SEQUENCE OF MORPHOLOGICAL CHANGES IN EPITHELIAL CELL CULTURES INFECTED WITH POLIOVIRUS*

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(Received for publication, May 12, 1956)

The cytopathic changes induced by poliovirus in cell cultures, first described by Robbins, Enders, and Weller (1), have since been observed by many workers. The description of the morphological changes however, has been limited to the late stages in the swollen and rounded cells, when the changes were so pronounced that they could be detected in the unstained preparations. In a preliminary investigation, it was noted that virus production occurred several hours before the cytopathic effect could be detected in living cultures. The possibility was then considered that a finer cytological study might show changes in the early stages of the cycle of virus multiplication in cultured cells. The present paper reports such a study, in which the sequence of intracellular changes occurring in monolayer cultures of monkey kidney cells after infection with poliovirus, has been related to the growth cycle of the virus. As the experiments were designed to determine the temporal relationship existing between the morphological changes in the infected cells and the appearance of newly formed virus within the cells and in the culture fluid, two conditions had to be fulfilled. Firstly, the cells in the culture should be infected within a brief period; ideally, simultaneous infection of all cells should occur. Secondly, a large proportion of the available cells should be infected with the original inoculum, to insure that the changes observed were actually induced by the seed virus.

Materials and Methods

Since the synthesis of poliovirus in monkey kidney cells in tissue culture is rapid (complete within 8 hours), a variable lag in the response of the cells, due to a delay in virus adsorption or other factors, could result in a wide variation in the morphological characteristics of the cells at a given time. It was also necessary to obtain a uniform distribution of

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

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infected cells in the culture, so that differential cell counts made on samples of a few thousand cells in different areas of the monolayer would be representative of the entire culture. Cells grown in wide flat containers, such as Petri dishes, were used. During the adsorption period of the virus, the cultures were rocked gently to permit an even distribution of infective material. Under such conditions the percentages of infected cells in the same stage of cytopathic degeneration, in different areas of the monolayer, were similar. Areas ranging from 1 to 4 mm.^{2} were used for counting cells in this evaluation. In some cases, cultures grown on coverslips placed in flat-sided tubes were also used.

Tissue Culture Methods.—Monolayer cultures of kidney epithelial cells from young rhesus monkeys were used in their second passage *in vitro* (2, 3). The nutrient medium consisted of 0.5 per cent lactalbumin enzymatic hydrolysate in Hanks's balanced salt solution, to which 2 per cent calf serum was added. For cytological studies the cultures were grown in test tubes that had one side flattened near the base over an area of 15 × 40 mm.[1] and in which a coverslip, 11 × 22 mm., had been placed before seeding with 1 ml. of suspension containing 300,000 to 400,000 of the passage cells. For experiments in which the rate of virus production was also determined, the cultures were grown in Petri dishes, 4 cm. in diameter, seeded with 4 ml. of the cell suspension. The Petri dish cultures were kept in a humidified incubator flushed with a mixture of air and 5 per cent CO_2. Both types of culture usually formed a uniform monolayer of epithelial cells after 2 days incubation.

Virus.—The following strains were used: Brunhilde (Type 1), Y-SK (Type 2), and Saukett (Type 3). All had been passed through several transfers in monkey kidney cultures. Brunhilde strain was used in most of the studies but the pattern of morphological changes was confirmed with the other strains as well.

Infection of Cultures.—The nutrient medium was removed from the cultures which were then washed with pH 7.3 phosphate buffered saline (PBS). The cultures were inoculated with 0.5 ml. of virus suspension. The dose of virus was calculated so that several infectious units were available per cell, of which only a small proportion were expected to be adsorbed (4). The cultures were reincubated for 1 hour at 37°C. They were rocked every 5 minutes to allow an even adsorption of virus throughout the monolayer. The inoculum was then drawn off and saved for titration of unadsorbed virus. Each culture was washed five times with 5 ml. of ice cold PBS to remove most of the unadsorbed inoculum. Fresh nutrient medium prewarmed at 37°C. (1 ml. for tube cultures and 3 ml. for Petri dish cultures) was then added to the cultures and they were reincubated at 37°C. Infected cultures were harvested every 30 or 60 minutes for a period of 8 to 10 hours after virus inoculation. At least two cultures were removed at each selected interval, one for histological observation and another for titration of extracellular and intracellular virus.

Titration of Virus.—The supernatant fluids (extracellular virus) were removed from the tubes or Petri dishes and centrifuged for 15 minutes at 1000 R.P.M. in order to remove any cells that had come loose from the glass. They were then frozen at −20°C. and stored until titrated by the plaque technique of Dulbecco and Vogt (5).

After the supernatant fluids had been removed, the cells were scraped from the glass with a rubber policeman. They were then resuspended in 5 ml. of fresh nutrient (chilled medium) and frozen for titration of intracellular virus. Before assay, the cells were disrupted by sonic vibration. The details of the assay procedure and a report on the kinetics of virus production and release will appear separately (4).

Preparation of Specimens for Cytological Observation.—Two different procedures were used, depending on whether the cells had been grown on coverslips placed in flat-sided tubes, or directly on the bottom surface of Petri dishes. In the first case the coverslips were removed from the tubes with the aid of a metal spatula; they were then fixed for 1 hour either

[1] Leighton tubes obtained from Microbiological Associates, Bethesda.

in Bouin, Carnoy, or Zenker fluid, and kept in 70 per cent alcohol until stained. When the fixative employed was Zenker fluid, the cultures were washed several times in water before placing them in the 70 per cent alcohol. Bouin- and Carnoy-fixed cultures were transferred directly from the fixative to the alcohol.

Cultures grown in Petri dishes were fixed and handled in a similar manner in the same containers in which they were grown, and then peeled from the glass after embedding in collodion as described by Enders and Peebles (6). In such cultures, the presence of the collodion film obscured a clear image at high power, and for this reason it was found desirable to dissolve away the film before staining. This was done in the following manner: portions of the films were pressed down with filter paper on clean glass slides, keeping the surface which contained the cells in direct contact with the glass. The slides were then immediately covered with oil of cloves to clear the collodion membrane. After a contact period of 1 to several hours, depending on the thickness of the film, the slides were washed in several changes of absolute alcohol and transferred to alcohol-ether for several hours to remove the collodion. The preparations were stored in 80 per cent alcohol until stained.

