Poliomyelitis Viruses in Tissue Culture. IV. Protein-Free Nutrient Media in Stationary and Roller Tube Cultures.* (19823)

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The multiplication of poliomyelitis virus in roller tube cultures of human and monkey tissues is usually accompanied by degenerative changes in the developing outgrowth (cytopathogenic effect)(1-6). For such cultures, tissue fragments are imbedded in a plasma clot attached to the inner wall of a test tube and after incubation in the presence of a suitable medium in a rolled drum at 35°C for several days, cellular proliferation is usually quite marked. If poliomyelitis virus is added to the cultures at this time, subsequent cytopathic changes in the outgrowth serve as an *in vitro* indicator of virus proliferation.

The present report is concerned with the simplification of the medium in which cells grow out from the tissue fragments and in which virus may be grown in tube cultures. Also, in confirmation of Li and Schaeffer(7), and Scherer and Syverton(6), satisfactory results may be obtained with tubes which are not rolled. The nutrient media in current use for work with poliomyelitis virus tube cultures contain serum or embryonic extracts in addition to serum ultrafiltrate and isotonic salt solution(1-6). In the present study, protein-containing, cellular growth stimulants (serum or embryonic extract) have been successfully replaced by a protein hydrolysate (lactalbumin)† and the roller drums have been replaced by racks for holding the tubes on their sides with the fragments submerged in the nutrient medium.

Materials and Methods. The technic of preparing roller tube cultures of monkey testicular tissue as used in this laboratory has been described in detail, together with the behavior of selected strains of poliomyelitis virus in tissue culture(3,5). These conditions have been followed in the present experiments except that the acetone-extracted chick embryo (ACE) in the medium has been replaced by a lactalbumin enzymatic hydrolysate.‡ The powdered hydrolysate (5 g) was mixed with Hank's salt solution without sodium bicarbonate (95 ml) and dissolved by autoclaving at 10 lb pressure for 10 minutes. The solution is stable when kept frozen, and presumably at 4° also. If a slight precipitation occurs upon thawing, it can be brought into solution by heating in lukewarm water. The pH of the solution is 6.8 and it is brought to 7.5 with sterile 0.1 N NaOH before using. The *complete medium* which is added to the imbedded tissue fragments during outgrowth of cells consists of Hanks-Simms solution (3 parts of Hanks' balanced salt solution plus 1 part of Simms' ultrafiltrate) 9 parts, and 5% lactalbumin hydrolysate, 1 part. After the addition of virus, a 1% lactalbumin hydrolysate solution is used, as this concentration is sufficient to maintain the cells during the subsequent observation period. In each of the experiments to be described half of the tubes were rolled in a drum(5) and half were held stationary on their side in a rack or in a cardboard carton which was slightly inclined, so that 1 ml of nutrient fluid would form a shallow layer and reach not over 2/3 of the distance from the bottom to the opening of the 6 x 150 mm test tube. The 6 to 10 tissue fragments were placed in a line on one side of the test tube as recommended by Youngner, Ward, and Salk(4). This was particularly useful in the present experiments to insure that all fragments were submerged in the fluid medium. All tubes were held at 35°. They were removed every few days for microscopic examination of the cultures and for changing nutrient fluids.

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† Protein digests had already been used to stimulate cell proliferation in tissue culture in 1928(9).

‡ The hydrolysate was obtained from Nutritional Biochemical Corp., Cleveland, Ohio. A typical analysis, according to the producer, yielded total nitrogen 12%, amino nitrogen 7.3%, ash 3.6%, sodium chloride 0.9%, and moisture 6.8%.

TABLE I. Effect of Lactalbumin Hydrolysate on Growth of Fibroblasts and Their Destruction by Poliomyelitis Virus.

	Growth of fibroblasts		Extent of degeneration on 10th day*		
	4th day	6th day	10^{-2} virus	10^{-3} virus	10^{-4} virus
LACT.,† 0.5% final conc.	Luxuriant	Luxuriant	3+	1+	—
0.1%	Good	"	3+	1+	—
0.01%	Beginning	Good	2+	1+	—
Chick embryo extr.	"	"	2+	1+	—

* 10th day of exp., or 4 days after addition of virus. Extent of fibroblast degeneration is given in arbitrary units which have been defined(5). 10^{-2} virus indicates tissue culture Y-SK virus, diluted 1 to 100, added to the tubes on the 6th day; 10^{-3} virus, diluted to 1 to 1000; etc.

