STUDIES ON THE PROPAGATION IN VITRO OF POLIOMYELITIS VIRUSES

IV. Viral Multiplication in a Stable Strain of Human Malignant Epithelial Cells (Strain HeLa) Derived from an Epidermoid Carcinoma of the Cervix*

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The propagation of poliomyelitis virus in tissue cultures prepared from primary explants derived from a variety of extraneural tissues from man (1–9) and monkeys (5, 7–17) has given new impetus to the development of methods for the production of poliomyelitis virus without resort (beyond the original explant) to man or to an experimental animal. A wide variety of tissues, normal and malignant, have been employed (16, 18) over several years in attempts to obtain strains of cells adaptable to cultivation in tissue culture beyond the first several generations. These attempts at the University of Minnesota met with failure until recently when success was achieved by the propagation of poliomyelitis virus in cultures of monkey testicular "fibroblasts" maintained in series (16). Contrariwise, stable strains of animal cells were developed by Gey and coworkers (19–22). Some of these strains have since been tested for susceptibility to a variety of viruses (23–26). The obvious need for a highly susceptible strain of human cells capable of yielding poliomyelitis virus in quantity in from one to several days led to the employment of a strain of malignant epithelial cells adapted to cellular cultivation *in vitro* (21, 22). It is the purpose of this paper to present the observations and data that relate to the employment of a stable strain for the successful propagation of poliomyelitis virus, Types 1, 2, and 3. This cellular strain, designated as strain HeLa by one of the authors (G. G.) when he obtained it from an epidermoid carcinoma of the cervix

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has been maintained in continuous serial culture passage *in vitro* from February 8, 1951, until the present. Cultures of this cell have now been shown in this laboratory to provide an inexpensive and effective means (*a*) for the assay of poliomyelitis viruses, (*b*) for the assay of antibodies by the neutralization technique, (*c*) for the production of virus in quantity, and (*d*) for the rapid isolation and identification of poliomyelitis viruses. These findings will be reported in a series of papers.

Materials and Methods

Virus.—Five strains representative of three immunological types of poliomyelitis virus were employed. The descriptive data that relate to the viruses of Type 1, Mahoney strain, and to Type 2, Yale-SK strain, have been presented previously (11). Type 3, Saukett strain, (tissue culture pool 10, March 24, 1952) was kindly supplied by Dr. Jonas E. Salk. In addition to these three established laboratory strains, two field strains of Type 3 virus, 50-M57, T3 and 52-M662, T3 were utilized immediately after their isolation from fecal samples of paralytic poliomyelitic patients. The immediate source of virus for the present experiments was supernatant liquid from infected cultures of monkey testicular tissue (11) or of strain HeLa cells. Virus was stored as described previously (11).

Assays for Virus.—Titrations for virus were performed by the transfer of serial tenfold dilutions of viral material to duplicate 6 to 10 day old cellular cultures which were kept at 36°C. Maintenance solution, MS, (27) a synthetic fluid, was employed as the liquid phase.[1] The quantities of cells used in the cultures will be stated for each experiment. Either Hanks's balanced salt solution (28) or MS at pH 7.0–7.4 was used for dilution of the virus in suspension. Microscopic observations for effects of the virus on the cells were made either daily, or twice daily. The highest final dilution of virus in the culture vessel which showed cytopathogenic effects within 5 days after inoculation was taken as the titration end-point.

Antisera.—Type-specific poliomyelitis antisera were kindly supplied by Dr. Jonas E. Salk. The labels for these antisera were as follows: Type 1—"Pool A-453, 1/23/50." Type 2—"Pool A-300, 20 June '49", Type 3—"Polio antiserum Type III Rebottled 7/18/52."

Cell Strain.—Tissue from an epidermoid carcinoma of the human cervix was placed in roller tube cultures on February 8, 1951, with result in a strain of malignant epithelial cells described as strain HeLa. The author (G. G.) who first cultivated this strain of cells maintained it by serial transfer through 30 cultural passages between February 8, 1951, and May 31, 1952, when cultures of these cells were sent to Minneapolis to serve as source material for the establishment of stock cultures of strain HeLa cells at the University of Minnesota.

Methods for Cellular Cultivation.—Strain HeLa cells for this study were cultivated *in vitro* on a glass substrate to provide surface for cellular multiplication by using a nutritive medium composed of either human placental serum (HPS), human adult serum (HAS), or human ascitic fluid (HAF), 50 per cent; chicken embryonic extract (EE), 2 or 5 per cent; and balanced salt solution, Hanks's (H), 48 or 45 per cent. The method for the preparation of chick embryo extract has been described (11). Hereafter, the designation of this medium and of similar media will be abbreviated, *i.e.*, HPS-50, EE-2, H-48. For the maintenance of cells, a mixture

[1] It was learned after these studies were completed, that a mixture of 10 per cent chicken serum and 90 per cent MS was superior to MS-100 for the maintenance of normal cellular structure. Titration end-points of poliomyelitis virus, Type 3, Saukett strain were identical in MS-100 and CHS-10, MS-90. However, the end-point in CHS-10, MS-90, was more easily determined microscopically, since non-specific degenerative changes did not occur within the 5 day period of cultivation.

