GROWTH CHARACTERISTICS OF POLIOMYELITIS VIRUS IN HELA CELL CULTURES: LACK OF PARALLELISM IN CELLULAR INJURY AND VIRUS INCREASE*

By W. WILBUR ACKERMANN, PH.D., ALAN RABSON, M.D.,‡ AND HILDA KURTZ

(*From the Department of Epidemiology and Virus Laboratory, School of Public Health, Ann Arbor*)

PLATES 48 AND 49

(Received for publication, June 25, 1954)

The response of tissues *in vivo* to virus infection consists frequently of an inflammatory reaction and in addition certain specific changes in the host cell which may be characteristic of the pathogen. The former is considered generally to be a secondary reaction, while the latter are primary changes (1). Since tissue cultures of cells of a single type cannot exhibit the inflammatory response, the cytological changes induced in them by virus infection represent the primary pathology. The present experiments are concerned with the relation of the primary pathology to the viral synthetic mechanism. Two possibilities have been considered. One is that the progressively developing visible cytopathology is the result of a progressively developing insult associated with a gradual increase in viral material. The second is that the essential injury is completed close upon the initiation of infection, in which case the visible pathology could develop independent of viral multiplication.

In previous studies of this series, techniques were developed for demonstrating and blocking specific phases of viral development (2, 3). The same methods were exploited in the present investigation to determine the relation of various phases of viral development to cellular injury. By experiment the cellular destruction has been distinguished from viral synthesis. The pathology of the HeLa cell induced by poliomyelitis virus was found to proceed under metabolic conditions which inhibit viral increase. It seems likely that the two processes operate independently and the yield of virus is determined by the relative rates.

Methods and Materials

Virus.—The virus chosen for these studies was the Saukett strain of Type III poliomyelitis virus. It was isolated by Dr. J. Salk in tissue culture, and in our laboratory has been passaged serially in HeLa cells.

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

‡ Communicable Disease Center, Public Health Service, United States Department of Health, Education, and Welfare, Atlanta.

p-Fluorophenylalanine.—We are indebted to Dr. H. O. Halvorson at the University of Michigan for samples of this material.

Tissue Cultures.—The line of HeLa cells used in all experiments was obtained from Dr. J. T. Syverton. The culture was maintained in as close an approximation to the method described by Syverton, Scherer, and Elwood (4) as possible. Tube cultures of HeLa cells were used for most experiments and for viral titrations. These were prepared by the method described in detail by the above authors (4).

Growth Medium.—The medium used to propagate the HeLa cells was a fluid composed of human serum (40 per cent), chick embryo extract (3 per cent), and balanced salt solution (4). All cultures contained as routine penicillin and streptomycin.

Maintenance Medium.—For the propagation of virus a medium composed of 10 per cent chicken serum, balanced salt solution, amino acids, vitamins, and some carbohydrate intermediates was used (4).

Viral Titrations.—The amount of virus was estimated by determining the limiting dilution which would initiate infection in 50 per cent of the tube cultures of HeLa cells inoculated. Serial 10-fold dilutions of virus were prepared in maintenance solution and 0.1 ml. aliquots of each dilution were added to 4 tube cultures. The cultures were incubated at 37°C. and examined for cytological changes characteristic of infection each day for 7 days. The dilutions recorded are always final dilutions. Since all tube cultures contain 1 ml. of fluid, all titers are expressed as the number of tissue culture infectious doses ($TCID_{50}$) per ml. of sample. The 50 per cent endpoints were calculated by the method of Reed and Muench (5).

EXPERIMENTAL

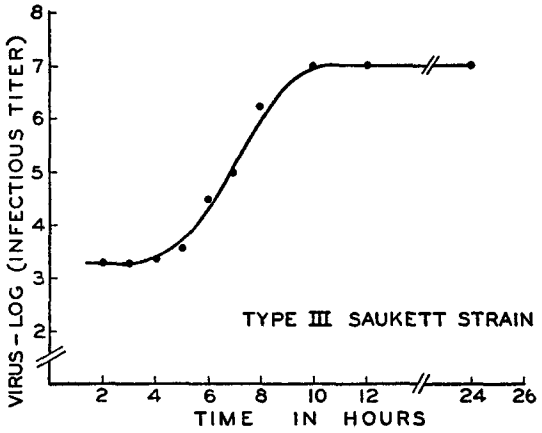
Characteristics of Infection of HeLa Cells by Poliomyelitis Virus

Viral Multiplication.—If one is to arrange in the order of their occurrence the various cytological changes observed in multicellular cultures and attributed to viral infection, it is important that all cells be in nearly the same stage of infectious development at the same time. Further, it is desirable that the development of the virus as measured by infectivity or other means be followed also to insure that one is dealing with a single sequence of infection. Such an infection of HeLa cells with poliomyelitis virus can be produced by the use of a massive inoculum which gives an initial high incidence of infection and leaves a minimum of susceptible cells. In this manner successive sequences of infection can be eliminated.

A tube culture containing approximately 10^5 cells arranged in a monolayer attached to the glass wall was exposed to 1 ml. of tissue culture fluid containing $10^{7.5}$ $TCID_{50}$ for 1 hour at 37°C. The residual inoculum was then removed and the culture was washed five times with maintenance solution. After washing, the cells were covered with 1 ml. of nutrient medium and incubated further at 37°C. At hourly intervals 0.1 ml. aliquots of fluid were removed for titration in other cultures of HeLa cells. The aliquots were replaced each time with the same volume of fresh medium.

