity by injecting them intramuscularly with 0.01 cc. of virulent rabies virus in graded doses. They were observed for signs of rabies for 4 weeks.

The results of this experiment are shown in Table III. The titre of the virus before irradiation was 0.03 cc. of the $10^{-3}$ dilution. Following irradiation it failed to kill the inoculated mice. In the immunity test, the virus in unvaccinated mice titred 1 to 400. Mice dying from causes other than rabies are not included in the table. All mice receiving 49,500 mouse doses in a 1.5 cc. volume (group 1) and in the concentrated 0.15 cc. volume (group 2) remained well. Mice receiving 16,600 doses in 0.5 cc. (group 3) showed little immunity, whereas those receiving 166,000 doses in 0.5 cc. (group 4) remained well. Finally, the mice receiving only 3,300 doses were not immunized. 50,000 or more mouse doses, even in a ten times concentrated volume, remained capable of conferring a high grade immunity. Similar tests have not been performed on dogs.

### *Minimum Volume and Virus Content of Mouse Brain Tissue Virus Required for Immunization of Mice*

Known sources of rabies virus other than tissue culture are limited largely to mammalian brain tissue. Brain tissue has the advantage of affording the largest yield of virus and consequently is the standard source of vaccines, yet it has the concomitant disadvantage of accompanying the virus in concentrations of from 4 to 33 per cent. Experiments were undertaken, therefore, to determine whether virus could be readily separated from brain tissue without loss of titre or immunizing potency.

Comparative titrations on supernatants and sediments of centrifuged mouse brain preparations indicated that speeds of 3,000 R.P.M. for 20 minutes removed a large part of the brain tissue without appreciable loss of virus.

*Experiment 5.*—The Pasteur strain of rabies virus was injected intracerebrally into the brains of four 3 weeks old W-Swiss mice. 6 days later, when they became prostrate, the brains of two were removed, ground in a mortar, and made up to a 10 per cent suspension in water. The material was then centrifuged 20 minutes at 3,000 R.P.M. in a Swedish angle centrifuge. The supernatant was removed and the sediment brought back to its original volume in serum water. Further dilutions of supernatant and sediment were made and titred intracerebrally in Swiss mice.

The results of the titrations are shown in Table IV. Both the supernatant and sediment titred as usual through the $10^{-7}$ dilution, indicating that as much titrable virus per cc. was contained in the relatively clear supernatant as in the thick brain-tissue-containing sediment.

Having learned that suspensions of mouse brain virus can be cleared by centrifugation without demonstrable loss of virulence, experiments were made to determine the relative immunizing potency of uncentrifuged and cleared vaccine.

*Experiment 6.*—9 cc. of 33 per cent commercial chloroformized antirabies canine vaccine were diluted with 81 cc. of horse serum water to make a 3.3 per cent suspension. 40 cc. of this suspension were used as a vaccine on one batch of mice. The remaining 50 cc. were centrifuged at about 2,500 R.P.M. for 15 minutes, after which the supernatant became lightly opalescent and largely free of gross particles. This supernatant was used as a vaccine on the second batch of mice.

TABLE IV

*Comparative Titre of Rabies Virus in Supernatant and Sediment of Brain Tissue Suspensions Centrifuged at 3,000 R.P.M. for 20 Minutes*

| Material | Fate of mice injected intracerebrally with virus suspensions (0.03 cc.) in dilutions | | | |
|---|---|---|---|---|
| | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ |
| Supernatant | 4/4 | 4/4 | 4/4 | 3/4 |
| Sediment | 4/4 | 4/4 | 2/4 | 3/4 |

Each mouse received a total of 1 cc. of the given vaccine—one-half of each batch in a single dose and the other in five doses of 0.2 cc. each. 3 weeks later the four batches of vaccinated plus a fifth batch of unvaccinated mice were tested for their immunity to virulent virus injected in 0.01 cc. doses in twofold dilutions into the gastrocnemius muscle.