of human serum or ascitic fluid, 40 per cent, and salt solution, 60 per cent, was employed. Four types of cultural vessel were used: test tubes, 16 by 150 mm. and 13 by 100 mm., Porter flasks, and square, screw cap bottles, 200 ml.[2] The test tubes, 16 by 150 mm., were either rolled at 10 to 12 R.P.H., or slanted in a stationary position. The test tubes, 13 by 100 mm., Porter flasks, and bottles were kept stationary at a slant of approximately 5 degrees. Cells were transferred serially by the employment of two technics, (*a*) the transfer of cellular clumps or explants, 0.5 to 1.5 mm. in average diameter, and (*b*) the transfer of cells suspended in liquid medium. The former technic was used until it became apparent that strain HeLa cells in suspension could be handled with greater efficiency. To carry out the transfer of cells as explants, an area of cellular growth was scraped from the wall of a cultural vessel with a bent tip glass pipette, placed in a Petri dish, and cut into fragments by the scissors-like action of two scalpel blades.[3] These fragments were placed on the glass wall of a cultural vessel wet with medium. After from 10 to 20 minutes the fragments were sufficiently adherent to the glass to permit covering them with liquid medium. When cells were transferred in liquid suspension, they were scraped from the glass either with a glass bent tip pipette or a platinum spatula, suspended in 0.5 per cent trypsin[4] at a pH of 7.4–7.6, incubated at 37°C. for 60 minutes, agitated to break cellular clumps and disperse the cells, and incubated for an additional 30 minutes. The volumes of trypsin solution employed were 8 ml. for the 200 ml. bottle, 1 ml. for the 16 by 150 mm. test tubes and Porter flasks, and 0.5 ml. for 13 by 100 mm. test tubes. The use of trypsin was essential to overcome the cohesive properties of strain HeLa cells, and to obtain cells in uniform suspension as single cells or clumps of several cells (29). Following centrifugation at 1000 R.P.M. for 10 minutes and removal of the supernatant trypsin solution, the cells were resuspended in a liquid medium, HPS-50 or HAF-50, EE-2, H-48, and dispensed in 0.25 ml. aliquots into cultural vessels, by employment of an apparatus similar to that described by Evans, Earle, *et al.* (30) for the preparation of replicate cultures. Cultures prepared by this method were slanted in a stationary position as described above. For cellular growth, cultures were incubated at 36 to 37°C.; for the mere maintenance of cells, a temperature range of 30 to 31°C. was frequently employed.

Method for Cellular Enumeration.—The concentration of cells in suspensions treated with trypsin was measured by direct microscopic enumeration of the cells in an aliquot of suspension, placed in a hemocytometer. The total number of cells in four chambers was counted, and the number of cells per milliliter of suspension was calculated by employment of the following formula:—

$$\frac{\text{Total cells in four chambers (3.6 c. mm.)}}{\text{3.6 c. mm.}} \times 1000 = \text{cells per cc.}$$

Method for Viral Cultivation.—After from 3 to 10 days of cultivation, more commonly 7, the cellular population usually sufficed for the support of viral growth. The liquid medium

[2] Kimble neutraglas, serum and sampling bottle.

[3] Bard-Parker No. 11.

[4] Two preparations of trypsin were employed, Armour, recrystallized trypsin and Difco trypsin 1:250. These were dissolved in MS-100. Recrystallized trypsin was found to be nontoxic for strain HeLa cells in concentrations less than 3 per cent. A concentration of 0.5 per cent was adequate for dispersing the cells. On the other hand, Difco trypsin, a less expensive preparation, was found to be toxic for strain HeLa cells in concentrations above 0.5 per cent. Accordingly, 0.5 per cent Difco trypsin was employed as routine to obtain maximal proteolytic activity without toxic effects. It was realized that the proteolytic activity for 0.5 per cent recrystallized trypsin is greater than of 0.5 per cent Difco trypsin, but the difference in cost made the Armour preparation less desirable for routine purposes.

was assumed to contain antibodies against poliomyelitis virus, since human serum or ascitic fluid was employed for the growth of cells. Therefore, this liquid was removed and replaced by an equal amount of MS-100. Since one replacement with MS-100 resulted in a dilution of from 1 to 20 or from 1 to 40 in the serum portion of the medium, a second replacement of the supernatant fluid, several hours after the first, was effected with result that the serum component of the medium used for cellular growth had a final dilution of from 1 to 400 to 1 to 800. These dilutions exceed the usual titer of poliomyelitis antibodies that might be encountered in human serum. The cultures that contained virus were incubated at 36–37°C.

Method for Staining Cells.—When preparations of fixed and stained cells were desired for photography or for later study, cells were grown on coverglasses, 11 by 22 mm., in Porter flasks, fixed with Bouin's solution, stained with Harris' hematoxylin, and mounted on microscopic slides.

EXPERIMENTAL RESULTS

Almost at once two facts became plain: (*a*) cells of the cultures infected with virus soon underwent degeneration and death, and (*b*) the virus multiplied.

The Destructive Effects of the Virus on Strain HeLa Cells

Six experiments (Experiments 1 to 6) were performed to learn whether poliomyelitis viruses, Types 1, 2, and 3 are destructive for strain HeLa cells.

Experiments 1 and 2.—Duplicate experiments were carried out on separate occasions with poliomyelitis virus, Type 1, Mahoney strain. For each experiment, strain HeLa cells were cultivated on coverglasses, 11 by 22 mm., in Porter flasks. Each culture contained 2 explants which were incubated for from 5 to 8 days in HAF-50, EE-2, H-48, 0.75 ml. Before virus was added, the liquid medium was replaced with MS-100, 0.9 ml. Virus, 0.1 ml., which had been harvested from monkey testicular tissue cultures (11) was transferred to each of 6 flasks. For control purposes, no virus was added to one pair of flasks and to a second pair, virus only was inoculated. For a third set of tubes Type 1 antiserum, 0.1 ml. of 1:2 dilution, was mixed with the contents. The fourth pair received Type 2 antiserum, 0.1 ml. of 1:2 dilution. For Experiment 2 a different pool of monkey testicular tissue culture Mahoney virus was used. Microscopic observations of the cells were made daily. When cellular degeneration was seen culturing was terminated, for fixation and staining of the cells. The appearance of cells affected by virus in the presence and absence of neutralizing antibodies are shown in Figs. 4 to 12.

It was observed microscopically that HeLa cells infected with poliomyelitis virus underwent progressive destruction. The cells became rounded and contracted, with pyknosis of the nucleus and granularity of the cytoplasm. Other evidence for involvement of the cytoplasm consisted of shrinkage, the detachment of many cells from the glass substrate, and total rupture ultimately. This sequence of changes has been termed the cytopathogenic effect of poliomyelitis virus (1, 3). The time required for cellular destruction varied with the size of the inoculum. The cytopathogenic effect was observed regularly, total destruction in from 12 to 120 hours resulting in the case of laboratory strains of virus. Field isolates more commonly required from 36 to 48 hours for the destruction of all cells.

Photomicrographs to illustrate the findings in Experiments 1 and 2 are presented in Figs. 1 to 12. When compared with the appearance of normal strain HeLa cells (Figs. 1 to 3), it is apparent that cells infected with poliomyelitis virus, Type 1, Mahoney strain were destroyed (Figs. 4 to 6). On the other hand, the presence in the cultures of type-specific antibody, prevented the cytopathogenic effect of this virus (Figs. 7 to 9), whereas the presence of antibody for Type 2 poliomyelitis virus did not effect the cytopathogenicity of Type 1 virus (Figs. 10 to 12). Complete cellular destruction commonly occurred within 24 to 72 hours after virus inoculation.