The data obtained are plotted in Text-fig. 1. It will be noted that there is a constant or latent period of nearly 4 hours followed by a release period of 5 to 6 hours. The virus is released at what appears to be an exponential rate. The maximum yield is reached about 10 to 11 hours after infection at which time

$10^{7.5}$ $TCID_{50}$ are usually produced with this viral strain. During the latent period there is present approximately 0.01 $TCID_{50}$ per cell which is presumably the residual inoculum which was attached superficially to the vessel or the cells and was not washed away. When the maximum yield is reached at the 11th hour, there are present about 300 $TCID_{50}$ per cell. The general appearance of the curve is reminiscent of those seen when a single sequence of infection was produced in multicellular cultures with either influenza (2) or equine encephalomyelitis viruses (6).



TEXT-FIG. 1. Growth pattern of poliomyelitis virus in HeLa cells. The culture was infected with a large inoculum of virus ($10^{7.0}$ $TCID_{50}$). After 1 hour of incubation at 37°C., the residual inoculum was removed by washing five times with maintenance solution. The values for each point represent averages of two closely comparable experiments.

Cytopathology of a Normal Infection.—Since the cells are growing in a monolayer and are readily viewed in the tube cultures under low magnification, it is possible to test further the completeness of the initial infection by direct observation. However, the recognition of infected cells is facilitated by using stained preparations under high magnification. By a modification of the technique described above, one can obtain stained preparations under conditions comparable to those produced in tube cultures. In this way, it has been possible to identify infected cells, to describe the sequence of the cytological changes, and to estimate the initial degree of involvement of the culture.

Two ml. of growth medium containing 400,000 HeLa cells were dispensed into a Porter flask containing a small coverslip. After 4 days of incubation at 37°C., a continuous sheet of cells was generally present on the coverslip. The flasks were then washed with maintenance solution in the manner described for tube cultures to prepare them for virus inoculation. After the viral inoculum was added, the flasks were again incubated at 37°C. until the infection had proceeded to the desired point. The medium was then removed and the culture washed with buffered saline to remove residual serum prior to fixation. Absolute methyl alcohol (ace-

tone-free) was then added directly to the Porter flask, thus rapidly fixing the culture. After 24 hours of fixation, the coverslips were removed from the Porter flask and stained with Giemsa.

In Fig. 1 a group of HeLa cells are seen which were fixed and stained before infection with virus. The cells show considerable pleomorphism. They have pale blue-staining, somewhat vacuolated cytoplasm and, though not well illustrated in this figure, areas of sparse cell growth frequently show well defined interlacing cytoplasmic processes. The nuclei vary in size and shape and contain finely granular red-purple chromatin. There may be present one to six deep blue–staining nucleoli which often are large and bizarre in shape. Multinucleated giant cells are common, and mitotic figures are seen with varying frequencies in different cultures. Abnormal mitoses including tripolar figures can be found.

Fig. 2 shows a culture of HeLa cells infected 7 hours previously and fixed and stained as described above. Scattered through the culture are cells showing a marked increase in cytoplasmic basophilia with early nuclear pyknosis. Some of the cells show a superficial resemblance to early mitosis, but the irregular pyknotic nucleus can be distinguished from clumped chromosomes. It should be emphasized that deeply basophilic cells with dark staining, irregularly shaped, shrunken nuclei can also be seen occasionally in non-infected cultures, especially after prolonged incubation, and may represent spontaneously degenerating cells. In the cultures 7 to 9 hours after infection, however, these cells are seen with greater frequency and such infected cultures cannot be confused with young control cultures of equal age.

By 10 to 12 hours after infection, the majority of the cells shows marked loss of cytoplasmic processes and further increased cytoplasmic basophilia. The nuclei are very dark staining (Fig. 3).

A culture 21 hours after infection is shown in Fig. 4. Most of the cells no longer adhere to the coverslip. The few that do show marked nuclear pyknosis, and a striking finding is the presence of many deep blue–staining cytoplasmic granules which are irregular in size and shape. These are consistently found in HeLa cells undergoing cytopathology after infection with a number of strains of Type I and Type III poliomyelitis virus. They have not been seen in control cultures, and only rarely in cultures incubated for long periods without change of medium or in cultures injured by various chemical agents or "toxic" stool suspensions.

Normal cells can be seen occasionally in cultures even 21 hours after infection. Their presence and the observation that all cells in a culture infected for 7 hours do not begin to show cytopathology at the same time illustrate the lack of uniformity in response of the HeLa cell to viral infection. In other host virus systems in which there is excellent evidence to believe there is a single sequence of infection, this difference in response of individual cells has been deduced from other considerations (2, 6). The problem of synchronizing viral infections in tissue cultures has been discussed in detail elsewhere (7). It is only of interest to remark that the phenomenon can be observed directly.

Despite these individual differences in a small percentage of cells, it is important to note that with this technique the vast majority of cells are infected simultaneously and are progressively undergoing cytological changes in a

rather uniform manner. These observations further support the conclusion that the growth curve of Fig. 1 is the result of a single sequence of infection.