The results of the test (Table V) show that the test virus in unvaccinated mice titred 1 to 1,600. The mice given uncentrifuged material withstood at least twenty times and those given centrifuged material withstood five and twenty times this amount of virus respectively. Apparently the centrifuged supernatant confers an immunity of the same order as that of uncentrifuged material.

Further experiments showed that virus-containing supernatants prepared as above could be readily irradiated until virulence was destroyed and yet retain their immunizing potency.

*Experiment 7.*—Fifteen 3 weeks old W-Swiss mice were inoculated intracerebrally with 0.03 cc. of the rabies Pasteur strain diluted 1 to 100 in serum water. 6 days later the mice were prostrate, sacrificed, and their brains removed. The brains were emulsified

with diluent to make a 10 per cent suspension. This suspension was then titrated for virulence.

18 cc. were placed in a quartz flask, 9 cc. plus 9 cc. of diluent in a second flask, and finally, 1.5 cc. plus 13.5 cc. in a third flask to give final dilutions of 10 per cent, 5 per cent, and 1 per cent for irradiation. Irradiation was then commenced and samples were

TABLE V

*Immunization of Mice with Graded Doses of Centrifuged and Uncentrifuged Commercial 33 Per Cent Chloroformized Rabies Vaccine*

*Immunity of Vaccinated Mice*

| Dose of vaccine | Mortality of mice given test virus intramuscularly (0.01 cc.) in dilutions | | | | | | | Titre* of virus in mice | Difference in titre of virus in vaccinated and non-vaccinated mice |
|---|---|---|---|---|---|---|---|---|---|
| | 1/80 | 1/160 | 1/320 | 1/640 | 1/1,280 | 1/2,560 | 1/5,120 | | |
| Group 1: 1 cc., 1 dose | 0/4† | 0/4 | 1/4 | 0/4 | 0/4 | — | — | <80 | 20+ |
| " 2: 0.2 cc., 5 doses | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | — | — | <80 | 20+ |
| " 3: 1 cc., 1 dose (supernatant) | 3/4 | 0/4 | 1/4 | 1/4 | — | 1/4 | — | 320 | 5 |
| " 4: 0.2 cc., 5 doses (supernatant) | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | — | — | <80 | 20+ |
| " 5: No vaccine | — | — | 3/4 | 3/4 | 3/4 | 0/4 | 2/4 | 1,600 | |

* Estimated by method of Reed and Muench (2).

† 0/4 = none of 4 mice injected died of rabies.

TABLE VI

*Virulence of Mouse Brain Rabies Virus Following Irradiation with a Quartz Mercury Vapor Lamp*

Virulence of 10 per cent emulsion before irradiation $10^{-5}$ - 4/4*:$10^{-6}$ - 4/4:$10^{-7}$ - 0/4:$10^{-8}$ - 0/4.

| Concentration | Fate of mice injected with undiluted material irradiated: | | | | | | |
|---|---|---|---|---|---|---|---|
| | 20 min. | 30 min. | 40 min. | 50 min. | 60 min. | 70 min. | 80 min. |
| *per cent* | | | | | | | |
| 10 | 4/4 | 4/4 | 3/4 | 2/4 | 0/4 | 0/4 | 0/4 |
| 5 | 1/3 | 0/2 | 0/3 | 0/4 | 0/4 | 0/4 | 0/4 |
| 1 | 0/4 | 0/4 | 0/4 | — | — | — | — |

* 4/4 = 4 of 4 mice injected died of rabies.

— = material not tested.

withdrawn for virulence tests at 20 minutes and at 10 minute intervals thereafter for a total of 80 minutes.

The results of the various virulence tests are shown in Table VI.

The virulence end point of the material before irradiation proved to be 0.03 cc. of the $10^{-6}$ dilution. The 10 per cent emulsion proved virulent after 50 minutes' but not longer irradiation; the 5 per cent after 20 but not

longer, and the 1 per cent not after 20 minutes' irradiation. Both the 5 per cent and the 1 per cent preparations appeared to be as readily inactivated by ultraviolet light as the tissue culture materials.