† LACT. = Lactalbumin hydrolysate.

Results. When lactalbumin hydrolysate is used in place of ACE, fibroblasts from monkey testicular tissue grow out more rapidly. By the 4th day after the fragments are implanted in the tubes, the outgrowth is sufficiently luxuriant so that the tubes may be inoculated with virus. As shown from Table I, this is about 2 to 3 days earlier than seen with ACE. Such tubes respond to tissue culture adapted virus in similar fashion to ACE-containing tubes, and titration results similar to those shown in Table I have been obtained with stationary as well as rolled tubes. Photomicrographs in Fig. 1 and 2 illustrate the clear nature of the normal fibroblasts and also the extensive degeneration produced by the action of the virus, in both rolled and stationary tubes. For purposes of comparison, tubes containing ACE were set up at the same time and typical fragments are also shown.

A number of such experiments have been carried out in which virus has been added 4 days after the tissue fragments have been implanted in the tube. With slower growing fragments (ACE medium), it has been customary to change fluid on the 3rd or 4th day after implantation and add virus on the 6th or 7th day. Because cells grow out faster with hydrolysate in the medium, virus may be added at the time of the first fluid change. This is particularly convenient in carrying out neutralization tests for poliomyelitis antibodies in tissue cultures, for the tubes need be handled only on the 4th day when fluid is changed and the virum-serum mixture is added, and on the 8th day when the tubes are read(5).

Virus growth was supported as well, and apparently for longer periods, in the hydrolysate tubes than in the ACE tubes. A typical experiment showing the titrations of fluids harvested at various intervals from ACE and lactalbumin hydrolysate cultures, both rolled and stationary, is shown in Table II. Although virus could still be detected in all tubes 30 days after their inoculation, virus production fell off markedly in the ACE after the 18th day and in the hydrolysate tubes after the 23rd day or perhaps even later. The degeneration observed in the hydrolysate tubes was specific, for not only was it prevented by homotypic antiserum, but also fluid from tubes showing degeneration produced a similar effect when titrated in ACE tubes. The degeneration produced in titrating the hydrolysate tubes was also proven to be specific. In tubes which yielded an endpoint at the 10^{-4} concentration, fluid was harvested from the last tubes in the series showing degeneration (10^{-4}) and from the tubes at the next dilution also (10^{-5}). These fluids were transferred to a new series of ACE roller tubes, half of which had each received 0.1 ml of 1:10 Brunhilde antiserum. The results, listed below, indicate that Brunhilde virus was present in the tubes at the titration endpoint (read by cytopathic changes), but not in the next tubes in the series which failed to show degenerative changes:

	Results of tests in ACE roller tubes, degeneration
Harvest from 10^{-4} tubes	4+
10^{-4} tubes + BA*	0
10^{-5} tubes	0
10^{-5} tubes + BA	0

* BA = Brunhilde antiserum.

After the 16th day or so, the fibroblasts in the hydrolysate control tubes tend to shrink or wander in toward the tissue fragment and