Influence of a Metabolic Inhibitor on Cytopathology and Viral Multiplication

Effect of p-Fluorophenylalanine on Uninfected HeLa Cell.—A variety of analogues of essential metabolites have been shown to inhibit viral multiplication in isolated surviving tissues. In most instances it has been impossible to observe the concurrent effect upon cytopathology for a number of reasons. However, with the techniques described in the previous section and by using a host-virus system showing marked characteristic cytological changes, it has been possible to correlate the effect of FPA (fluorophenylalanine) both upon viral multiplication and upon cytopathology. This inhibitor is a metabolic antagonist of PA (phenylalanine) which was found ideally suitable for these studies. Preliminary to interpreting its effects upon a viral infection, however, it may be of value to consider its influence upon the uninfected HeLa cell, upon cellular viability, multiplication, and morphology.

A mature, fully grown bottle culture of cells was exposed to FPA at a concentration of 0.1 mg. per ml. for 3 days. The fluid overlay was removed and the cells were suspended in a trypsin solution. A total of 1.34×10^6 cells were recovered. A control bottle not treated with FPA yielded 1.29×10^6 cells. The cells previously treated with FPA were then washed and used to prepare two new bottle cultures with 6.68×10^5 cells each. After 8 days of incubation with nutrient medium, 4.16×10^6 cells were recovered from these cultures, showing nearly a 3-fold increase. This is considered the normal rate of multiplication under our conditions.

It is apparent that exposure for a period of 3 days to the concentration of FPA mentioned did not destroy the viability of the HeLa cell. However, when 0.1 mg. per ml. of FPA was added to a culture freshly seeded with HeLa cells, it was found that the cells remained attached to the glass surface but multiplication did not proceed. The effect of the analogue at that concentration may thus be described as cytostatic but not cytocidal.

When tube cultures are treated with FPA (0.1 mg./ml.) and examined with the microscope without staining, a characteristic change in morphology is seen. The processes of the cells become rounded and are longer and narrower than normal. The general impression is that of a network rather than a sheet of cells. Slightly lower concentrations (0.06 to 0.04 mg./ml.) of FPA do not produce the effect while higher concentrations (0.2 to 0.4 mg./ml.) will cause the cells to round and escape from the glass surface. The morphological changes seen with 0.1 mg. of FPA cannot be confused with injury due to virus.

HeLa cells grown on coverslips were exposed to FPA (0.1 mg./ml.) for 24 hours and carefully examined under high magnification after fixation and staining. A typical group of cells is shown in Fig. 5. The prominent cytoplasmic processes are readily seen but there are no other significant morphologic alterations.

Effect of FPA on Poliomyelitis Virus in Vitro.—Before considering the influence of FPA on the course of the viral infection, not only was its action upon the uninfected cell studied but also its effect *in vitro* upon the virus.

Three 0.5 ml. portions of infected tissue culture fluid were placed into separate test tubes. To one of these, FPA (0.1 mg./ml.) was added. The remaining two contained no supplements. One of the latter was stored at 4°C. for 24 hours while the other two were incubated at 37°C. for the same time. After 24 hours all three samples were titrated for virus in HeLa cultures. The titers of the control tubes incubated at 4°C. and 37°C. and the FPA-treated sample were found to be respectively $10^{-6.3}$, $10^{4.7}$, and $10^{5.5}$.

It is clear that while the virus shows some instability upon incubation for 24 hours at 37°C., the maximum concentration of FPA which was used in subsequent experiments has no pronounced effect upon this instability. Hence, the effect of FPA directly upon the virus is not a consideration in interpreting its influence on viral multiplication.

Relation of Concentration of Viral Inoculum to Effect of FPA on Pathology.—In the first section of this paper the course of the cytological changes subsequent to a large viral inoculum was described. However, if a small inoculum was added to a culture of HeLa cells, initially only a few cells were infected and several days were required for the infection to spread successively from cell to cell. Ultimately the destruction was just as complete as in the case in which the massive inoculum was used. The effect of FPA upon the course of the cytopathology produced in these different situations will now be described.

Thirty-two tube cultures were prepared and inoculated with varying dilutions of a tissue culture fluid containing virus. The range of dilutions extended by 10-fold increments from 10^{-1} to 10^{-8}. The original titer of the fluid for HeLa cells was $10^{-6.5}$ $TCID_{50}$. Four cultures were inoculated with each dilution and two of these received 0.1 mg. per ml. of FPA before the addition of virus while the remaining two served as controls. At daily intervals the cultures were examined under the microscope and the progress of the cytological changes was followed.

The experiment is summarized in Table I, in which the resulting data are recorded. The general impression gained by visualization as to the degree of involvement of the culture was expressed by a designation of (−), (+−), or (+). The negative designation was used when all cells had rounded and left the wall of the culture vessel, while the (+) symbol indicated a gross appearance of normality.

With a 1 to 10 dilution of the inoculum, the cytological changes were rapid and complete in both control and treated cultures. When the inoculum had been diluted 10^{-2}, destruction of the control culture resulted as it did under the influence of a 10^{-5} or 10^{-6} dilution of virus. In the presence of FPA, however, the 10^{-2} dilution of virus produced only partial visible destruction of the culture and many cells remained unaffected. With the high dilutions of virus (10^{-3} to 10^{-6}) the FPA appeared to give complete protection.

Since it could be seen clearly that some cells were destroyed and others not when a 10^{-2} dilution of virus was employed in the presence of FPA, it was considered possible that a small number of cells might also be lost even when a 10^{-4} or 10^{-5} dilution of virus was used as inoculum. In order to search for such cells, a culture of HeLa cells was grown upon a coverslip, infected with 10^{-3} dilution of virus, and incubated for 15 hours in the presence of FPA, after which the preparation was fixed and stained. Careful microscopic examination revealed a low proportion of cells with typical cytological changes characteristic of infection with poliomyelitis virus. Apparently some cells were infected initially and were destroyed by the infection, but the infection, as detected by cytopathology, did not spread to other cells (Fig. 6).