For routine observation the fixed cultures were stained with hematoxylin and eosin. The intranuclear inclusions stained bright red, purple, or blue, with hematoxylin and eosin, depending on the staining and fixation procedure. To have them appear bright red, Zenker fixation was used, and the following precautions taken. The slides were well washed after fixation, and stored in 70 per cent alcohol. Before staining, they were treated with iodine and sodium thiosulfate, and washed thoroughly in distilled water. They were stained with Harris hematoxylin to which 5 per cent acetic acid was added just before using. The intensity of the staining was controlled by microscopic observation, and differentiation with dilute hydrochloric acid carried out if overstaining occurred. After treatment with hematoxylin, the desired blue color was achieved by leaving the slides for 10 to 20 minutes in distilled water to which enough lithium carbonate had been added to raise the pH to about 7. The slides were washed in water for at least half an hour, and then counterstained in an 0.5 per cent aqueous solution of eosin Y followed by an 0.5 per cent solution of the same dye in 95 per cent alcohol. If necessary, the excess stain was removed in 70 per cent alcohol before completing the dehydration. The Feulgen reaction was performed on cultures fixed in Carnoy fluid according to the technique described by Stowell (7).

Cultures were also grown in the chamber described by Rose (8) for continuous observation of living cells under the phase microscope.

RESULTS

Several experiments were performed with the technique outlined under Materials and Methods; one is described below.

Thirty-two cultures were inoculated with about 8×10^6 plaque-forming units (PFU) of Brunhilde virus. From the difference in titers of virus in the original inoculum and in the fluid after the 1 hour contact period, it was found that approximately 16 per cent of the inoculated virus had been adsorbed to the cells. The number of cells per culture were determined both by direct count of the cells in suspension after trypsinization of several uninoculated cultures, and by photographic count of the number of cells in a given area whose ratio to the total area of the plate was known. Both methods yielded a similar figure, 2.2×10^6 cells per plate. A multiplicity of approximately 0.6 infectious units per cell was obtained.

After removal of the inoculum the cultures were incubated as described above. Two cultures were harvested at hourly or half hourly intervals up to 10 hours after inoculation, for virus titrations and for cytological observations.

The cultures were observed at 100 magnification before each harvest. No change could be detected in the cells until 8 hours after virus inoculation, when groups of round, swollen, and highly refractile degenerating cells appeared in a fairly regular pattern throughout the monolayer. However, when the cells which were peeled from the culture dishes were fixed and stained with hematoxylin and eosin, typical alterations could be detected in the cells as early as 4 hours after virus inoculation. The first changes occurred in the nuclei of the infected cells and consisted in a patchy disappearance of the chromatin network located in the central part of the nucleus. This process finally extended to the whole central zone. There was also an increase of the basophilia of the nuclear outlines, and at the same time, one or more distinct type B acidophilic inclusion bodies were seen in the nucleus. When Carnoy fluid was used as a fixative, these inclusions appeared slightly basophilic, as also reported by Cowdry (9) for the type A inclusions of herpes fixed in Carnoy fluid. Because of their shape and staining characteristics, they could not be mistaken for nucleoli. These intranuclear inclusions can be seen in Figs. 8, 9, 15 to 17, and 18. The nucleus underwent a slight wrinkling, which took place before any cytoplasmic changes could be detected in the cells, at least by the methods that we have used. The next stage in the process of cellular degeneration was observed about 5 hours after inoculation, and consisted in the appearance of an eosinophilic mass in the cytoplasm of the infected cells. This mass was virtually in contact with the distorted nucleus (Figs. 9, 16, 17, and 18). Shortly after the cytoplasmic mass was first noted, the cells began to pull away from the glass surface, and slight swelling occurred (Figs. 17 and 18). At this time, cellular changes could be distinguished with the phase microscope, as shown in Fig. 2. A marked increase in the basophilia of the peripheral zone of the cytoplasm occurred in the cells at this stage, as seen in fixed and stained preparations (Fig. 19). This was followed by further distortion and wrinkling of the nucleus and by rounding up of the cell. In spite of the coarse nuclear alterations, the nucleolus persisted at this stage in many of the nuclei (Figs. 10 and 13). In some cells, it was difficult to distinguish the nucleolus because of the shrivelling of the nucleus and the condensation of chromatin about the nuclear membrane, but if the cells were carefully stained and differentiated the nucleolus could be seen in most cases.

Once rounded, the cells peeled easily from the glass and for this reason the next stage, characterized by disappearance of the cytoplasmic basophilia (cells in the top half of Fig. 20), and pycnosis and fragmentation of the nuclear chromatin (Fig. 14) could only be observed in a small fraction of the cell population, since most of the cells at this stage had already come free into the culture fluid.

The sequence of changes reported above was determined by observing their time of appearance in the stained preparations. At each period of observation,

cells of the types which had appeared earlier were seen, together with new cell types characteristic of the later stages of infection. In this particular experiment, almost half of the available cells in the culture failed to show cytopathic changes in the 10 hour period of observation. This is understandable when we consider the low multiplicity of infection, about 0.6 infectious unit per cell. In order to evaluate the morphological data on a more quantitative basis, the cells were classified into seven different types, depending on the degree of cytopathic change, and differential cell counts were made on a number of areas in each culture. The morphological characteristics of the different cell types observed in preparations fixed in Zenker, Carnoy, or Bouin fluid and stained with hematoxylin and eosin, are given below:—

Type I.—Normal cells, with pale cytoplasm containing mitochondria and fat droplets. The nucleus exhibited a fine chromatin network, and contained one or two nucleoli. (Figs. 1, 7, and two cells shown in the right half of Fig. 15).

Type II.—Cells with typical early nuclear changes. There was loss of chromatin in the central zone of the nucleus, and in most cases, condensation of chromatin-like material about the nuclear membrane. One or more small acidophilic intranuclear inclusions were present. These inclusions differed from the early stages of herpes inclusions in that they were darker and their shape did not follow as closely the contours of the chromatin-free central areas, the poliomyelitis inclusions being more rounded and smaller. The cytoplasm appeared normal (cell at top of Fig. 8).

Type III.—The nucleus contained one or more acidophilic inclusions, and nucleoli were still present. The nucleus appeared wrinkled and somewhat distorted. No cytoplasmic changes were seen (cell at bottom of Fig. 8 and left of center of Fig. 15).

Type IV.—Cytoplasmic changes occurred, consisting of the appearance of an eosinophilic paranucleus mass. The nuclear changes were essentially the same as those observed in Type III cells, but the distortion of the nucleus was more marked (Figs. 9, 16, and 17).

Type V.—The cells began to pull away from the glass, and a great increase in the basophilia of the peripheral zone of the cytoplasm was observed. The cells did not appear fully rounded, but under the phase microscope their outlines appeared much sharper than those of uninfected cells. (Figs. 17 and 18).