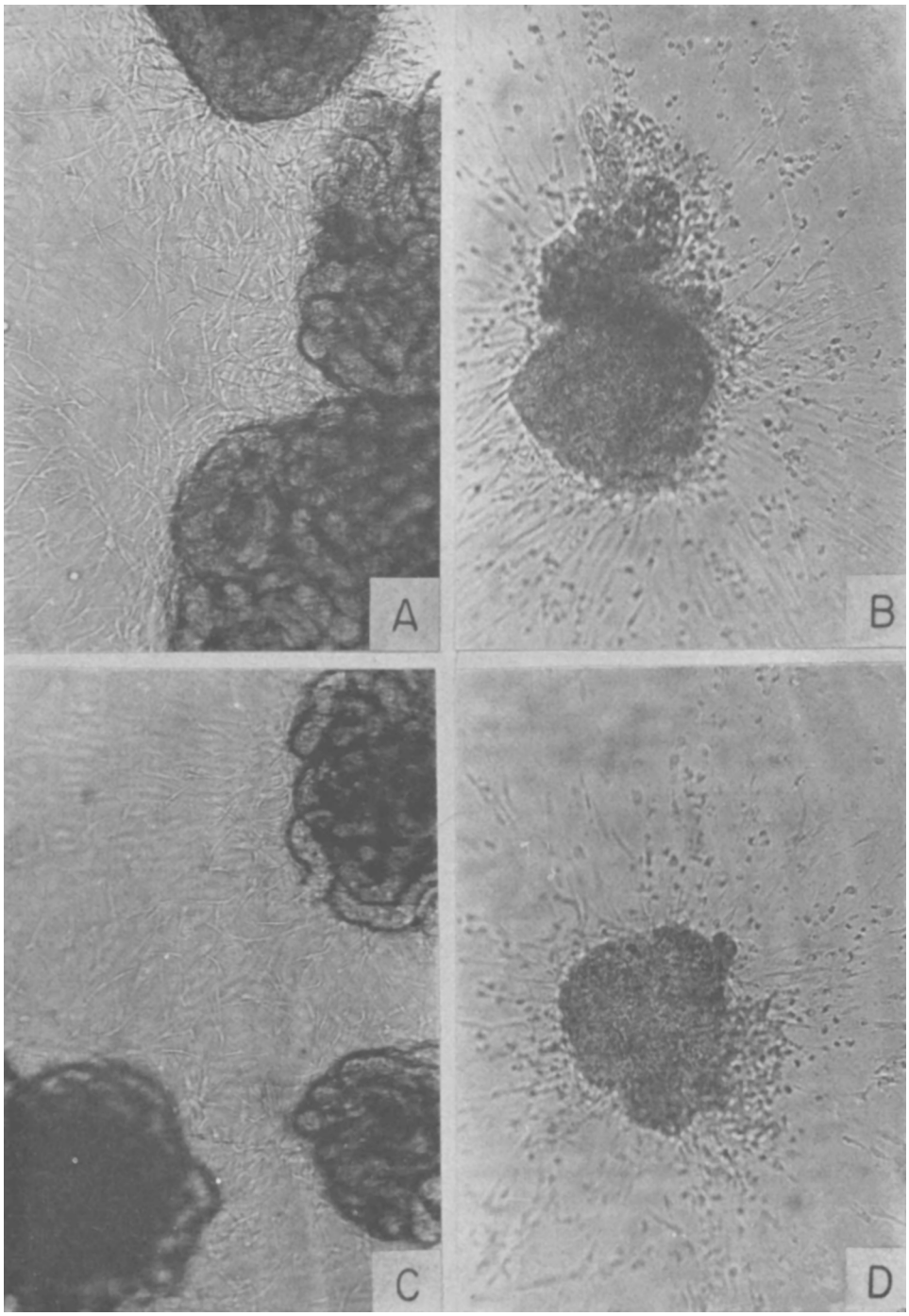

FIG. 1. Comparison of rolled and stationary cultures of monkey testicular tissue grown in *lactalbumin hydrolysate medium*. (A) Uninoculated, rolled control tubes showing clear, fibroblastic outgrowth on the 8th day after implantation of tissue fragments. (B) Roller tube culture inoculated on the 4th day with 100 tissue culture doses of Brunhilde (Type 1) virus. As shown, 4 days later, extensive degeneration is evident. (C) Uninoculated stationary control tube, similar to A. (D) Virus treated stationary culture, similar to B.

