FURTHER STUDIES ON THE TITRATION AND NEUTRALIZATION OF THE WESTERN STRAIN OF EQUINE ENCEPHALOMYELITIS VIRUS IN TISSUE CULTURE*

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PLATES 1 AND 2

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In the course of a study on cultivation of the Western strain of equine encephalomyelitis virus (W.E.E.), it has been found that tissue heavily infected with virus fails to grow. This phenomenon seemed to be a direct contradiction to the observation of Feller, Enders, and Weller (1) who noted that tissue was able to multiply in roller tube cultures infected with vaccinia virus. Following the observation of the failure of the tissue to grow when infected with the W.E.E. virus, an attempt was made to determine how delicate is the balance between cellular growth and presence of virus. Could the technique be utilized for titration and neutralization of virus? The results of such a study were briefly presented in a previous paper (2). Since then further studies have been directed toward the simplification of the different procedures, the improvement of the neutralization potency, and the clarification of some of the mechanisms governing the neutralization. These form the subjects of the present report.

Materials and Methods

Culture Medium and the Care of Cultures.—Serum ultrafiltrate diluted in "X_6" physiological solution (UF-X_6) has been used for the cultivation of the virus. This medium had previously been utilized for tissue cultures by Simms and Sanders (3) and for the propagation of viruses by Sanders and his coworkers (4, 5). The procedures for the preparation of this medium and the care of the cultures as given in these papers have been followed.

Simms' Solutions.—The composition of the two physiological solutions "X_6" and "Z_2" is given in Table I. Physiological solution "Z_2" (method of preparation, see Simms and Sanders (3)) has been used for the temporary bath of the tissue and the dilution of the virus.

Tissue.—Minced skeletal muscles of 9 day developing chick embryos were used. The pieces of tissue after mincing measured about 0.5 to 1 mm. in diameter. 10 to

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15 pieces were suspended in 1 cc. UF-X_6 in separate tubes and were used immediately.

In the earlier experiments the tissue was carefully minced with a cataract knife in such a manner that each piece was divided into two by a single chop. This method is tedious: it takes about 1 hour to mince 300 pieces of tissue. It was later found unnecessary to divide the tissue carefully. Tissue obtained by a rapid chopping on a sterile glass plate, irrespective of whether or not individual pieces of tissue were well separated by any particular chop, has proved to be equally viable with the tissue obtained from the previous method of mincing. In this way, it takes only 5 to 10 minutes to mince 300 pieces of tissue. The tissue after mincing was twice washed with 20 cc. of "Z_2" solution. Larger pieces of tissue were pipetted out and discarded.

Extract of Embryonic Tissue.—8 to 9 day developing chick embryos with eyes removed were ground in a pyrex glass tissue grinder[1] with 1 cc. UF-X_6 per embryo. The material was centrifuged for 30 minutes at 4,410 R.P.M. The supernatant fluid carefully removed and rapidly frozen in alcohol and dry ice mixture was then melted and recentrifuged. The supernate was finally stored at -72°C.

TABLE I

Composition of the X_6 and Z_2 Physiological Solution

	"X_6" solution		"Z_2" solution	
	gm. per liter	*mM per liter*	*gm. per liter*	*mM per liter*
NaCl	8.00	137.00	8.00	137.00
KCl	0.20	2.70	0.20	2.70
$CaCl_2 \cdot 2H_2O$	0.147	1.00	0.147	1.00
$MgCl_2 \cdot 6H_2O$	0.203	1.00	0.203	1.00
$NaHCO_3$	1.01	12.00	—	—
Na_2HPO_4	0.213	1.50	0.190	1.35
$NaH_2PO_4H_2O$	—	—	0.021	0.15
Dextrose	1.00	5.50	1.00	5.50
Phenol red	0.05	0.15	—	—

Plasma.—From the wing veins of a 1½ year old chicken, 10 cc. of blood was drawn in a syringe containing 1 cc. of a 0.1 per cent heparin. The blood was centrifuged and the plasma so obtained was stored in the ice box. The plasma was diluted with an equal amount of UF-X_6 before being used in the experiments.

Virus.—The Western strain of the equine encephalomyelitis virus was used throughout the experiment. Unless specified, the virus used was cultivated in chick embryo tissue and UF-X_6 medium at 37.5°C. for 30 hours. Serial dilutions of the infectious supernatant fluid were used in titration and neutralization experiments.

Antiserum.—Hyperimmune anti-W.E.E. horse serum prepared by the Lederle Laboratories, Inc., New York, N. Y., was used.

Control Serum.—Antimeningococcus horse serum prepared by the Division of Laboratories and Research, New York State Department of Health, in the year 1931 was used as a control.

[1] Obtained from Scientific Glass Apparatus Co., Bloomfield, New Jersey.

Experimental Results of the in Vitro Titration

Method 1. Titration in Tubes.—Sigurdsson (6) has drawn attention to the observation of Albert Fischer that when two tissue cultures are excised and transferred to a tube containing physiological salt solution, they coalesce in their growth to form one spherical mass of tissue. It was thought possible to apply this finding for the *in vitro* titration of the W.E.E. virus.