TABLE I

Relation of Concentration of Viral Inoculum to Effect of FPA on Cell Pathology

Dilution of inoculum*		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Pathology‡	Control	−	−	−	−	−	−	+	+
		−	−	−	−	−	+	+	+
	FPA§	−	+−	+	+	+	+	+	+
		−	+−	+	+	+	+	+	+

* The inoculum consisted of infected tissue culture fluid which undiluted had a viral titer of $10^{-6.5}$ ID_{50} for HeLa cells. Four cultures were infected with each dilution, two received FPA and two were used as controls.

‡ The designations (−), (+−), (+) represent the estimated degrees of involvement of the cultures as seen on the 3rd day under the microscope without staining. When all cells are off the glass the (−) designation is given.

§ The concentration of FPA was 0.1 mg./ml. and was added before the addition of virus.

Similar stained preparations were also prepared from cultures which contained FPA and which were infected with a massive inoculum. In this case, nearly all cells were infected and showed the typical degenerative changes characteristic of the infection (Fig. 7). The course of the cytological changes was similar both in character and in the rate of occurrence to that observed on infection under ordinary circumstances as already described.

Effective Range of Concentrations of FPA.—If a small inoculum of virus is used to infect a culture, the total destruction of the culture, which under normal circumstances would eventually ensue, is prevented by FPA. Indeed, it appears as if only those cells initially infected are lost. The following experiment was designed to determine the range of concentrations of FPA which is effective in producing the phenomenon.

Fourteen tube cultures of HeLa cells were prepared and overlaid with 1 ml. of medium containing 10 $TCID_{50}$. Concentrations of FPA in the range of 0.1 to 0.01 mg./ml. were added. The cultures were incubated at 37°C. and observed with the microscope each day. The general impression gained as to the degree of involvement of the culture was expressed by the designations (−), (+−), or (+) (*cf.* Table I).

The results of the observations are recorded in Table II. A concentration of 0.04 mg. per ml. of FPA produced partial protection of the culture while 0.06 mg. was completely effective as were concentrations up to 0.1 mg. When less than 0.04 mg. of FPA was used, complete deterioration of the culture resulted by the 3rd day.

TABLE II

*Relation of Concentration of FPA to Cell Pathology Induced in Cultures by Small Inocula**

Concentration of FPA (*mg./ml.*)‡	0.1	0.08	0.06	0.04	0.02	0.01	0.00
Pathology§	+	+	+	+−	−	−	−
	+	+	+	+−	−	−	−

* The inoculum used was a 10^{-5} final dilution of tissue culture fluid of titer $10^{6.0}$ ID_{50} per ml.

‡ The FPA was added before the addition of the viral inoculum. Two cultures were prepared with each concentration of FPA.

§ The meaning of the designations (−), (+−), (+) are given in Table I and represent differing degrees of involvement of the culture, (−) being the most severe. Readings were made on the 3rd day.

TABLE III

Reversal by PA of the Inhibition of Cell Pathology Effected by FPA

Cultures*	Estimated visible pathology‡				
Control	−	−	−	−	−
FPA§	+	+	+	+	+
FPA + PA§	−	−	−	−	−

* Fifteen culture tubes were inoculated with tissue culture fluid (original titer $10^{-6.5}$) to give a final dilution of 10^{-5}.

‡ The designations (−), (+) represent the degree of involvement of the culture. When all cells are off the glass the (−) designation is given. Readings were recorded on the 5th day after infection.

§ Five tubes received 0.1 mg. of FPA (fluorophenylalanine) before the addition of virus while five tubes received both 0.1 mg. FPA and 0.2 mg. of PA (phenylalanine) at the same time.

Reversal by PA of the FPA Effect.—It is well known that the inhibitory effect of FPA toward adaptive enzyme formation or cell multiplication is reversed by PA (phenylalanine) (8). This antagonism of the inhibitor by the metabolite is also characteristic of the phenomenon just described. It is demonstrated in the following experiment.

Fifteen tube cultures of HeLa cells were prepared overlaid with medium and infected with 30 $TCID_{50}$ of virus. Five cultures were retained as controls, five cultures received 0.1 mg. of

FPA, while both 0.1 mg. of FPA and 0.2 mg. of PA were added to the remainder. After 5 days of incubation at 37°C., the cultures were studied with the microscope under low magnification. The results can be found in Table III.

The five control cultures and those receiving both analogue and amino acid showed complete destruction. The cultures receiving only FPA presented a general impression of complete normality. The ability of FPA to protect a culture against the effect of a small inoculum of virus probably lies in the power of the analogue to prevent the functioning of PA normally present in the culture and required for the viral synthetic process.