Type VI.—The cells appeared fully rounded with an eccentric nucleus, pushed aside by the eosinophilic cytoplasmic mass. In many cells of this type, the nucleolus and one or more acidophilic inclusions could be distinguished in the nucleus (Figs. 10 to 13, 19, and 20.).

Type VII.—The cells were characterized by an acidophilic cytoplasm, nuclear pycnosis, and fragmentation of the nuclear chromatin (karyorrhexis) (Fig. 14).

The Feulgen reaction was performed on some of the cultures. Feulgen-positive

material was found to follow the same pattern of distribution in the cell nucleus as in the chromatin. The wrinkled nuclear membrane of the distorted nuclei gave a much more intense Feulgen reaction than the normal ones, but the central zone of the infected nuclei was Feulgen-negative. The nucleolus and intranuclear inclusions were Feulgen-negative at all times. The pycnotic nuclei seen in type VII cells appeared intensely Feulgen-positive, as has already been reported by others (10) for nuclei which became pycnotic due to other influences. Feulgen-positive cytoplasmic granules (11) were not seen, although the slides were carefully examined.

Two other specific changes were observed which have been deliberately excluded from the classification above, as they were present in only a small proportion of these cells. Basophilic granules, about 1 μ in diameter, as described by Ackermann, Rabson, and Kurtz (12) in cultures of HeLa cells inoculated with poliovirus, were observed in a small percentage of cells belonging to types IV, V, VI, and VII; they can be seen in Figs. 12, 13, and 20.). These granules appeared to be Feulgen-negative. They were associated with cells whose cytoplasm appeared otherwise very acidophilic, thus suggesting that these granules could represent a condensation of the diffuse cytoplasmic basophilic material of early stages. The number of cells of different types containing these granules at different times is given in Table I. It can be seen from these data that the number of cells with basophilic granules increased gradually from the 7th to the 10th hour, and the number of such cells was higher among the more advanced pathologic cell types. They did not appear to play a role in the process of virus production since they made their appearance largely after the titers of both intra- and extracellular virus had reached their peaks. The small percentage of cells containing the granules suggests that they are either not a constant feature of the poliovirus-infected cells or else that they appear and disappear within a very brief period.

The other cellular alteration observed as the presence of characteristic vacuoles (Figs. 5, 11, and 20) in the periphery of the cytoplasm in some cells, in the late stages of infection, as has already been reported (13–15). These vacuoles appeared to arise from hyaline areas in the vicinity of the nucleus, moving later to the peripheral zone of the cytoplasm, and they were seen in some of the type V and VI cells. They could be followed under the phase microscope, but their short life and the difficulty of distinguishing them in some cases after staining, prevented us from including them in the differential cell counts. It has been suggested by Lwoff *et al.* (14) that these vacuoles play an important role in the mechanism of virus release.

The results of the differential cell counts in this experiment have been plotted in Text-figs. 1 and 2. Examination of the results revealed that the appearance of infective virus within the cells occurred approximately at the same time that the first nuclear alterations were observed. In this experiment it was ob-

served that virus release occurred just before the first type VI cells were seen. In other experiments we observed that the time of appearance of infective virus inside the cells was usually coincident with the time of appearance of type II cells, with intranuclear inclusions, but poliovirus release occurred at variable times, always before the rounded type VI cells appeared. It is suggested (4) that external factors which vary the permeability of the cell membrane may play a role in the release process.

TABLE I

Distribution of Cells with Basophilic Cytoplasmic Granules among the Different Cell Types as Determined by Differential Cell Counts in Samples of 1000 Cells. (Experiment 1)

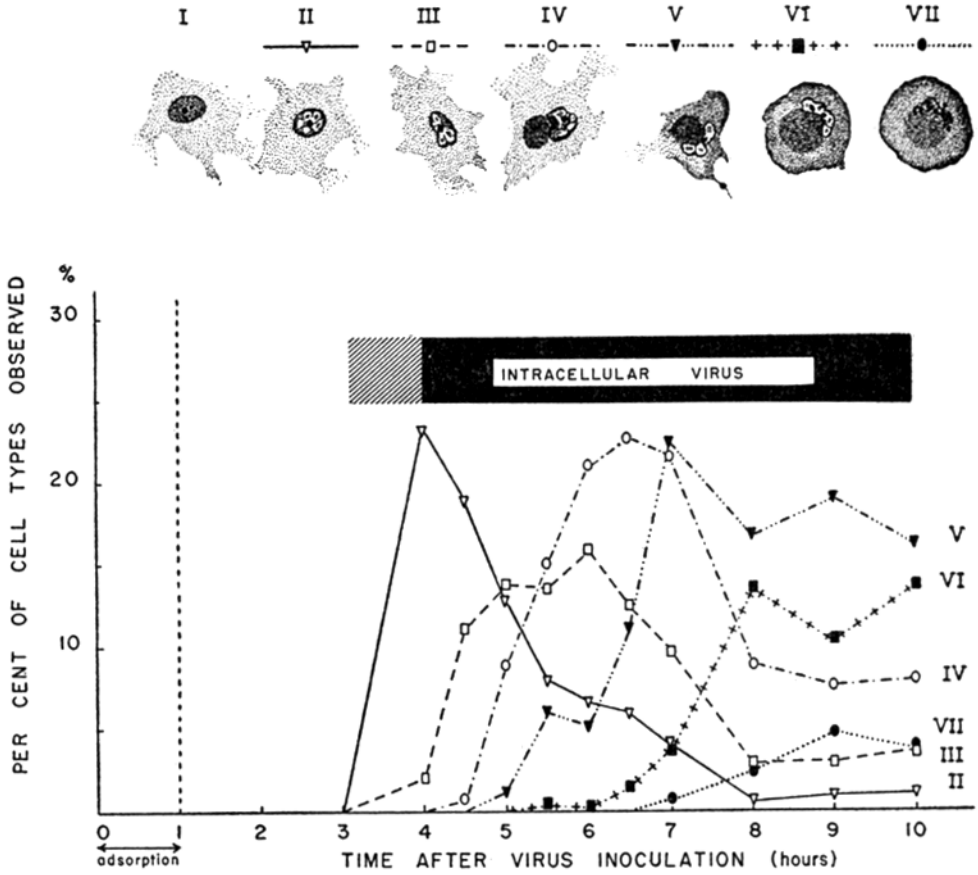
Time after virus inoculation	Cell types observed														Total of altered cells	
	I (Normal cells)		II (Inclusions in otherwise normal nuclei; normal cytoplasm)		III (Inclusions in wrinkled nuclei; normal cytoplasm)		IV (Same as III, plus eosinophilic mass in cytoplasm)		V (Same as IV, plus slight rounding of cells)		VI (Round cells; eccentric nuclei)		VII (Round cells; karyorrhexis)			
	With basophilic cytoplasmic granules	Without basophilic cytoplasmic granules	With basophilic cytoplasmic granules	Without basophilic cytoplasmic granules	With basophilic cytoplasmic granules	Without basophilic cytoplasmic granules	With basophilic cytoplasmic granules	Without basophilic cytoplasmic granules	With basophilic cytoplasmic granules	Without basophilic cytoplasmic granules	With basophilic cytoplasmic granules	Without basophilic cytoplasmic granules	With basophilic cytoplasmic granules	Without basophilic cytoplasmic granules	With basophilic cytoplasmic granules	Without basophilic cytoplasmic granules
hrs.																
0	0	1000	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	1000	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	1000	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	748	0	232	0	20	0	0	0	0	0	0	0	0	0	252
4½	0	693	0	189	0	112	0	6	0	0	0	0	0	0	0	307
5	0	632	0	129	0	137	0	90	0	12	0	0	0	0	0	368
5½	0	569	0	80	0	136	0	151	0	60	0	4	0	0	0	431
6	0	510	0	66	0	158	0	212	0	52	0	2	0	0	0	490
6½	0	464	0	60	0	125	0	227	0	112	0	12	0	0	0	536
7	0	378	0	40	0	98	4	212	4	220	0	38	0	6	8	614
8	0	548	0	5	0	28	4	86	33	135	33	103	15	10	85	367
9	0	542	0	8	0	30	8	69	39	152	25	79	18	30	90	368
10	0	532	0	10	0	37	8	73	65	98	57	82	7	31	137	331