*Experiment 8.*—A 1 per cent suspension of rabies virus was prepared as above and tested for virulence. A portion was set aside for inactivation with chloroform (Experiment 10) and a portion irradiated, and samples were tested for virulence at 10, 20, and 30 minutes. The bulk of the vaccine after 30 minutes' irradiation was stored in the

TABLE VII

*Immunization of Mice with Graded Doses of 1 Per Cent Irradiated and 1 Per Cent Chloroformized Mouse Brain Rabies Virus*

Virulence before irradiation (0.03 cc. in dilutions intracerebrally) $10^{-5}$ – 4/4*:$10^{-6}$ – 4/4: $10^{-7}$ – 3/3:$10^{-8}$ – 2/4.

" following " (0.03 cc. undiluted " ) 10 minutes – 5/5:20 minutes – 5/5:30 minutes – 2/10:30 plus 5 minutes – 0/5.

*Immunity of Vaccinated Mice*

| Dose of vaccine | Mortality of mice given test virus intramuscularly (0.01 cc.) in dilutions | | | | | | | | | Titre† of virus in mice | Difference in titre of virus in vaccinated and non-vaccinated mice |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1,280 | 1/2,560 | | |
| *Experiment 8.* Irradiated vaccine | | | | | | | | | | | |
| Group 1: 1 per cent 0.5 cc. (1,650,000 M.D.‡) irradiated | 1/5 | 2/5 | 0/5 | 0/5 | 0/5 | 1/5 | — | — | — | 10 | 80 |
| " 2: 1 per cent 0.1 cc. (330,000 M.D.) irradiated | 2/5 | 0/5 | 1/5 | 0/5 | 1/5 | 0/5 | — | — | — | 11 | 72 |
| " 3: 0.1 per cent 0.1 cc. (33,000 M.D.) irradiated | 1/5 | 0/5 | 1/5 | 2/5 | 2/5 | 2/5 | — | — | — | 16 | 50 |
| " 4: No vaccine | — | — | 4/5 | 4/5 | 3/5 | 4/6 | 4/5 | 4/5 | 1/4 | 800 | |
| *Experiment 11.* Chloroformized vaccine | | | | | | | | | | | |
| Group 1: 1 per cent 0.1 cc. (330,000 M.D.) | 2/4 | 2/4 | 0/4 | 3/5 | 1/5 | 1/5 | — | — | — | 31 | 26 |
| " 2: 0.1 per cent 0.1 cc. (33,000 M.D.) | 4/5 | 3/5 | 2/5 | 2/5 | 2/5 | 1/5 | — | — | — | 48 | 16.5 |

Footnotes the same as in Table I.

ice box to await the outcome of the virulence test. The material before irradiation titred very high,—0.03 cc. $10^{-8}$—and following 30 minutes' irradiation was still fatal to two of ten injected mice. Consequently it was given a second irradiation of 5 minutes, 8 days after the first. This time the preparation proved non-virulent.

The vaccine, then 13 days old, was given to mice intraperitoneally as follows: One group received 0.5 cc. of the 1 per cent suspension; a second group, 0.1 cc. of the 1 per cent, and a third group, 0.1 cc. of the 1 per cent diluted ten times to make a 0.1 per cent suspension. 3 weeks later these mice, together with controls, were tested for resistance to an intramuscular injection of virulent virus administered as described above.

The results are shown in Table VII. According to the high titre of the virus before irradiation, the mice given 0.5 cc. of the 1 per cent suspension

received at least 1,650,000 doses; those given 0.1 cc., 330,000 doses, and those given 0.1 cc. of the 0.1 per cent suspension, 33,000 doses. In the immunity test, challenge virus in the unvaccinated mice titred to an end point of 0.01 cc. of 1 to 800 dilution; the mice vaccinated with 1,650,000 doses withstood eighty times as much virus, those vaccinated with 330,000 doses, seventy-two times, and with 33,000 doses, fifty times as much virus. Apparently irradiated mouse brain virus gives clear-cut protection in doses of 33,000.

