Fine Structure of Changes Produced in Cultured Cells Sampled at Specified Intervals During a Single Growth Cycle of Polio Virus*

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ABSTRACT

Primary suspended cultures of rhesus monkey kidney cells were infected with poliomyelitis virus, type 1 (Brunhilde strain). The release of virus from these cells over a one-step growth curve was correlated with their change in fine structure, as seen in the electron microscope. Most of the cells were infected nearly simultaneously, and morphological changes developed in the cells were sufficiently synchronous to be classified into three stages. The earliest change (stage I) became visible at a time when virus release into the culture fluid begins, some 3 hours after adsorption. Accentuation of the abnormal characteristics soon occurs, at 4 to 7 hours after adsorption, and results in stage II. Stage III represents the appearance of cells after their rate of virus release had passed its maximum, and probably the abnormal morphology of these cells reflects non-specific physiological damage. There seems to be consistency between the previously described cellular changes as seen under the light microscope and the finer scale changes reported here.

Cytoplasmic bodies, called *U bodies*, were seen in large number at the time when the virus release was the most rapid (stage II). While these bodies are not of proper size to be considered polio virus, they seem to be specifically related to the infection. No evidence was found for the presence of particles that could even be presumptively identified with those of polio virus.

INTRODUCTION

Numerous publications have appeared in the past few years describing detailed observations obtained with the light microscope of cytopathologic changes produced in cell cultures by infection with polio virus (1, 2, 6–9, 13, 14). The work presented here extends this kind of study to an investigation by electron microscopy of the fine structure of changes produced by polio virus in tissue cultures of monkey kidney. Ultrathin sections of samples of cells taken from suspended cell cultures infected with type 1 (Brunhilde strain)

virus were examined. To help insure that each sample uniformly represented a stage of infection, and that each stage could be related to virus release, two conditions were fulfilled in the culture system which have rarely been met in previous electron microscope studies of virus infection. One was that samples were taken at specified times during development of a one-step growth curve of virus release, while the other was that nearly one hundred per cent of the cells were infected at nearly the same time; *i.e.* by the end of the adsorption period. The sudden rise of the one-step growth curve, combined with the almost universal initial infection, tends to preclude the likelihood of one cell being infected by virus released from other cells.

Our electron microscope observations correlate well with those that have been obtained with the

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light microscope. Of primary interest were cellular changes that appeared to be specific to the infection with polio virus. Other less specific cellular changes apparently continued after the virus release curve had leveled off. No discrete particles that could be identified as polio virus were seen. The probable causes of this failure to find intracellular virus, and the more general problem of the identification of virus particles within cells, will be discussed subsequently.

Materials and Methods

Stock Cultures.—The cells were taken from 5-day old, primary cultures of kidney epithelium of the rhesus monkey, grown in a medium consisting of 0.5 per cent lactalbumin hydrolysate in Earle's saline supplemented with 6 per cent ox serum.

Stock Virus.—The polio virus was type 1 (Brunhilde strain), passed through several transfers in monkey kidney cultures, and stored frozen in tissue culture fluid. The culture fluid used for the growth of the virus stocks was 0.1 per cent yeast extract in Earle's saline supplemented with 10 per cent monkey serum. This infected fluid was centrifuged to remove debris just before being used to infect other cultures.

Infection, Sampling, and Titration.—Three experiments were performed in which the following procedure was adopted. The nutrient medium was removed from the stock culture, and the cells were washed with two quick changes of versene (1/5000) made up in phosphate buffered saline from which Ca^{++} and Mg^{++} salts had been omitted (PD). A third change of versene was then added, and the cultures were incubated 30 minutes at 37°C. Subsequently the cells were collected, centrifuged, resuspended in PD, and counted. (All centrifugations were for 2½ minutes at 450 R.P.M. in a clinical centrifuge.) PD was added until a concentration of 2.5×10^6 cells/ml. was obtained. A volume of 12 ml. of the final suspension was then centrifuged and resuspended in 3 ml. phosphate buffered saline (PBS) to a concentration of 10^7 cells/ml. One ml. aliquots were placed in three paraffined tubes (4). One ml. of Brunhilde stock virus containing approximately 1.7×10^8 plaque-forming units (PFU) was added to one tube. To the second tube was added 1 ml. of Brunhilde stock virus which had been mixed 1 hour previously with 0.1 ml. high titer anti-Brunhilde serum. To the third was added 1 ml. of 24 hour culture fluid (0.1 per cent yeast extract in Earle's saline plus 10 per cent monkey serum) from uninfected kidney tissue cultures.