In evaluating differential cell counts from cultures fixed in advanced stages of degeneration (later than 7 hours), we must consider the fact that cells begin to come free into the fluid at this time. This phenomenon invalidates a straight forward differential cell count taken at this stage, and it accounts for the apparent decrease in the percentage of altered cells observed.

Observations under the Phase Microscope

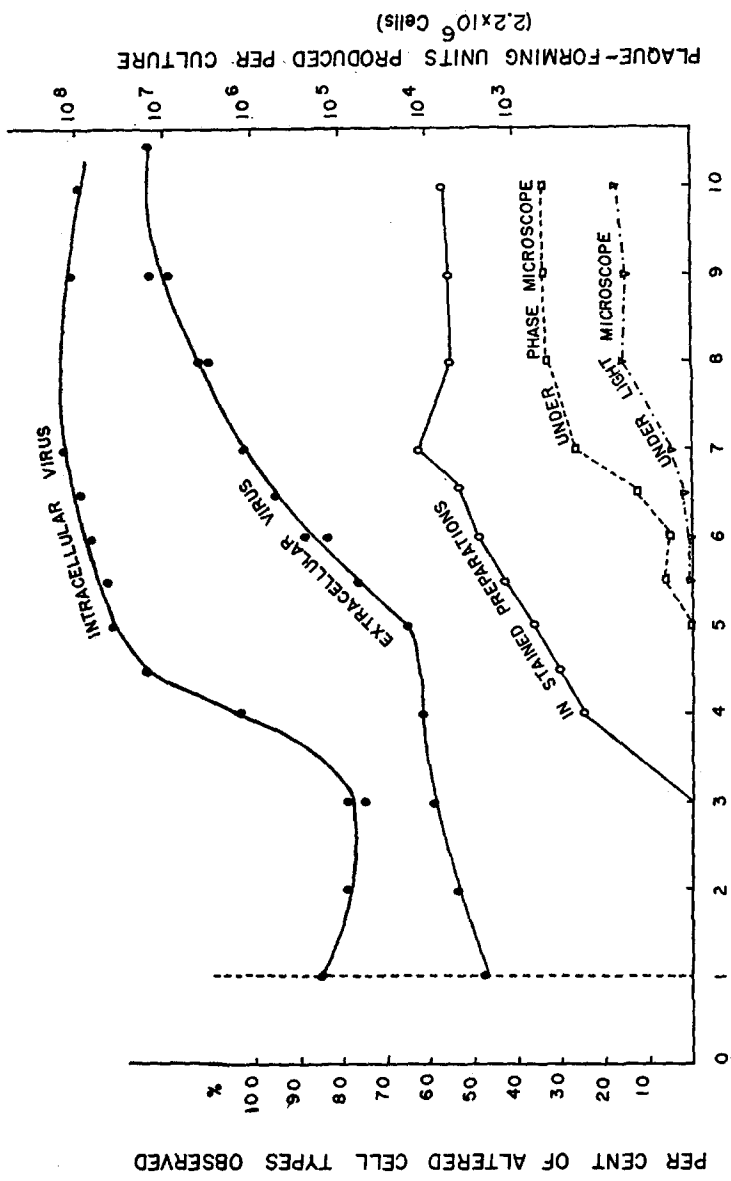
The first changes clearly observed were those corresponding to type V (Figs. 2 and 3). When the same cell was followed throughout the cycle of virus

growth, type IV cells could be distinguished in many cases. However, as some cells in an uninoculated culture may have abnormal cytoplasmic masses and distorted nuclei, it was difficult to determine in a quick survey with the phase microscope, whether a cell showed changes earlier than the type V stage.