Two pieces of tissue were introduced into each of a number of small tubes[2] containing one drop of embryonic tissue extract with two drops of UF-X_6, and then one drop of serially diluted virus was added. As control, one drop of physiological solution "Z_2" was added to tubes containing UF-X_6, tissue, and no virus. Care was taken to shake the tube in such a manner that the two pieces of tissue came in contact with each other. The preparations were then incubated at 37.5°C. To assure a firm union, the test was read in 36 to 48 hours.

The results of the preliminary experiments were not satisfactory because pieces of tissue were still able to grow together in the early phase of the infection. The union was not firm in the case of the heavily infected tissue and it could be broken up by slight agitation, but in the lightly infected tissue the union was firm and thus the sensitivity of the test was masked.

Since the principal inadequacy of the method appeared to be lack of opportunity for tissues to become heavily infected with virus, another method was introduced as follows:

(*a*) To a series of tubes with a diameter of 1.5 cm., 10 pieces of tissue and 1 cc. of UF-X_6 were added; (*b*) one drop of tenfold dilutions of the virus was added to each tube; (*c*) the tubes were slightly agitated manually so that the majority of tissue segments remained separated; (*d*) the different mixtures were incubated at 37.5°C.; (*e*) at the end of 48 hours of incubation two pieces of tissue from each tube were transferred to a small tube containing the embryonic tissue extract and UF-X_6 as described above; and finally these tubes were incubated at 37.5°C. for 36 hours before the readings were made.

For a comparison of the sensitivity of the test, the same tenfold dilutions of the virus that were inoculated into the tubes were injected intracerebrally into Swiss mice, weighing 10 to 12 gm.

The results (Table II) show that the titre is higher in the *in vitro* test (10^{-6}) than in the *in vivo* experiment (10^{-5}). There are irregularities in the result which may be attributed to technical difficulties. It is believed that such difficulties can be overcome. However, the results of repeated tests suggest that even in its present form the technique gives constant results and is dependable so far as the demonstration of virus is concerned.

[2] The diameter of the tube is 5 mm. and the bottom of the tube was drawn out and sealed in the flame, a procedure which helped to bring the two pieces of tissue in close contact. Glass tubing can be used for this purpose.

To prove that the tissue was really infected after this period of incubation, the supernatant fluid of the cultures inoculated with different dilutions of the virus was injected intracerebrally into mice soon after the pieces of tissue were transferred into small tubes. The results as shown in Table III bear out the findings in Table II.

TABLE II

Comparison of in Vitro and in Vivo Titration of W.E.E. Virus: Tube Method

Titrated in	Virus dilution tested							
	No virus	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Mice	A A A	2 2 2	2 2 2	2 2 2	2 2 2	A A A	A A A	A A A
Tissue culture	G G 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	G G 0	G G 0

A = mouse remained alive 5 days.

Each number indicates day of death of the animal after the infection and presumably represents a virus death.

G = union of the two pieces of tissue.

0 = the two pieces of tissue showed no union.

TABLE III

Confirmation by Mouse Test of the in Vitro Titration of W.E.E. Virus: Tube Method

Virus dilution tested	Results of titration in tissue cultures	
	Union of tissue	Supernatant fluid inoculated into mice intracerebrally
No virus	G G 0	A A A
10^{-2}	0 0 0	2 2 2
10^{-3}	0 0 0	2 2 2
10^{-4}	0 0 0	2 2 3
10^{-5}	0 0 0	2 2 2
10^{-6}	0 0 0	2 2 3
10^{-7}	G G 0	A A A
10^{-8}	G G 0	A A A

Legend as in Table II.

A new method of titration, utilizing a hanging drop preparation, was also introduced. This method proved to be highly satisfactory and the tube method was temporarily dropped.

Method 2. Hanging Drop Preparation.—The procedure was essentially the same as in the tube method just described except that the pieces of tissue after infection and incubation were transferred to either micro-culture slides or Carrel dishes. For two pieces of tissue one drop of embryonic tissue extract and one drop of diluted plasma were used. When in Carrel dishes all the pieces of tissue from the same tube could be

plasma-patched in one dish. After the plasma coagulated the slides or dishes were incubated at 37.5°C. The readings were made 48 hours after incubation under the low power microscope. Growth of the fibroblasts from the explant, if it occurred, was noticeable overnight but abundant growth usually required 48 hours of incubation.

TABLE IV

Comparison of in Vitro and in Vivo Titration of W.E.E. Virus: Hanging Drop Preparation

Titrated in	Virus dilution tested							
	No virus	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Mice	A A A	2 2 2	2 2 2	2 2 2	2 3 3	A A A	A A A	A A A
Tissue	G G	0 0	0 0	0 0	0 0	0 0	G G	G G
culture	G G	0 0	0 0	0 0	0 0	0 0	G G	G G

A = mouse remained alive 5 days.