In the first two experiments, the tube containing *stock virus plus anti-Brunhilde serum* was not prepared. All tubes were incubated 40 minutes at 37°C., with occasional shaking to prevent settling of the cells.

The cells were washed free from the virus as follows: 2 ml. PBS were added to each cell suspension in the paraffined tubes, and the suspension was transferred

to centrifuge tubes containing 4 ml. PBS. After centrifuging, the cells were resuspended in 8 ml. fresh PBS, centrifuged again, resuspended in 12 ml. PBS, incubated 5 minutes at 37°C., centrifuged again and resuspended in 3 ml. PBS, and counted. The count was approximately 2.5×10^6 cells/ml. The larger part of the cell suspension was then distributed in 0.2 ml. quantities to paraffined flasks containing 10.0 ml. of growth medium (0.5 per cent lactalbumin hydrolysate *plus* 0.1 per cent yeast *plus* 10 per cent monkey serum). The end of this operation was taken as zero time, *from which all times after infection were measured.* A separate flask was prepared for each of the several infected and control specimens that were to be subsequently sampled for virus assay and fixed for electron microscopy. The flasks were incubated at 37°C.

A portion of the cells not distributed to flasks was plated on monolayer cultures for determination of the percentage of cells which had been successfully infected at zero time. This percentage was determined as the ratio of the number of plaques obtained by plating the cells on Petri dish monolayer cultures immediately after sampling to the total number of cells in the same sample as counted in the hemocytometer.

One ml. samples of supernatant fluid from the infected cells were withdrawn from the flasks for virus assay at 0, 2, 4, 6, 7½, and 9 hours. The samples were stored at—15°C., until subsequently titered by the plaque technique (4, 5). At hourly intervals from 0 to 6 hours, and at 7½ and 9 hours, the infected cells in the flasks were fixed for electron microscopy. (In addition, some cells were prepared for electron microscopy just after adsorption, but before washing. These were found to be identical in both gross and fine structure with those prepared at zero time. For this reason these will not be referred to subsequently.) Uninfected cells were fixed at 0, 3, 6, and 9 hours, while cells treated with *stock virus plus anti-Brunhilde serum* were fixed at 2, 4, 5, and 7½ hours.

Preparation of Specimens for Electron Microscopy.— To each flask containing infected cells was added 0.5 ml. of cold, 1 per cent OsO_4 in veronal buffer (pH 7.5). The cells were promptly centrifuged. After the overlying medium was removed, 1 ml. of fresh fixative was added, and the cells were kept at 4°C. for 15 to 20 minutes. The cells were then washed twice in distilled water and dehydrated step-wise to 70 per cent alcohol, in which medium they were stored for 24 hours. Complete dehydration and infiltration with methacrylate were then performed. The cells finally were embedded in methacrylate (75 per cent butyl, 25 per cent methyl) in rectal gelatin capsules and polymerized at 48°C.

Sectioning and Electron Microscope Examination.— Sections were cut with a Porter-Blum microtome provided with a diamond knife.[1] Serial sections were

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[1] The authors are deeply grateful to Dr. Umberto Fernández-Morán, Instituto Venezolano de Neurologia

floated on water, picked up on formvar films, and mounted on single-slot grids by a method previously described (17). Microscopic examination was performed with an RCA electron microscope, type EMU-3B. Polystyrene latex particles, approximately 260 mμ in diameter, were sprayed on most specimens before examination as an index of magnification, and as an aid in focussing and assessing the quality of the micrographs.

