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STUDIES ON THE CULTIVATION OF POLIOMYELITIS VIRUSES IN TISSUE CULTURE

I. The Propagation of Poliomyelitis Viruses in Suspended Cell Cultures of Various Human Tissues[1]

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I. INTRODUCTION

As more fully reviewed in the preface to this series of papers (1), various workers have attempted in the past to obtain evidence for the *in vitro* multiplication of the agent of poliomyelitis. This paper is concerned with a presentation of the procedures for the cultivation of the poliomyelitis viruses in suspended cell cultures as employed in this laboratory, and with a description of representative experiments on the behavior of these viruses in such cultures as observed during the past four years. The basic technique here described was developed in connection with studies on the *in vitro* cultivation of mumps virus (2). The observation that a simple modification of the conventional culture procedure, consisting of the replacement of the nutrient fluids at 3 to 4-day intervals with infrequent passages to new cultures, would permit demonstration of the multiplication of this agent, suggested that the technique be applied to other viruses, as yet not cultivable. Attempts were first made to isolate the agent of varicella in suspended cell cultures of human embryonic skin, fascia, and muscle tissue (3). The accumulating indications that poliomyelitis virus may multiply in extraneural tissues *in vivo*, led to the inoculation of available skin-muscle cultures with the Lansing strain. It soon became apparent that such extraneural tissues did, in fact, support the growth of poliomyelitis virus (4). Investigations were then carried out with suspended cell cultures of human embryonic intestine and brain tissue and mature preputial tissue (5) and subsequently expanded as herein outlined.

II. MATERIALS AND METHODS

A. Strains of poliomyelitis virus.

The Lansing, Brunhilde and Leon strains have been employed as representatives of the three known antigenic types (6).

Two lines of the Lansing strain have been studied. One of these, and that on which most of the observations have been made, was obtained in 1943 from Dr. Sidney Kramer,

[1] This study was aided by a grant from The National Foundation for Infantile Paralysis, Inc.

who had maintained it in his laboratory for at least 3½ years. In his hands the LD_{50} of infected mouse cord-medulla suspensions for 10-g mice proved to be constant at approximately 10^{-3}. We have subjected this strain to 11 mouse passages. Inocula for tissue cultures were derived from 6 pools of mouse brains. The mouse LD_{50} of these pools has varied between $10^{-2.7}$ and $10^{-3.7}$. The other line, which has exhibited the same degree of mouse pathogenicity, was obtained from Dr. Charles Armstrong in 1948 and has since been passed serially 6 times in mice.

The Brunhilde strain was supplied by Dr. Howard Howe in 1948 as a suspension of monkey cord. We have passed this virus twice in rhesus monkeys.

The Leon strain was received from Dr. Jonas Salk in 1950 as a suspension of monkey cord. This material has been used as the original inoculum for tissue cultures.

B. Preparation and storage of infected materials

The Lansing viruses used as primary inocula for tissue cultures were prepared as 10% suspensions in isotonic phosphate buffer solution[2] of pools of infected mouse brains. When contaminating bacteria were shown to be absent by appropriate procedures, aliquots were preserved in the CO_2 cabinet in glass-sealed vials. Stock 10% suspensions of monkey cord infected with the Brunhilde and Leon viruses were treated in the same manner. Dilutions of virus were usually prepared in buffer solution.

TABLE I

Summary of mouse infectivity titers (LD_{50}) of Lansing tissue-culture virus after storage under various conditions

PERIOD OF STORAGE	STORED AT −15–20 C		STORED IN CO_2 CABINET	
	HS	HS + milk	HS	HS + milk
0 days	$10^{-2.6}$	$10^{-2.3}$	$10^{-2.6}$	$10^{-2.3}$
12 days	$10^{-2.4}$	$10^{-2.3}$	$10^{-2.0}$	$10^{-2.5}$
7 months	$10^{-2.0}$	$10^{-2.4}$	$10^{-2.4}$	$10^{-2.6}$
17 months	$10^{-1.6}$	$10^{-2.1}$	$10^{-1.7}$	$10^{-2.0}$

Fluids from tissue cultures were preserved in one of two ways. In most instances those reserved as inocula for subsequent passages were sealed in Pyrex glass ampules and stored in the CO_2 cabinet. Fluids to be used for other purposes were placed in Erlenmeyer flasks or test tubes, which were plugged with cotton, sealed with Parafilm or with a rubber stopper superimposed over the cotton plug, and kept at −15 to −20 C.

Data have been obtained in respect to the stability of Lansing tissue culture virus[3] under these conditions of storage and in respect to the stabilizing effect of milk under the same conditions when added to the virus. Aliquots of the virus suspension were diluted with equal parts of HS medium (see below) or skimmed milk,[4] sealed in glass ampules, and stored either at −20 C or in the CO_2 cabinet. At intervals the infectious titer of these materials was determined in mice. The results are summarized in Table I. After 7 months

[2] A stock buffer solution is prepared by dissolving 55.2 g $NaH_2PO_4 \cdot H_2O$, 58.5 g NaCl, and 12.2 g NaOH in 1 l of distilled water; the working solution is prepared by adding 9 parts of distilled water to 1 part of this stock solution. Both solutions are sterilized in the autoclave. The pH of the working solution (7.1–7.2) should be checked at the time of its preparation.

[3] In this and other papers in this series poliomyelitis virus propagated in tissue culture will be referred to as "tissue culture virus."

[4] Prepared by autoclaving 10 minutes at 10-lb pressure; the pH is then adjusted to 7.0 with NaOH and HCl.

no significant decrease in infectivity under any of the conditions was observed. During the ensuing 10 months the infectivity of the virus preserved in HS (Hanks-Simms medium) appeared to have diminished somewhat, as compared with that stored in milk. In no instance, however, did the maximal decrease in titer exceed 1 log.

C. Methods for the detection and assay of viral activity

1. *The mouse.* Titration of the content of Lansing virus in pooled mouse brains or in the supernatant fluids removed from cultures was carried out by the intracerebral inoculation of etherized white mice with 0.03 ml of tenfold dilutions in isotonic phosphate buffer. It should be emphasized that in determinations of the virus content of tissue culture fluids by any of the techniques we have employed, such fluids had been previously centrifuged as described below. White Swiss mice, approximately 21 days old, obtained from the Harvard Medical School colony, were used throughout. Groups of 6 mice were inoculated with each dilution and then observed daily for 30 days for signs of paralysis or death. The LD_{50} was calculated according to the method of Reed and Muench (7).

2. *The monkey.* Monkeys were anesthetized with sodium pentobarbital injected intraperitoneally and a blood specimen was taken from the femoral vein. The inoculum in two divided doses totaling 1 ml was introduced into the brain as described by Bodian (8). During a period of 30 days monkeys were observed daily and the rectal temperature was usually recorded. Animals that became paralyzed were bled, and killed by injection of ether into the heart. As routine, the spinal cord was removed and portions from 4 to 6 levels were fixed in 10% formalin. Sections were stained with hematoxylin and eosin. Occasionally, tissues from the motor area of the cerebrum were likewise examined. The spinal cords of those animals alive at the end of 30 days were also examined histologically.

3. *The tissue culture.* It has been shown that poliomyelitis virus may exert *in vitro* a cytopathogenic effect on the cells that support its multiplication. This phenomenon can be recognized in at least three ways that have been described elsewhere (9). These are:

a. The failure of infected tissues to maintain a metabolic rate, as measured by acid production, comparable to that of uninfected control tissues. The difference in acid production can be discerned by comparing the pH of the medium of infected and uninfected cultures during the period of cultivation. A difference of 0.2 pH unit or greater is considered to be significant. For purposes of convenience this procedure is hereafter referred to as the "pH-differential test."

b. The failure of infected tissue fragments to develop a characteristic outgrowth of cells when explanted from flask to plasma hanging-drop cultures. This is referred to as the "explant test."

c. The degeneration of new cell outgrowth in infected roller-tube cultures as multiplication of the virus proceeds.

All three methods of detecting the cytopathogenic effect have been employed in this study as means of determining the presence of virus in various materials. They have also been applied to the titration of infectivity. The titers so determined have been expressed as 50% infectivity endpoints for the tissue culture (ID_{50}) as described in the third paper of this series (10).

D. Technique of suspended cell culture

1. *Glassware and other apparatus.* Pyrex glassware is employed except for pipettes. In the experiments described in this series of papers, three different methods of cleaning glassware have been used: a. treatment with sulfuric acid-potassium dichromate cleaning fluid; b. washing with a detergent; c. boiling in soap solution. The last procedure has now been adopted as routine and is carried out in the following manner.[5] A stock 15% solution of Ivory soap is made by boiling soap fragments in water. When it is cooled a gel is formed.

[5] This method may not be satisfactory in areas where the water supply has a high mineral content.

For use, this material is diluted to yield a 0.5% soap solution. Contaminated glassware is autoclaved, and scrubbed with a brush. It is then placed in soap solution and boiled for 15 minutes. While still hot the pieces are rinsed individually in 15 changes of *hot* tap water and then 5 times with distilled water. New glassware is soaked for 1 hour in 25% sulfuric acid, rinsed 10 times in tap water, and then boiled in the soap solution and treated as described above. All glassware is dried by draining in the inverted position, plugged with gauze-covered stoppers made from bleached absorbent cotton, sterilized in the autoclave at 15-lb pressure for 30 minutes, and redried under reduced pressure in the autoclave.