Experiments 3 and 4.—Two experiments similar in design to Experiments 1 and 2, were carried out with poliomyelitis virus, Type 2, Yale-SK strain. Virus from different groups of monkey testicular tissue cultures was employed for these experiments. The results are illustrated by the photomicrographs in Figs. 13 to 21.

Figs. 13 to 24 show photomicrographs representative of the microscopic observations for Experiment 3. It can be seen that poliomyelitis virus, Type 2, Yale-SK strain, caused destruction of strain HeLa cells (Figs. 13 to 15). When antiserum to Type 1 virus was present in the cultures, the cytopathogenic effect of Type 2 virus was unmodified (Figs. 16 to 18). However, antibodies to Type 2 virus effectively prevented the destruction of strain HeLa cells by Yale-SK virus (Figs. 19 to 21). Cultures kept for control purposes without either virus or antiserum retained their normal appearance throughout the period of study. For examples, normal cells and extraordinary cellular arrangements are shown in Figs. 22 to 24. Frequently coalescense of cells occurred to form epithelial sheets (Fig. 23), and occasionally unusual aggregations of cells were observed (Fig. 24), perhaps resulting from abnormal cellular division.

Experiments 5 and 6.—Two experiments were carried out with strain HeLa cells and poliomyelitis virus, Type 3, Saukett strain. Experiment 5 was similar to Experiment 1 except that groups of three, instead of pairs of Porter flask cultures were employed, and antibodies to Type 3 virus as well as to Types 1 and 2 virus were used. Virus from the seventh passage in strain HeLa cells was utilized. Experiment 6 was performed with test tube and Porter flask cultures and virus from the fifth passage in strain HeLa cells. Otherwise Experiment 6 was similar in design to Experiment 5. The findings in these experiments are indicated in Figs. 25 to 36.

It can be seen from the photomicrographs of strain HeLa cells (Figs. 25 to 27) that poliomyelitis virus, Type 3, Saukett strain was cytopathogenic for these cells. Frequently with potent Saukett strain virus observable cellular destruction was evident in less than 24 hours following the inoculation of virus into cultures. The inability of heterotypic antibodies to protect strain HeLa cells from Type 3 virus was established for Type 1 antiserum (Figs. 28 to 30) and for Type 2 antiserum (Figs. 31 to 33). In contrast, cells protected from the destructive effects of Type 3 poliomyelitis virus by the presence of homotypic neutralization antibodies retained their normal appearance (Figs. 34 to 36).

The Propagation of Poliomyelitis Virus in Strain HeLa Cells

Six experiments were carried out to learn how well poliomyelitis virus multiplies in strain HeLa cells.

Experiments 7 and 8 were designed to measure the growth of poliomyelitis virus, Type 1, Mahoney strain by differential titrations of the viral inoculum and of the harvest following a single passage in roller tubes.

TABLE I

Evidence for the Multiplication of Poliomyelitis Virus, Type 1, in Cultures of Strain HeLa Human Epithelial Cells (20 Explants per Culture)

(Results of Experiment 7)

Concentration of virus in liquid phase of culture as measured by the ability of successive decimal dilutions of supernatant fluid to destroy strain HeLa cells		
Duration of cultivation in hrs.		
0	48	72
1 (undiluted)		$10^{-6 \text{ to } 7}$
10^{-1}	10^{-6}	
10^{-2}	$10^{-5 \text{ to } 6}$	
10^{-3}	10^{-5}	
10^{-4}	$10^{-4 \text{ to } 5}$	
10^{-5}	$10^{-4 \text{ to } 5}$	

Experiment 7.—Cultures in test tubes, 16 by 150 mm., which contained 20 explants in each tube were incubated in HAF-50, EE-2, H-48, 1 ml. The medium was replaced on the 4th day by the same mixture. On the 7th day, the liquid was replaced with MS-100, 0.9 ml., and an aliquot, 0.1 ml. of each serial tenfold dilution of poliomyelitis virus, Type 1, Mahoney strain, was inoculated into each of two cultures. When degeneration of cells had become evident the supernatant liquids from each pair of cultures were pooled and stored in sealed glass ampules at −70°C. Subsequently, these pools of virus were assayed by titration in strain HeLa cultures made up of two areas of 7 to 10 day cellular outgrowth in test tubes, 13 by 100 mm. The results from Experiment 7 are shown in Table I.

Table I presents the data recorded for Experiment 7. It can be seen that when the initial titration end-point of virus in the cultures was from 1 to 10^{-3}, virus multiplied from 100- to 1,000,000-fold in 48 to 72 hours of cultivation. It is noteworthy that the final titers for virus in the liquid phases of the cultures were in the range of from 10^{-4} to 10^{-6} regardless of the amount of virus inoculated, provided the cultures were incubated until cellular degeneration occurred.

Experiment 8 was planned to learn the effect of utilizing for viral cultivation approximately one-tenth as many strain HeLa cells as were employed in Experiment 7.

Experiment 8.—The procedure for Experiment 8 was identical with that for Experiment 7 with the exception that each cultural tube contained 2 rather than 20 explants. The results from Experiment 8 are presented in Table II.

The results of Experiment 8 (Table II) established two points: (*a*) relatively small numbers of strain HeLa cells (*i.e.*, two areas of cells no greater than 5 mm. in diameter) readily supported the growth of poliomyelitis virus, Type 1, to result in a high titer, 10^6, within a period of from 48 to 72 hours; (*b*) the smallest inoculum of virus that was employed resulted in a final yield as great as that attained from an inoculum 10 to 100 times greater, but with a small inoculum degeneration of cells was delayed (*i.e.*, from 72 to 120 hours).

The comparative findings of Experiments 7 and 8 (Tables I and II), show that the titer of virus from cultures with 2 explants of cells approximated the yield of virus that resulted from cultures with 10 times as many cells. These

TABLE II

Evidence for the Multiplication of Poliomyelitis Virus, Type 1, in Cultures of Strain HeLa Human Epithelial Cells (2 Explants per Culture)

(Results of Experiment 8)

Concentration of virus in liquid phase of cultures as measured by the ability of successive decimal dilutions of supernatant fluid to destroy strain HeLa cells			
Duration of cultivation in hrs.			
0	48	72	120
Undiluted		$10^{-5 \text{ to } 6}$*	
Undiluted			$10^{-3 \text{ to } 4}$*
10^{-1}	$10^{-5 \text{ to } 6}$		
10^{-2}	$10^{-5 \text{ to } 6}$		

* Results at 72 hours and 120 hours refer to different cultures inoculated with the same virus.

results may reflect the employment of tenfold increments for the titration of virus.