Each number indicates day of death of the animal after the infection and presumably represents a virus death.

G = growth of fibroblasts from explants noted.

0 = no growth from the explants.

Each "G" or "0" represents growth activity of a single explant. Four explants were used for each plasma preparation.

TABLE V

Confirmation by Mouse Test of the in Vitro Titration of W.E.E. Virus: Hanging Drop Preparation

Virus dilution tested	Results of titration in tissue cultures	
	Growth of fibroblasts from the explant	Supernatant fluid inoculated into mice intracerebrally
No virus	G G G G	A A A
10^{-2}	0 0 0 0	2 2 2
10^{-3}	0 0 0 0	2 2 2
10^{-4}	0 0 0 0	2 2 2
10^{-5}	0 0 0 0	2 2 3
10^{-6}	0 0 0 0	2 2 3
10^{-7}	G G G G	A A A
10^{-8}	G G G G	A A A

Legend as in Table IV.

Activity of the virus in tissue culture was at all times compared and confirmed by mouse inoculation.

The results of the comparison of the *in vitro* and the *in vivo* titration are shown in Table IV. They show that the method is again more sensitive than the *in vivo* titration, the latter giving a titre of 10^{-5} as compared to 10^{-6} in the *in vitro* test.

The test was repeated 10 times with similar results except that in 5 instances

the titre obtained in the tissue culture method was the same as in the animal test.

The results of the confirmation of the *in vitro* titration are shown in Table V and, as was to have been expected, whenever the tissue was infected the virus was detected in the supernatant fluid by mouse test.

Interpretation of the results based on the confirmatory mouse test indicated that abundant growth of tissue occurred when the cells were not infected by the virus or had overcome the infection of the virus. Absence of growth indicated that the tissue was infected with the virus.

Demonstration of Neutralizing Antibodies by the in Vitro Test

Following several successful repetitions of the *in vitro* titrations the logical next step appeared to be the application of the method to the neutralization test.

The hanging drop preparation was used for the neutralization test. The immune serum as well as the control serum was mixed with an equal amount of a tenfold dilution of the virus. The mixtures of serum and virus were incubated in the water bath at 37.5°C. for 1 hour. Two drops from each mixture were then introduced into separate tubes containing 10 to 15 pieces of tissue. The mixtures of serum, virus, and tissue were kept in the incubator for 15 minutes in order to allow the virus to become associated with the cells. The tissue was then washed three times, each time with 5 cc. of Z_2 solution. The washed tissue from each tube was then transferred to separate tubes containing 1 cc. of UF-X_6 and the entire preparation was incubated at 37.5°C. At the end of 48 hours about 10 pieces of the tissue from each tube were transferred by means of a capillary pipette to separate Carrel dishes. Two drops of embryonic tissue extract and five drops of diluted plasma were added and mixed. The dishes were incubated and the growth of the tissue was studied under the low power microscope at the end of 48 hours.

For comparison of the sensitivity of the test similar mixtures of serum and virus were inoculated intracerebrally into mice.

The results of this procedure as shown in Table VI indicate that the amount of neutralization (10,000 neutralizing doses) in the *in vitro* test is the same as that obtained on the animal inoculation. As in the usual neutralization test in which animals are used, computation of neutralizing doses was reached by subtracting the titre of the immune serum-virus preparation from the titre of the control. Thus, it will be seen that in the present experiment, growth of tissue in the immune serum-virus group occurred at a 10^{-2} dilution as compared with 10^{-6} in the control. Interestingly enough the serum-virus mixture which proved to be neutral when injected into mice was not neutral when inoculated into the tissue cultures. The explanation of this discrepancy will be discussed later.

For confirmation of the results of the neutralization test the supernatant

TABLE VI

Comparison of the in Vitro and in Vivo Neutralization Tests of W.E.E. Virus (Preliminary Method)

Serum used	Titrated in	Dilution of the virus tested								Neutralizing doses*
		10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
Immune	Mice	2 2 2 2	A A A A	A A A A	A A A A	A A A A	A A A A	A A A A	‡	10,000
	Tissue culture	00000 00000	00000 00000	GGGGG GGGGG	GGGGG GGGGG	GGGGG GGGGG	GGGGG GGGGG	GGGGG GGGGG	‡	10,000
Control	Mice	‡	‡	2 2 2 2	2 2 2 2	2 2 3 3	A A A A	A A A A	A A A A	
	Tissue culture	‡	‡	00000 00000	00000 00000	00000 00000	00000 00000	GGGGG GGGGG	GGGGG GGGGG	

Legend as in Table IV except that ten explants were used for each plasma preparation.
* = calculated on 50 per cent end point titres.
‡ = not done.