TABLE VIII

*Immunization of Mice with Graded Doses of 1 Per Cent Irradiated Mouse Brain Rabies Virus*

Virulence before irradiation (0.03 cc. in dilutions intracerebrally) $10^{-5}$ – 4/4*:$10^{-6}$ – 4/4: $10^{-7}$ – 2/4:$10^{-8}$ – 0/4.

" following " (0.03 cc. undiluted " ) 35 minutes – 0/6.

*Immunity of Vaccinated Mice*

| Dose of vaccine | Mortality of mice given test virus intramuscularly (0.01 cc.) in dilutions | | | | | Titre† of virus in mice | Difference in titre of virus in vaccinated and non-vaccinated mice |
|---|---|---|---|---|---|---|---|
| | 1/10 | 1/40 | 1/160 | 1/640 | 1/2,560 | | |
| No vaccine (A) | 5/5 | 5/6 | 5/6 | 1/6 | 2/6 | 368 | |
| " " (B) | 5/5 | 5/5 | 4/5 | 1/5 | 1/5 | 384 | |
| 1 per cent 0.2 cc. (660,000 M.D.†) | 1/6 | 0/6 | 1/6 | 0/6 | 0/6 | <10 | 37.5+ |
| 1 per cent 0.1 cc. (330,000 M.D.) | 3/5 | 3/5 | 0/5 | 1/5 | 0/5 | 40 | 9.6 |
| 0.1 per cent 0.1 cc. (33,000 M.D.) | 5/5 | 4/5 | 3/5 | 0/5 | 0/5 | 160 | 2.4 |
| No vaccine (C) | 5/7 | 4/5 | 2/5 | 0/5 | — | 40 | |
| 1 per cent 0.1 cc. (330,000 M.D.) | 1/7 | 0/6 | 1/6 | 0/6 | — | <10 | 4+ |

Footnotes the same as in Table I.

Further tests showed that the critical dose for immunizing these mice was roughly in the neighborhood of 50,000, an amount similar to that found necessary for tissue culture virus.

*Experiment 9.*—A 1 per cent suspension of rabies virus was prepared as above and tested for virulence. It was then irradiated 35 minutes and tested again for virulence.

15 days later the vaccine was given intraperitoneally to mice as follows: One batch received 0.2 cc. of the 1 per cent vaccine and a second batch (A) was left unvaccinated as controls. 5 days later, a second batch of mice was given 0.1 cc. of the vaccine, a third batch, 0.1 cc. of the 1 per cent vaccine diluted ten times to make a 0.1 per cent preparation, and a fourth batch (B) was left unvaccinated as controls. At the same time another batch was given 0.1 cc. of the 1 per cent vaccine and a final batch (C) set aside as controls.

3 weeks following vaccination, the mice were tested for immunity as follows. The first two series prepared on the 15th and 20th days were tested intramuscularly with a virulent passage strain and the final series with a street strain which had received no laboratory passages.

The results are shown in Table VIII. According to the virulence tests, the vaccinated mice received 660,000, 330,000, and 33,000 doses of irradiated virus respectively (Table VIII). The immunity test showed that the test virus in the non-vaccinated mice (A and B) titred to an end point of 0.01 cc. of the 1 to 368 and 384 dilutions respectively; the mice receiving 660,000 and 330,000 doses of vaccine withstood 37.5 and 9.6 times this amount respectively, indicating a considerable degree of immunity. Mice vaccinated with 33,000 doses withstood 2.4 times as much virus as non-vaccinated mice. Finally, street virus in non-vaccinated mice titred to the 1:40 dilution and mice vaccinated with 330,000 doses withstood at least four times as much virus, indicating a well marked immunity. In short, 33,000 doses barely immunized the mice, whereas more than this amount gave good protection.