Capillary pipettes. These are manufactured from 8-inch lengths of 8-mm soft glass tubing, which are treated as new glassware, plugged with cotton at both ends, autoclaved and stored in cans. Pipettes made from this tubing are used once and discarded.

Rubber stoppers. New black rubber stoppers are boiled for 15 minutes in N/2 NaOH, rinsed thoroughly in tap water, boiled in 4% HCl, again rinsed in tap water and finally in 5 changes of distilled water. Used stoppers are scrubbed and washed with water. Rubber stoppers fabricated from a special nontoxic stock[6] have recently been employed, a substitution that appears to be desirable (11).

2. *Preparation of medium.* The standard medium, hereafter referred to as "HS" (Hanks-Simms) used in suspended cell cultures consists essentially of Hanks' balanced salt solution (12) and Simms' ox-serum ultrafiltrate (13).

Hanks' balanced salt solution. All water employed is twice distilled. The second distillation is carried out in a glass still. Salts of analytical reagent grade are employed; at present, sodium chloride analytical reagent grade for biological work (Merck) is being used. Stock solutions, A and B, are prepared.

Solution A. Weigh out, 160 g NaCl; 8 g KCl; 2 g $MgSO_4 \cdot 7H_2O$; and 2 g $MgCl_2 \cdot 6H_2O$.[7] Dissolve these salts in approximately 800 ml H_2O. Dissolve 2.8 g $CaCl_2$ in approximately 100 ml H_2O. Combine these two solutions and make up to 1000 ml with H_2O. Add 2 ml chloroform as a preservative, stopper, and store at 5 C. *Solution B.* Weigh out, 3.04 g $Na_2HPO_4 \cdot 12H_2O$; 1.2 g KH_2PO_4, and 20 g dextrose and dissolve in approximately 800 ml H_2O. Add 100 ml 0.4% phenol red solution[8] and make up to 1000 ml with H_2O. Add 2 ml chloroform, stopper, and store at 5 C. Hanks' balanced salt solution is prepared by adding 1 volume of stock solution A and 1 volume of stock solution B to 18 volumes of H_2O. It is then autoclaved at 9-lb pressure for 10 minutes. This solution is kept at 5 C. Immediately before use, 0.5 ml of sterile 1.4% $NaHCO_3$ solution[9] is added to each 20 ml of Hanks' solution.

Ox-serum ultrafiltrate. The other major component of the medium, ox-serum ultrafiltrate,[10] is furnished in 100-ml ampules under an atmosphere of 5% CO_2; unused portions are preserved at 5 C in stoppered flasks after the introduction of 5% CO_2 in air.

Composition of the standard medium (HS). One part of ox-serum ultrafiltrate is combined with 3 parts of Hanks' balanced salt solution. For each ml of this mixture, 50 units of penicillin and 50 μg of streptomycin base are then added to prevent bacterial contamination. As routine, HS has been prepared on the day it is used. The antibiotics are diluted either with normal saline solution or distilled water. At present, crystalline penicillin G

[6] Supplied by The West Co., Phoenixville, Pa. of Stock #124.

[7] Hanks now recommends the use of 4 g $MgSO_4 \cdot 7H_2O$, and omits $MgCl_2$ (Proc. Soc. Exp. Biol. & Med. **71**: 196, 1949); we have had no experience with this modification.

[8] The phenol red indicator is prepared as follows. One gram of phenol red is placed in a flask and N/20 NaOH is added slowly and with agitation until the powder is almost dissolved. Dropwise addition of NaOH is then continued until solution is complete at which time the color is a deep red. Sufficient distilled H_2O is added to bring the volume to 250 ml. Addition of excess NaOH gives a purple solution that is not satisfactory.

[9] The bicarbonate solution is sterilized by autoclaving for 10 minutes at 9 lbs. pressure and stored in the ice box.

[10] May be procured from Microbiological Associates, Bethesda, Maryland.

potassium is used, diluted to a concentration of 10,000 units per ml. This concentrated solution is stored at −15 C for an interval not exceeding 1 week. Streptomycin is now employed as the calcium chloride complex, diluted to a concentration of 50,000 μg per ml. The concentrated solution is kept at 5 C for a period not exceeding 1 month.

Modifications of the medium. Various modifications of this basic medium have been made in attempts to improve the yield of virus. None has proved of value. These have included: a. incorporation of 0.2% human albumin in the HS medium; b. the addition of plasma according to the technique of Plotz (14); c. the addition of 5% inactivated (55–56 C) normal horse serum; d. the addition of 20% inactivated horse serum and 20% beef embryo extract; e. substitution for the HS medium of the complex medium developed for the cultivation of malaria parasites (15). It is also of interest to record that, in one experiment, the omission of the ox-serum ultrafiltrate from the basic medium resulted in the apparent failure of virus growth.

3. *General procedures for maintaining sterile technique.* The advent of antibiotics has eliminated the need for many of the precautions to exclude bacterial contamination formerly essential in tissue culture work. All manipulations, however, are performed in a chamber or "hood." Before use, the hood is wiped out, a towel wet with lysol placed on the work surface, and the interior of the chamber "steamed" for about 10 minutes with the door closed. Mycotic or bacterial contaminants have been encountered only rarely when this simple procedure has been followed and antibiotics have been included in the medium.

4. *Tissues.*

a. *Preparation of different types of tissue.* A variety of tissues from man and from other mammals have been used.

Human embryonic tissues. This material was employed in most of the experiments. It was obtained under sterile precautions at the time of abdominal hysterotomy for therapeutic indications. Embryos of between 12 and 18 weeks gestation have been utilized. Rarely tissues were obtained from stillborn fetuses, or from premature infants at autopsy.

In the experiments on prolonged propagation of virus three sorts of embryonic materials were used: elements of skin, connective tissue, and muscle; intestinal tissue; brain tissue. Embryonic tissues were prepared in the following manner. Whenever possible the embryo was removed from the amniotic sac under sterile precautions, transferred to a sterile towel and kept at 5 C until dissected; usually dissection was carried out 1 to 3 hours after hysterotomy, but on one occasion virus was successfully propagated in tissues from an embryo that had been stored *in toto* for 24 hours at 5 C.

The skin, muscle, and connective tissues, hereafter referred to as "skin-muscle," employed in these experiments were dissected from the arms and legs. It was considered, therefore, that such tissues did not contain intact nerve cells. Depending upon the size of the embryo, either all of the brain, or an unselected portion thereof, was used. Similarly, in the case of small embryos the entire intestine was minced, while in the case of larger specimens only a portion was employed. No attempt was made to eliminate the meconium.

Mincing of tissues. Tissues whether embryonic or of other origin are cut into large pieces (±1 cm^2) and transferred in amounts not exceeding 10 g to a 50-ml round-bottom centrifuge tube for mincing. A small quantity of HS medium is added to prevent dessication of the tissues during mincing. Mincing is accomplished by repeated cutting with long-handled scissors (Simm's uterine scissors) until the pieces are reduced to approximately 1 to 2 mm in diameter. A volume of HS medium equivalent to that of the minced tissue is then added. Cultures are usually prepared as soon as mincing is completed. The minced tissue can, however, be preserved for varying periods in the manner described subsequently.

Nonembryonic human tissues. Propagation of virus has also been accomplished in several types of nonembryonic human tissues. Preputial tissues were obtained from individuals ranging in age between 3 months and 11 years. Circumcision was performed by the dorsal slit method following preparation of the skin with tincture of green soap and zephiran. Twenty specimens were used; all yielded cultures that were characterized by active metabolism and were not contaminated with bacteria. Our limited experience with prepuces obtained from newborn infants by the Gomco clamp technique makes it impossible to state whether they are also satisfactory. The prepuce was covered with sterile moist gauze and kept at 5 C until it was minced, a procedure usually carried out within 4 hours of removal. The ring of tissue was opened, washed in 3 changes of HS medium each consisting of about 15 ml, and then minced. In the case of tissues, including foreskin, known to be contaminated with bacteria, the antibiotic content of the medium used for storage as well as that first added to the cultures was increased to 100 units of penicillin and 100 μg of streptomycin base per ml.

Normal kidney tissue from children ranging in age between 1 and 9 months was obtained as a byproduct of the operation of uretero-arachnoid anastomosis for communicating hydrocephalus (16). Portions of the kidney cortex and medulla were minced within a few hours of removal. Since the resulting tissue suspension was usually very turbid, the minced tissue was washed 3 or 4 times with several volumes of HS. Occasionally, kidney tissue was obtained at autopsy from children between the ages of 1 week and 7 months. Three specimens of this sort have provided satisfactory cultures; four other kidneys, however, were contaminated with bacteria that proved resistant to the antibiotics. In recent experiments a Fischer press (17) has at times supplanted the use of scissors for mincing kidney tissue.

Other types of human tissues obtained at operation have been investigated. Fragments of thyroid, testis, and uterus were prepared in the manner already described. In single experiments tissues from an embryoma and from a neuroblastoma were employed. Others were carried out with placental tissue.