TABLE VII

Confirmation by Mouse Test of the in Vitro Neutralization Tests with W.E.E. Virus (Preliminary Method)

Serum used	Dilution of the virus	Results of neutralization in tissue cultures	
		Growth of fibroblasts from the explant	Supernatant fluid inoculated into mice intracerebrally
Immune	10^{-0}	0000000000	2 2 2 2
	10^{-1}	0000000000	2 2 3 3
	10^{-2}	GGGGGGGGGG	A A A A
	10^{-3}	GGGGGGGGGG	A A A A
	10^{-4}	GGGGGGGGGG	A A A A
	10^{-5}	GGGGGGGGGG	A A A A
	10^{-6}	GGGGGGGGGG	A A A A
Control	10^{-2}	0000000000	2 2 2 2
	10^{-3}	0000000000	2 2 2 2
	10^{-4}	0000000000	2 2 2 3
	10^{-5}	0000000000	2 2 3 3
	10^{-6}	GGGGGGGGGG	A A A A
	10^{-7}	GGGGGGGGGG	A A A A

Legend as in Table IV except that ten explants were used for each plasma preparation.

fluid of the cultures was injected intracerebrally into mice soon after the pieces of tissue were transferred to Carrel dishes. The results as shown in Table VII suggest that the interpretation of the *in vitro* study is correct.

This experiment was repeated thrice and the degree of neutralization obtained was 100 neutralizing doses in one case and 1,000 and 10,000 neutralizing doses in the other two.

Although definite neutralization was demonstrated in the above experiments, the technique was not satisfactory. In the first place washing the tissue was time consuming. In the second place the neutralizing titre was not consistent and was inferior to that obtained in the animal test by the intraperitoneal route of inoculation (7). Attempts were directed therefore toward improvement of the technique and explanation of the inconsistency of the results.

As there is sufficient evidence that many viruses in neutral mixtures can be reactivated by dilution (8–19), it seems logical to expect an increase in the neutralizing potency in the *in vitro* experiment if the process of washing is eliminated from the procedure. The introduction of the washing procedure in the previous method of neutralization test was due to the preliminary finding of the failure of two pieces of chick embryo tissue to coalesce in the presence of anti-W.E.E. immune horse serum. It was not known at that time whether or not these pieces of tissue after prolonged contact with the anti-W.E.E. immune horse serum would grow subsequently when patched in plasma. Studies along this line led to the finding that if one drop of a 1:1 dilution of anti-W.E.E. immune horse serum was added to a tissue culture containing 1.5 cc. of UF-X_6 and incubated for 48 hours, the tissue when subsequently patched in plasma was able to grow. This finding permitted elimination of the washing procedure from the test and thus the dilution effect of the neutral mixture was minimized. The following procedures were then adopted.

The preparation of the serum-virus mixtures was the same as in the previous experiments except that the virus used was obtained from the mouse brain after repeated passages in adult Swiss mice instead of from the supernatant fluid of tissue culture. After the mixtures were incubated for 1 hour, one drop of the fluid from each of the serum-virus mixtures was then introduced into separate tubes each containing 10 to 15 pieces of tissue. 1.5 cc. of UF-X_6 was immediately added to each tube. The entire preparation was incubated at 37.5°C. At the end of 48 hours of incubation the tissue was patched with plasma and returned to the incubator. Readings were made 48 hours later under the low power microscope.

Again the activity of the virus in tissue culture was at all times compared and confirmed by mouse inoculation both intracerebrally in adult Swiss mice and intraperitoneally in 12 to 14 day old unweaned mice (Tables VIII and IX).

In Table VIII, it can be seen that the amount of neutralization obtained by the tissue culture method (1,000,000 neutralizing doses) is 200 times greater than that observed by intracerebral mouse inoculation (5,000 neutralizing doses) and is slightly greater than that noted on intraperitoneal inoculation. It is interesting to note that the serum-virus mixture which was neutral by both

TABLE VIII

Comparison of the in Vitro and in Vivo Neutralization Tests of W.E.E. Virus (Present Method)

Serum used	Titrated in		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Neutralizing doses*
Immune	Mice	Intracerebrally	2 3 3 3	2 3 3 3	3 3 3 3	A A A A	A A A A	‡	‡	‡	‡	5,000
		Intraperitoneally	4 A A A	A A A A	A A A A	A A A A	A A A A	‡	‡	‡	‡	750,000
	Tissue culture		0 0 0 0	G G G G	G G G G	G G G G	G G G G	‡	‡	‡	‡	1,000,000
Control	Mice	Intracerebrally	‡	‡	‡	2 2 2 3	2 2 3 3	3 3 3 3	3 4 A A	A A A A	A A A A	
		Intraperitoneally	‡	‡	‡	3 4 4 4	4 4 4 4	4 4 4 5	A A A A	A A A A	A A A A	
	Tissue culture		‡	‡	‡	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	G G G G	G G G G	

Legend as in Table IV except that mice in the intraperitoneal group were observed for 10 days.

* = calculated on 50 per cent end point titres.

‡ = not done.