RESULTS

I. Biological Assay

In the three experiments the number of plaques produced by the cells plated out at zero time for infected centers was 86, 85, and 87 per cent, respectively, of the number of cells counted. Thus, we may believe that an average of 86 per cent of the cells have started the infection process by the end of the adsorption period. In the third experiment, the sample of cells to which *stock virus plus anti-Brunhilde serum* had been added produced 10 per cent as many infected centers as cells counted. The purpose of adding the *stock virus plus anti-Brunhilde serum* was to furnish a check on the possibility of infection by a contaminant virus, by making possible a comparison between the number of plaques formed when the anti-serum was added and the number formed when the anti-serum was not added (normal infection). Since about ten times as many plaques were produced in the latter case, as in the former, it can be concluded that at least 90 per cent of the plaques formed from normally infected cells were not due to a contaminating virus. It is very likely that the remaining 10 per cent of plaque formers were due to infections by polio virus particles that escaped neutralization.

The amounts of virus released during the course of infection are shown in Text-fig. 1. In each experiment it will be seen that the virus release started between 2 and 4 hours, and that the virus titer reached a maximum after about 6 hours. The average final yield of virus was 70 PFU (plaque-forming units) per infected cell in experiment I, 100 in experiment II, and 94 in experiment III.

II. Cytological Observations

Morphology of Control Cells.—Before considering the changes produced by type 1 polio virus in cultured cells of monkey kidney tissue, we shall

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e Investigaciones Cerebrales, Caracas, Venezuela, for making the diamond knife available.
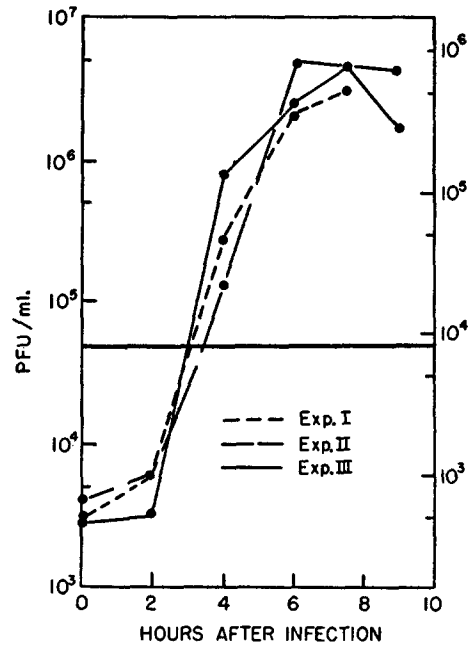
TEXT-FIG. 1. One-step growth curves of poliomyelitis virus type 1 (Brunhilde strain), assayed from supernatant fluid from monkey kidney cells maintained over a 9-hour period in nutrient culture medium containing 10 per cent monkey serum. Three separate experiments were performed. Time 0 (approximately 50 minutes after mixing the cells with virus) corresponds to the moment when, after adsorption and washing, cells were diluted in nutrient medium. The scale on the left is for experiments I and II, and that on the right for experiment III. The solid horizontal line indicates the number of cells which, when plated out at time 0, subsequently released virus and formed plaques. Similar samples taken for electron microscope examination allow a correlation of morphological change in the cell with virus release from these cells.

describe the range in appearance of the control cells; *i.e.*, the uninfected ones and those treated with *stock virus plus anti-Brunhilde serum*. Thin sections revealed the control cells to be generally singly dispersed within the methacrylate. They were ovoid in contour, with small cytoplasmic projections non-uniformly placed along their surfaces. The nucleus, located toward the center of the cell, was rounded in contour, but with occasional indentations that were rather pronounced in the area of the centrosphere. The grainy nucleoplasm was more electron dense toward the periphery of the nucleus than toward its center. When sectioned at a fortuitous angle, the nuclear membrane appeared double in places. One or more dense

nucleoli were seen in sections cut at an appropriate level.

Mitochondria containing well defined cristae and dense granules were widely dispersed in the cytoplasm. These, as well as rounded lipide inclusions, were most prevalent toward the center of the cell in the area of the centrosphere. The lipide bodies were seen in cross-section to have uneven contours at their edges. Vacuoles devoid of content permeated the cytoplasm, even in the vicinity of the nucleus. Vacuoles filled with heterogeneous contents were also seen. Both circular and elongate profiles of the rough walled elements of the endoplasmic reticulum (11) were present, as well as fine, dense aggregations of this system in the centrosphere region. Elements of a smooth walled vacuolar system seen as profiles of highly variable diameter were abundantly present (Fig. 2, *CV*). It is not certain whether these are related to the smooth walled elements of the endoplasmic reticulum or whether they represent a separate system. Occasional sections revealed large bodies of distinct outline in the cytoplasm (Fig. 3) which may be similar to the "dense bodies" described by Clark (3) in developing kidney.

