TABLE I. The Effect of Mixing Plasmas of Newborn and Adult on Prothrombin Time.

Fresh cord plasma	cc	.1	.09	.08	.07	.06	.05	.04	.03	.02	.01	.0	
" adult "	cc	—	.01	.02	.03	.04	.05	.06	.07	.08	.09	.1	
Prothrombin time*	sec.	12	12	12	12	12	12	12	12	12	12	12	
Stored cord plasma	cc	.1	.1	.09	.08	.07	.06	.05	.04	.03	.02	.01	— —
" adult "	cc	—	—	.01	.02	.03	.04	.05	.06	.07	.08	.09	.1 .1
Deprothrombinized rabbit plasma	cc	—	.02	.02	.02	.02	.02	.02	.02	.02	.02	.02	.02 —
Prothrombin time*	sec.	24	12	10½	10	9½	9	8½	8½	8¼	8	8	8 35

* The method employed has been previously described(3).
The mothers were supplied with vit. K (synkayvite) prepartally.

newborn baby as in that of the adult.

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Growth of Poliomyelitis Viruses in Large Stationary Cultures.* (20144)

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The first reported methods for propagating poliomyelitis viruses in tissue cultures utilized implants of the suspended cell type(1). Subsequently roller tubes in which the proliferating fragments of tissue were imbedded in plasma clots were used(2). In addition to permitting direct microscopic visualization of cultures for cytopathogenic effect, this method had the advantage of giving an early growth of virus(3). Recent reports have indicated that these same advantages obtain if culture tubes are kept stationary instead of rotated(4,5).

In this report are presented results obtained with the growth of poliomyelitis viruses in large stationary flask cultures. A simplified medium (Hanks-Simms) and fragments of monkey testes imbedded in plasma clots were used. The work was done in order to obtain large quantities of supernatant fluid of sufficiently high virus titer for complement-fixing antigen preparation according to the method of Svedmyr and Enders(6) without having to maintain many small culture units and without having to use special equipment for rotating large culture bottles.

Materials and methods. Viruses. Brunhilde (type 1), Y-SK (type 2) and Saukett† (type 3) strains of poliomyelitis viruses were used in titered pools of supernatant fluid from infected roller tube cultures of monkey testes. Each virus strain had been through at least three consecutive tissue culture passages, and was specifically neutralized by prototype antiserums. *Chicken plasma.* Dehydrated chicken plasma (Difco) was reconstituted and carbonated in the manner reported by Melnick (7). *Monkey testes.* Testes from 3 to 5 normal immature *rhesus* (*M. mulatta*) monkeys were minced together and used for cultures on the same day they were obtained. *Nutrient fluid.* Two kinds of fluid were used. One referred to as "complete medium" contained Hanks and Simms solutions and acetone-extracted chick embryo extract(7). The

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† We are indebted to Dr. Jonas Salk for supplying us with the Saukett strain.

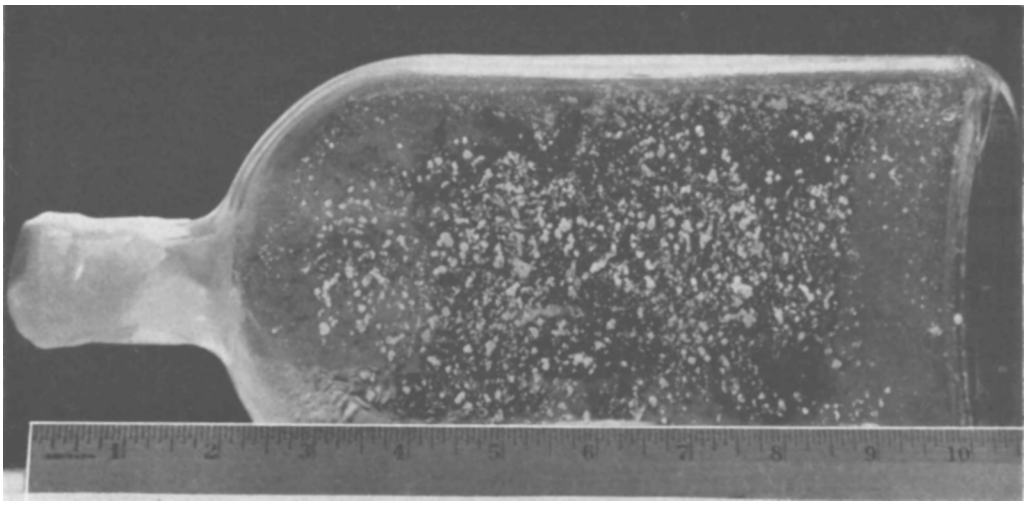

FIG. 1. Culture in Roux flask illustrating the amount and distribution of tissue.

complete medium was used for all fluid changes in roller tube cultures and for fluid changes in flask cultures during the preinoculation growth phase. Another medium referred to as "simplified medium" contained only Hanks and Simms solutions in ratio of 3:1. The simplified medium was used for fluid changes in flask cultures at the time of virus inoculation and thereafter. All nutrient fluids contained 50 units of penicillin and 50 μg of streptomycin per ml.