TABLE IX

Confirmation by Mouse Test of the in Vitro Neutralization Tests of W.E.E. Virus (Present Method)

Serum used	Dilution of virus	Growth of fibroblasts from the explant	Supernatant fluid inoculated into mice: Intracerebrally	Supernatant fluid inoculated into mice: Intraperitoneally
Immune	10^{-1}	0 0 0 0	2 2 2 3	4 4 4 5
	10^{-2}	G G G G	3 3 3 3	A A A A
	10^{-3}	G G G G	A A A A	A A A A
	10^{-4}	G G G G	A A A A	A A A A
	10^{-5}	G G G G	A A A A	A A A A
Control	10^{-4}	0 0 0 0	2 2 3 3	4 4 4 4
	10^{-5}	0 0 0 0	2 2 2 3	4 4 4 5
	10^{-6}	0 0 0 0	2 3 3 3	4 4 4 5
	10^{-7}	0 0 0 0	3 3 3 3	4 4 4 4
	10^{-8}	G G G G	A A A A	A A A A
	10^{-9}	G G G G	A A A A	A A A A

(The three data columns in Table IX sit under the header "Results of neutralization in tissue cultures".)

Legend as in Table IV.

the intraperitoneal mouse inoculation and the tissue culture method was apparently not neutral when inoculated intracerebrally into mice (Tables VIII and IX).

This experiment was repeated 10 times with approximately similar results which are summarized in Table X. Because of the shortage of unweaned mice the intraperitoneal mouse inoculation test was not carried out.

TABLE X

Summary of the Neutralizing Potency Obtained from Tissue Culture Method and from Intracerebral Mouse Inoculation Test

Experiment No.	Titrated in	Neutralizing potency
1	Tissue culture	1,000,000
	Mice	10,000
2	Tissue culture	1,000,000
	Mice	1,000
3	Tissue culture	1,000,000
	Mice	1,000
4	Tissue culture	1,000,000
	Mice	5,000
5	Tissue culture	100,000
	Mice	500
6	Tissue culture	1,000,000
	Mice	10,000
7	Tissue culture	100,000
	Mice	1,000
8	Tissue culture	1,000,000
	Mice	10,000
9	Tissue culture	1,000,000
	Mice	10,000
10	Tissue culture	100,000
	Mice	1,000

The growth and absence of growth of the tissue treated with the different serum-virus mixtures were photographed and are shown in Figs. 1 to 8.

The decrease in the neutralizing potency in the preliminary neutralization test was most probably due to the dilution effect obtained during the process of washing. In order to determine the matter, an experiment was conducted with both types of neutralization carried out at the same time. The results of such a study again showed similar differences in the degree of neutralizing activity.

In the course of the study on *in vitro* neutralization two phenomena were observed. First, it was consistently noted that pieces of tissue in the tubes containing antimeningococcus immune horse serum and virus tended to coalesce when care was not taken to separate them before or during the incubation. On the other hand, pieces of tissue in the tubes containing anti-W.E.E. immune horse serum and virus remained separated. Second, when these pieces of tissue were plasma-patched, the tissue, if not infected, grew much more readily when it was obtained from the tubes containing antimeningococcus immune horse serum than when it was derived from the tubes containing anti-W.E.E. immune horse serum. These discrepancies were later found to be due to the presence of anti-chick embryo tissue components in the anti-W.E.E. immune horse serum. Evidently, the immunization of the horse against W.E.E. virus was performed with infected chick embryo tissue.

The question then arose whether or not the sensitizing reaction of the tissue had any influence on the neutralizing potency of the W.E.E. virus. An *in vitro* neutralization experiment was, therefore, performed with the anti-W.E.E. immune horse serum having the anti-chick embryo tissue components previously absorbed by the chick embryo tissue. The absorption was carried out as follows: To 4 cc. of the serum, minced tissue from three 10 day developing chick embryos was added. The mixture was incubated at 37.5°C. for 1 hour and then placed in the ice box overnight. A similar test with anti-W.E.E. immune horse serum unabsorbed by the chick embryo tissue was carried out at the same time for comparison. The results of such a study showed no difference in the neutralizing potency of the two sera. This experiment was repeated and similar results were obtained.

DISCUSSION

Titration of virus potency and demonstration of viral neutralizing antibodies are procedures not only involving large numbers of animals but also presenting disadvantages which make quantitative interpretation difficult. The present study of titration and neutralization of the virus in tissue culture suggests a simplified approach and in particular removes the variable of individual animal reactivity.

The *in vitro* titration and neutralization of the W.E.E. virus by means of tissue culture appears to be a feasible procedure. It is hardly necessary to speak of the economy of the method since one chick embryo can be used for a complete titration experiment. All procedures are quite simple and can be applied to mass study. It is estimated that the actual time consumed in a titration experiment is about $1\frac{1}{2}$-hour and in a neutralization test it is about 2 hours.

The tube method of titration has not been perfected. However, it seems worthwhile to present the incomplete study since the method might be found useful when homologous plasma for the growth of tissue is not available.