The purpose of Experiment 9 was to learn the ability of this stable strain of human epithelial cells to support the growth of the laboratory-adapted Mahoney strain upon its transfer in series.

Experiment 9.—2 explants after growth for 7 days in HPS-50, EE-2, H-48 provided cells. A viral inoculum of 0.1 ml. of a dilution ranging from 10^1 to 10^5 was added to the fluid content, 0.9 ml., in each cultural tube, 16 by 150 mm. Incubation at 37°C. was interrupted temporarily at intervals of 12 to 24 hours for microscopic examination. When destruction of the cells had occurred, transfer to the next cultural generation was made. The data for this experiment are presented in Table III.

It can be seen from Table III that propagation of the Mahoney strain was carried out successfully throughout 50 successive tissue changes, to result in a final cumulative log dilution of 10^{58} for the HeLa cellular culture fluid employed as the starter inoculum. The presence of virus in the original inoculum

and in aliquots of the tenth and twentieth tissue passage fluids was established by the production in monkeys of paralysis and/or death and histological evi-

TABLE III

Propagation in Vitro of Poliomyelitis Virus in Cultures of Human Epithelial Cells, Strain HeLa

(Results of Experiments 9, 10, 11, and 12)

Experiment	Poliomyelitis virus		No. of culture passages of virus	Results as indicated by		
				Cumulative log of dilution of virus	Infectivity of supernatant fluid for	
	Type	Strain			HeLa cells as log	Monkey*
9	1	Mahoney	1	1		Yes
			10	10	5	"
			20	20		"
			30	34	4	"
			50	58	5	N.T.
10	3	Saukett	1	1	6	Yes
			10	11	N.T.	"
			20	35	6	"
			30	53	5	N.T.
			40	66	5	Yes
			50	75	5	"
			60	89	6	"
			80	115	6	N.T.
11	3	M50-57	1	1	6	Yes
			10	10	4	"
			20	22	5	"
			40	48	5	N.T.
12	3	M52-662	1	1	6	Yes
			10	15	5	"
			18	23	5	"
			40	53	5	N.T.

* The monkeys were observed for a 28 day period. "Yes" indicates the occurrence of paralysis and/or death; the presence of histological evidence of poliomyelitis infection in the central nervous system or evidence for specific homotypic antibodies; "N.T." signifies not tested.

dence of poliomyelitis. Finally, the identity of an aliquot of supernatant fluid from the twentieth passage was established as Type 1 poliomyelitis virus by neutralization of its cytopathogenic effects with Type 1 antiserum. The findings of Experiments 7 to 9 established conclusively the propagation *in vitro* of the Mahoney strain of poliomyelitis virus.

Experiments 10 to 12 provide further unequivocal evidence for the propaga-

tion *in vitro* of poliomyelitis virus. It was found early in these studies that HeLa cells readily support the serial propagation of the laboratory-adapted strain of poliomyelitis virus, the Mahoney strain of Type 1. Evidence for infection was manifested, following inoculation within a 24 to 48 hour interval, by total destruction of cells. Virus was then found to have been produced in high titer. For comparative purposes, three strains of poliomyelitis virus, Type 3, one a laboratory strain, Saukett strain, and two strains directly upon their isolation from stool specimens of paralytic patients were kept in serial transfer.

Experiments 10, 11, and 12.—The experimental procedure for the three experiments was similar. 2 explants, or approximately 25,000 HeLa cells in suspension, were transferred to tubes, 16 by 150 mm., and 0.25 ml. of HPS-50, EE-2, H-48 was added for incubation at 37°C. On day 3, 0.25 ml. of HPS-80, EE-10, H-10 was added. Sufficient cells for viral propagation were commonly present on day 7. The tubes were made ready for inoculation with virus by the replacement of supernatant fluid twice at intervals of 1 hour by MS-100, 0.9 ml. Thus removal of human serum was effected by dilution.

The initial inoculum for Experiment 10 consisted of a 10^1 dilution of Dr. Salk's tissue culture pool 10, Saukett strain, March 24, 1952. Thereafter at 1 to 3 day intervals, serial transfer was effected by employing as the inoculum pooled supernatant fluid from the preceding culture generation in a dilution of from 10^1 to 10^5. To date, successful propagation throughout 80 generations has resulted in a cumulative log dilution of 10^{115}.

The starter virus suspension for Experiment 11 consisted of a 10^1 dilution of the second tissue culture passage of a field strain of Type 3 poliomyelitis virus. This strain was designated M50–57, T3 upon its isolation from the stool of a patient ill with paralytic poliomyelitis in 1950.

The starter inoculum employed for Experiment 12 was a 10^1 dilution of pooled supernatant fluid from a tissue culture isolate, M52–662, T3. This isolate was procured from the fecal sample of a paralytic poliomyelitic patient in 1952. Each strain of virus was maintained by transfer to two fresh HeLa cultures at intervals of 24 to 48 hours.

The salient findings for Experiments 10 to 12 are summarized in Table III.

The results of Experiments 10 to 12 (Table III) provided conclusive evidence that human epithelial cells which had already been maintained *in vitro* by serial passage for 18 months retained during the present studies fully their ability (*a*) to support the growth of laboratory and field strains of poliomyelitis virus and (*b*) to yield large quantities of virus, as reflected by the cumulative log of the dilution. Moreover, the serial transfer of four strains of virus for from 40 to 80 passages again provided unequivocal evidence for the propagation of virus in strictly extraneural cells. The cumulative log of virus dilution at the 80th passage was at least 115.

Further Applications of Strain HeLa Cellular Cultures to the Study of Poliomyelitis Viruses

Strain HeLa cells were utilized in a variety of studies involving poliomyelitis viruses in attempts to demonstrate properties of theoretical interest or of possible practical application.

Comparative Studies with Monkey Testicular Tissue.—For virus assay, titrations of poliomyelitis virus were carried out in strain Hela cellular cultures and the results were compared with titrations performed simultaneously in cultures of monkey testicular tissue (Experiments 13 and 14).