### *Relative Immunizing Potency for Mice of Irradiated and Chloroformized Vaccines*

The relative immunizing potencies of irradiated and chloroformized vaccines have been compared in five tests. Three of these showed no striking differences, whereas two showed a superiority of irradiated vaccines.

*Experiment 10.*—A 1 per cent suspension of mouse brain virus was divided into two parts; one was spun in a Swedish centrifuge at 3,000 R.P.M. for 30 minutes and the other in a horizontal centrifuge at 500 R.P.M. for 5 minutes. The supernatants were both removed, titrated for virulence, exposed to ultraviolet light for 20 minutes, and 18 days later 0.25 cc. was injected as a vaccine into mice every other day until four doses had been given. 3 weeks later the vaccinated plus the control mice were tested for immunity to an intracerebral injection of virulent street virus.

Both the Swedish and horizontal centrifuge supernatants titred 0.03 cc. of $10^{-7}$ dilution and were non-virulent after 20 minutes' irradiation. The results of the immunity test are shown in Table IX.

This experiment shows that 1 per cent brain virus supernatants, following centrifugation at 500 or 3,000 R.P.M., remain equally virulent; that they may be exposed to ultraviolet light, and rendered avirulent in 20 minutes; and finally that they immunize mice in 1 cc. doses against at least 10,000 intracerebral lethal doses of street virus.

*Experiment 11.*—This experiment was run in conjunction with Experiment 8. The portion of 1 per cent virus set aside for treatment with chloroform received chloroform to make a 1 per cent concentration. The suspension was then shaken for 2 minutes in a mechanical shaker, and for 2 minutes daily thereafter. The material proved virulent after 6 days when injected intracerebrally into mice but not after 11 days.

Batches of mice were injected with 0.1 cc. and 0.1 cc. of a 0.1 per cent suspension of

TABLE IX

*Immunization of Mice with Graded Doses of 1 Per Cent Irradiated Mouse Brain Rabies Virus against Intracerebral Test Infection*

Supernatant—horizontal centrifuge

Virulence before irradiation (0.03 cc. in dilutions intracerebrally) $10^{-5}$* – 4/4:$10^{-6}$ – 4/4: $10^{-7}$ – 3/4:$10^{-8}$ – 0/4.

" following " (0.03 cc. undiluted " ) 20 minutes – 0/5.

Supernatant—Swedish centrifuge

Virulence before irradiation (0.03 cc. in dilutions intracerebrally) $10^{-5}$ – 4/4:$10^{-6}$ – 3/4: $10^{-7}$ – 2/3:$10^{-8}$ – 0/4.

" following " (0.03 cc. undiluted " ) 20 minutes – 0/5.

*Immunity of Vaccinated Mice*

| Dose of vaccine | Mortality of mice given test virus intracerebrally (0.03 cc.) in dilutions | | | | | Titre† of virus in mice | Difference in titre of virus in vaccinated and non-vaccinated mice |
|---|---|---|---|---|---|---|---|
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | | |
| No vaccine | — | 3/3 | 4/4 | 1/4 | 3/4 | $10^{-5}$+ | |
| 1 per cent 0.25 cc., 4 doses (3,300,000 M.D.‡) | | | | | | | |
| "Horizontal" supernatant | 0/4 | 0/4 | 0/4 | — | — | $<10^{-1}$ | 10,000+ |
| "Swedish" " | 0/4 | 0/4 | 0/4 | 0/2 | — | $<10^{-2}$ | 10,000+ |

Footnotes the same as in Table I.

TABLE X

*Immunization of Mice with Graded Doses of Irradiated and Chloroformized Mouse Brain Rabies Virus*

Virulence before irradiation (0.03 cc., $10^{-7}$ dilution intracerebrally).

" following " (0.03 cc. undiluted " ) 35 minutes—0/6.