Feller, Enders, and Weller (1) found no difference in the growth of tissue in infected and non-infected cultures of vaccinia virus but if the W.E.E. virus is substituted for vaccinia cessation of tissue growth occurs when the tissue is sufficiently infected. It is felt that the difference in the effect of the two viruses on chick embryo tissue lies in the degree of susceptibility of the cells to the virus, or perhaps in a difference of cellular reaction to the presence of virus. Thus, in fertilized egg preparations, vaccinia infection produces a local chorioallantoic lesion, whereas the W.E.E. virus produces death of the embryo within 24 hours. On the basis of these findings it would be interesting to repeat the *in vitro* and *in vivo* titrations with other viruses that have similar effects on embryos, *e.g.* Eastern equine encephalomyelitis virus, fowl plague, and Newcastle disease.

If a highly susceptible tissue be used, not necessarily chick embryo tissue, it is possible that other viruses might be similarly employed in the *in vitro* titration. For instance, if the tissue from embryonic mouse were used, many other viruses might conceivably be titrated *in vitro*, *e.g.* St. Louis encephalitis virus, Japanese encephalitis virus, lymphocytic choriomeningitis virus, acute keratoconjunctivitis virus, Jungeblut-Sanders mouse virus, etc. In this connection, it seems opportune, at this time, to mention preliminary successful *in vitro* titration experiments on both the St. Louis encephalitis virus and the Jungeblut-Sanders mouse virus by the use of embryonic mouse tissue. These studies will be reported in a separate paper.

It is felt that if human tissue were employed for the study, the field of investigation might be further enlarged to include virus infections which have not been thoroughly studied owing to difficulty of transmission to experimental hosts (*e.g.* measles, chickenpox, and trachoma). In addition the possibility may also be considered that the status of human infectious diseases of suspected virus etiology might be clarified by the use of such methods as have been presented here (*e.g.* infectious mononucleosis and epidemic encephalitis lethargica). This possibility has already been pointed out by Goodpasture and his coworkers (20–23) following upon their successful attempts to graft human skin on the chorio-allantoic membrane of the developing chick embryo.

It is further suggested that the method hereby presented may possibly be applied to the titration and neutralization of toxin and antitoxin of bacterial origin and to the evaluation and standardization of the toxicity of biological and chemical products.

A number of viruses in neutral mixtures have been found in animal experiments to be reactivated by simple dilution with 0.85 per cent saline solution (8–19). This dilution phenomenon is also demonstrated in the *in vitro* neutralization tests described above. There is however one point which requires further comment. In animal experiments (8–19), the reactivation of virus was demonstrated when the dilution of the serum-virus mixture was as

low as 1:10. Whereas when the present method of *in vitro* neutralization test is employed, there is hardly any noticeable dilution effect even though the dilution of the serum-virus mixture in the UF-X_6 medium used is as high as 1:15 (Table VIII). It is felt that this discrepancy can be explained on the basis that a similar dilution, or an even higher dilution occurs in the blood and body fluid before the brain is reached, when the neutral mixture is inoculated intraperitoneally. In other words, the so called just neutral mixture (*i.e.* the highest concentration of virus that can be neutralized by the same amount of immune serum) based on the animal inoculation test is in a sense an over-neutral mixture before injection. In this connection, it is interesting to point out the possible dilution effect of bacterial toxin and antitoxin mixtures by the blood and body fluid of animals. Otto and Sachs (24) working on mixtures of botulism toxin and antitoxin found that a considerably higher toxicity is noticeable when the mixture is injected intravenously instead of subcutaneously. It seems likely that this phenomenon is due to an immediate dilution effect by the blood when the mixture is injected intravenously. This phenomenon has also been described in the case of diphtheria toxin-antitoxin mixtures. Glenny (25) in this connection states that "it is possible to produce a mixture of toxin and antitoxin causing. . . . no edema when injected subcutaneously into guinea pigs, but after absorption from the site of injection sufficient toxin becomes free to kill the animal in 5 days."

The results of the *in vitro* titration also suggest that tissue culture may be a more sensitive method for the detection of the virus. A dilution of the virus which was innocuous when injected intracerebrally into mice was detected in the tissue culture. This study confirms the observation of Sanders (26) who found that keratoconjunctivitis virus was more readily isolated in tissue culture than in the animal. The superiority of tissue culture over animal inoculation in the detection of viruses can be easily appreciated if one remembers that in tissue cultures we are dealing with tissue and virus, while in animals we are dealing not only with tissue and virus but also with natural immunity. Sanders (27) has pointed out another fact which may account for the superiority of tissue culture over animal inoculation in the primary isolation and detection of viruses. The amount of material that can be inoculated into a mouse by the intracerebral route is limited to 0.03 to 0.05 cc. On the other hand, a large amount of material can be introduced into a tissue culture. If the amount of the supernatant fluid is large the amount of tissue employed can be proportionally increased. The possibility of bacterial contamination in the tissue culture can be lessened if small amounts of sulfadiazene are added. Preliminary experiments by Sanders, Simms, and myself have shown that the addition of sulfadiazene does not interfere with the growth of the W.E.E. virus.

