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Propagation of Rabies Virus in Tissue Culture.*

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Over the past five years, several strains of rabies virus have been propagated for varying lengths of time in tissue culture in an effort to discover the conditions under which an increased concentration of this and other neurotropic viruses might be obtained. Previously, Kanazawa,[1] Webster and Clow,[2] and Kligler and Bernkopf[3] have reported the successful cultivation of rabies virus *in vitro*. Kanazawa employed rabbit embryo brain and Tyrode's solution without serum. Webster and Clow employed mouse or chick embryo brains together with Tyrode's solution containing 10% monkey serum, and were able to produce typical rabies in mice with 1/33,000 cc of the culture material. Kligler and Bernkopf reported that human or monkey serum seemed to be essential for the growth of the virus; when these were replaced by other sera, the virus failed to grow.

Materials and Methods. In the present study, use has been made of different strains of virus that have been carried for various periods in mice. The best culture results were obtained with a virus strain isolated in 1935 from a dog brain sent from Alabama and carried since then in the Connaught Laboratories through 106 intracerebral mouse passages.† Inasmuch, however, as this strain was used in only the more recent experiments, it is quite possible that the improved results may have been due in part, at least, to other factors.

Of the twelve series of culture experiments that were made, one series was carried through 57 passages, another series through 24 passages, one series through 19, one through 12, two through 10, and the others for shorter periods. Two passages of 3 and 4 days, respectively, were made each week. As a rule, the tissues were ground at transfer and for animal inoculation. In a few of the experiments, however, direct inoculations were made from culture to culture; and in these experiments the material for mouse titrations was made from cell-free supernatant obtained by centrifuging the unground tissue-fluid mixture.

All of the more recent experiments have been made in 125 cc Erlenmeyer flasks which, in certain instances, were fitted with stoppers accommodating inlet and outlet tubes[4] for continuous gassing with a mixture of oxygen (21 or 80%), carbon dioxide (8%) and nitrogen. In six of the earlier culture series a 2-armed, 5-cm Carrel flask was used and the cultures were gassed continuously through filter bulbs inserted in the rubber stoppers.[5]

Finely chopped embryo mouse brain was used as the tissue component of the medium, one large brain or its equivalent being used for each culture. In the early experiments, the fluid portion of the medium consisted either of heated (1 hr at 56° C) rabbit serum (¼ or ⅓) or of ox serum ultrafiltrate[6] (⅓), together with 1.4% sodium bicarbonate

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2 Webster, L. T., *Rabies*, New York, Macmillan, 1942; Webster, L. T., and Clow, A. D., *Science*, 1936, **84**, 487; *J. Exp. Med.*, 1937, **66**, 125.

3 Bernkopf, H., and Kligler, I. J., *Brit. J. Exp. Path.*, 1937, **18**, 481; Kligler, I. J., and Bernkopf, H., *Brit. J. Exp. Path.*, 1938, **19**, 378; *Am. J. Hygiene, Sec. B*, 1941, **33**, 1.

4 Parker, R. C., and Hollender, A. J., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 88.

5 Parker, R. C., *Tissue Culture*, in Glasser, O., *Medical Physics*, Chicago, The Year Book Publishers, Inc., 1944, p. 1568.

6 Simms, H. S., and Sanders, M., *Arch. Path.*, 1942, **33**, 619. *Cf.* Sanders, M., *J. Exp. Med.*, 1940, **71**, 113; Sanders, M., and Molloy, E., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 327.

(1/6), and, to make up the balance, Tyrode's solution containing 4 times the usual amount of glucose. Because these mixtures tend to be quite alkaline, the pH was adjusted to 7.2, by means of a gas mixture, before the tissue was added, and thereafter, the cultures were gassed continuously, day and night. In the more recent experiments, the fluid medium consisted either of heated rabbit serum or ox serum ultrafiltrate (1/3) combined with Simms' X7 solution.[6] As before, the pH of the medium was adjusted before the tissues were added. As an indicator of pH, all cultures contained either 0.001% or 0.005% phenol red.

Squibb Strain of Fixed Virus. In the longest culture series, an early experiment of 57 passages, the titer was never unusually high, but the virus was retained throughout the entire period (28½ weeks). In the 41st passage, the material killed mice through 10^{-5}. Thereafter, the killing power of the virus dropped off until, near the end of the experiment, the mouse titer had fallen to 10^{-2}. In this series, 2-armed Carrel flasks were used with 4 cc of medium, which consisted of rabbit serum (1/4), 1.4% sodium bicarbonate (1/6), and Tyrode's solution containing extra glucose. The virus used was a Squibb fixed rabies strain in its 12th mouse passage; and the cultures were gassed continuously with a mixture containing 80% O_2, 8% CO_2 and 12% N_2.