Growth of virus. a) *In 125 ml Erlenmeyer flasks.* The necks of 125 ml Erlenmeyer flasks were bent so that the plane of the flask mouth was vertical in order to minimize the risk of air-borne contamination. The flasks were cleaned and sterilized according to described procedures(7) for tissue culture use. Reconstituted chicken plasma, 0.25 ml, was spread by agitation over the bottom of each flask. A fine mince of monkey testes was distributed in the plasma film with a pipette until about 10-12 fragments of tissue were contained in each square centimeter on the bottom of the flask. The plasma was clotted with 0.2 ml of chick embryo extract. After half an hour 10 ml of complete medium were added. The flasks were stoppered with gauze-covered cotton, sealed with parafilm‡ and incubated at 35°C. Nutrient medium was changed every 3 days. Simplified medium was used for the second fluid change and for all subsequent ones. The virus to be tested for growth (100 or 1000 roller tube infecting doses) was added with the second fluid change. All supernatant fluids after that were saved and stored at −70°C. The cultures were discontinued one month after the virus had been added. b) *In Roux flasks.* Roux culture bottles with a capacity of 1000 ml were cleaned and sterilized. One ml of reconstituted plasma was spread by agitation over one side of the flask. A fine mince of monkey testes was distributed with a long pipette through the plasma film until there were about 8-12 fragments per square centimeter (Fig. 1). One ml of chick embryo extract was added to clot the plasma. After half an hour 75 ml of complete medium were added, the flask was stoppered with gauze-covered cotton, sealed with parafilm and incubated at 35°C. For the first fluid change complete medium was used. Simplified medium was used when the virus was introduced at the second fluid change and for all changes thereafter. After virus inoculation (750 roller tube infecting doses) all supernatant fluids were saved and stored at −70°C. Cultures were discontinued thirty days after virus inoculation. c) *In roller tubes.* Roller tube cultures were prepared in the manner reported by Melnick(7). Supernatant fluids were replaced with complete medium when the tubes were three and five days old. Virus was

‡ Parafilm may be obtained from the Marathon Corporation, Menasha, Wis.

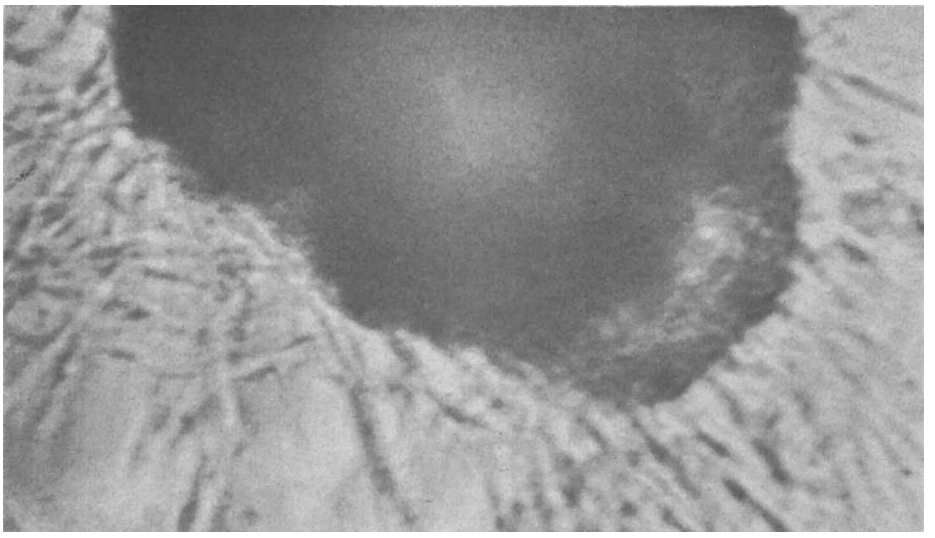

FIG. 2. Cellular proliferation from an implant of monkey testis in Roux flask culture, 6 days old. Microscopic detail of culture was quite poor because of the two thick layers of irregular glass, through which light was transmitted.

added at the time of the second fluid change. Fluids were changed every three days thereafter. The supernatant fluids were obtained and stored at −70°C. Later, virus growth curves were determined. *Titrations.* Supernatant fluids containing poliomyelitis virus were diluted serially in half logarithm increments (serial 3.2 dilutions) with Hanks solution. Each dilution was tested in 0.1 cc amounts in two 5-day-old roller tubes. The