*Immunity of Vaccinated Mice*

| Dose of vaccine | Mortality of mice given test virus intramuscularly (0.01 cc.) in dilutions | | | | | | | Titre† of virus in mice | Difference in titre of virus in vaccinated and non-vaccinated mice |
|---|---|---|---|---|---|---|---|---|---|
| | 1/10 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1,280 | | |
| Group 1: No vaccine | — | — | 4/6 | 4/6 | 2/6 | 1/6 | 2/6 | 250 | |
| " 2: 0.5 cc., irradiated (1,650,000 M.D.‡) | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | — | <10 | 25+ |
| " 3: 0.1 cc., irradiated (330,000 M.D.) | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | — | <10 | 25+ |
| " 4: 0.5 cc., chloroformized (1,650,000 M.D.) | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | — | <10 | 25+ |
| " 5: 0.1 cc., chloroformized (330,000 M.D.) | 1/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | — | <10 | 25+ |

Footnotes the same as in Table I.

the vaccine respectively. 3 weeks later these mice, together with those given the irradiated virus and the controls, received the test virus intramuscularly in twofold dilution.

The results of the test are shown in Table VII. The mice given chloroformized vaccine withstood 26 and 16.5 times as much virus as non-vaccinated mice but only one-third as much as the mice given equivalent doses of irradiated vaccine respectively.

*Experiment 12.*—A 1 per cent mouse brain virus was prepared as in Experiments 7 and 9. One portion was irradiated 35 minutes and proved non-virulent by mouse inoculation; a second portion was treated with 1 per cent chloroform and proved non-virulent after 15 days. 48 days later, batches of mice received a single intraperitoneal injection of 0.5 or 0.1 cc. of the irradiated or chloroformized vaccine respectively. 6 weeks later, all mice, together with unvaccinated controls, were given a test intramuscular injection of virulent virus in a dose of 0.01 cc. in graded twofold dilutions.

The results are shown in Table X. Test virus in the non-vaccinated mice showed a titration end point of 0.01 cc. of 1 to 250 dilution, as contrasted with less than 1 to 10 in all vaccinated mice. The twenty-fivefold difference indicates considerable immunity in both irradiated and chloroformized vaccine groups.

### *Relative Immunizing Potency for Mice of Irradiated Mouse and Dog Brain Vaccines*

The next step in developing a vaccine was to compare the titres of virus in infected brains of young mice with those in young guinea pigs, rabbits, and dogs. Repeated tests showed that dogs alone, injected with virus intracerebrally when 1 month old, yielded brain tissue with virus titres equal to those in infected mouse brains, namely, 0.03 cc. of the $10^{-6}$ or $10^{-7}$ dilution. Vaccines were prepared with infected dog brains and results obtained which paralleled those with mouse brains.

*Experiment 13.*—An infected dog brain weighing 64 gm. was homogenized in a mechanical shaker, diluted with buffer to form a 1 per cent emulsion, titrated for virulence, spun in a horizontal centrifuge at 500 R.P.M. for 5 minutes, distributed in 35 cc. quantities in quartz flasks, irradiated 20 minutes with ultraviolet light, tested again for virulence, and stored in the ice box. 3 weeks later, groups of mice were vaccinated as follows: Group 1 received 0.2 cc. intraperitoneally and group 2 received 0.2 cc. of the 1 per cent vaccine diluted ten times. A third group received 0.2 cc. of a commercial chloroformized vaccine, a fourth 0.2 cc. of the same vaccine diluted ten times, and a final group was left unvaccinated as controls. 3 weeks later the immunity of the mice was tested by giving them 0.01 cc. of street virus intramuscularly in graded doses.

The 1 per cent centrifuged supernatant titred 0.03 cc. of the $10^{-7}$ dilution and, following irradiation, failed to kill mice. Results of the immunity test are shown in Table XI. The titre of street virus in the unvaccinated was approximately 0.01 cc. of the 1 to 160 dilution, whereas that in mice

TABLE XI

*Immunization of Mice with Graded Doses of Irradiated and Chloroformized Dog Brain Rabies Virus*

Virulence before irradiation (0.03 cc., $10^{-7}$ dilution intracerebrally).
" following " (0.03 cc. undiluted " ) 20 minutes—0/6.