Figs. 1 and 2 illustrate a general similarity in appearance between cells examined 7½ hours after being treated with *stock virus plus anti-Brunhilde serum* (Fig. 1), and those treated with infected tissue culture fluid at zero time (Fig. 2). No qualitative differences were noted between the infected material at zero time and either of the two different kinds of control samples. As the experiment progressed, fat bodies accumulated and empty vacuoles extending into the cytoplasm increased in size and number in the control samples. Each of the control samples presented a gratifying homogeneity in the appearance of the cell population, although an occasional cell was seen which appeared to be qualitatively different from the majority. These differences may be due either to the physiological state of the cell or to the kind of cell type from which it was derived.

Morphology of Infected Cells.—It is artificial to separate the cellular changes brought about by infection with polio virus into discrete stages, but it is useful, and perhaps permissible, to do so if this separation is understood to be an exaggeration of what must be a gradual change in each cell. We believe that the rate of change from cell to cell was sufficiently similar to make it possible to assign a period after infection during which the majority of cells exhibit a classifiable morphological stage.

Stage I.—The earliest distinct change which polio-infected monkey kidney cells exhibited is designated stage I, and a typical example is presented in Fig. 5. The nucleus is displaced to the side of the cell, and the cytoplasm has increased in density toward the center of the cell. The outlines of the nucleus are more generally irregular. Dense material has accumulated at the nuclear margin of many such cells. Mitochondria generally appear normal in this stage. Elements of the endoplasmic reticulum appear to be present in the peripheral cytoplasm, although the over-all density of this region is somewhat reduced compared to the more central regions. Cells in this stage of development are seen in greatest abundance between 3 and 4 hours after adsorption of the virus.

Fig. 4 represents a cell which may be typical of a still earlier phase of infection, although such cells are not seen with sufficient frequency to be classed as a distinct stage. In circumscribed areas of the cytoplasm there are many small, smooth walled vacuoles, or canaliculi, which extend from the nuclear area to the cell membrane, and which may be elements of the endoplasmic reticulum. They differ from vacuoles seen in control cells in that they are more localized in distribution and smaller in size. It is questionable if these structures are specific to polio-infected cells, although their accumulation may represent a physiological change induced by the virus.

Fig. 6 shows an area (*Y*) in the cytoplasm of relatively homogeneous content seen in a rather small proportion of infected cells at stage I. Since it is not common to the majority of cells observed, its relevance to infection is unknown.

Stage II.—Cells exhibiting the characteristics classified as stage II are represented in Figs. 7 to 9. This stage differs from the one before it by the presence of discrete dense bodies in the cytoplasm, as well as by an accentuation of the characteristics present in the earlier stage. The nucleus, displaced to the side of the cell, has become increasingly infolded, and masses of dense material, presumably chromatin, are aggregated at its periphery.

The dense cytoplasmic bodies characteristic of this stage, and of unknown nature, are here designated *U bodies*. They are seen in varying numbers in different cells, and within those cells in stage II they apparently increase in number as the time after infection increases. They may be seen dispersed throughout the cytoplasm, in small accumulations (Fig. 7), or in large masses, almost

inclusions (Fig. 8). They are rather variable in size when seen in section, and are occasionally surrounded by a dense membrane. Their diameter is most commonly about 120 to 130 mμ. No particles of a size appropriate to be polio virus (27 mμ) appear to be associated with them.

In the cells of stage II the mitochondria appear normal. The endoplasmic reticulum is not radically altered in the peripheral regions of the cell, although its disposition in the central regions is hard to assess. Smooth walled vacuoles invade the cytoplasm in increasing number.

Stage II cells were occasionally found in samples infected for only 4 hours, but were increasingly common in the 5- and 6-hour samples. Many stage II cells were also seen in the 7½-hour sample.