Experiment 13.—Roller tube cultures of testicular tissues were prepared from immature monkeys, animals with a dentition corresponding to 8 to 12 years in man. Each tube contained 5 explants embedded in chicken plasma, and a liquid medium composed of horse serum-50, EE-10, H-40, 1.5 ml. When sufficient cellular outgrowth had occurred after 7 days of incubation, the liquid medium was replaced with MS-100, 2 ml., which contained virus, Type 1, Mahoney strain, in known dilutions. The cells were observed at 1, 3, 6, and 12 days for the occurrence of viral cytopathogenicity. The same dilutions of virus in MS-100, 0.4 ml., were placed in cultures of strain HeLa cells (2 explants per 13 by 100 mm. test tube). Daily observations were made for cellular destruction.

TABLE IV

Comparative Infectivity End-Points of Poliomyelitis Virus Procured from Cultures of Strain HeLa Cells and Monkey Testicular Tissues

(Results of Experiments 13 and 14)

Virus		Titer of virus in liquid phase of cultures	
Type	Strain	Monkey testis*	Strain HeLa‡
1	Mahoney	10^{-5}	$10^{-5 \text{ to } 6}$
3	Saukett	$10^{-5 \text{ to } 6}$	10^{-6}

* Results by 12 days after viral inoculation. The same results were present at 6 days for Type 1 virus; for Type 3 virus, the second of two tubes inoculated with 10^{-5} concentration of virus became positive between the 6th and 12th days of viral cultivation.

‡ Results by 5 days after viral inoculation.

Experiment 14.—In this experiment poliomyelitis virus, Type 3, Saukett strain was employed as procured from the twentieth passage of virus in cultures of strain HeLa cells. The methods described for Experiment 13 were used for titration of virus in monkey testicular cultures and in strain HeLa cultures, except that the latter cultures contained 2 explants per 16 by 150 mm. test tube.

The results from Experiment 13 and 14 are shown in Table IV.

Table IV presents the results of assays of poliomyelitis virus in monkey testicular cultures and strain HeLa cultures (Experiments 13 and 14). It can be seen that poliomyelitis viruses, Types 1 and 3, produced slightly higher titration end-points when measured in cultures of strain HeLa cells than when measured by the accepted tissue culture method that utilizes monkey testicular tissue. Since virus was detected by strain HeLa cells in slightly higher dilutions, and the effects of virus in all dilutions were evident earlier with strain HeLa cells (1 to 5 days) than with monkey testicular cultures (6 to 12 days), it appears that strain HeLa cells were more sensitive to the cytopathogenic effects of poliomyelitis virus than monkey testicular cells.

Experiments 7 to 12 (Tables I, II, and III) yielded evidence for the applicability of the cellular strain to possible mass production of virus for serologic complement fixation tests or other purposes.

It can be seen from the results presented in the tables that cultures of strain HeLa cells are capable of producing large quantities of poliomyelitis virus. The amount of virus was as great as, or greater than, that produced by the inoculation of animals or of other cellular cultures. The virus is obtained in high titer and in clear solutions that have relatively low contents of protein, lipids, or lipoproteins when compared with virus suspensions prepared from nerve tissue.

TABLE V

The Survival after Shipment by Mail of Strain HeLa Cells in Culture

(Results of Experiment 15)

Time in transit	Type of shipment	Type of culture	Survival incidence
days			
3	Airmail and mail truck	Explant	2/2*
3	" " " "	Cell suspension	19/20
4	" " " "	" "	2/2
6	" " " "	" "	2/2
6	Railroad and mail truck	" "	1/1
7	" " " "	" "	2/2

* The numerator indicates the number of cultures which showed an abundance of healthy cells when incubated in liquid medium (HPS-50 or HAF-50, EE-2, H-48) for 2 to 5 days after shipment. The denominator signifies the number of cultures sent.

A single experiment (Experiment 15) was carried out in anticipation of the possible use of the HeLa strain of cells in cultural tubes designed for field diagnostic and epidemiologic studies. It was performed to learn the effect upon strain HeLa cells of adverse environmental conditions that may occur during shipment by plane or train for long distances.

Experiment 15.—To learn whether strain HeLa cells withstand shipment by mail, a series of cultures were sent during October and November by either air mail or regular mail, over a distance of approximately 2500 miles; *i.e.*, from Minneapolis, Minnesota, to Norwich, New York, and return. The cultures contained cells which had been grown on the glass wall of test tubes, 16 by 150 mm. or 13 by 100 mm. for from 7 to 10 days in HPS-50 or HAF-50, EE-2, H-48 medium. For shipment, all but 1 drop of liquid medium was removed and the tubes were sealed tightly with a rubber stopper about which was placed adhesive tape. The mailing cartons commonly employed for the shipment of test tubes were used, and the instruc-

tion "Keep from Heat" was placed on the package. For the first shipment, "Live Cells" was also noted. To prolong the period in transit, several shipments were kept at room temperature for 1 or 2 days before being returned to Minneapolis. Upon return of the cultures to this laboratory, fresh medium, HPS-50 or HAF-50, EE-2, H-48, was added and the tubes were incubated at 36 to 37°C. The results from Experiment 15 are given in Table V.

The data that pertain to the shipment of cells (Experiment 15) are presented in Table V. It is apparent that shipment of strain HeLa cells was successful by air mail or regular mail. The viability of the cells usually was established within 24 hours after incubation by the development of cellular processes; the cellular populations in the cultures were replenished in from 2 to 5 days.

Shipment of strain HeLa cells was much less successful when the cells had been incubated in maintenance solution prior to shipment. A group of 10 cultures kept at 36°C. in MS-100 for 5 days before shipment showed survival of cells in only three cultures.

It was desirable to learn the applicability of this cellular strain, the cultural media employed, and the techniques utilized to the isolation of field strains of virus and to the specific immunologic identification of such strains. By the methods here described virus was readily recovered from patients with paralytic poliomyelitis, during the summer of 1952, and 100 strains were quickly identified. These findings (18) are included in a paper to appear later.

DISCUSSION

The present work shows that a strain of malignant human epithelial cells (strain HeLa, Gey) maintained by serial cultivation *in vitro* since February, 1951, will support the multiplication of poliomyelitis virus. The propagation of each of the three types of virus was accompanied by progressive destruction of the cells. These destructive effects were readily prevented by the employment of the homotypic poliomyelitis antibody though not by heterotypic antibodies. The experiments demonstrate further that strain HeLa cells can easily, inexpensively, and effectively be employed (*a*) for the quantitation of virus, (*b*) for the measurement of specific antibody to poliomyelitis virus by the tissue culture–neutralization technic, and (*c*) for the production of virus for a variety of purposes, for example, to serve as antigens for complement fixation.