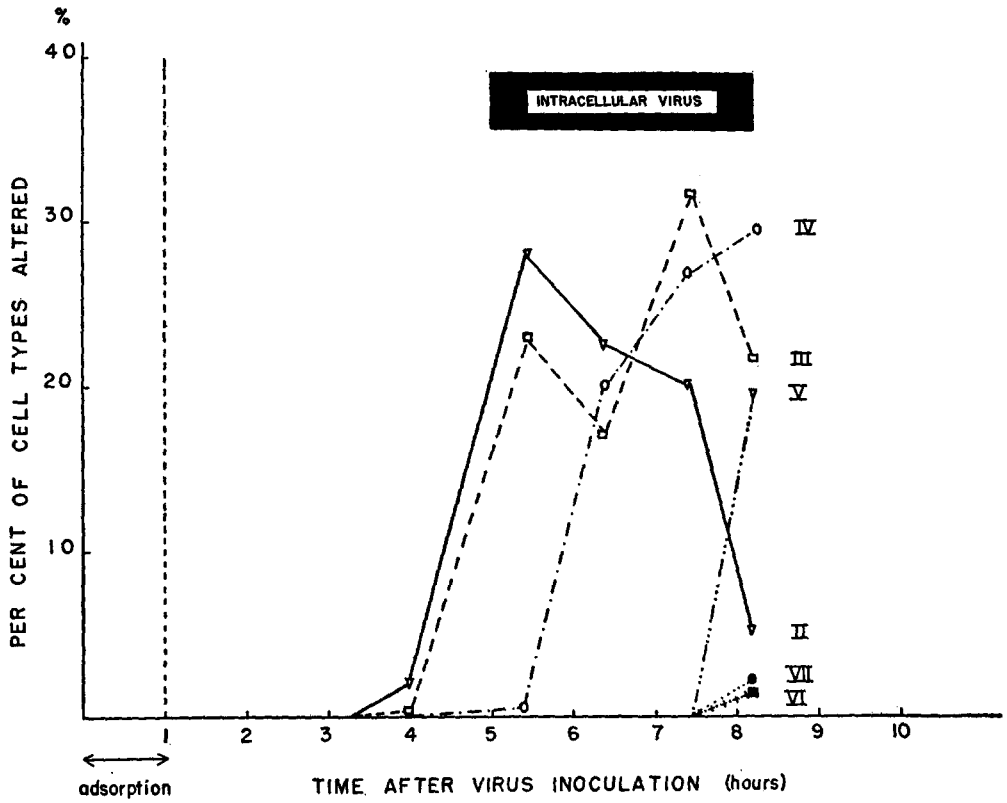
TEXT-FIG. 1. Schematic diagram to illustrate the succesive changes experienced by monkey kidney cells in culture after inoculation with poliovirus, and the correlation of those changes with the appearance of infective virus within the cells. See further explanation in text.

Intranuclear inclusions could not be seen with the phase microscope, and the distorted nucleus and the paranuclear mass were often masked by a cluster of fat droplets and mitochondria. However, the phase microscope was found to be particularly useful for detecting the characteristic vacuoles which appeared in the last stages (14–16). These vacuoles originated in hyaline perinuclear areas and underwent constant changes in shape and location, until



TEXT-FIG. 2. Schematic diagram of the same experiment as shown in Text-Fig. 1 to illustrate the differences in percentages of altered cells detected in living, unstained cultures and in fixed and stained cultures after poliovirus inoculation, and the relationship of their appearance to the time of virus production and release. The apparent decrease in the percentages of altered cells in cell counts taken later than 7 hours after virus inoculation is due to the fact that cells in advanced stages of degeneration begin to come free into the fluid at this time.

they finally develop in to clearly defined vacuoles in the peripheral zone of the cytoplasm. The span of existence of the vacuoles was brief, for they disappeared from the cell within a hour or so of their formation, so that the rounded cell of the last stage no longer contained them. The phase microscope also revealed that some of the type V and VI cells presented long cytoplasmic projections,



TEXT-FIG. 3. Schematic diagram of experiment similar to the one in Text-fig. 1, in which there was a slight delay both in the appearance of infective virus and in morphological changes. See Text-fig. 1 for explanation of cell types.

frequently with dense thick nodules (Figs. 4 and 5); they were never seen in the final stage (Fig. 6).

Several experiments were performed in the manner described above, and essentially the same results were obtained. Text-fig. 3 gives the result of an experiment in which, for unknown reasons, the morphological changes appeared slightly later than usual. It is noteworthy that there was a corresponding delay in the appearance of intracellular virus.

DISCUSSION

When monkey kidney epithelial cells in cultures were infected with poliovirus, typical morphological changes were found to occur in stained preparations several hours before they could be seen in the living cultures. Thus, the usual criterion for ascertaining the cytopathic effect of the virus in tissue cultures—the rounding of infected cells—was not adequate to allow us to detect these early changes. This may be unimportant for determining the production of virus in many of the cell systems currently used, because generally a few hours after the early changes occur, the cells become swollen and rounded and they can be easily distinguished even in the unstained preparations. However, it is conceivable that in certain virus-host cell systems, the cells might undergo changes in the pattern of distribution of their nuclear chromatin and either recover or fail to degenerate further. If such cases occur, observation of the living, unstained cells under ordinary light microscope would fail to show the effect of the virus.

Examination of Text-fig. 1 shows that transition from one cell type to the next occurred rapidly, and that the whole sequence of changes ending with death of the cell was completed in about 7 hours after virus inoculation. Although a large number of the involved cells were at the same stage of infection at a given time, a small proportion responded earlier, and another small number later than the majority. Variations up to 1 hour may have been due to differences in the time of infection, but greater delays must be due to variations in the response of individual cells. The exhaustive washing away of the inoculum at the end of the contact period would eliminate the possibility of virus from the original inoculum adsorbing at a later time to a significant number of cells.

In all of our experiments it was observed that infective virus was formed in the cells some time before they rounded. It appears that virus had already been released from cells before the typical pycnotic changes were seen, as such changes were first noted after the peak of virus release had occurred. This is in agreement with the observations of Lwoff *et al.* (16) in their studies of poliovirus yields from single cells. It was not possible in our experiments to establish a clear correlation between virus release and a certain stage in the morphological process. Variations in the medium and other factors (4) seem to play a role in the process, perhaps by causing changes in the permeability of the cell membrane.

The present data suggest that the nucleus participates in the process of poliovirus synthesis. The first morphological changes observed occurred in the cell nucleus in which loss of chromatin took place in the central areas, and material within the staining properties of chromatin was deposited about the nuclear membrane. At the same time, eosinophilic type B intranuclear inclusions appeared, similar to the early stage inclusions observed by Sabin and Ward (17) and by Bodian (18) in the motor neuron of monkeys infected with poliovirus. Our observations are in line with the findings of Caspers-

son (19) and of Kaplan and Melnick (20) in nerve cells infected with poliovirus. From studies of infected nerve cells by ultraviolet microspectrography, Caspersson suggested that the cell nucleus plays an important role in the virus multiplication process. Kaplan and Melnick in their studies of cell fractions of nervous tissues from infected animals, found that a significant part of the virus in the cell was associated with the nuclear fraction, especially in the early stages of infection.

Cytoplasmic changes were seen only after the nuclei had undergone considerable distortion. This is at variance with the situation in nerve cells of infected monkeys, in which Bodian (18) observed chromatolysis of the cytoplasmic Nissl bodies as the first morphological change. It is possible however, that the two sets of observation in different cell types are not contradictory, as the Nissl substance is extremely sensitive to slight physical, chemical, and physiological changes of the cell. Such cytological changes might perhaps have occurred in the cultured cells before the nucleus became altered. If so, the histological techniques employed were incapable of revealing them in the supposedly less sensitive cytoplasm of the monkey kidney cells in culture. Early cytological alterations after poliovirus infection have also been reported by Hogue *et al.* (21) in cultured nerve cells. They were observed in living, unstained cultures and consisted in retraction of cell processes as early as 1 hour after inoculation of the virus.