Webster Strain. Three experiments, two of 7 passages and one of 8, were made with the Webster strain of virus‡ that had been passed through 126 mouse passages. In these experiments, 2-armed Carrel flasks were used with 3 cc of medium consisting of 25% rabbit serum, and the cultures were gassed continuously in the manner already described. The chopped brain tissue from two embryos was added to each culture. But at the termination of the experiments, the culture virus failed to kill mice in amounts less than 1/330,000 cc (0.03 cc of 10^{-4}).

Alabama Strain. The most satisfactory results were obtained in some of the more recent culture series in which the Alabama strain of virus was used. In the first of these experiments, the medium (3 cc) consisted of heated rabbit serum (1/3) and Simms' X7 solution (2/3). The original virus inoculum (1:1000), introduced as 1/10 of the medium, was prepared from a brain of the 109th mouse passage. The brain, which had been kept in a CO_2-ice box at —60° C for 32 days, was weighed, ground with quartz sand (60-80 mesh) and diluted 1:10 (10^{-1}) with distilled water. After it had been allowed to stand in an ice bath for 45 min, it was centrifuged 10 min at 2000 rpm and the clear supernatant was again diluted, 1:10) with glucosol (Tyrode's solution without bicarbonate) containing 10% heated rabbit serum for inoculation to the cultures. The cultures were prepared in 125 cc Erlenmeyer flasks and were incubated for 24 hours before the virus was added. The tissues were ground at transfer (and for mouse titrations) with Tenbroeck grinders that were kept chilled in an ice bath during the grinding. After low-speed centrifugation, the tissue was resuspended in the cell-free supernatant, and transferred in 0.03 cc amounts to the new cultures. At the 19th transfer (passage), the culture virus killed mice (Swiss strain) at a dilution of 10^{-6} (1:1,000,000). The original inoculum, titrated at the beginning of the experiment, killed four out of four mice that had been inoculated with virus at 10^{-7}. Unfortunately, higher dilutions were not tested. A separate pipette was used for each dilution.

Effect of Gassing and Cultures. An important feature of the 19-passage experiment just described was the attempt to determine the effect, if any, of continuous gassing upon the multiplication of the virus. For the first six passages, none of the cultures was gassed continuously. Thereafter, the series was broken into two parts, one of which was placed on the gas manifold for each successive passage, the other not. Comparative mouse titrations were made at the end of the 8th, 10th, 11th, 12th and 14th passages. Two of

‡ This virus was supplied through the courtesy of Dr. L. T. Webster, to whom the culture virus was eventually submitted for verification of identity. On the basis of complement fixation tests, Dr. Webster concluded that the culture virus behaved like typical rabies.

these tests showed the gassed cultures to be slightly better than the ungassed ones, but in the others the results were quite similar. Hence, it would seem that, for this particular virus, no real advantage is to be gained by supplying the tissues with extra oxygen provided, of course, that the buffering system in the medium is capable of maintaining the pH at an optimal level.

Effect of Grinding the Culture Tissues. Another feature of the 19-passage experiment was the titration in mice of culture virus prepared by grinding the tissues with quartz sand (60-80 mesh) and with the Tenbroeck grinders; and the results so obtained were compared with the results of mouse titrations made with the cell-free culture fluid after low speed centrifugation of the unground culture material. Tests made with culture material of the 15th and 19th passages showed grinding to be quite necessary in order to release the main bulk of the virus from the tissues, but there was little to choose between the two methods of grinding.

Rabbit Serum and Serum Ultrafiltrate. In another culture series, one of 12 passages, a comparison was made of the effect of heated (1 hr at 56° C) rabbit serum and ox serum ultrafiltrate when each of these were used as ⅓ of a medium, the balance of which consisted of Simms' X7 solution. From the beginning, the culture series was divided into two parts that were kept separate and distinct for each successive passage. The medium of one part of the series contained heated rabbit serum; the other, ultrafiltrate. Otherwise, the two halves of the experiment were comparable in every respect; and 3 cc of medium were used throughout. The virus inoculum was prepared from a mouse brain of the Alabama strain in the 109th mouse passage. The brain, which had been kept frozen in a dry ice box at —60° C for 5 days, was weighed, ground with quartz sand and made up to 10^{-1} with distilled water. After having been allowed to stand for 1 hour in an ice bath, the emulsion was centrifuged for 10 min at 2000 rpm and made up to 10^{-2} with distilled water. Subsequent 10-fold dilutions, up to and including 10^{-8}, were prepared in beef broth containing 10% horse serum (heated 1 hr at 56° C). Each culture was inoculated with 0.3 cc of the virus material at 10^{-3}. The potency of the original virus inoculum was determined by injecting mice intracerebrally with 0.03 cc of each of the higher dilutions. At 10^{-8}, one mouse of the three that were injected died with typical rabies. At each of the lower dilutions, all three mice succumbed. Throughout the experiment, the cultures were transferred twice a week at 3- and 4-day intervals, respectively. Also, the pH of the cultures was adjusted to pH 7.1 with an appropriate gas mixture and the flasks were closed with rubber stoppers before being set to incubate at 37° C.