Effect of FPA on Viral Propagation.—From direct observations of monolayer cultures, it is clear that while a small inoculum of virus will initiate infection of a limited number of cells, infection in the presence of FPA is restricted to those initial sites which show cytopathology, and involvement of the remaining susceptible cells does not occur. When a massive inoculum is used, complete disintegration of the culture occurs, simply because nearly all cells are infected at the start. These results would be expected if FPA were able to inhibit multiplication of the virus but not the cytopathogenic effect. The incipient stages of infection may proceed in the presence of fluorophenylalanine in a manner similar to the initiation of infection with influenza virus in the presence of methoxinine (9). In this event the reaction between cells and virus would proceed in the presence of FPA to an extent determined by the relative concentrations of the reactants. The cells which were infected would be destroyed but would produce no new virus to spread the infection. The extent of involvement of the culture would depend upon the size of the viral inoculum; however, the effect of FPA upon multiplication should be independent of it. This explanation of the action of FPA was tested and the analogue was found to be effective as an inhibitor of viral multiplication without regard to the size of the inoculum.

Experiments were also designed to determine whether FPA inhibited an early or late stage of viral development, the object of the latter observation being to estimate how far infection must advance in order to initiate the chain of events which ultimately result in visible cytopathology.

Six tube cultures of HeLa cells were treated for 1 hour at 37°C. with 1 ml. each of tissue culture fluid containing $10^{7.5}$ $TCID_{50}$. The residual inoculum was then removed and the culture washed five times with maintenance solution and overlaid with 1 ml. of fresh medium. To one culture no further additions were made, but to the remaining ones a single addition of 0.1 mg. of FPA was added at various intervals. When the addition was made to a particular culture a 0.1 ml. sample of fluid was removed and titrated for virus. After 24 hours all cultures were sampled for titration.

By this procedure it was possible to determine if viral increase occurred after the addition of the inhibitor.

The data from two closely comparable experiments are included in Table IV.

When FPA was added 1 or 2 hours after the addition of virus, complete suppression of viral multiplication resulted. The inhibitor was much less effective when added to a 3 hour old infection, and when added at the 4th or 5th hour it produced no inhibition. These data demonstrate not only that FPA inhibits viral multiplication but also that it is phase-specific. A relatively early stage of viral development is involved. The analogue must be added nearly 7 hours before the major part of the yield is produced (*cf.* Text-fig. 1) in order to be effective. Presumably phenylalanine functions in some process required for viral production which is completed by the 2nd hour for all the virus particles

TABLE IV

Relation of Duration of Infection to Inhibitory Effectiveness of FPA

Time* of addition of FPA	Titer at time* of addition		Titer at 24 hrs.	
	I‡	II‡	I‡	II‡
hrs.				
None	3.8§	2.8§	7.5	6.5
1.3	—	2.5	—	2.8
2.0	3.7	3.5	3.5	1.8
3.0	3.8	3.5	5.5	5.2
4.0	3.8	4.5	6.8	6.8
5.0	4.0	4.7	6.8	6.8

* Time is measured in hours from the addition of virus to cells. In each case the addition was 0.1 mg. of FPA.

‡ The data under I and II represent those obtained from two separate but closely comparable experiments.

§ These two values are for titers 1.3 hours after addition of virus to the cultures. Each culture was infected with undiluted tissue culture fluid having a titer of $10^{-6.5}$. After 1 hour the residual inoculum is removed and the culture washed with maintenance solution.

which will appear many hours later. In this respect PA is reminiscent of the role of methionine in the development of influenza virus (9).

Relation of Cytopathology to the Ability of the Cell to Support Viral Synthesis. —The evidence of the stained preparations revealed a typical progressively developing pathology of infected cells in which, because of the presence of FPA, no mature virus was being produced. This experimental situation offered an opportunity to study the cytopathogenic effect of the virus as an independent process and to determine its relation to the ability of the cell to support viral synthesis. The experimental approach to the latter point was made in the following manner: A series of parallel cultures of HeLa cells was infected with a massive viral inoculum; FPA was added to inhibit viral multiplication, and the cytopathology was allowed to develop; at various stages of cytopatho-

logical development, PA was added to reverse the effect of FPA and to determine if the cells still retained their viral synthetic ability.

Seven tube cultures of HeLa cells were infected with 1 ml. of tissue culture fluid containing $10^{7.0}$ $TCID_{50}$ of virus and 0.1 mg. of FPA; the cultures were washed five times with maintenance solution after 1 hour and overlaid with 1 ml. of fresh medium containing 0.1 mg. FPA and incubated again at 37°C.; to all the cultures but one PA was added at 0, 2, 4, 6, 8, or 10 hours.

The experiment is summarized in Table V. From the titers which were obtained for the 2 and 24 hour samples, it was possible to decide in which cultures viral multiplication occurred. The data show that PA would reverse the effect of FPA if the multiplication of the virus had been suspended for 6 hours. How-

TABLE V

Relation of Time to Susceptibility to Reversal of an Inhibited Infection

		Time of addition of phenylalanine*, *hrs.*						
		None	0	2	4	6	8	10
Viral titers	2 hrs.	4.0	3.5	2.5	3.5	4.0	3.0	3.5
	24 "	3.0	6.0	6.0	6.5	6.0	3.5	3.5

* Time measured in hours after the addition of virus to cells. The FPA (0.1 mg./ml.) was present before the addition of virus and throughout the experiment. A control culture containing no FPA had titers at 2 and 24 hours of 3.5 and 6.5 respectively. All cultures were infected with large inocula, titer $10^{-6.5}$.

ever, if the inhibition had been in operation for more than 6 hours, the capacity for reversal was lost. The ability of the cell to function in viral production probably survived longer than the data indicate. The cell must function for at least 5 hours before that functioning is evidenced by mature virus (*cf.* Text-fig. 1). For example, if the additional productive period of the cell at 6 hours were 4 hours and the development of the virus required a latent period of 4 hours, very little new virus would ever be formed upon the addition of PA at 6 hours.