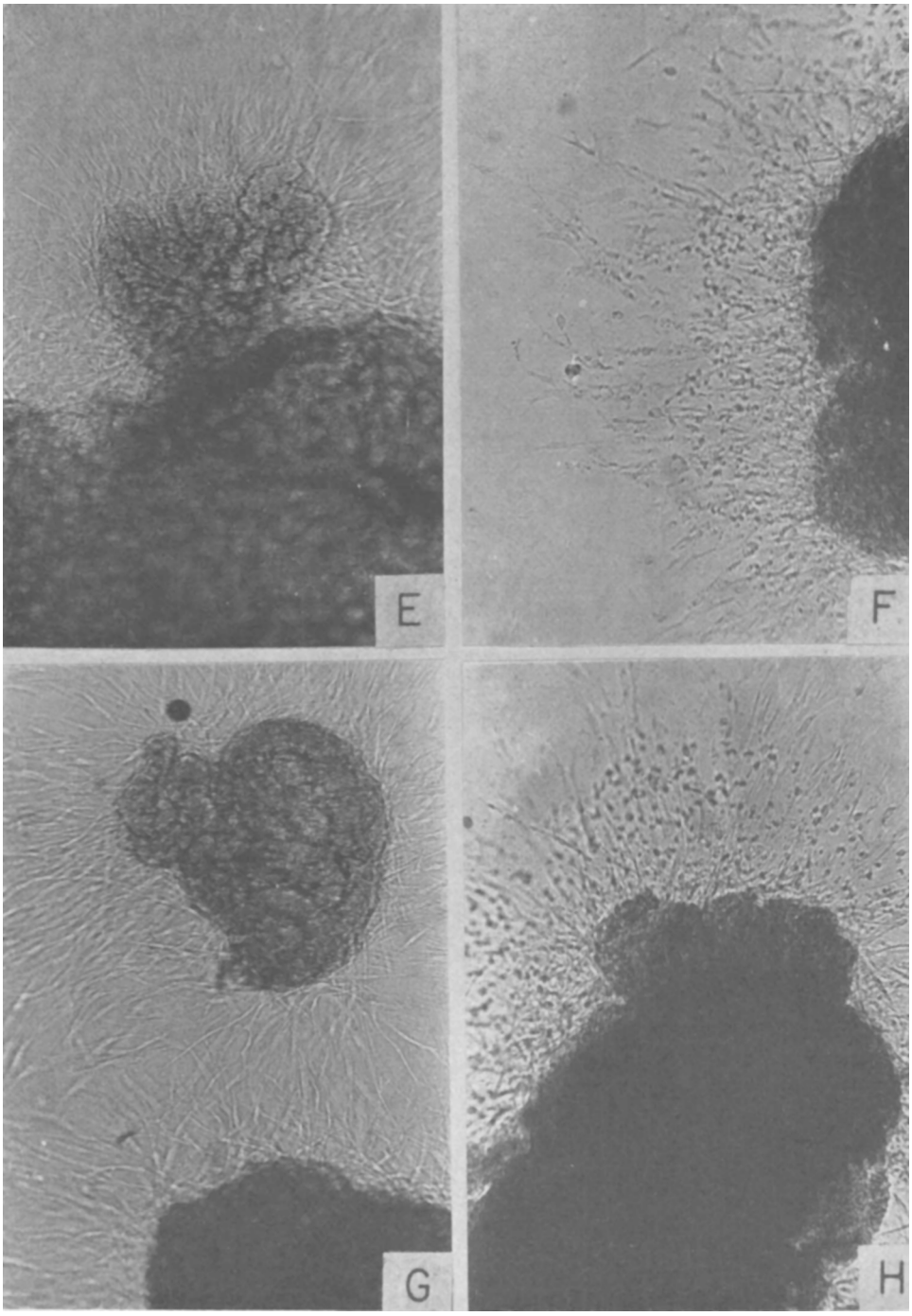

FIG. 2. Comparison of rolled and stationary cultures of monkey testicular tissue grown in *acetone-extracted chick embryo medium*. (E) Uninoculated, rolled control tubes showing clear, fibroblastic outgrowth on the 8th day after implantation of tissue fragments. (F) Roller tube culture inoculated on the 4th day with 100 tissue culture doses of Brunhilde (Type 1) virus. As shown, 4 days later, extensive degeneration is evident. (G) Uninoculated stationary control tube, similar to E. (H) Virus treated stationary culture, similar to F.

TABLE II. Titrations of Harvests from Stationary and Rolled Tubes Containing Acetone-Extracted Chick Embryo (ACE) or Lactalbumin Hydrolysate (LACT.)*

Day of harvest	Type tube culture in which virus was grown† ACE rolled	ACE stationary	LACT. rolled	LACT. stationary
4	10^{-3}	10^{-3}	10^{-4}	10^{-3}
9	10^{-4}	10^{-3}	10^{-4}	10^{-3}
13	10^{-2}	10^{-1}	10^{-4}	
18	10^{-2}	10^{-2}	10^{-3}	10^{-2}
23	10^{-1}	10^{-1}	10^{-3}	10^{-3}
30	10^{0}	10^{0}	10^{-2}	10^{-1}

* All titrations were carried out in rolled tubes containing ACE, using 0.1 ml per tube as in inoculum, and serial 10-fold dilutions. Results are given as the least concentration of fluid producing a cytopathogenic effect in at least one of two tubes during an observation period of 8 days. For further details see Ledinko, Riordan, and Melnick(5).

† Three tubes of each type indicated were inoculated with 100 tissue culture doses of Brunhilde (Type 1) virus, 8th tissue culture passage. Fluids from each set of three tubes were pooled when harvested.

parallel the periphery of the fragment. Such fibroblasts are not suitable for microscopic examination, which must be discontinued. However, the control tissue continues to metabolize well for over 30 days, at this time still bringing the pH of the medium from 7.5 to 7.0 in a few days. As shown in Table II, virus continues to multiply for sometime after the fibroblasts have degenerated. This is true for stationary as well as rolled tubes.