Mouse titrations were made at each transfer. The greatest concentration of culture virus was obtained in the 6th passage. It was also in the 6th passage that the greatest difference was found to exist between the amount of virus contained in the rabbit serum cultures and in the ultrafiltrate cultures. In this passage, the rabbit serum cultures killed two out of three mice at 10^{-7} and all mice injected with lower dilutions. The ultrafiltrate cultures, on the other hand, failed to kill mice in dilutions higher than 10^{-4}. Beyond the 6th passage, the ultrafiltrate cultures improved considerably, just as the rabbit serum cultures showed a slight decrease in potency. And by the end of the experiment, it was not possible to detect any appreciable difference between the two sets. It should also be noted that the culture virus from both sets was frozen in a CO_2-ice cabinet for 11 days between the 5th and 6th passages.

Temperature of Incubation. Because it was found in a series of experiments[4] with Theiler's GD-VII mouse virus that virus production was more abundant at 35°C than at 37.5°C, a similar test was carried out with the rabies virus (Alabama strain). In an experiment of 9 passages, half of the cultures were carried at the one temperature, half at the other, and the results were determined by injecting mice with the ground culture material in dilutions ranging from 10^{-3} to 10^{-7}, inclusive. For three passages (2, 4 and 7), the virus yield was slightly greater at 37.5°C than at 35°C. For three passages (3, 5 and 8), the yield was slightly greater at 35°C. In two pas-

TABLE I.
Propagation of Rabies Virus in Cultures of Brain Tissue from Mice of Increasing Ages.

Passage	Culture Nos.	Tissue (age of mouse)	Mouse titer*	Passage	Culture Nos.	Tissue (age of mouse) days	Mouse titer*
1	13990-93	embryo	10^{-4}	13	14156-61	5	10^{-5}
2	13999-02	"	10^{-5}	14	14166-71	5	10^{-5}
3	14007-10	"	10^{-4}	15	14186-89	5	10^{-4}
4	14045-48	"	10^{-6}	16	14194-97	5	10^{-5}
5	14053-56	"	10^{-5}	17	14202-05	5	10^{-5}
6	14067-70	1 day	10^{-6}	18	14214-17	5	10^{-5}
7	14079-82	1 "	10^{-6}	19	14227-30	5	10^{-5}
8	14103-06	1 "	10^{-5}	20	14240-43	5	10^{-6}
9	14119-22	3 days	10^{-5}	21	14269-72	14	10^{-2}†
10	14137-39	3 "	10^{-5}	22	14302-05	14	10^{-4}
11	14141-44	5 "	10^{-5}	23	14316-18	14	10^{-4}
12	14145-50	5 "	10^{-5}	24	14324-27	14	10^{-1}

* Highest dilution of culture fluid that killed at least two of the three mice that were injected with each of the various dilutions tested.

† One of the three mice injected was killed both at 10^{-3} and at 10^{-4}.

sages (1 and 9), the results were identical at the two temperatures. And in the remaining passage, complete mouse titrations were not made. The cultures comprising the first 5 passages of the 24-passage culture series described in the final section of this report were the cultures that were carried at 37.5° C for the first 5 passages in this experiment.

Chick Embryo Brain Cultures. During the early part of the work, various unsuccessful attempts were made to cultivate the Squibb fixed virus in chick embryo brain cultures. The chick culture series were inoculated with virus that had been carried by intracerebral passage in mice and also with virus that had been carried for several passages in mouse embryo brain cultures. Unsuccessful attempts were also made to cultivate, in chick embryo brain cultures, mouse virus that had been adapted to chick tissue by serial intracerebral passage in embryonated eggs. To this end, the mouse virus was carried by the method of Dawson[7] through 23 chick embryo passages (187 days) during which time the virus continued to kill mice at dilutions of 10^{-3}. But when the virus was transferred to chick embryo brain cultures, it survived only a few passages.