No effort has been expended yet to determine with precision the rate at which the viral synthetic ability is lost in an inhibited infection, but from the considerations above and the data of several experiments, it is estimated at 10 to 12 hours. This corresponds approximately to the productive period seen in the normal course of the infection (Text-fig. 1). Thus, in an inhibited infection after 6 hours not only is the host cell lost but also the infectious element in whatever form it may exist in the latent period. The duration of the viral productive period of the cell seems predetermined by some event early in the

infectious process. Like the visible pathology, it proceeds with some degree of independence of viral multiplication.

DISCUSSION

A single sequence of infection can be produced in a multicellular culture of HeLa cells by the use of a massive inoculum of poliomyelitis virus. From direct visualization it was clear that nearly all cells in the culture for which the growth curve of Fig. 1 was obtained were infected.

There are differences clearly discernible in the rate at which cytopathology develops in individual cells, which are not attributable to secondary spread of infection. The differences in response are indications of the lack of synchronization of infection of individual cells. The problem of synchronizing the virus increase in multicellular cultures has been considered in detail elsewhere and has been an important consideration in the analysis of the kinetics which produce the growth curve which now seems typical of the infection of animal cells (7).

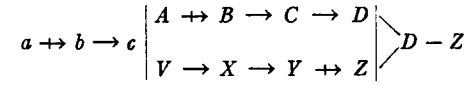
It should also be noted that while striking cytological changes accompany the appearance of virus, the preponderance of cells still retain a remarkable amount of morphological integrity 10 hours after the maximum yield of virus is released. Furthermore, limited experiments indicate that these injured cells also retain some ability to function metabolically. They take up oxygen and can reduce tetrazolium dyes (10).

In devising, from experimental data, any scheme to trace the origin of a single viral particle one must be cognizant not only of the individual variation of cells, but of the fact that each cell yields many virus particles which appear over an interval of time. When certain reactions have been completed in a single cell for the formation of the first viral particle, they must be repeated for the production of successive infectious units. Nevertheless, the pertinent data indicate that some reactions are completed during one interval for the production of all the virus which may subsequently appear.

There is a concept that a sequence of reactions composed of many steps all operating simultaneously produce a continuous metabolic flow with the development of fixed steady-state concentrations of substrates which are the viral intermediates. Superimposed upon this is a second concept of stages representing successive time intervals in which particular portions of the metabolic sequence become essential in the viral synthetic process. When the development of virus reaches a stage at which it is no longer sensitive to the influence of a certain phase-specific inhibitor, this is not evidence as to whether the sensitive reaction is occurring at that time but only whether it is important at that time.

A third coexisting concept is that of essential sequences of reactions in parallel, several independent sequences of reactions occurring during the same phase of viral development.

For example, in the accompanying diagram the reaction $Y \rightarrow Z$ may occur during a later phase than reaction $A \rightarrow B$, yet when the latter reaction is inhibited in an infected cell the formation of Z may proceed. However, if reaction $a \rightarrow b$ were inhibited one might not anticipate that either $A \rightarrow B$ or $Y \rightarrow Z$ would occur.



The technique of determining whether two inhibitors act upon reactions in sequence or in parallel has been illustrated previously in a virus infection (9).

Fluorophenylalanine (FPA) inhibits a reaction of an early phase of viral development. The function of this reaction seems to be completed about 7 hours before the major portion of the viral yield appears. When this reaction is blocked by FPA, viral multiplication does not occur, but the cytopathological process develops. It is clear from the evidence that the cytopathology develops independently of a major portion of the viral synthetic process. Possibly FPA inhibits one of a series of parallel reactions as $A \rightarrow D$, and it is not proved that others as $V \rightarrow Z$ do not proceed independently with the gradual accumulation of non-infectious viral material. The latter reactions may produce the progressively developing cytopathology by competing with normal cellular reactions.

It is also possible that the cytopathology may result from some metabolic injury occurring close upon initiation of infection. The biochemical lesion may be the activation of some catabolic enzyme or some other more superficial alteration which impairs cellular function and produces only, in time, the visible cytological alterations.

The present data disclose a degree of autonomy of the processes leading to viral increase and to cellular injury. The actual extent of this autonomy can be determined only by a consideration of more data than are presently available.

SUMMARY

A detailed study of the cytological changes which are induced in HeLa cells by the Saukett strain of Type III poliomyelitis virus has been made. The observations were of cultures in which a single sequence of infection was induced. The cytological changes were examined in relation to the growth curve of the virus in the same type of culture. This curve showed a latent period of 4 to 5 hours, followed by a gradual release of virus over an interval of 6 to 7 hours. Changes in the staining character of the cells occurred before the major portion of the viral yield appeared. The infected cells exhibited a striking cytopathology with increased basophilia, nuclear pyknosis, and basophilic cytoplasmic granules. Individual cells showed characteristic differences in the rate at which the cytopathology progresses.

The multiplication of the virus in HeLa cells was inhibited by fluorophenyl-

alanine. The inhibitory effectiveness of the antimetabolite was related to the age of the infection. It apparently inhibits only an early stage of viral development. The inhibition is completely reversed by phenylalanine if the amino acid is added within 6 hours, not later, after the induction of virostasis. The data are interpreted in terms of the rate at which the ability of the infected cell to support viral synthesis was lost. Flurophenylalanine also inhibited the multiplication of HeLa cells; however, the effect upon the uninfected cell was reversible after 3 days, as indicated by viability after such treatment.