The capacity of the following tissues of nonhuman origin to support multiplication of virus was also investigated: embryonic chick skin-muscle and brain; adult and embryonic mouse skin-muscle, brain and intestine; beef embryonic skin-muscle; rabbit kidney; and monkey kidney and testis.

b. *Storage of tissue.* After mincing with scissors or passage through the Fischer press, fragments are placed in 250-ml Erlenmeyer flasks containing 20 ml of HS medium. Sufficient tissue is added to cover the bottom of the flask with a single layer of fragments. The flasks are tightly fitted with rubber stoppers and kept at about 5 C. Virus may be propagated in cultures prepared with tissues kept for periods as long as 19 days under these conditions.

It has also been found feasible to store tissues in the form of finished cultures. Suspended cell cultures of embryonic skin-muscle prepared with HS containing 0.2% human albumin and stored at 5 C for as long as 28 days have subsequently supported the multiplication of Lansing virus. Fragments removed from these cultures at intervals during the period of storage and explanted in plasma drops exhibited cell migration—a fact indicating that cell viability was maintained during this extended period.

5. *Preparation of cultures.* As routine, suspended cell cultures are prepared in 25-ml Erlenmeyer flasks. Three ml of HS is added to each of a series of flasks which are then loosely closed with #0 rubber stoppers. With a large bore capillary pipette ($\pm$3 mm internal diameter), 3 or 4 drops of the minced tissue suspension are placed in each flask. With practice the number of fragments added can be kept fairly uniform by inspection. The flasks are agitated to distribute the tissue in the medium, and *tightly* stoppered. Unless the flasks are tightly stoppered, the pH of the medium of individual cultures inoculated with the same material may vary widely. Cultures are incubated at 35 C. After 1 to 4 days the first change of HS medium is made and the virus inoculum is added. This procedure was

adopted with the thought that it might eliminate traces of extracellular antibody specific for poliomyelitis virus. In each experiment a minimum of 3, and usually 4, cultures were inoculated with aliquots of a given suspension of virus. Three or 4 cultures to which virus was not added were always included as controls.

6. *Maintenance of cultures.* Cultures are maintained for periods varying from 2 weeks to 1 month by changing the medium at intervals of 3, 4, or 5 days. This is accomplished by removing the medium as completely as possible with a capillary pipette after the tissue has settled. A new pipette is used for each culture. It has been determined that the volume of residual medium is somewhat less than 0.2 ml on the average. Three ml of fresh HS are then added to each flask, using a clean serologic pipette for each set of comparable cultures, e.g., cultures inoculated with the same dilution of virus.

Between changes of the fluid phase the metabolic activity of the tissue is reflected by the rapidity with which the pH of the medium declines. Immediately after the cultures are prepared the pH is usually about 7.4. In the presence of actively metabolizing tissue, the pH falls to 6.8 or lower during the ensuing 3 or 4 days. The pH of each culture is recorded just before the stopper is removed prior to each change of medium.

To follow changes in pH colorimetrically a series of KH_2PO_4-NaOH buffers are prepared, covering the range from 6.8 to 7.6 with an interval of 0.2 pH unit. The pH of each buffer is checked with a pH meter and phenol red is added in a concentration of 0.002%. These standards are sealed in 3-ml amounts in 25-ml Erlenmeyer flasks by drawing out the necks in a gas-oxygen burner. The pH of cultures is estimated by direct comparison with these standards.

7. *Passage of virus in tissue cultures.* Certain of the strains were propagated serially in tissue cultures for many months. Serial passage is effected by transferring at intervals 0.1 ml of the pooled supernatant fluids removed from one group of flasks to each of a set of newly prepared cultures. The frequency with which passage can be made depends therefore upon the availability of tissue. When tissues are not available, the fluid to be used as inoculum is temporarily stored.

The inocula for all passages are rendered essentially free of cells by centrifugation at 2000 rpm for 15 minutes in an International No. 2 centrifuge equipped with a 250A head. The supernatant fluid is removed with a capillary pipette. Samples of all fluids used as inocula are cultured in thioglycollate broth.

III. EXPERIMENTAL

Multiplication of Poliomyelitis Viruses During a Single Passage

Evidence for the multiplication of a virus in tissue culture is provided by the demonstration in the *same* culture of a significant increase in the quantity of the virus. Such evidence has been obtained in the case of poliomyelitis viruses and will be presented first. Subsequently data will be described which indicate that these viruses may be propagated through many serial passages in tissue culture.

A. Multiplication of the Lansing strain

1. *In human embryonic skin-muscle.* The details of a typical experiment with the Lansing strain will illustrate the procedures generally employed and will show that multiplication of this agent occurred in a single set of comparable cultures.

Exp. 7, Lansing virus. Cultures of human embryonic skin-muscle tissue were prepared and incubated at 35 C. Forty-eight hours later the fluids were changed. Four of these cultures were then each inoculated with 0.1 ml of the fluid removed on the 16th day from the preceding passage of the Lansing strain in the same type

of culture. The mouse infectivity titer of this inoculum was $10^{-0.5}$. To each of 4 additional cultures was added 0.1 ml of the pooled fluids from the control cultures included in the preceding passage. The fluids were changed at 4-day intervals and the cultures maintained for 20 days. The mouse infectivity titers of the pooled fluids removed every 4 days from the flasks inoculated with virus were determined.

The mouse infectivity titers of the pooled fluids thus obtained in Exp. 7 are recorded in Fig. 1*A*. In Fig. 1*B* the calculated number of mouse LD_{50} doses per ml of these fluids is given and in addition the number calculated to be present in each ml of fluid at the start of the experiment after addition of the inoculum.

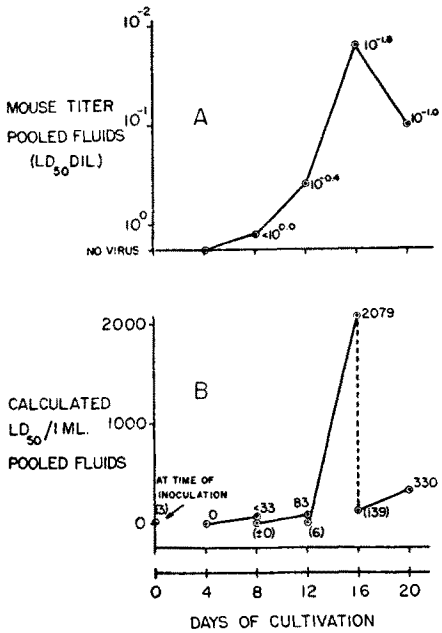


FIG. 1. *Exp. 7.* Mouse infectivity of pools of fluids removed at 4-day intervals from 4 suspended cell cultures of human embryonic skin-muscle tissue inoculated with Lansing tissue culture virus.

It is apparent that virus was not detected in significant quantities until the 12th day following inoculation. During the succeeding 4-day period a marked increase occurred and the peak of virus output as indicated by this method of assay was attained. The data presented in part B also enable one to estimate quantitatively the total amount of virus emerging in the fluid during the 20-day experimental period since they provide information in respect to the quantity of virus removed by the recurrent changes of the medium. As indicated above it was determined that 0.2 ml or less of fluid remains after each withdrawal. Therefore, $\frac{1}{15}$ (or less) of the virus previously present is redistributed in 3 ml of the fresh medium at each change. This reduction in virus content is indicated in part B of fig. 1 by the broken lines. The calculated quantities of residual

virus per ml immediately after the addition of fresh medium are given in parentheses. This residual virus must be deducted in calculating the amount released during any 4-day period. Since 9 LD_{50} were inoculated into each flask and a total of about 6900 LD_{50} were released into the medium during 20 days the increase of viral infectivity was of the order of 770×. Considered in the light of the results of control experiments that will be described, this factor is regarded as sufficient to warrant the conclusion that multiplication of the virus had taken place.

The results of a similar experiment in which infected mouse brain was used as the inoculum indicate that the Lansing strain adapted to the mouse multiplies

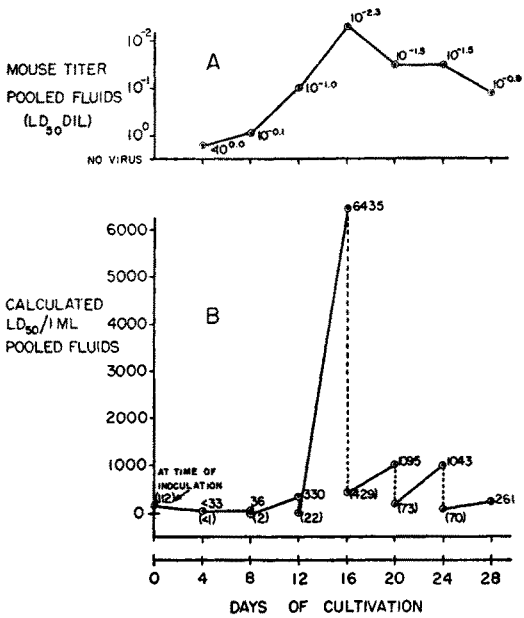


FIG. 2. *Exp. 13.* Mouse infectivity of pools of fluids removed at 4-day intervals from suspended cell cultures of human embryonic skin-muscle tissue inoculated with Lansing mouse brain virus.

in cultures of human tissue in a comparable manner as shown in Fig. 2. The details of experimental procedure were as follows:

> *Exp. 13.* A group of 32 cultures of human embryonic skin-muscle was prepared and incubated at 35 C. Twenty-four hours later the fluid was changed and to each of 16 flasks was added 0.1 ml of a 1:50 dilution of mouse brain suspension (LD_{50} = $10^{-3.7}$). The medium was changed at 4-day intervals. At each change, aliquots of the fluid were pooled and the mouse infectivity titer of the pool was determined. To provide material for studies on tissue changes described elsewhere (10), groups of 4 cultures were removed from the experiment on the 8th, 16th, and 24th days of cultivation.