*Immunity of Vaccinated Mice*

| Dose of vaccine | Mortality of mice given test virus intramuscularly (0.01 cc.) in dilutions | | | Titre† of virus in mice | Difference in titre of virus in vaccinated and non-vaccinated mice |
|---|---|---|---|---|---|
| | 1/10 | 1/40 | 1/160 | | |
| Group 1: 1 per cent, 0.2 cc. irradiated | 1/9 | 0/8 | 0/8 | <10 | 16+ |
| " 2: 0.1 per cent, 0.2 cc. irradiated | 5/9 | 4/8 | 1/8 | 40 | 4 |
| " 3: 20 per cent, 0.2 cc. chloroformized | 2/8 | 0/8 | 0/8 | <10 | 16+ |
| " 4: 2 per cent, 0.2 cc. chloroformized | 2/8 | 2/8 | 1/8 | 10 | 16 |
| " 5: No vaccine | 7/9 | 5/8 | 3/8 | <160 | |

Footnotes the same as in Table I.

TABLE XII

*Immunization of Mice with Graded Doses of Irradiated Dog Brain Rabies Virus after 9 Months' Storage*

Virulence before irradiation (0.03 cc., $10^{-7}$ dilution intracerebrally).
" following " (0.03 cc. undiluted intracerebrally) 30 minutes—0/6.

*Immunity of Vaccinated Mice*

| Dose of vaccine | Mortality of mice given test virus intracerebrally (0.03 cc.) in dilutions | | | | | | | Titre† of virus in mice | Difference in titre of virus in vaccinated and non-vaccinated mice |
|---|---|---|---|---|---|---|---|---|---|
| | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | | |
| Group 1: No vaccine | — | — | 4/4 | 4/4 | 4/4 | 1/4 | 0/4 | $10^{-6}$ | |
| " 2: 1 per cent, 0.25 cc., 4 doses, irradiated 15–30 min | — | 1/4 | 1/4 | 1/4 | 0/4 | — | — | $10^{-4}$ | 100 |
| " 3: 1 per cent, same dose, irradiated 90–120 min | 0/2 | 1/4 | 0/4 | 0/4 | 0/4 | — | — | $<10^{-2}$ | 10,000+ |
| " 4: 6.6 per cent, same dose, chloroformized | — | 2/3 | 2/4 | 3/4 | 1/4 | — | — | $10^{-5}$ | 10 |

| Dose of vaccine | Mortality of mice given test virus intramuscularly (0.01 cc.) in dilutions | | | | | Titre† of virus in mice | Difference in titre of virus in vaccinated and non-vaccinated mice |
|---|---|---|---|---|---|---|---|
| | 1/10 | 1/40 | 1/160 | 1/640 | 1/2,560 | | |
| Group 5: No vaccine | 3/3 | 4/4 | 1/4 | 1/4 | 2/4 | 640 | |
| " 6: 1 per cent, 0.2 cc., irradiated 15–30 min | 0/4 | 1/4 | 0/4 | 0/4 | 0/4 | <10 | 64+ |
| " 7: 1 per cent, same dose, irradiated 90–120 min | 2/4 | 0/4 | 0/4 | 0/4 | 0/4 | 10 | 64 |
| " 8: 6.6 per cent, same dose, chloroformized | 3/4 | 1/4 | 1/4 | 0/4 | 1/4 | 35 | 18 |

Footnotes the same as in Table I.

receiving the 1 per cent irradiated or 20 per cent chloroformized vaccines was at least sixteen times, and in mice receiving 0.1 per cent irradiated or 2 per cent chloroformized vaccine, four to sixteen times greater, demonstrating considerable immunizing potency of both the 1 per cent irradiated dog brain and the 20 per cent chloroformized preparations.