While the fluoro derivative completely inhibited viral multiplication, it did not prevent the cytopathogenic effect of the virus. In the presence of fluorophenylalanine, the disintegration of an infected cell proceeded at what appeared to be the ordinary rate, without any increase of the infectious agent. Experimentally the processes leading to viral increase and to cellular injury have been shown to possess a significant degree of autonomy.

BIBLIOGRAPHY

1. Rivers, T. M., *in* Viral and Rickettsial Infections of Man, (T. M. Rivers, editor), Philadelphia, J. B. Lippincott Company, 1952, 2nd edition, 7.
2. Ackermann, W. W., and Maassab, H. F., *J. Exp. Med.*, 1954, **99**, 105.
3. Ackermann, W. W., *J. Exp. Med.*, 1951, **93**, 337.
4. Syverton, J. T., Scherer, W. F., and Elwood, P. M., *J. Lab. and Clin. Med.*, 1954, **43**, 286.
5. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.
6. Dulbecco, R., and Vogt, M. *J. Exp. Med.*, 1954, **99**, 167.
7. Ackermann, W. W., and Francis, T. F., *Advances Virus Research*, **2**, in press.
8. Halvorson, H. O., and Spiegelman, S., *J. Bact.*, 1952, **64**, 207.
9. Ackermann, W. W., and Maassab, H. F., *J. Exp. Med.*, in press.
10. Rabson, A., and Ackermann, W. W., unpublished results.

EXPLANATION OF PLATES

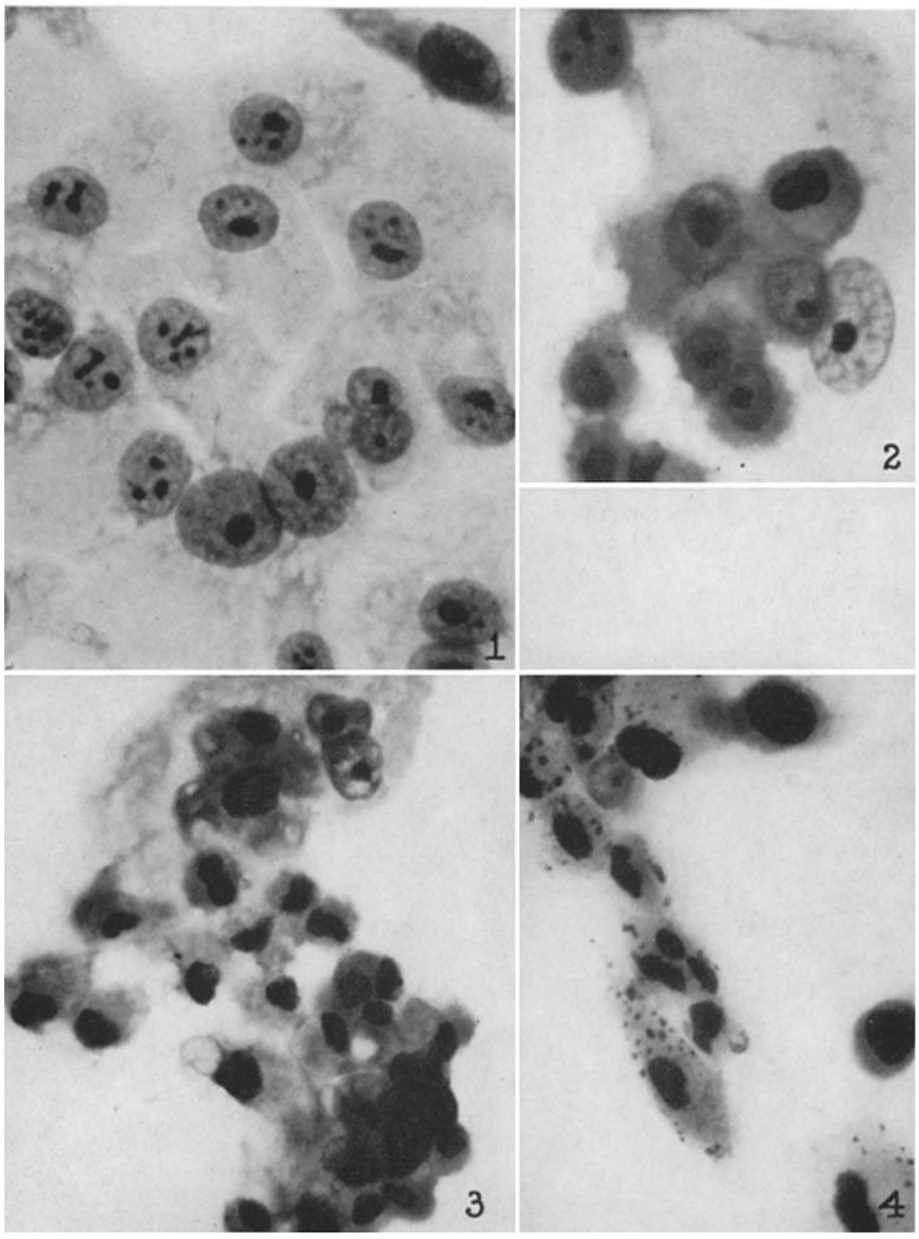
PLATE 48

FIG. 1. Uninfected culture of HeLa cells; stained with Giemsa. × 511.