It remains unexplained why there is a difference between the neutralizing potency obtained when the serum-virus mixture was injected into the peri-

toneal cavity and when the mixture was inoculated into the brain of mice. Can this be due to a difference in the behavior of the nervous and non-nervous cells? It is felt that a comparison of the titration activity and neutralization potency of W.E.E. virus when associated with fibroblasts and brain cells respectively *in vitro* might shed some light on this matter.

SUMMARY

1. Titration and neutralization of the Western strain of equine encephalomyelitis virus can be carried out *in vitro* by means of tissue culture.

2. The *in vitro* titration test as presented is a more sensitive method than animal inoculation.

3. Tissue culture may be better than animal inoculation for the detection of small amounts of virus.

4. The neutralization obtained in tissue culture is 100 to 1,000 times greater than that observed in the intracerebral test in mice and is comparable to the potency obtained by the intraperitoneal route of inoculation.

5. The possibility of applying this method to the study of other viruses, known and unknown, is discussed.

6. Further applications of the method described above to evaluation and standardization of bacterial toxins and antitoxins and to the testing of the toxicity of biological and chemical products are suggested.

7. Evidence for the reactivation *in vitro* as well as *in vivo* of virus in neutral mixtures by dilution is presented.

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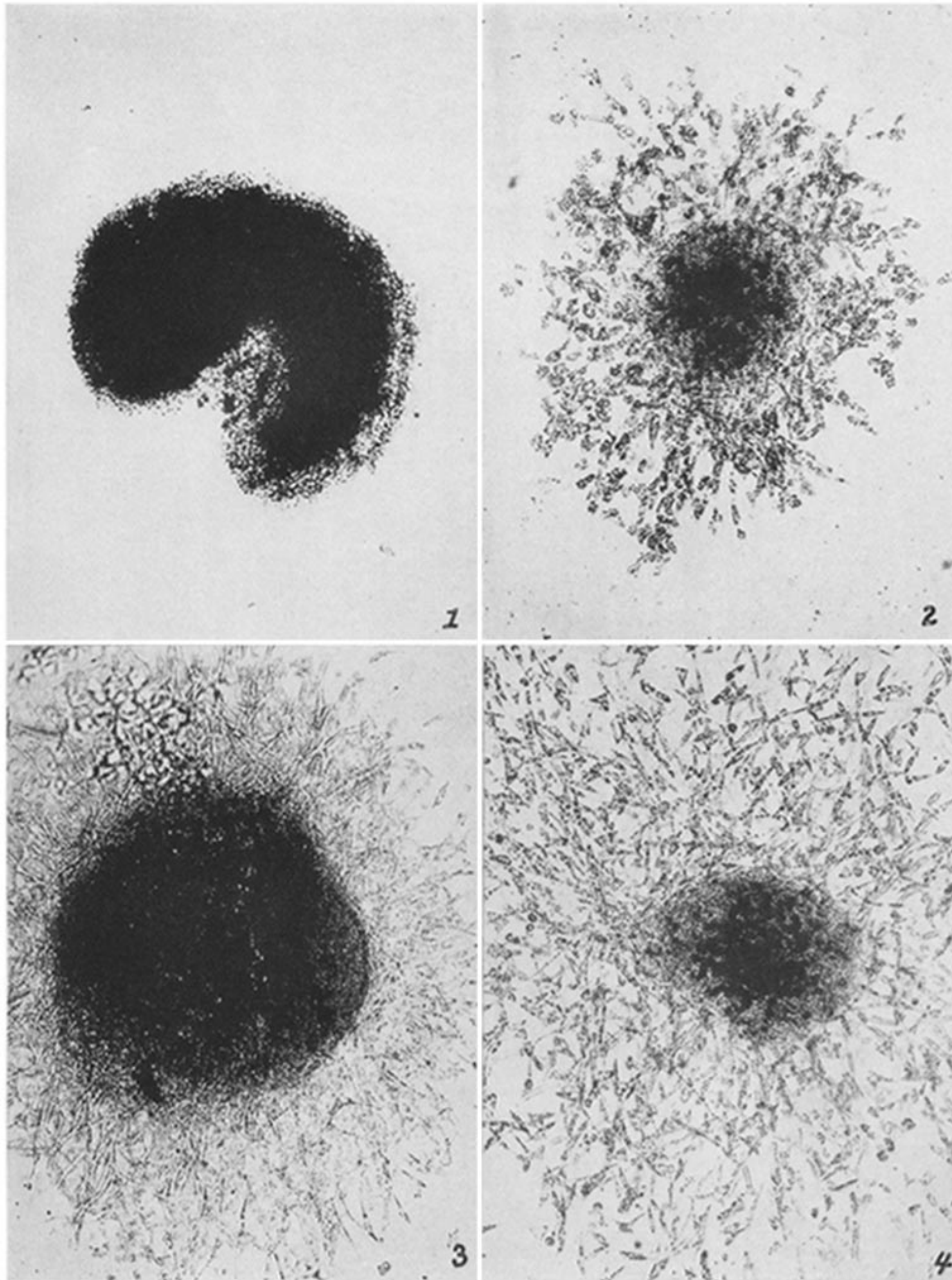
EXPLANATION OF PLATES

Figs. 1 to 8 show growth or absence of growth of tissue in a plasma patch. The tissue was derived from cultures infected with different mixtures of serum and W. E. E. virus as indicated below. Approximately × 55 in all figures. These photographs were taken of explants without stains or fixatives.