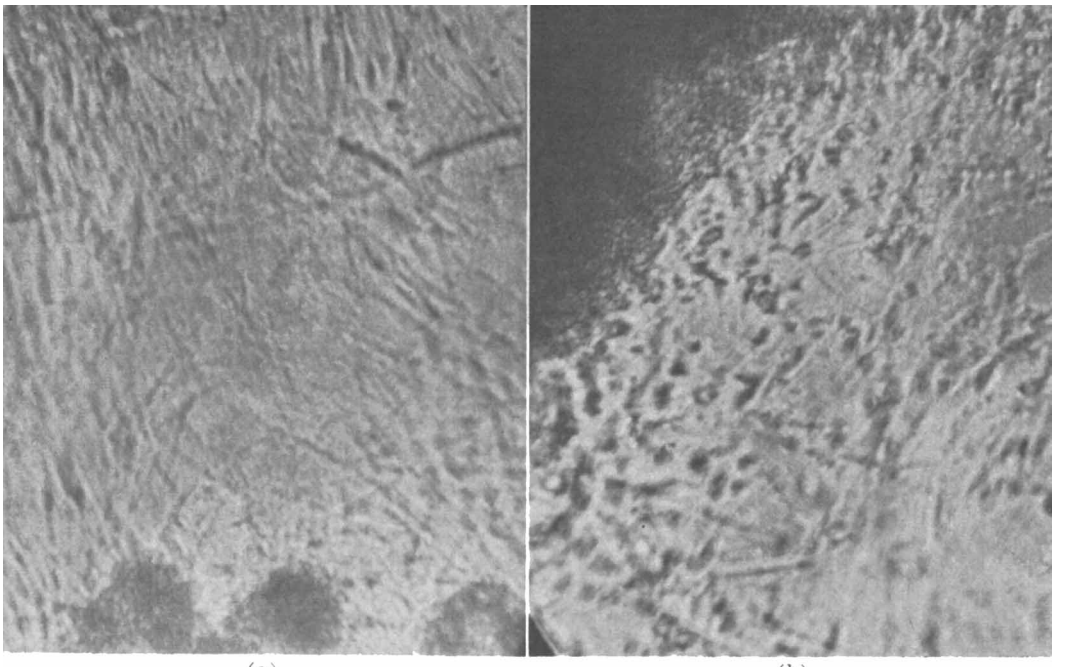

FIG. 3. (a) Cellular proliferation in a Roux flask culture at 12 days without virus inoculation. (b) Twelve-day-old Roux flask culture showing cytopathogenic effect after Brunhilde virus inoculation 6 days previously.

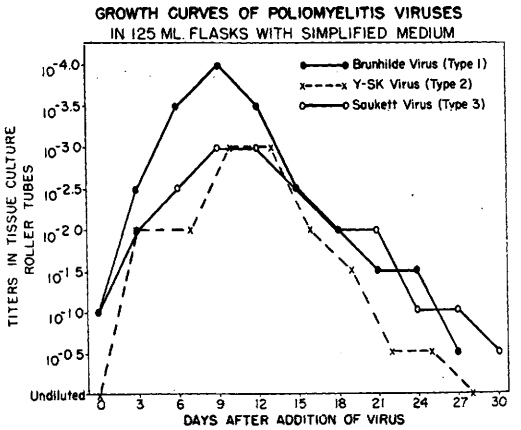

FIG. 4. Titers at zero days indicate virus concentration in flask fluids immediately after adding the virus inoculum.

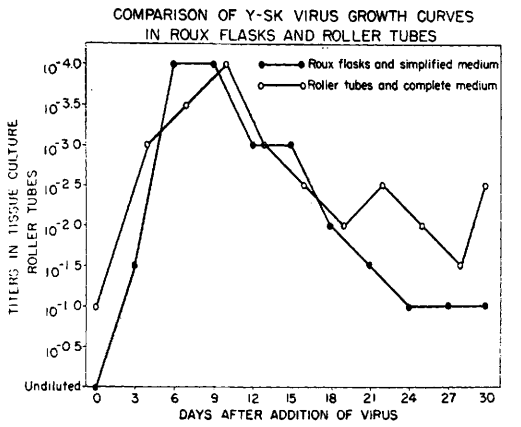

FIG. 5. Titers at zero days indicate virus concentration in flask fluids immediately after adding the virus inoculum.

tubes were read for cytopathogenic effect 6 days after inoculation (time of the second post-inoculation fluid change). The endpoint was taken as the greatest dilution which caused a cytopathogenic effect in both tubes. The dilution of virus that occurred in the roller tubes at the time of inoculation was not taken into account in figuring final titers.