FIG. 2. Culture of HeLa cells 7 hours after infection with a large inoculum of Type III poliomyelitis virus; stained with Giemsa. Many cells show increased basophilia and early evidence of nuclear pyknosis. One cell with normal appearance can be seen in the group. × 511.

FIG. 3. Culture of HeLa cells 10 hours after infection with large inoculum of Type III poliomyelitis virus; stained with Giemsa. Nuclei are pyknotic, strongly basophilic and cytoplasmic processes have retracted. × 511.

FIG. 4. Culture of HeLa cells 21 hours after infection with large inoculum of Type III poliomyelitis virus, stained with Giemsa. The cytoplasm shows deeply staining irregular shaped granules. One normal appearing cell is surrounded by others showing cytopathology. Considerable morphological integrity still remains. × 511.



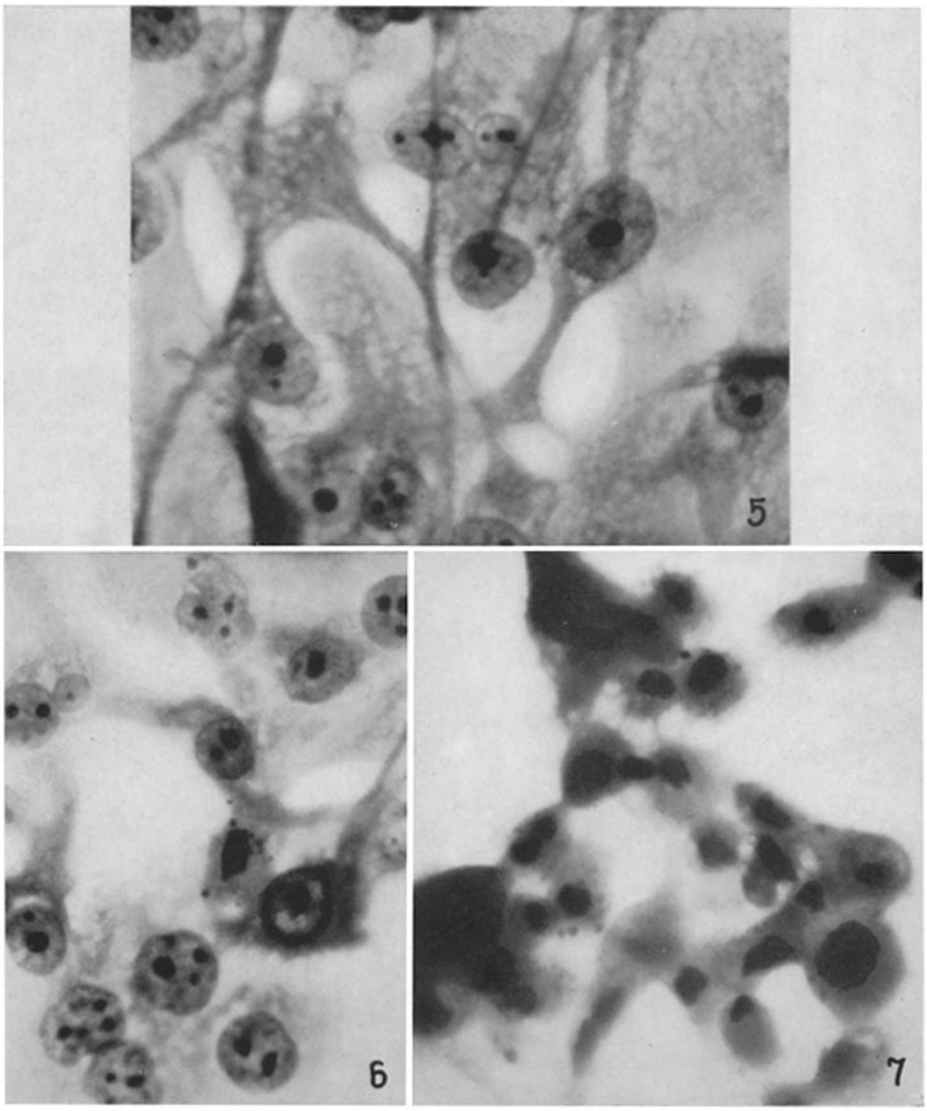
(Ackermann *et al.*: Poliomyelitis virus in HeLa cell cultures)

PLATE 49

FIG. 5. Culture of uninfected HeLa cells after 24 hours in 0.1 mg./ml. of *p*-fluorophenylalanine; stained with Giemsa. The cells show prominent cytoplasmic processes which are longer and more slender than normal, giving the appearance of a network of cells. × 511.

FIG. 6. A culture of HeLa cells 15 hours after infection with 10^{-3} dilution of Type III poliomyelitis virus (original titer $10^{-6.5}$ TC ID_{50}); the culture medium contained *p*-fluorophenylalanine (0.1 mg./ml.). Stained with Giemsa. A single cell can be seen showing cytopathology with pyknotic nucleus and deep staining cytoplasmic granules surrounded by many normal cells. × 511.

FIG. 7. A culture of HeLa cells 15 hours after infection with a large inoculum of Type III poliomyelitis virus; the culture medium contained *p*-fluorophenylalanine (0.1 mg./ml.). Stained with Giemsa. Typical widespead cytopathology is seen, with increased basophilia, nuclear pyknosis and cytoplasmic granules. × 511.



(Ackermann *et al.*: Poliomyelitis virus in HeLa cell cultures)