Postembryonic Mouse Brain Cultures. Rabies virus (Alabama strain) derived from a mouse brain frozen at —60° C for 60 days was cultivated for 24 successive passages in cultures of brain tissue from embryo mice (5 passages), 1 day old mice (3 passages), 3 day old mice (2 passages), 5 day old mice (10 passages) and 14 day old mice (4 passages), respectively. The results are summarized in Table I. At the end of the 5th passage in the embryo brain cultures, the mouse titer was 10^{-5}. At the end of 20 passages, the last 10 of which consisted of brain cultures from 5 day old mice, the titer was still 10^{-5}. In this instance, then, the virus multiplied quite as readily in cultures prepared from the brains of 5 day old mice as in cultures prepared from embryo mice. In cultures prepared from the brains of 14 day old mice, the virus multiplied less abundantly than in the 5 day old brain cultures from which the virus was derived; and at the end of the 4th passage in cultures prepared from the older mice, the titer had dropped to 10^{-1}. At this point, the series was discontinued.

Before the 20th passage culture virus was transferred to cultures prepared from 14 day old mouse brains (Table I), two attempts were made to cultivate the virus in cultures prepared from the brains of 28 day old mice. Thus, one series of such cultures was inoculated with fresh culture virus that had been carried previously for 6 passages in cultures of brain tissue from 5 day old mice; another series, of three successive passages, was initiated with fresh culture virus that had been carried for 7 passages in cultures of the 5

[7] Dawson, J. R., Jr., *Am. J. Path.*, 1941, **17**, 177.

day brains. But the results were negative throughout.

Finally, an attempt was made to discover whether embryo mouse brain culture virus could be cultivated directly in cultures prepared from the brains of 5 day old mice, or whether it was necessary to adapt the virus gradually by passing it through an intervening series of cultures prepared from the brains of 1 and 3 day old mice. Accordingly, virus from embryo mouse brain cultures and from brain cultures from 5 day mice were each transferred directly to sister cultures prepared from the brains of 5 day old mice that were litter mates. The mouse titrations made at the end of the experiment gave identical results. It would appear, therefore, that the intermediate culture passages were quite unnecessary as a means of adapting the embryo brain virus to brain cultures from 5 day mice.

Summary and Conclusions. (1) The rabies virus has been propagated for as long as 57 passages (transfers) in cultures of chopped mouse embryo brain suspended in rabbit serum and buffered salt solutions. Under conditions developed in shorter, more recent experiments, the virus multiplied to the extent that mice were killed regularly by the culture material in dilutions as high as 10^{-6} (1:1,000,000); and it was possible to produce typical rabies in mice with 1/33,000,000 cc of the culture virus. When ox serum ultrafiltrate was substituted for rabbit serum, the virus titer was somewhat less. (2) Comparative experiments showed the importance of grinding the culture material for mouse titrations. But no significant increase in virus output was ever obtained by subjecting the cultures to continuous gassing with mixtures of O_2, CO_2 and N_2. (3) A duplicate series of cultures incubated for 9 passages at 35° C and at 37.5° C, respectively, showed no consistent difference in the amount of virus produced. (4) Attempts to cultivate the rabies virus in chick embryo brain cultures were unsuccessful even when the virus had been adapted to chick tissue by serial intracerebral passage in embryonated eggs. (5) Rabies virus from a mouse brain frozen at —60° C for 60 days has been cultivated for 24 successive passages in cultures of brain tissue from embryo mice (5 passages), 1 day old mice (3 passages), 3 day old mice (2 passages), 5 day old mice (10 passages) and 14 day old mice (4 passages), respectively. Culture virus developed in brain material from 5 day old mice, failed to multiply in cultures of brain tissue from 48 day old mice.

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Age and Autonomic Balance.*

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It has been shown by Feldman, Cortell, and Gellhorn[1] that anoxia causes an excitation of autonomic centers leading to a discharge of the vago-insulin (v.i.) and sympathetico-adrenal (s.a.) systems. If the responsiveness of these centers to anoxia or similar procedures is taken as a standard the question may be investigated as to whether external or internal factors alter the reactivity of these structures, and thereby influence the central autonomic balance. The effect of temperature[2] and fever[3] and of changes in the endo-

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1 Feldman, J., Cortell, R., and Gellhorn, E., *Am. J. Physiol.*, 1940, **131**, 281.

2 Gellhorn, E., and Feldman, J., *Am. J. Physiol.*, 1941, **133**, 670.

3 Feldman, J., and Gellhorn, E., *Endocrinology*, 1941, **29**, 141.