The changes seen after poliovirus inoculation of cells in culture have generally been called pycnotic degeneration. The present study has shown that, although the final stage is similar to classical pycnosis, great differences exist in the intermediate steps. Pycnotic degeneration is a well known process which has been studied by cytologists, pathologists, and histochemists, and is defined as shrinkage of the cell nucleus accompanied by loss of structural differentiation. These changes have been reported to occur when the cell is damaged by x-rays or a variety of toxic substances, or when starvation or senescence of the cell occurs. The sequence of changes due to these factors, known as autolytic degeneration, has been the subject of detailed cytochemical studies (10); it is very different from the one described for the poliovirus-infected cell.

The differential characteristics of both processes are given in the table below:

Poliovirus-induced degeneration	Toxic or senile pycnosis
Distortion and wrinkling of the nucleus	Nuclear shrinkage with persistence and even exaggeration of the spherical shape
Persistence of the nucleolus until the last stages	Early disappearance of the nucleolus
The nuclear membrane and its folds sharply outlined; disappearance of chromatin in the central areas of the nucleus	Nucleus homogeneous, dark, and lacking internal structure
Small acidophilic intranuclear inclusions	No intranuclear inclusions

In spite of these marked differences in the intermediate stages of the process leading to pycnosis and destruction of the cell nucleus, the last stage of the poliovirus-infected cell (type VII of our classification) does not differ significantly from the last stages of toxic or senile pycnosis. However, type VI and earlier phase cells could not be mistaken for cells undergoing non-specific degeneration. These differences emphasize the fact that the cytopathic process is much more complex than that of simple cellular degeneration.

SUMMARY

A sequential study is reported of the cytological changes induced in cultures of monkey kidney epithelial cells by poliovirus. The pattern of cytological changes was followed through a single cycle of virus multiplication. Morphological alterations were correlated with the appearance of new infective virus within the cells and in the culture fluid.

Alteration of the chromatin pattern of the nucleus, and Type B acidophilic intranuclear inclusions, were seen as early as 4 hours after virus inoculation. Later wrinkling and shrivelling of the nucleus occurred, and eosinophilic cytoplasmic masses appeared. The rounded, pycnotic cell, customarily used as an index of the cytopathic response, was found only during the last stages of the infective process. On the basis of these changes, infected cells could be classified into six different types. Differential cell counts were made on the stained cultures, and the stage of cytopathic degeneration was correlated with the appearance of virus in the cells and in the culture fluid. Newly formed virus could be detected within the infected cells at about the same time that the first nuclear alterations and intranuclear inclusions were seen.

The virus-induced morphological changes exhibited a specificity distinct from the classical pycnosis of autolytic degeneration.

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EXPLANATION OF PLATES

Key to figures:—

N—cell nucleus
m—mitochondrion
P—cytoplasmic acidophilic mass
i—acidophilic intranuclear inclusion
n—nucleolus
V—cytoplasmic vacuole
b—basophilic granules

PLATE 24

FIGS. 1 to 6. Living cells taken with the phase microscope, × 625 except Fig. 2, which is at × 540.

FIG. 1. Normal monolayer cell culture of monkey kidney epithelium.

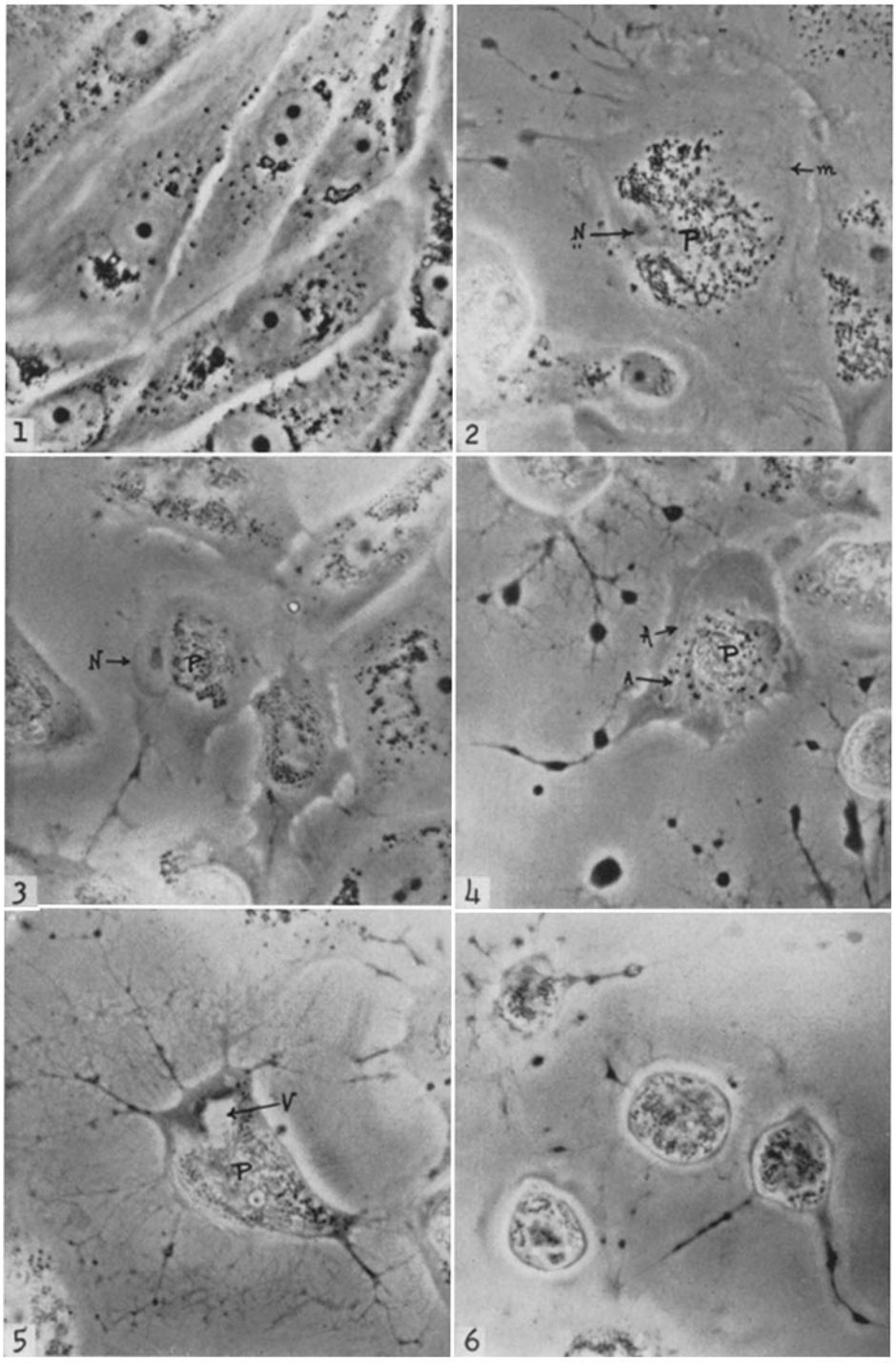
FIG. 2. Cell in the early phase of infection with Type 1 poliovirus, Brunhilde strain. The cell in the center of the field corresponds to type IV of Text-fig. 3. The nucleus appears to have been forced aside to the left edge of the cell by a cytoplasmic clump of material.