The usefulness of normal cells of extraneural origin in tissue culture for the study of poliomyelitis viruses has been previously established (1–17). The results of the present study make it plain that cancerous cells of strain HeLa have much in common with the cells previously employed. For example, they share the following properties with monkey testicular fibroblasts and cells from a variety of human and monkey tissues: (*a*) in cultures they enable laboratory and field strains of poliomyelitis virus of all three types to propagate, (*b*) in synthetic solution, they maintain viability and an unaltered

morphology, and (*c*) under the extraordinary environmental conditions that result from shipment by train and plane they possess hardiness and the capacity for renewed proliferation upon adding nutritive fluid.

It is especially noteworthy that epithelial cells of strain HeLa possess distinctive inherent qualities which make these cells excel in usefulness for the study of poliomyelitis virus. To cite examples that are especially noteworthy: (*a*) the stability of these cells while in continuous serial passage for more than 2 years makes it apparent that the strain can be maintained in perpetuity by continuous transfer in series; (*b*) the capacity to grow in fluid media on glass simplifies cultivation and eliminates chicken plasma, a source of foreign protein; (*c*) the ease with which strain HeLa cells can be procured free in suspension by treatment of cellular sheets with trypsin and quantitation by direct enumeration makes available replicate cultures *en masse* and single cells for intimate host cell-virus studies. A further advantage of the use of this cell in poliomyelitis studies consists in the elimination of the inconvenience of obtaining tissues at need from human embryos, patients, or monkeys. Moreover, the cost of a large monkey colony and the hazard of inadvertently acquiring from man or monkey an extraneous virus or other infectious agent contained in tissue explants are excluded.

Foreign proteins and other antigenic materials constitute a risk and nuisance in the production of specific antigens for serologic studies. This problem for man could be simplified by the utilization of a nutritive complex of human derivation for the propagation *in vitro* of virus. Strain HeLa cells cultivated on glass in a synthetic solution in the absence of chicken plasma and embryonic extract constitute such a complex. Moreover, satisfactory cellular multiplication can be achieved in diluted human serum without chick embryo extract whenever it becomes desirable to grow cells in a medium made entirely from materials of human derivation.

The extraordinary susceptibility of strain HeLa cells to infection with poliomyelitis virus is reflected by the rapidity after infection of degenerative changes and death of the cells and the production of virus. This susceptibility permits the detection of virus in field specimens in from 12 to 96 hours. Moreover, the yield of virus from cultures of strain HeLa cells is as great in magnitude as that from animals or cultures of human or monkey tissues. This strain of cells should therefore have many uses in the study of poliomyelitis.

The evidence that poliomyelitis virus propagates in extraneural cells *in vitro* is unequivocal. This evidence was obtained by studies that employed mixed cell cultures from tissue explants (1–15, 17) and serial cultures of fibroblasts derived from monkey testicle (16). The results of the present work made it apparent that the virus also is epitheliotrophic, a malignant epithelial cell from an epidermoid carcinoma of the human cervix providing a cellular substrate fully adequate for multiplication of virus *in vitro*.

Ultimately it will be desirable to employ for the detection of human viruses or other obligate cellular parasites, not only strains of human fibroblasts and epithelial cells but also cultures of other cells that have been or can be continuously cultured. Further efforts are under way to develop cellular strains of these types for studies with human infectious agents.

The findings as a whole serve to demonstrate many distinctive properties of a stable strain of human epithelial cancer cell that can be applied advantageously to the study of the poliomyelitic group of viruses and possibly to many viruses encountered in man.

SUMMARY

The cells of a human epithelial cancer cultivated *en masse* have been shown to support the multiplication of all three types of poliomyelitis virus. These cells (strain HeLa of Gey) have been maintained *in vitro* since their derivation from an epidermoid carcinoma of the cervix in February, 1951. As the virus multiplied it caused in from 12 to 96 hours degeneration and destruction of the cancer cells. The specific destructive effect of the virus was prevented by adding homotypic antibody to the cultures but not by adding heterotypic antibodies.

Methods for the preparation of large numbers of replicate cultures with suspensions of strain HeLa cells were described. The cells in suspension were readily quantitated by direct counts in a hemocytometer. A synthetic solution that maintains cellular viability was employed for viral propagation.

The experimental results demonstrate the usefulness of strain HeLa cells for (*a*) the quantitation of poliomyelitis virus, (*b*) the measurement of poliomyelitis antibodies, and (*c*) the production of virus.

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EXPLANATION OF PLATES

All the photographs were made by Mr. Henry Morris. The magnifications are 65, 160, and 250.