From the results of the titrations of materials from Exp. 13 which are presented in Fig. 2*A*, it is clear that increase in virus began approximately at the

same time as in the previous experiment and again reached its maximum on the 16th day after inoculation. The calculated total increase in virus based on the data given in Fig. 2*B* was of the order of 77× (i.e., calculated virus content per flask immediately after inoculation = 336 LD_{50}; accumulated virus in supernatant fluids, about 25,800 LD_{50}). This factor of increase is smaller than that noted in the previous experiment. The difference is probably to be attributed to the larger inoculum, since the total yield of virus was greater.

2. *The decline of viral infectivity in the culture medium at 35 C.* In the calculation of the total amount of virus entering the fluid phase the assumption was tacitly made that virtually all of the virus accumulating during a 4-day period remained in the active state and could therefore be assayed by tests for infectivity. This assumption is supported by the results of an experiment to determine the rate of inactivation of the virus in HS medium in the absence of tissue.

Exp. 125. To 1 part of Lansing tissue culture virus 2 parts of HS were added and 3 ml of the mixture were placed in each of four 25-ml flasks. The pH was adjusted to approximately 7.0 with 5% CO_2 in air. The flasks were tightly stoppered and incubated at 35 C. At intervals, 0.15 ml of fluid was withdrawn from each, the pH readjusted with the gas mixture to pH 7, and the flasks resealed and again incubated. The infectivity titers of the pooled fluids were determined in mice. It should be noted that in this experiment, the nutrient fluids were not changed and the frequent readjustment of pH simulated the changes that occur in the tissue cultures. The results are summarized in Table II.

TABLE II

Summary of results of experiment on inactivation of infectivity of Lansing virus at 35 C (Exp. 125)

DAYS INCUBATION	AVERAGE pH OF HS	MOUSE TITER
0	6.9	$10^{-1.4}$
2	7.1	$10^{-1.1}$
4	7.0	$10^{-1.4}$
6	6.9	$10^{0.5}$
9	7.0	$10^{0.6}$
12	ND	$10^{0.0}$

ND = not done.

From these results it is evident that no significant reduction in viral infectivity occurred during the first 4 days. Although it is realized that such conditions are not entirely comparable to those provided by the presence of living cells, these observations suggest that the quantity of active virus recovered at the end of each 4-day period in the experiments described above represented essentially all of the agent that was released into the fluid during that time. As shown in Table II, between the 6th and 9th day the titers were lower and by the 12th day only the undiluted fluid infected mice. This decrease in infectivity contrasts with the increase observed in the cultures containing human tissue and so may

be regarded as indirect evidence that multiplication of virus occurred in the experiments previously described.

3. *Failure of virus to persist in cultures of tissues from certain other species.* The failure to obtain any indication of multiplication of virus in suspended cell cultures of tissues from mice and chick embryos likewise supports the conclusion that the virus multiplied in human embryonic skin-muscle tissue. Seven experiments have been carried out in which mouse embryonic brain, intestine and skin-muscle, adult mouse intestine and chick embryonic skin-muscle were employed in tissue cultures and inoculated with Lansing mouse-brain virus. In none was virus detected in the pooled supernatants after the 4th day. In certain cases tests for virus in the tissues likewise were negative.

4. *The relationship of the size of the inoculum to viral multiplication in human embryonic skin-muscle cultures.* After the fact had been established that the virus could be propagated in cultures of non-nervous tissue, it became of interest to determine a. the relationship between the size of the inoculum and virus

TABLE III

Mouse infectivity titers of fluids removed from groups of human embryonic skin-muscle cultures inoculated with varying dilutions of Lansing tissue-culture virus

DILUTION OF INOCULUM	EFFECT OF INOCULUM IN MICE	TITER OF POOLED FLUIDS					
		Day fluid removed					
		4	8	12	16	20	25
10^{0}	6/6	$10^{0.1}$	$10^{-1.7}$	$10^{-1.7}$	$10^{-1.4}$	$<10^{0.0}$	N.V.*
10^{-1}	4/6	N.V.	$10^{-1.2}$	$10^{-1.4}$	$10^{0.5}$	$10^{0.4}$	N.V.
10^{-2}	1/6	N.V.	N.V.	N.V.	$10^{0.3}$	$10^{0.0}$	N.V
10^{-3}	0/6	N.V.	N.V.	$<10^{0.0}$	$10^{0.0}$	N.V.	N.V.
10^{-4}	0/6	N.V.	N.V.	N.V.	N.V.	N.V.	N.V.

* N.V. = No virus detectable by mouse inoculation.

multiplication and b. the "sensitivity" of the tissue culture as compared with the mouse in the detection of small quantities of virus. The results of the following experiment furnish a certain amount of information on these points, which is in general agreement with other findings that will be subsequently discussed.

Exp. 52. Twenty-four suspended-cell cultures were prepared with skin-muscle tissue from a human embryo. Twenty-four hours later the HS was changed. Tenfold falling dilutions of Lansing tissue-culture virus were prepared in phosphate buffer. Three flasks and 6 mice were then inoculated with each dilution, employing 0.03 ml as the inoculum for both cultures and mice. The mouse infectivity of the pooled fluids removed from each group of cultures at intervals of approximately 4 days was determined.

The data derived from this experiment and summarized in Table III indicate clearly that the time at which virus appears in the fluid of the cultures varies directly with the amount of virus in the inoculum. With small inocula the interval may be as long as 16 days. Furthermore, the maximal yield of virus in

the fluid was obtained with the largest inoculum. In respect to this finding, it is important to note that virus in suspended-cell, as compared with roller-tube, cultures appears to behave differently. In the latter type of culture the yield of virus following inoculation of small quantities usually equals that following the introduction of larger amounts (18). This difference in behavior will be subsequently discussed.

It is also evident from Table III that the tissue culture in this instance was at least comparable to the mouse in its capacity to reveal the presence of small quantities of virus. Thus only one of six mice died following inoculation of the virus diluted 10^{-2}, whereas multiplication of the agent was observed in cultures inoculated with virus diluted 10^{-3}.

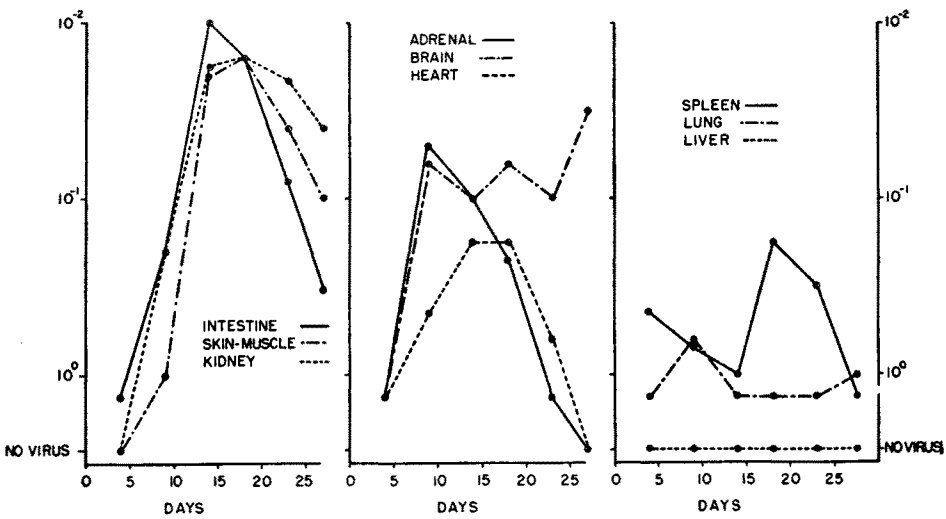


FIG. 3. *Exp. 62.* Multiplication of the Lansing strain in suspended cell cultures of 9 different tissues derived from a single human embryo. Mouse-infectivity titers (LD_{50} dilution) of pools of fluids removed from the cultures at 4-day intervals.

5. *Multiplication of Lansing virus in other types of human embryonic tissue.* The capacity of other human embryonic tissues to support the growth of the Lansing strain in suspended cell cultures has been investigated. In one experiment, tissues of 9 different varieties, all derived from a single embryo were utilized.

Exp. 62. Tissue suspensions were prepared from the intestine, brain, skin-muscle, lung, liver, both adrenals, heart, both kidneys, and spleen. From each, 3 cultures were made, except in the case of spleen tissue which was sufficient for only 2 flasks. After 24 hours' incubation at 35 C, the HS was changed and each culture inoculated with 0.1 ml of a 1:100 dilution of infected mouse brain (mouse titer $10^{-3.5}$). As indicated in Fig. 3, fluids were harvested at intervals for 27 days, the fluids from each set of flasks were pooled, and the mouse infectivity of each pool was determined.