PLATE 1

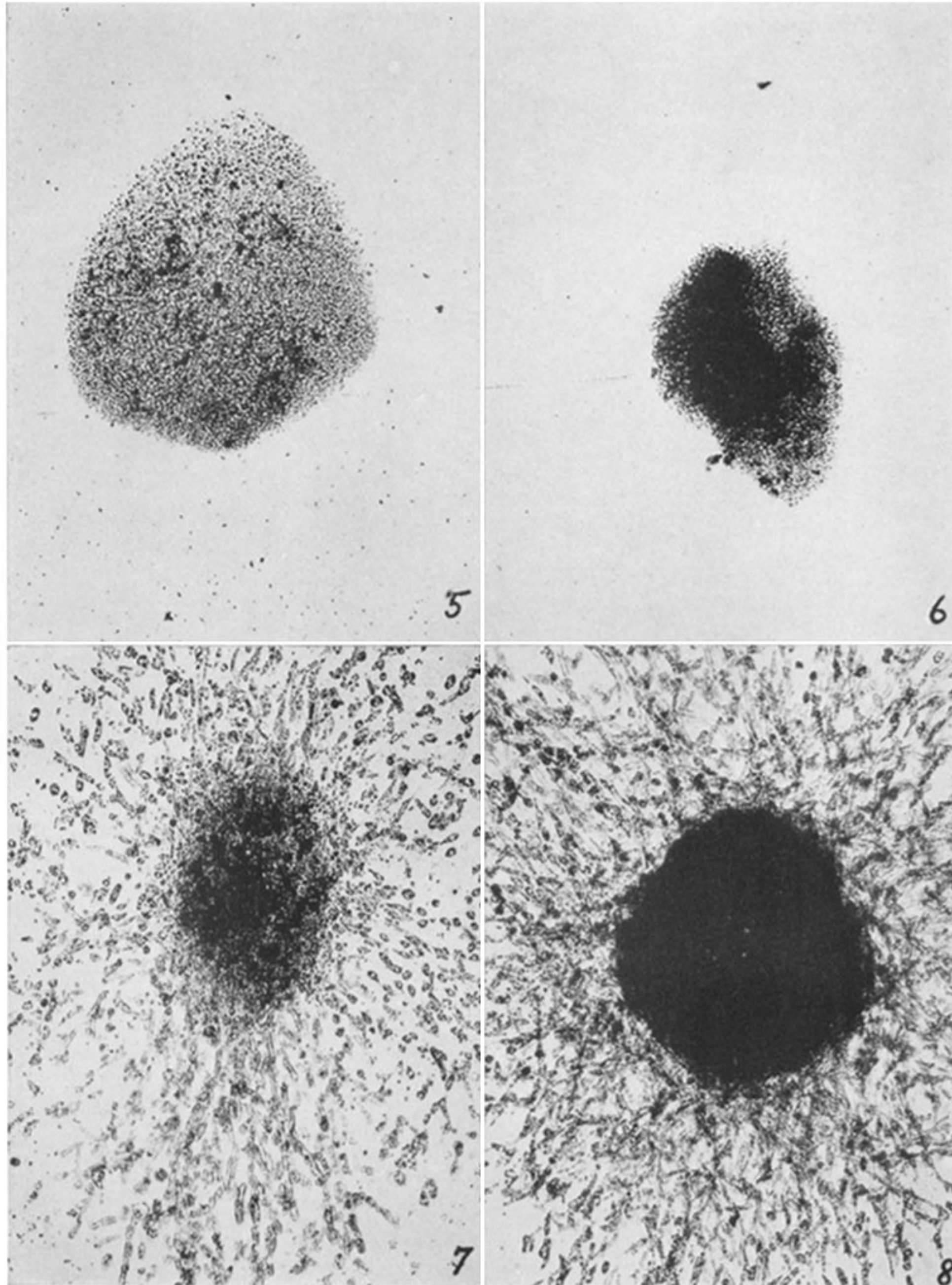
FIG. 1. Immune serum and 10^{-1} virus = no growth.
FIG. 2. Immune serum and 10^{-2} virus = growth.
FIG. 3. Immune serum and 10^{-3} virus = growth.
FIG. 4. Immune serum and 10^{-4} virus = growth.



(Huang: Neutralization test of W.E.E. virus in tissue culture)

PLATE 2

FIG. 5. Control serum and 10^{-6} virus = no growth.
FIG. 6. Control serum and 10^{-7} virus = no growth.
FIG. 7. Control serum and 10^{-8} virus = growth.
FIG. 8. Control serum and 10^{-9} virus = growth.



(Huang: Neutralization test of W.E.E. virus in tissue culture)