PLATE 39

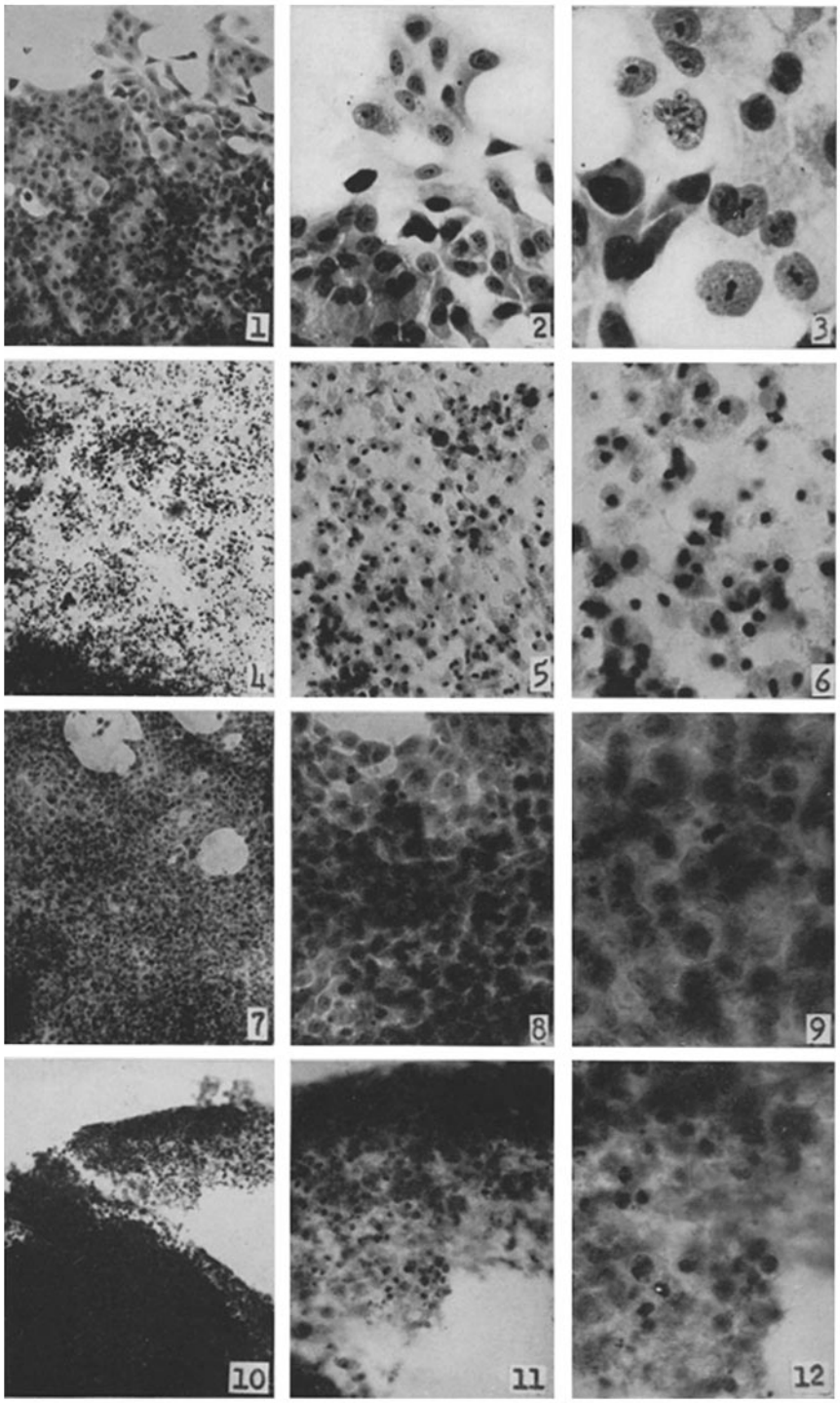
Results of Experiment 1. Evidence of the pathogenicity of poliomyelitis virus for human epithelial cells, strain HeLa, and of the protective effect of homotypic antibody. Harris's hematoxylin stain.

FIGS. 1 to 3. Normal cells from strain HeLa.

FIGS. 4 to 6. Degenerated strain HeLa cells; photographed 2 days after the inoculation of poliomyelitis virus, Type 1.

FIGS. 7 to 9. Normal appearing cells from strain HeLa, protected from the effects of virus by homotypic antibody. The photographs were made 2 days after inoculation of the cultures with virus and antibody.

FIGS. 10 to 12. Degenerated strain HeLa cells; 2 days after the inoculation of poliomyelitis virus, Type 1 together with antibody for Type 2 virus.



(Scherer *et al.*: Propagation *in vitro* of poliomyelitis viruses)

PLATE 40

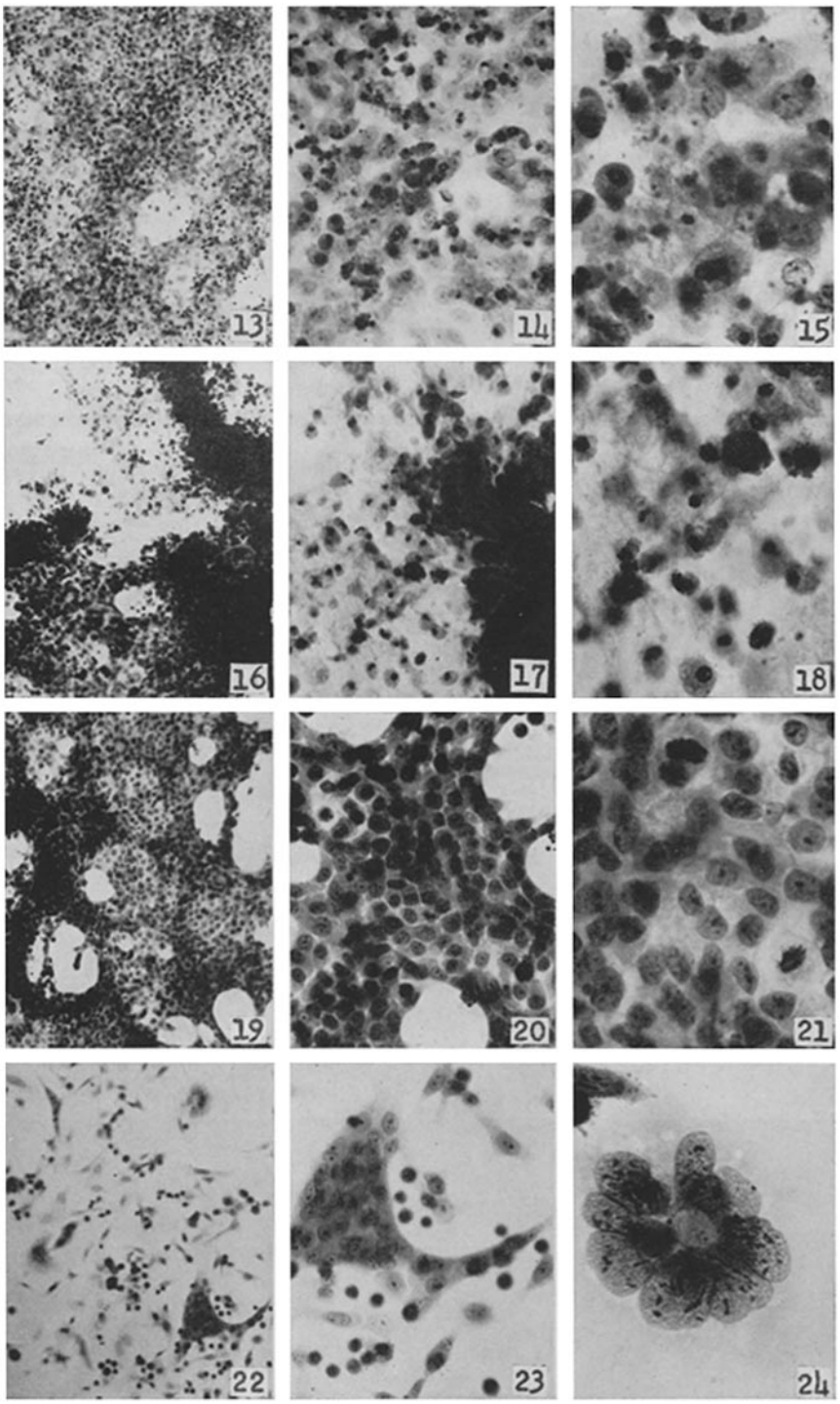
Results of Experiment 3. The destruction of human epithelial cells, strain HeLa, by poliomyelitis virus, Type 2, Yale-SK strain; to illustrate the neutralization of viral cytopathogenicity by homotypic antibody. Harris's hematoxylin stain.