The results presented in Fig. 3 are thought to be representative of the multiplication of mouse-adapted Lansing strain in the various sorts of tissue employed. Except in this experiment, however, relatively few cultures have been prepared with tissues other than skin-muscle, intestine, kidney or brain. These 4 tissues yielded the highest titers. The failure of virus to proliferate in liver tissue may be attributed to the fact that this tissue did not metabolize well. On the other hand, the relative incapacity of lung tissue to support virus multiplication cannot be explained in this manner.

To supplement the data already presented with respect to the titer of virus to be expected in the fluid phase of suspended cell cultures, the results of other experiments may be summarized. The Kramer line of the Lansing strain in the absence of any marked change in mouse virulence, usually exhibited a mouse

TABLE IV

Summary of experiments on the propagation of the Lansing strain in suspended cell cultures of human tissues removed at operation

TISSUE	SOURCE	RESULTS
Thyroid	33-yr female with primary hyperthyroidism	$7/10^{-1.0}$ *; $10/10^{-1.7}$; $14/10^{-1.6}$; $17/10^{-1.6}$ $21/10^{-1.4}$; $24/10^{-1.4}$; $29/10^{-0.6}$.
Foreskin	4-yr male	$4/<10^{0.0}$; 8/N.V‡; $12/10^{-0.4}$; $16/10^{-0.5}$; $20/10^{-1.0}$.
Kidney	7-mo male	7, 10, 14, $18/10^{-2.3}$ †
Testis	70-yr male	$16/10^{-2.3}$
Embryoma of kidney	15-mo male	8, 12, $15/10^{-1.2}$
Neuroblastoma	10-mo female	$16/10^{-1.4}$

* $7/10^{-1.0}$ = pool of fluids collected on 7th day after inoculation of cultures; mouse infectivity titer $10^{-1.0}$.

† 7, 10, 14, $18/10^{-2.3}$ = pool of fluids collected on 7th, 10th, 14th, 18th days; mouse infectivity titer $10^{-2.3}$.

‡ N.V. = no virus detectable by mouse inoculation.

infectivity titer between 10^{-1} and 10^{-2} in cultures of embryonic skin-muscle; in this medium the highest titer ever observed was 10^{-3}. Comparable titers of the virus in fluids from kidney cultures have been recorded. Titers obtained with intestine and brain tissue have in most instances ranged between $10^{0.5}$ and $10^{-1.5}$, with a maximum of $10^{-1.5}$ for the former and $10^{-1.8}$ for the latter. Similar data for the Armstrong line of the Lansing strain were derived from one experiment in which this agent was carried through 9 subcultures in skin-muscle; during this time 17 titrations were done. Maximum mouse infectivity titers generally ranged between $10^{-1.5}$ and $10^{-2.5}$.

6. *Multiplication of the Lansing strain in human tissues removed at operation.* The Lansing strain may also be propagated in cultures composed of various mature human tissues and in certain tumors. In Table IV are summarized the results of such experiments in which the inoculum consisted of infected mouse

brain. It is clear that tissues of widely different origin are capable of providing conditions for virus multiplication.

Attempts to cultivate this virus in suspended cell cultures of human uterus starting with infected mouse brain were unsuccessful. In only 1 of 3 experiments with suspended cell cultures of uterus was virus found to be present in low titer after several changes of HS. In no case could the agent be propagated in serial passage. Whether or not tissue-culture virus will multiply in uterus cultures of this type remains to be determined.

The results of two experiments with human placental tissue were equivocal since virus in low titer was demonstrable after 12 and 21 days of cultivation. Serial passage in this tissue was not attempted.

7. *Cultivation of the Lansing strain in tissues of the rhesus monkey.* Three experiments were carried out with kidney tissue from the rhesus monkey. In two, the animals were killed by intracardial injection of ether. The tissue failed to metabolize and no evidence of virus multiplication was obtained. A third experiment was performed using tissue obtained from an animal sacrificed by a blow on the head. The metabolism of this tissue was active and virus was demonstrable in high titer during a period of 20 days. At the end of this time the titer was $10^{-1.9}$. These observations may be compared with those of other workers (19, 20), who have employed monkey testicular tissue.

B. Multiplication of Brunhilde virus.

1. *Multiplication in human embryonic brain tissue.* The Brunhilde strain appears to behave like the Lansing strain in a single culture passage, although, because of the number of monkeys required, experiments comparable to those with the latter have not been done. The results of one experiment in which the tissue culture method of viral assay was employed, will serve to support this statement.

> *Exp. 95.* The inoculum consisted of Brunhilde virus that had been passed serially in tissue culture and its titer determined by the tissue culture method (roller tube). To each of 3 cultures of human embryonic brain sufficient virus was added to yield a final titer in the fluid phase of these cultures of $10^{-0.5}$ immediately after the addition of the inoculum. The fluids removed at each change of medium were pooled and the virus content of each pool determined by titration *in vitro*. This was accomplished by preparing tenfold dilutions and inoculating 0.1 ml of each into each of 3 cultures of human embryonic brain tissue. These cultures thus employed for titration were maintained for a period of 30 days. The endpoint of viral infectivity was determined by the pH differential test. The results are recorded in Table V.

The virus present in the fluid apparently attained its maximum by the 7th day following inoculation and remained at this level during the ensuing 23 days. In evaluating these data, it should be recalled that a difference of 0.2 pH unit between control and inoculated cultures was arbitrarily selected as significant of a cytopathogenic effect and the titers given in the table are based on this criterion. It is possible, however, that the slightly higher pH values of

the 7th and 12th day fluids in the cultures inoculated with fluid diluted 10^{-4} reflect virus proliferation. If so, the output of virus attained a maximum between the 7th and 12th days and then declined in a manner similar to that of the Lansing strain.

The findings, then, show that multiplication of the Brunhilde strain occurs in a single passage in tissue culture. Moreover, they make it evident that such multiplication can be demonstrated without recourse to the experimental animal. No comparable experiments with the Leon strain have as yet been performed.

TABLE V

The multiplication of the Brunhilde strain in cultures of human embryonic brain tissue as determined by titration of the supernatant fluids in tissue culture

MATERIAL TITRATED: POOLED FLUID HARVESTED ON	DILUTION OF POOLED FLUID ADDED TO CULTURES USED IN TITRATION						TITER OF POOLED FLUID BY "pH-DIFFERENTIAL" TEST†
	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
	pH*	pH	pH	pH	pH	pH	
3rd day	7.3	7.2	6.9	ND	ND	ND	10^{-1}
7th day	7.0	7.1	7.2	7.2	7.0	6.9	10^{-3}
12th day	7.4	7.2	7.2	7.2	7.0	6.9	10^{-3}
16th & 20th day	ND	7.3	7.2	7.1	6.9	6.9	10^{-3}
26th & 30th day	7.3	7.3	7.1	7.2	6.9	6.9	10^{-3}
pH of control cultures on 24th day	6.9						

* Mean pH of cultures used in titration on 24th day after inoculation.

† Endpoints based on a difference of 0.2 pH unit or greater between control and inoculated cultures.

Multiplication of Poliomyelitis Virus During Serial Passages in Tissue Culture

A. Lansing strain

Serial propagation of Lansing, Brunhilde, and Leon viruses has been accomplished in cultures of a variety of tissues. The results of two long-term experiments, one with the Kramer line of the Lansing strain and the other with the Brunhilde strain, will be given in detail. These will illustrate the procedure employed. They also provide experimental data that relate to the change in virulence of the agents which has been noted in the course of prolonged cultivation. Other experiments on serial propagation will be summarized briefly.

A summary of the successful experiments on the serial propagation of Lansing virus in cultures of various human tissues is presented in Table VI; except as noted, the Kramer line of virus was used. The inocula employed to initiate these experiments consisted of a 1:50 or a 1:100 dilution of infected mouse brain. It should be noted that whereas the mouse infectivity of tissue culture material was frequently determined, only occasionally were fluids tested for monkey pathogenicity.

From the information assembled in Table VI it is evident that the Lansing strain can be maintained for long periods of time in cultures of various types of embryonic and mature tissues. The observations recorded in one experiment (Exp. 7) are presented in more detail in Table VII. Certain of the data from this table are recorded graphically in Fig. 4 which further illustrate the technical procedures and also the relationship between the size of the inoculum and multiplication of the virus. In this experiment the virus was cultivated for 23 passages in embryonic skin-muscle tissue during a period of 396 days. The inoculum employed in the original passage consisted of a 1:50 dilution of infected mouse brain (mouse titer $10^{-2.7}$). It is to be noted that, however calcu-

TABLE VI

Experiments on serial propagation of the Lansing strain in suspended cell cultures of various human tissues

EXP.	TISSUE	PASSAGES IN TISSUE CULTURE	CHANGES OF FLUID EXCLUSIVE OF PASSAGE	CUMULATE DAYS IN CULTIVATION	MOUSE TITER OF LAST PASSAGE	FLUIDS TESTED AND POSITIVE ON MONKEY INOCULATION†
1	Embryonic skin-muscle	19	61	331	$10^{-0.5}$	14th & 19th pass.
7	Embryonic skin-muscle	23	78	396	$10^{-0.7}$	7th, 8th, 11th, 14th, 18th, 23rd pass.
13	Embryonic skin muscle	4	14	72	$10^{-2.6}$	ND
41-L*	Embryonic skin-muscle	10	37	191	$10^{-2.7}$	3rd & 7th pass.
6A	Embryonic intestine	15	43	231	$10^{-0.6}$	5th, 11th & 15th pass.
45	Embryonic intestine	4	12	65	V	ND
6B	Embryonic brain	15	43	231	$10^{-1.6}$	3rd, 11th & 14th pass.
43*	Embryonic brain	5	14	76	V	ND
22	Foreskin	14	46	229	$10^{-1.3}$	4th, 7th, 14th pass.
93	Mature kidney	9	15‡	78	$10^{-0.6}$	9th pass.