Fig. 3. Cells from the same culture at a slightly more advanced stage. The cell at the left of the center corresponds to the transition between types IV and V of Text-fig. 3. Cells are beginning to pull away from each other.

FIG. 4. Another field of the cultures at a more advanced stage. The cell in the center of the field corresponds to type V of Text-fig. 3. The outlines of the cell nucleus are obscured by the paranuclear mass. Clear, filamentous areas are seen in the cytoplasm (*A*). They appear to arise in the central zone of the cytoplasm and they constantly change their shape and location, moving towards the clear peripheral zone. The cell has lost contact with its neighbors and exhibits thread-like cytoplasmic extensions which contain dense swellings.

FIG. 5. Culture at a slightly more advanced stage (type V). The cell has begun to round up and large clear vacuoles are seen in the upper left of the cytoplasm. The nucleus is masked by the cytoplasmic paranuclear mass.

FIG. 6. The last stages of cytopathic degeneration induced by poliovirus.



(Reissig *et al.*: Epithelial cell cultures infected with poliovirus)

PLATE 25

FIGS. 7 to 14. Cells fixed in Zenker solution, stained with hematoxylin and eosin. Figs. 7 and 8 at magnification of 925. Figs. 9 to 12 × 1200.

FIG. 7. Control; uninoculated culture of monkey kidney epithelial cells.

FIG. 8. Cells after inoculation with poliovirus Type 3, Saukett strain. The cell at upper center corresponds to type II of Text-fig. 3: it has lost most of its nuclear chromatin, which only remains in a small sector in the left half of the nucleus. The nucleus contains two acidophilic inclusions. The cell at lower right corresponds to type III; it has lost most of its chromatin and has several intranuclear inclusions which can be clearly differentiated from the nucleolus. The two nuclei at lower left appear unaltered:

FIG. 9. Cells from the same culture but showing a more advanced degree of degeneration, (type IV). The distorted nucleus shows several inclusion bodies and two nucleoli; it appears to be forced aside by a cytoplasmic acidophilic mass.

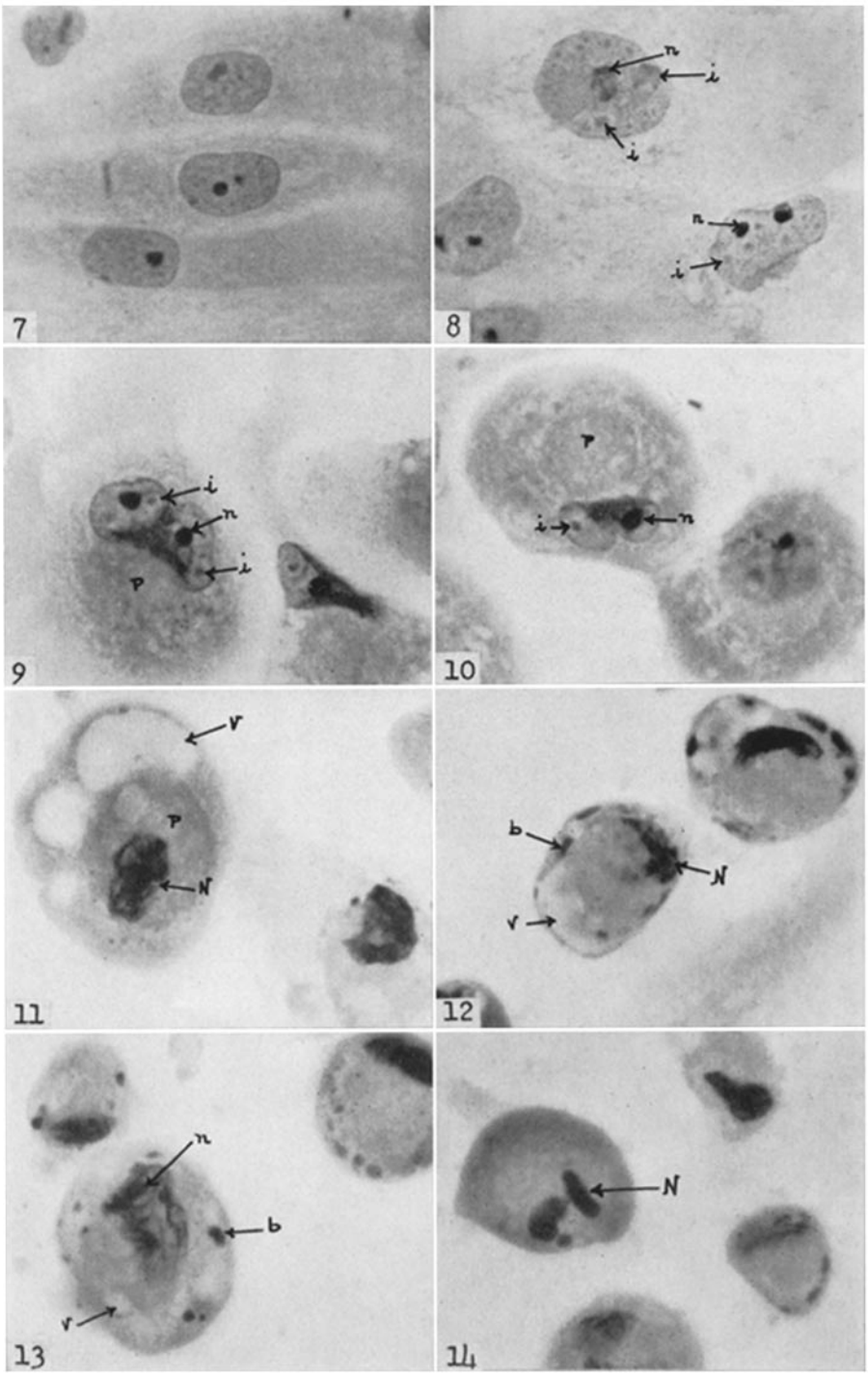
FIG. 10. Cell from a similar culture at a more advanced stage (type V). The cell at left center shows a well defined cytoplasmic eosinophilic mass. The nucleolus can be clearly distinguished in the distorted nucleus which also contains an acidophilic inclusion body.