FIGS. 13 to 15. Degenerated strain HeLa cells; 2 days after the inoculation of poliomyelitis virus, Type 2.

FIGS. 16 to 18. Degenerated strain HeLa cells; photographed 2 days after inoculation of poliomyelitis virus, Type 2, together with antibody for Type 1 virus.

FIGS. 19 to 21. Normal appearing cells from strain HeLa 2 days after inoculation of virus and antibody. These cells were protected from the effects of virus by homotypic antibody.

FIGS. 22 to 24. Normal cells from strain HeLa which had been maintained in MS-100 for 3 days. These cells were growing apart from the original explant, as isolated cells and small aggregates of cells.



(Scherer *et al.*: Propagation *in vitro* of poliomyelitis viruses)

PLATE 41

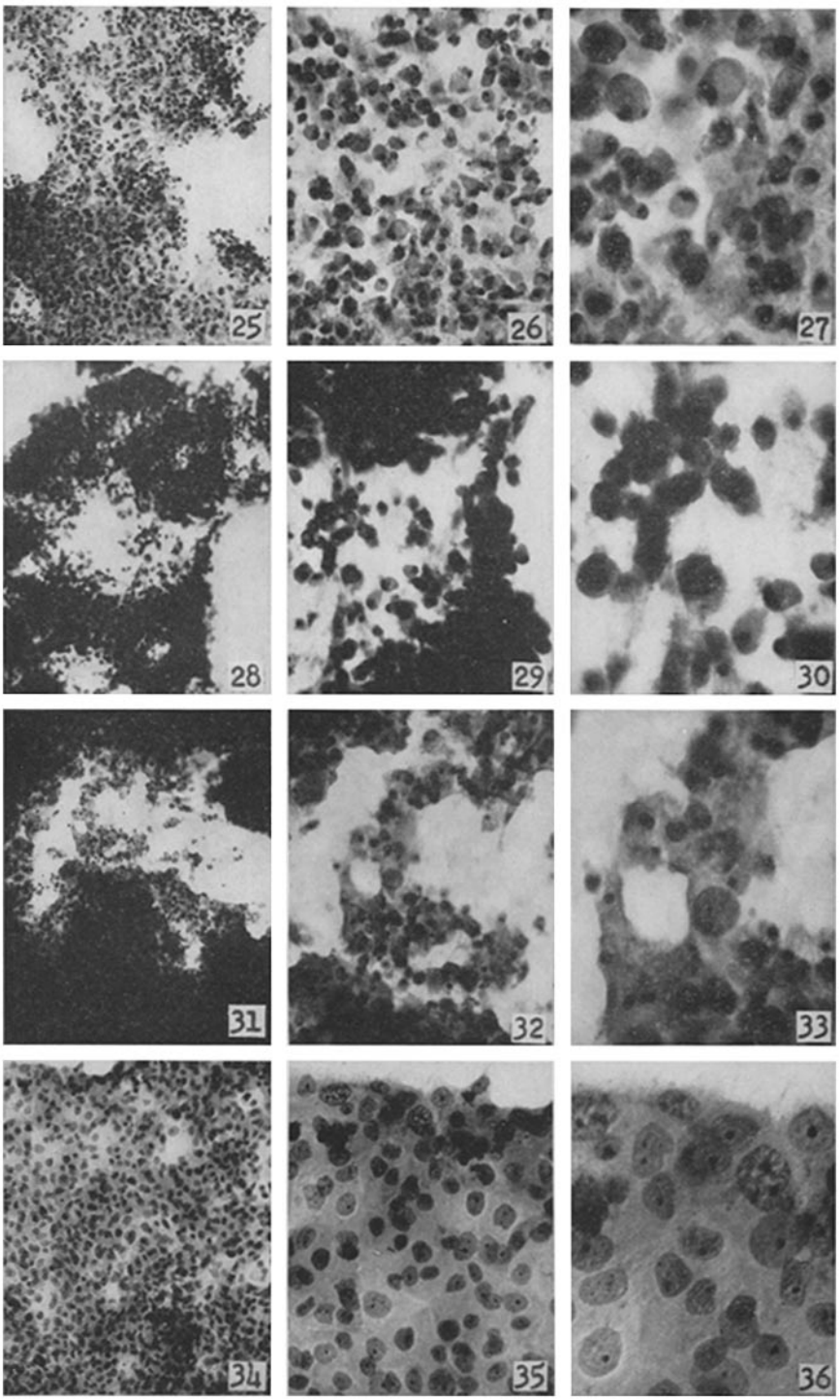
Results of Experiment 5. The destruction of human epithelial cells, strain HeLa, by poliomyelitis virus, Type 3, Saukett strain; the neutralization of viral cytopathogenicity by homotypic antibody. Harris's hematoxylin stain.

FIGS. 25 to 27. Degenerated strain HeLa cells 1 day after the inoculation of poliomyelitis virus, Type 3.

FIGS. 28 to 30. Degenerated strain HeLa cells, 1 day after inoculation of poliomyelitis virus, Type 3, together with antibody for Type 1 virus.

FIGS. 31 to 33. Degenerated strain HeLa cells, 1 day after inoculation of poliomyelitis virus, Type 3, together with antibody for Type 2 virus.

FIGS. 34 to 36. Normal appearing cells from strain HeLa, protected from the effects of virus by homotypic antibody; 1 day after inoculation of virus with antibody.



(Scherer *et al.*: Propagation *in vitro* of poliomyelitis viruses)