* Armstrong's Lansing virus.

† Fluids from passages indicated when tested in monkeys produced paralysis and typical pathology.

‡ Because of the use of combined fluids from several changes as inocula this value is low.

V = virus present; undiluted fluid produced symptoms in mice, but titration not done.

ND = not done.

lated, a very great dilution of this inoculum was achieved during these passages. As indicated in Table VI, material from 6 passages, including the 23rd, produced paralysis and characteristic pathology in monkeys. In two other experiments (Exp. 1 and 13) this virus was likewise carried for a long period in the same type of tissue. The Lansing strain obtained from Armstrong (Exp. 41-L) has similarly been maintained for 191 days through 10 passages in skin-muscle. In embryonic human brain and in intestinal tissue (Exp. 6A and 6B) the Kramer line of virus was passed 15 times during a period of 231 days.

Serial passage of this virus has likewise been accomplished in mature human kidney and preputial tissues. The former has yielded consistently good results. Passage of the virus, however, was not always successful in foreskin tissue

TABLE VII

Summary of data from Experiment 7 on the serial passage of the Lansing strain in cultures of embryonic skin-muscle tissue

PASS.	DAY OF CULTIVATION WITH TIME OF FLUID REPLACEMENT AND PASSAGE INDICATED																								DILUTION OF PRIMARY INOCULUM BY PASSAGE‡	DILUTION OF PRIMARY INOCULUM BY FLUID CHANGE AND PASSAGE§	CUMULATE DAYS IN CULTIVATION
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25			
1				C*				C				C				C				*1.3*							10^{-5}	20
2				0.5				1.3				*1.3*				0.7				0.3				0.0		10^{-1}	10^{-6}	32
3				C				C				C				C				*1.3*						10^{-2}	10^{-15}	52
4				1.4				1.5				1.0				*0.5*				0.3						10^{-4}	10^{-20}	68
5				NV				V				*0.4*				1.8				1.0						10^{-5}	10^{-23}	80
6				<0.0				0.3				0.7				1.0				*1.3*						10^{-7}	10^{-30}	100
7				1.8				3.0				2.3				2.3				*2.3*						10^{-9}	10^{-36}	120
8				1.4				1.4				*1.0*				1.5				0.3				0.7		10^{-10}	10^{-40}	132
9				C				C				C				*1.5*										10^{-12}	10^{-45}	148
10				C				C				1.6				*1.2*										10^{-13}	10^{-50}	164
11				NV				1.0				1.4				1.5				*1.6*					1.1	10^{-15}	10^{-55}	184
12				<0.0				0.8				0.7				*<0.0*					NV					10^{-16}	10^{-60}	200
13				NV					<0.0				0.3				0.0				*0.0*				<0.0	10^{-18}	10^{-66}	221
14				C				C				C				*0.0*										10^{-19}	10^{-71}	237
15					C				C				C				C				*0.0*					10^{-21}	10^{-77}	258
16				NV				V					NV				V				*<0.0*					10^{-22}	10^{-83}	279
17			C				C			C				C				*<0.0*								10^{-24}	10^{-89}	297
18				C				C				*0.2*					C				NV					10^{-25}	10^{-93}	309
19			C				C		C					C				*NV*								10^{-27}	10^{-99}	327
20				C				C				C			C				*NV*							10^{-28}	10^{-106}	346
21			C				C			C				*0.0*												10^{-29}	10^{-111}	360
22			C				C			C				C				*<0.0*								10^{-31}	10^{-117}	378
23			<0.0				0.3				0.7			0.4				*0.7*								10^{-32}	10^{-123}	396

* C = fluid changed, mouse titration not done.

† Number = mouse infectivity titer of the fluid removed at time of change expressed as the negative log of the LD_{50} dilution. Italics indicate fluid was used as inoculum for next passage.

‡ Calculated on basis of a dilution factor of 1:30 at each passage.

§ Calculated on basis of a dilution factor of 1:30 at each passage and an estimated dilution factor of 1:15 at each fluid change.

NV = no virus, i.e., none of 6 mice inoculated with undiluted fluid showed symptoms.

V = virus present; undiluted fluid produced symptoms in mice, but titration not done.

(Table VIII). On two occasions when the first attempt at passage failed, repetition at a later date succeeded. The reason for the two failures is not apparent; in both unsuccessful passages the tissue substrate metabolized actively. In this connection it is pertinent to record that in a single experiment the 3rd passage of the Lansing strain in cultures of human testicular tissue failed. Again there was no obvious explanation.

B. Brunhilde strain

Experiments in which the serial propagation of this virus was achieved are summarized in Table IX. The inoculum consisted of either a 1:10 or a 1:50 di-

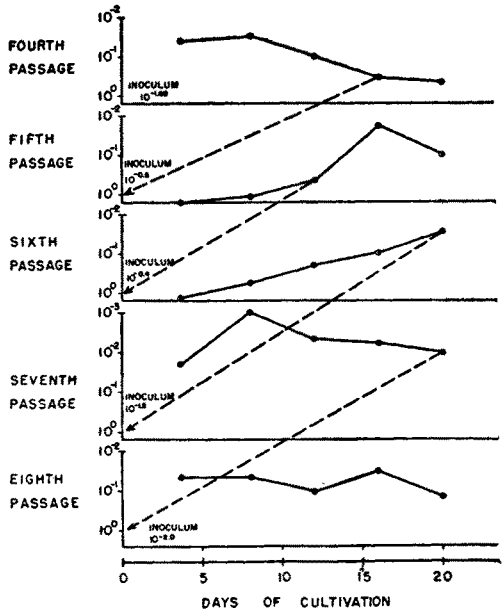


FIG. 4. *Exp.* 7. Mouse-infectivity titers (LD_{50} dilution) of pools of fluids removed at intervals during the 4th to 8th serial passages of the Lansing strain in suspended cell cultures of human embryonic skin-muscle tissue. The mouse infectivity of each pool of fluids employed as inoculum for passage is indicated.

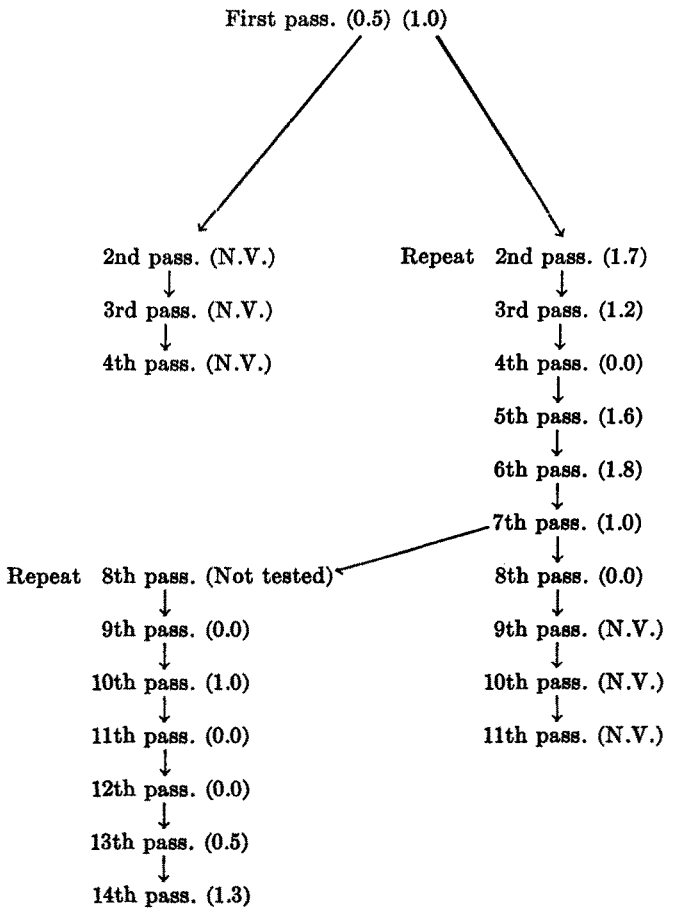
lution of infected monkey spinal cord. No difficulty was encountered in starting or maintaining these four lines, three of which were propagated in embryonic tissues and one in adult tissue. The manipulations employed in Exp. 41B in which the Brunhilde strain was cultivated for 15 passages in embryonic skin-muscle are summarized in Table X. It is apparent that great dilution of the original inoculum was achieved. Virus, however, was demonstrable by monkey inoculation in material obtained from the 15th tissue culture passage. The pathogenicity for the monkey of the virus so propagated will be considered subsequently.

In other types of tissue few attempts to propagate this strain in a series of

cultures have been made. Three trials to establish the agent in foreskin cultures were unsuccessful. In the first, no virus was demonstrable on inoculation of 5th passage material into monkeys. In the others no virus was demonstrable by the

TABLE VIII

*Summary of Experiment 22 on serial propagation of the Lansing strain in cultures of human foreskin tissue**



* Figures in parentheses indicate the negative log of the mouse-infectivity titer of the tissue culture fluid employed to inoculate the subsequent passage.