Results. Cultures in 125 cc Erlenmeyer flasks. Brunhilde, Y-SK and Saukett strains of poliomyelitis viruses were successfully propagated. With each strain, supernatant fluids from two or more consecutive fluid changes yielded titers of 10^{-3} or more in roller tube titrations (Fig. 4). Titers above 10^{-2} were obtained in supernatant fluids from 5 to 7 consecutive fluid changes. Maximum growth occurred between the third and eighteenth days. The highest titers were obtained 9 or 10 days after virus inoculation.

Cultures in Roux flasks. At the time of virus inoculation, when cultures were 6 days old, outgrowth of new tissue (Fig. 2) was comparable to that seen in roller tubes. Typical cytopathogenic effect occurred after virus inoculation (Fig. 3).

A growth curve was completed for Y-SK virus (Fig. 5). Virus titers greater than 10^{-3} were demonstrated for 4 consecutive supernatant fluid harvestings. Maximum growth occurred between the third and eighteenth days. The growth curve followed very closely that of the same virus strain in roller tubes with complete medium (Fig. 5).

Growth curves for Brunhilde and Saukett strains were not determined in Roux flasks. These strains multiplied and caused a cytopathogenic effect in the Roux flask cultures.

Discussion. The Erlenmeyer flask cultures were made as a pilot study for virus propagation. They were used to prepare small standard pools of virus. Their principal value was to indicate that stationary flask cultures might be used to prepare large amounts of fluid which contained virus in appreciably high titer. When the method was extended to the 1000 ml Roux flasks it was found that virus titers in supernatant fluids compared favorably with those obtained from roller tube cultures. In 15 days a single Roux flask culture infected with Y-SK virus provided 300 ml of supernatant fluid with a virus titer beyond 10^{-3}. Comparable yields with roller tubes required 75 cultures.

The use of a simplified medium appeared to make little difference as far as virus yield was concerned. Growth curves of Y-SK virus in Erlenmeyer flask cultures were compared using complete versus simplified medium. The duration and degree of maximum growth were identical. The only difference occurred in the fourth week after virus inoculation when fluid from the flasks containing complete medium gave a virus titer one logarithm higher than fluids from flasks containing simplified medium(8). The difference in titer between roller tube and Roux flask fluids, which occurred

during the fourth week of virus growth, may be the effect of different mediums rather than different culture technics.

Size of a culture unit did not appear to be a limiting factor in striving for large yields of virus. Cultures were successfully grown in six-liter rectangular culture bottles. These extremely large flasks were abandoned in favor of the Roux flasks, which were more manageable and not as prone to contamination with molds.

Summary. Poliomyelitis viruses were propagated in large stationary tissue cultures of plasma imbedded monkey testes. Large volumes of supernatant fluids with high virus titers were achieved. The virus yield, as measured by titer and duration of maximum growth, was almost identical with that obtained in roller tubes, and greater than that reported for suspended cell flask cultures. The method had another advantage in that equipment for rotating bottles was not required. Since the Roux flasks could be stacked one on top of another, a minimum of incubator space was required.

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Electron Microscopic Examination of Inclusion Bodies of Herpes Simplex Virus. (20145)

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Despite extensive investigation(1) there is still doubt concerning the relation of herpes simplex virus to the intranuclear inclusion bodies which are characteristically seen in infected tissues. Recent studies with the light microscope of such inclusions stained by the Feulgen method suggest that they contain virus and do not represent solely an abnormal product of cellular metabolism(2,3). This paper reports preliminary observations made with the electron microscope of thin sections of cells infected with herpes simplex virus.

Materials and methods. Chick embryo chorioallantoic membranes heavily infected with the HRE strain of herpes simplex virus (4) were ground with sterile sand in a mortar and suspended in nutrient broth containing penicillin and streptomycin. After centrifugation to remove large tissue particles, the supernatant fluid was diluted to contain approximately 300 ID_{50}/ml. Two-tenths milliliter of this suspension was inoculated onto the chorioallantoic membranes of 11-day-old chick embryos. Following 48 to 72 hours incubation at 35°C the membranes were removed and immediately fixed in 1% buffered osmium tetroxide, according to the method of Palade (5). Small pieces, showing pocks grossly, were excised from the fixed membrane, dehydrated in ethyl alcohol, embedded in methacrylate, and sectioned by the thermal expansion method. The sections were mounted on formvar screens and the methacrylate was removed by immersion in amyl acetate. After light shadowing with palladium the preparations were examined in an RCA type EMU electron microscope. Adjacent portions of membrane prepared by routine methods and