Several thousand tubes containing lactalbumin hydrolysate in the medium have been used in this laboratory, in which fibroblastic outgrowth was obtained in stationary tubes.

Discussion. This study indicates that the presence of protein in the *fluid* phase of the nutrient medium is not essential for the growth of fibroblasts or for the multiplication of poliomyelitis viruses in tube cultures. Thicke *et al.*(8) have recently found that synthetic medium "199"(10) will support the propagation of poliomyelitis virus in flask cultures, and Salk *et al.*(11) have also indicated that it is highly satisfactory in roller tube cultures. Future work will undoubtedly simplify the medium so that it will contain only those compounds essential for outgrowth of cells and for virus multiplication. The use of commercially available and inexpensive lactalbumin hydrolysate offers certain advantages in this direction, particularly as a substitute for the amino acid block used in synthetic media(10).

The benefits of protein-free media are great in subsequent work with tissue culture grown virus. To mention only a few: (i) foreign proteins can be eliminated from vaccines prepared from such fluids (except for the small amounts coming from the plasma clot and the tissue fragments), and (ii) complement-fixing antigens and purified virus preparations should be more easily obtained from starting material in which extraneous protein is absent or present in insignificant amounts. In their work on the preparation of complement-fixing antigens, Svedmyr, Enders, and Holloway(12) have recommended the replacement of the protein-containing medium by a dialyzable medium, but only after cellular outgrowth had occurred and after inoculation of the cultures with virus.

Stationary tubes are particularly useful in allowing poliomyelitis virus isolations, typing, and neutralization tests to be carried out in laboratories in which roller drums are not available. If tubes with tissue fragments imbedded in a plasma clot become available from a central supply agency, then any good hospital laboratory equipped with an incubator should be able to carry out diagnostic tests for poliomyelitis.

Summary. Poliomyelitis virus may be propagated in test tube, plasma-clot cultures of monkey testicular tissue in the absence of protein in the fluid phase of the nutrient medium. The tubes may be held stationary with the fragments submerged under a thin layer of medium, or they may be turned in a roller drum. The medium used contained lactalbumin enzymatic hydrolysate, serum ultrafiltrate, and balanced salt solution. With such a medium, fibroblasts grow out within 4 days after the cultures are made, and are then suitable for use in virus titrations and neutralization tests in which the presence of virus is indicated by its cytopathogenic effect on the fibroblasts. Also such cultures continue to produce virus for a period of about four weeks, long after the degeneration of the fibroblasts.

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Ineffectiveness of Aureomycin and Terramycin Against Rabies Street Virus in Mice.* (19824)

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There is no therapeutic material that is efficacious against infection with rabies virus. Therefore it was of interest to try terramycin for two reasons: a) concentration of ingested terramycin in the brain is reported to be relatively high(1), and b) conflicting evidence has been presented on the efficacy of terramycin against rabies virus. Quilligan *et al.*(2) showed no effect of terramycin on the titration of rabies virus in mice, while Azevedo and de Macedo(3) claimed 98% survival rates in rats and rabbits. This work was undertaken to secure more evidence on the action of terramycin on the rabies virus, and to compare it with aureomycin.

Method and materials. The virus material was rabies street virus in a 20% suspension of dog salivary gland.‡ The LD_{50} of this virus by intracerebral inoculation in 3-week-old white mice was $10^{-5.8}$ as calculated by the Reed-Muench method(4). The terramycin and aureomycin were incorporated into finely ground fox-chow at the rate of 100 mg of antibiotic per 100 g of feed. The mice were starved for a day prior to the experiment so that they would accept the ground mixture. In order to get some idea of how much drug the mice were ingesting, a weighed amount of feed and material was placed in a Petri dish and the material was weighed each day. The difference was then taken to be the amount consumed by the mice in the cage. The amount of aureomycin or of terramycin actually ingested by mice under this regimen is adequate to ensure a definite therapeutic response with murine and scrub typhus infections(5).

Procedure. Twenty mice divided into 2 cages with 10 mice in a cage were placed on the aureomycin feed mixture. Twenty mice divided into 2 cages with 10 mice in a cage were placed on the terramycin feed mixture. Twenty mice divided into 2 cages with 10

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