N.V. = no virus; i.e., none of 6 mice inoculated with undiluted fluid showed symptoms.

cytopathogenic test when skin-muscle roller-tube cultures were inoculated with fluid from the 2nd and 5th passages, respectively.

C. Leon strain

The Leon strain of poliomyelitis virus has been cultivated for 6 passages in cultures of human embryonic brain tissue. Pooled supernatant fluids from the

6th passage exhibited a titer of 10^{-3} when titrated in roller tubes. Similarly, this virus has been maintained for 5 passages in cultures of mature kidney tissue; the titer of the fluids from the 4th passage was 10^{-2} by the roller-tube method. Three passages have been accomplished in skin-muscle tissue. Monkeys have not been inoculated with any of these materials.

Identification of the Viruses Propagated in Tissue Cultures

The identity of the viruses thus maintained in a series of cultures was confirmed in several ways. The pertinent data may be summarized as follows. The Lansing lines when propagated for prolonged periods of time in various human tissues continued to produce paralysis and death in mice (Table VI), although on occasion they exhibited a reduced pathogenicity for this animal. Also, these agents, when tested, produced a paralysis in the monkey. The lines of the Brunhilde strain likewise continued to exhibit pathogenicity for the monkey (Table IX) although, here again, one line showed a reduction in pathogenicity.

TABLE IX

Experiments on serial propagation of the Brunhilde strain in suspended cell cultures of various human tissues

EXP.	TISSUE	PASSAGES IN TISSUE CULTURE	CHANGES OF FLUID EXCLUSIVE OF PASSAGE	CUMULATE DAYS IN CULTIVATION	FLUIDS TESTED AND POSITIVE ON MONKEY INOCULATION*
41B	Embryonic skin-muscle	15	54	267	2nd, 3rd, 4th, 7th, 10th, and 15th pass.
47	Embryonic intestine	4	11	62	4th pass.
43	Embryonic brain	6	16	89	6th pass.
93	Mature kidney	8	10†	63	8th pass.

* Fluids from passages indicated when tested in monkeys produced paralysis and typical pathology.

† Because of the use as inocula of combined fluids from several changes this value is low.

Further evidence for the presence of the Lansing and Brunhilde viruses was obtained in neutralization tests performed in roller-tube cultures. For example, the cytopathogenic effect of the virus obtained from the 23rd tissue culture passage of Exp. 7 (Lansing strain) and the virus of the 15th passage of Exp. 41B (Brunhilde strain) was in each case inhibited by homologous antiserum and not by antisera against the heterologous types. No evidence was obtained following inoculation of appropriate animals with materials from control cultures that a contaminating virus had been introduced during the course of these experiments. For instance, materials from the last passages of Exp. 7 and 41B were inoculated into suckling mice; no indication of the presence of a Coxsackie-like agent was obtained.

Two lines of the Leon strain also have been identified by neutralization tests in roller-tube cultures. The agents from the 5th passage in embryonic brain tissue and the 4th passage in kidney tissue were neutralized specifically by Leon antiserum.

TABLE X

Summary of data from Experiment 41B on serial passage of the Brunhilde strain in cultures of embryonic skin-muscle tissue

PASSAGE	DAY OF CULTIVATION WITH TIME OF FLUID REPLACEMENT AND PASSAGE INDICATED*																			FLUIDS TESTED AND POSITIVE ON MONKEY INOCULATION	DILUTION OF PRIMARY INOCULUM BY PASSAGE†	DILUTION OF PRIMARY INOCULUM BY FLUID CHANGE AND PASSAGE‡	CUMULATE DAYS IN CULTIVATION
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21				
1				C				C				C				*C*								10^{-4}	16
2				C				C				C				*C*						P§	10^{-1}	10^{-9}	32
3				C				C				C				C				*C*		12th-day fluid	10^{-3}	10^{-15}	52
4				C				C				C				C					*C*	21st-day fluid	10^{-4}	10^{-21}	73
5				C				C				C				C					*C*		10^{-6}	10^{-27}	94
6				C				C				C				*C*							10^{-7}	10^{-32}	110
7					C				C				C				C				*C*		P	10^{-9}	10^{-38}	131
8				C				C					C				C				*C*			10^{-10}	10^{-44}	152
9			C				C			C				C				*C*						10^{-12}	10^{-51}	170
10				C				C				*C*					C				C		P	10^{-13}	10^{-55}	182
11			C				C		C					C			*C*							10^{-15}	10^{-61}	198
12				C				C				C			C				C					10^{-16}	10^{-67}	217
13			C				C			C				*C*			C				C			10^{-18}	10^{-72}	231
14			C				C			C				C				*C*						10^{-19}	10^{-78}	249
15			C				C				C			C				*C*					P	10^{-21}	10^{-85}	267

* C = fluid changed. Italic indicates fluid was employed as inoculum for next passage.

† Calculated on basis of a dilution factor of 1:30 at each passage.

‡ Calculated on basis of a dilution factor of 1:30 at each passage and an estimated dilution factor of 1:15 at each fluid change.

§ P = pool of fluids removed on 3 different days.

Attempts to Cultivate Poliomyelitis Virus in Tissues of Certain Other Animals

A. In tissues from the mouse, chick, ox and rabbit

Certain of the unsuccessful experiments in which tissues of various species other than man were used have been mentioned already. In addition, 3 experiments with Lansing virus failed to provide any indication that multiplication occurred either in cultures of adult mouse brain or intestine. One attempt to cultivate this agent in adult mouse skin-muscle likewise failed, as well as two in which chick embryonic brain was used. Single attempts to cultivate Lansing virus in beef embryonic skin-muscle and in rabbit kidney were also unsuccessful.

B. Attempts to adapt Lansing virus to growth in mouse and chick tissues in the presence of human tissue

In an effort to adapt Lansing virus to growth in chick and mouse embryonic brain tissues, suspended cell cultures were prepared with each of these tissues and minced human embryonic brain tissue was incorporated prior to the addition of Lansing virus. Five passages in such mixed cultures were carried out. Fluids of the 5th passage of both lines contained Lansing virus. Infected fluids from this passage were then inoculated into cultures that consisted only of chick embryonic brain or only of mouse embryonic brain; no evidence of virus multiplication could be demonstrated following inoculation of mice with fluids removed at various intervals from these cultures.

Level of virus production during serial passage

A. Lansing strain

An apparent decrease in the production of virus occasionally was noted after serial passage of the Lansing strain in tissue culture. In 2 experiments the mouse infectivity titer of the fluid phase declined abruptly during the course of prolonged propagation of the agent in human embryonic skin-muscle tissue. The maximum mouse infectivity titers recorded for each passage of Exp. 7 (see Table VII) and similar data for Exp. 1 are summarized in Fig. 5. It is noteworthy that in Exp. 7 the maximum titers that were determined during the first 11 passages or 180 days of cultivation exceeded $10^{-1.3}$, whereas those during the subsequent 12 passages were $10^{-0.8}$ or lower. In Exp. 1, a similar reduction in titer was noted after only 3 passages.

It should be emphasized that the reduction in infectivity of the tissue culture virus for the mouse, as observed in Exp. 1 and Exp. 7, represented a loss of virulence of the agent for the animal, and was not a manifestation of an altered capacity of the virus to multiply *in vitro*. Thus a comparison of the results of repeated titrations performed in tissue culture of fluids obtained from the early and late passages revealed no significant differences in their infectivity for human cells. For example, fluids from the 8th and 23rd passages in Exp. 7 yielded an ID_{50} for the tissue culture of $10^{-3.5}$ in each instance. It is of interest to note that titrations of materials from Exp. 7 in monkeys have shown no reduction in virulence comparable to that observed for the mouse.

B. Brunhilde strain

In vitro titrations by the roller-tube method of fluids from early and late passages of the Brunhilde strain (Exp. 41B; see Table X) have likewise shown no significant change in the endpoint of viral infectivity. The titers of fluids from the second and tenth passages as determined by the cytopathogenic effect were both of the order of 10^{-4}. Titration of these same materials in monkeys ndicated that between the 2nd and 10th passages, the infectivity of the virus ior this animal declined approximately 100 times. These observations suggest fhat serial propagation of the Brunhilde strain, as in the case of the Lansing