A final experiment is submitted to show the effectiveness of the 1 per cent irradiated dog brain virus after 9 months at ice box temperature.

*Experiment 14.*—A 1 per cent dog brain virus was prepared and tested for virulence according to the method described in the previous experiment. Portions were exposed to ultraviolet light for 15 and 30 minutes, and for 90 and 120 minutes. Samples were tested for virulence at varying intervals from 3 to 30 minutes. The original material titred 0.03 cc. $\times 10^{-7}$ and at 30 minutes virus failed to kill mice. The various samples were then stored in the ice box at 40°F.

9 months later, mice were treated as follows: Group 1 remained unvaccinated. Other groups of mice were vaccinated intraperitoneally in the following manner. Group 2 was given 0.25 cc. of the pooled 15 and 30 minute vaccines every other day four times; group 3 received the same dose of pooled 90 and 120 minute vaccines; group 4 was given the same dose of a commercial chloroformized vaccine diluted five times; group 5 was left as a second batch of controls; group 6 was given a single dose of 0.2 cc. of the 15 minute irradiated vaccine, group 7 a single dose of 0.2 cc. of the 120 minute irradiated vaccine, and group 8 the same dose of the chloroformized vaccine diluted 1 to 5. 3 weeks later the first four groups were tested for immunity to an intracerebral injection and groups 5 to 8 were tested for immunity to an intramuscular injection of virulent virus.

Table XII shows that a total of 1 cc. of 1 per cent irradiated dog brain vaccine immunized mice against 100 to 10,000 intracerebral lethal doses and at least 64 intramuscular doses. The chloroformized vaccine immunized mice against 10 intracerebral test doses and 18 intramuscular doses.

## DISCUSSION

From a practical viewpoint, culture virus has not yet proved a satisfactory source of rabies vaccine, due chiefly to its low content of virus. To immunize, approximately 1 cc. is required for mice and 500 cc. for dogs,—about 5 per cent of the body weight.

The supernatant of centrifuged brain tissue virus, however, has proved a good source of vaccine. The virus content of infected brain tissue per unit volume is 1,000 times that of tissue culture. A 10 per cent emulsion can be centrifuged to sediment a large portion of the tissue fragments without lowering the titre of virus. The supernatant from a 1 to 5 per cent emulsion can be irradiated so as to destroy virulence without loss of immunizing potency. 0.1 cc. of such a preparation immunizes mice adequately,—0.5 per cent of the body weight.

Altogether the results of these experiments to date suggest that basically the immunizing potency of a vaccine is dependent upon virus content, that is, that the immunizing antigen is the virus particle. They indicate also that one intracerebral lethal mouse dose of a given strain of virus from tissue culture is equivalent in immunizing potency to one dose of the same strain from infected mammalian brain. Finally, the findings point to a relation between number of mouse lethal doses required to immunize and body weight.

The 1 per cent irradiated dog brain virus has proved an effective and practical vaccine for immunizing mice, and equal or superior to chloroformized vaccine. It is now being tested in dogs with promising results (4).

## SUMMARY

In the experiments described above, we found with respect to tissue culture rabies virus that 1 cc., which contains approximately 50,000 mouse intracerebral lethal doses, properly irradiated, was required to immunize a mouse; 500 cc., which contain 25,000,000 doses, were required to immunize a 20 pound beagle dog.

Tissue culture virus concentrated ten times proved capable of immunizing mice in a dose one-tenth as large as that required for unconcentrated culture virus.

Brain virus suspensions were centrifuged so as to remove a large part of the tissue particles without striking loss in the virulence of the supernatant. The centrifuged supernatants of 1 to 5 per cent brain virus suspensions were irradiated so as to destroy virulence and yet retain immunizing potency.

Irradiated supernatants of mouse brain virus proved capable of immunizing mice as well as or better than similar supernatants treated with chloroform.

0.1 cc. of a 1 per cent irradiated dog brain virus containing approximately 50,000 mouse intracerebral lethal doses immunized mice effectively.

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