FIG. 11. Cells in the last stages of virus degeneration corresponding to type VI of Text-fig. 3. Characteristic vacuoles are seen in the peripheral zone of the cytoplasm.

FIG. 12. Cell from the same culture showing basophilic cytoplasmic granules, which are found in some cells at this stage.

FIG. 13. Another field from the same culture. The nucleolus can still be seen in the wrinkled nucleus.

FIG. 14. Field from the same preparation showing a cell, the nucleus of which is already breaking up into fragments.



(Reissig *et al.*: Epithelial cell cultures infected with poliovirus)

PLATE 26

FIGS. 15 to 20. Cells fixed with Zenker solution, stained with hematoxylin and eosin. All × 925.

FIG. 15. Monolayer culture of monkey kidney epithelium 4 hours after inoculation with Type 3 poliovirus, Saukett strain. Two acidophilic inclusions can be seen in the slightly wrinkled nucleus of the cell slightly above and to the left of the center. The two nuclei at the right appear normal.

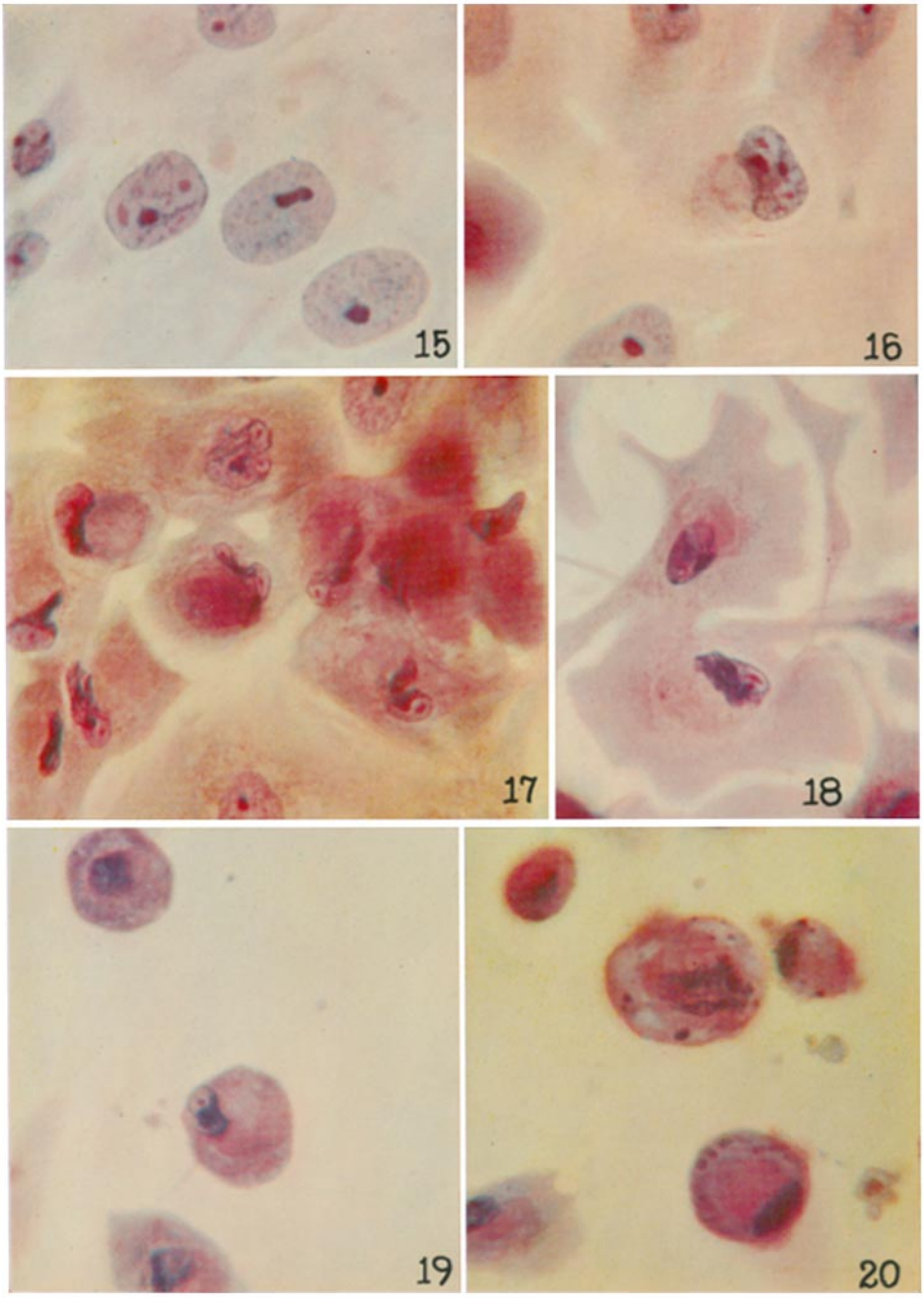
FIG. 16. Another field of the same culture. The cell at the center is at a slightly more advanced stage than the one in the preceding figure and has two small intranuclear inclusion bodies. An eosinophilic paranuclear cytoplasmic mass can be seen. The cell outlines appear normal.

FIG. 17. Cells from a similar culture at a more advanced stage. Retraction of the cell edges is now apparent, in several of the cells. Intranuclear inclusions can be seen in many cells. The cells in the left half and the one in the center of the field also show eosinophilic paranuclear masses. (Types IV and V.)

FIG. 18. Another field from the culture shown in Fig. 17. The cells are beginning to pull away from each other. (Type V.)

FIG. 19. Cells from a culture in the last stages of poliovirus degeneration. In the cell in the center an inclusion can be seen in the shrunken nucleus. The cytoplasm appears basophilic except for a paranuclear eosinophilic mass. (Type VI.)

FIG. 20. Another field from the culture shown in the preceding figure. Basophilic cytoplasmic granules can be seen in the cytoplasm of the cells. The large cell in the center also shows cytoplasmic vacuoles. (Type VI.)



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