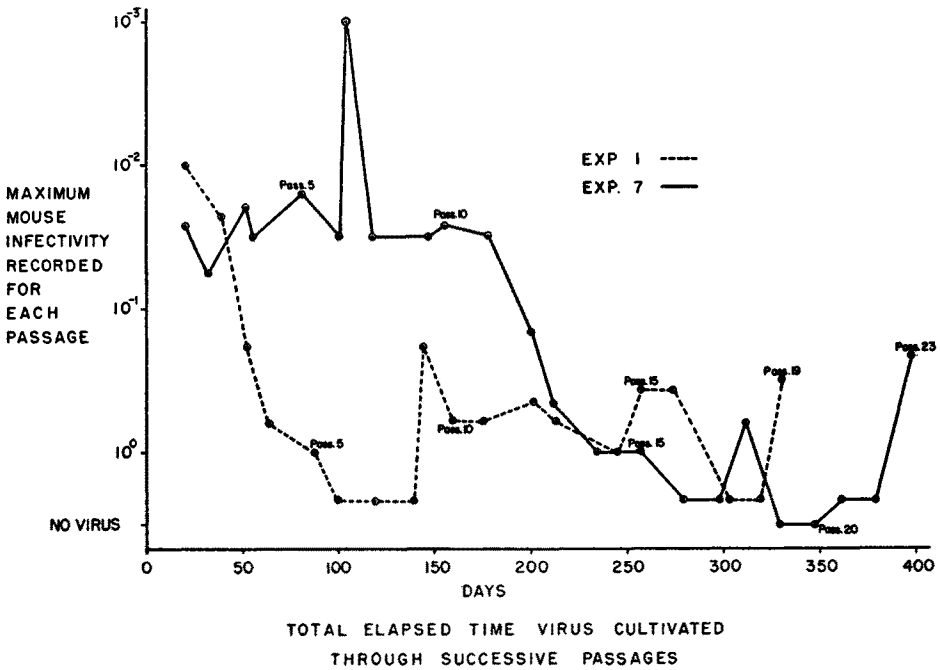


FIG. 5. The maximum mouse-infectivity titers (LD_{50} dilution) recorded for various pools of fluids from each passage during the maintenance of two lines of the Lansing strain in suspended cell cultures of human embryonic skin-muscle tissue.

strain, likewise was associated with an alteration in virulence of the agent for the animal although no change occurred in the capacity of the virus to multiply in tissue culture.

IV. DISCUSSION

Evidence has been presented that representatives of each of the three known antigenic types of poliomyelitis virus, i.e., the Lansing, Brunhilde and Leon strains, can be propagated for an indefinite period of time in suspended cell cultures of certain human tissues. Furthermore, it has been demonstrated that a variety of human tissues, both embryonic and mature, will, in cultures of this

type, support the multiplication of these agents. During the course of these experiments it has been shown that the same order of multiplication occurs in cultures of a non-nervous tissue such as human embryonic skin and muscle that is devoid of intact nerve cells as in comparable cultures of embryonic brain tissue.

These observations are at variance with those of earlier workers for reasons perhaps elucidated by a consideration of the technical procedures employed in the present study. The modified Maitland tissue-culture method we have used was developed for the cultivation of the mumps virus (2) which grows at a slower rate than many other viruses, e.g., influenza and equine encephalomyelitis. In the classical method subcultures are carried out at 2 to 4-day intervals. In contrast, the modified technique by frequent replacement of the nutrient medium permits maintenance of the tissue in an active metabolic state for prolonged periods of time. Therefore, passages need be carried out at relatively infrequent intervals. The results of our studies on the multiplication of the poliomyelitis viruses *in vitro*, suggest that these agents, like the mumps virus, multiply relatively slowly. Accordingly, it is likely that the modified culture technique permitted demonstration of the multiplication of poliomyelitis virus in situations where the classical procedure might yield negative results.

It is obvious that the use of human tissues per se influenced the outcome of these studies, although the type of human tissue employed in the cultures proved to be of less importance than might have been surmised. While subsequently other workers have extended our observations by showing that certain monkey tissues will likewise support the growth of the poliomyelitis viruses *in vitro*, to date they, as well as we, have failed in attempts to propagate the virus in tissues from animals other than the primates. The role of the ox-serum ultrafiltrate remains to be accurately defined. It has been shown, however, that the omission of the ultrafiltrate from the culture system has resulted in a reduction in virus proliferation.

In retrospect, it is of interest to consider the suggestive evidence of viral multiplication obtained by Flexner and Noguchi (21) and by Long and his coworkers (22). Human ascitic fluid was employed as a constituent of the medium and Long observed well preserved cells apparently derived from this source in the cultures. The irregular persistence of poliomyelitis virus recorded by these workers may have represented actual multiplication of the virus in these cells of human origin.

Certain characteristics of the suspended cell culture system as here employed deserve comment. In contrast to roller-tube cultures, little cell growth occurs, and serum and embryonic extract, classical sources of growth promoting materials, are lacking. The cultural conditions are favorable to cell survival rather than cell multiplication. Although many of the cells originally introduced remain viable for relatively long periods of time, there is a gradual decline in the functional cell population. This progressive reduction in the number of cells available for viral multiplication may well play a role in limiting the amounts of virus produced when small inocula are employed. Thus in the experiment recorded in

Table IV virus eventually appeared in those cultures inoculated with the higher dilutions, but only in small amounts and for a brief period at a time when the tissues were deteriorating.

A single determination only has been made of the quantities of virus present in the tissue fragments. The results, not described in the body of this paper, indicated that early in the period of cultivation somewhat larger amounts of virus per unit volume were present in the tissues than in the fluid phase. Later the amounts of virus in the fluid equalled or exceeded that found to be present in the tissues.

In suspended cell cultures infected with adequate inocula of the Lansing and Brunhilde strains, the maximum virus concentration achieved in the fluid phase of the cultures was considerable. Indeed on the basis of titrations performed by tissue-culture methods, the levels of virus in the fluid often appear to be comparable to those found in the nervous tissues of infected monkeys or mice.

Certain practical applications of the procedure for the cultivation of the poliomyelitis viruses here described have been already mentioned. The culture can be substituted for the monkey in the isolation of viruses. Heretofore, monkey pathogenicity has been a definitive attribute of this group of agents. It is possible that *in vitro* isolation studies will reveal poliomyelitis viruses that are essentially nonpathogenic for animals other than man.

Techniques for the assay of viral infectivity *in vitro* based on the cytopathogenic effect of the virus, are considered in detail in another paper of this series. Their development has made possible the recognition of the decrease in animal pathogenicity observed during prolonged cultivation. Had the classical method of titration in animals alone been employed it would have been impossible in the case of the Brunhilde virus and difficult in that of the Lansing strain to decide whether a reduction in pathogenicity had intervened or whether the capacity of the virus to multiply *in vitro* had decreased. Since pathogenicity may be depressed in certain instances after a few passages, it is possible that the apparent failure of earlier workers to propagate the virus *in vitro* may have depended in certain cases upon the lack of an alternative method of viral assay.

The observation that continued cultivation has repeatedly been accompanied by an alteration of the pathogenicity of poliomyelitis virus indicates that this attribute can be readily modified. This finding suggests that through future applications of the tissue-culture technique a variant might be obtained whose attributes of high antigenicity and low pathogenicity for man would render it suitable as an immunizing agent.

The suspended cell as compared with the roller-tube technique has certain advantages, as well as disadvantages. Among the latter are the gradual deterioration of the cell population, and the prolonged period of cultivation often required for demonstration of the cytopathogenic effect. The chief advantages offered by suspended cell cultures consist in the ease with which they may be prepared and maintained and the fact that large volumes of infected fluid, relatively free of serum or tissue elements, may be obtained.

Of general interest is the observation that under conditions of cultivation

in vitro poliomyelitis viruses exhibit no exclusive neurotropic character since they proliferate actively in extraneural tissues. This observation will be supported by additional evidence presented in other papers of this series. It cannot be directly inferred that, because of this behavior, multiplication in the living animal likewise occurs in these sites. Nevertheless, the demonstration that cells other than neurons provide conditions compatible with the growth of the virus affords indirect support for the hypothesis of extraneural viral proliferation as a factor in the pathogenesis of poliomyelitis.

SUMMARY

In 1949, in preliminary notes, we recorded the fact that the *in vitro* propagation of the poliomyelitis viruses may be accomplished in suspended cell cultures of various types of human tissues. The present paper details the procedures we have employed in the preparation and maintenance of suspended cell cultures for this purpose, and summarizes observations on the behavior of poliomyelitis viruses in such cultures, as observed during the past 4 years.

Each of 3 antigenic types of poliomyelitis virus has been propagated in suspended cell cultures of human embryonic skin and muscle tissue, in cultures of human embryonic brain tissue and in cultures of mature human kidney tissue. In addition it has been demonstrated that one or more of these 3 types of virus will multiply in cultures of various other human embryonic tissues, including intestine, adrenal and spleen. Likewise, mature human tissues including thyroid, foreskin, and testis have been successfully employed for the cultivation of the Lansing strain as well as materials from two tumors, an embryoma of the kidney, and a neuroblastoma. Poliomyelitis virus has also been cultivated in monkey kidney cultures.

Assay of the infectivity of materials removed from the tissue cultures was performed by the classical method of titration in animals and also by the technique of titration *in vitro* employing the cytopathogenic effect of the poliomyelitis viruses as an indicator of viral activity. Materials from cultures prepared with human embryonic skin-muscle tissues, kidney tissue, or brain tissues yielded the highest infectivity titers. Serial propagation in suspended cell cultures of human embryonic skin-muscle tissue was achieved for 23 passages with the Lansing strain, for a cumulate period of 331 days in cultivation, and for 15 passages with the Brunhilde strain, during a total period of 267 days. Throughout these prolonged periods of cultivation, virus production in each instance remained essentially constant as assayed by the *in vitro* technique. The titers as determined *in vitro* were in most cases equivalent to those observed in the central nervous system of the infected animal. However, in experiments with Lansing virus prolonged cultivation in human tissues was associated with a marked decline in apparent infectivity titer of the agent for the mouse. Likewise, in one experiment with Brunhilde virus, a significant decrease in the apparent infectivity titer for the monkey was observed.

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