Stage III.—A third set of characteristics comprising *stage III* in the infection process is represented by Fig. 10. The outlines of the cell are rounded and usually intact. Clear vacuoles (*CV*), devoid of content, both large and small, are seen to permeate the cytoplasm in increasing number. The nucleus is almost unrecognizable in that its outline is highly distorted and its interior contains dense accumulations of chromatin peripherally. This material may severely distend the nuclear membrane, and it may even break away from the rest of the nucleus and form isolated bodies (no examples shown). These changes are probably those described as one form of classic pyknotic degeneration of the nucleus, and are, therefore, not likely to be specific to the infection. In the cytoplasm the mitochondria are swollen and distorted, so that only remnants of the cristae serve to make them recognizable. The cytoplasm is filled with coarse and fine vacuolar elements whose relationship to the rough and smooth walled elements of the endoplasmic reticulum, and to the clear vacuoles (*CV*) becomes quite obscure. Recognizable *U bodies* are totally absent in this stage. Stage III cells are most commonly seen in 7½- and 9-hour samples.

DISCUSSION

The numerical data obtained by plating out individual cells to produce infective centers provide clear evidence that nearly 90 per cent of the cells have adsorbed virus at zero time. If it is assumed that infection, or virus development, is initiated at approximately the same time in the majority of cells, the next question is whether or not the process develops at the same rate in different cells. Lwoff, Dulbecco, Vogt, and Lwoff (9)

have found that the virus yield from *single* isolated cells was released over a period as short as 30 minutes, while our single-step growth curves show the virus release from the entire cell *population* to be extended over a 3-hour period. It would seem reasonable to expect that our one-step growth curve would rise more steeply (approaching 30 minutes for its completion) if the rate of virus development were synchronous throughout the population. Since there is good correspondence in morphological appearance from cell to cell in a given sample, however, we might infer that the departure from synchrony is not large.

The cultured monkey kidney epithelial cells examined here seem not to contain many of the structural arrangements seen in sections of other kinds of intact kidney epithelium (see for example Clark (3)). The cultured cells are probably phagocytic, and if they have this proclivity it may account for considerable variation in the appearance of vacuoles. The accumulation with time of lipides in the control cells is probably a reflection of less than optimal culture conditions in the suspensions.

Importance may be attached to the fact that the first distinct morphologic features that are related to poliomyelitis infection, stages I and II, occur during the time when virus release is in log phase in the culture. Stages I and II, following each other rapidly in time, may be considered together as representing the specific phases of cell change.

The general characteristics of stages I and II correspond well with light microscope studies of poliomyelitis infection. The central, dense cytoplasmic area is apparently the same as the immobile cytoplasmic body described by Barski, Robineaux, and Endo (2) in phase contrast cinematography of infected cells. This area is probably also the same as the eosinophilic mass described by Reissig, Howes, and Melnick (13). Our observations could not confirm the presence of a specific nuclear density, which appeared to these authors as an acidophilic inclusion, present in cells before the formation of the dense cytoplasmic area. Lwoff, Dulbecco, Vogt, and Lwoff (9) describe a hyalinization of the peripheral cytoplasm of single cells; this is probably seen in our sections as the decreased peripheral density (already described).

As reported by Palade and Siekewitz (12), the presence of RNA granules appears to be associated with the rough walled elements of the endoplasmic

reticulum, and these in turn are responsible for a strong basophilic staining reaction. This association is further borne out by our finding that the peripheral regions of the cell in stages I and II contain largely rough walled elements of the endoplasmic reticulum, and that these are found during a phase of infection similar to that in which Reissig, Howes, and Melnick (13) reported strong peripheral, cytoplasmic basophilia. In later stages of infection these authors found an acidophilic staining reaction of the cytoplasm. This observation is in accord with what we see in our stage III cells in which the rough walled elements of the endoplasmic reticulum are present in decreased amount.

It is impossible to reconstruct a three-dimensional picture of the *U bodies* seen in large numbers in stage II cells. At high magnification it is immediately evident that they are highly variable in morphology, and are even quite variable in diameter. In some sections it appears that they are surrounded by a membrane, although this membrane rarely appears to be a single one encircling the body (Fig. 9). From this evidence one might suspect that the *U bodies* represent a dense material which either forms or invades some kind of canalicular system in the cell.

It is interesting to note how slightly changed many of the normal morphologic elements of the stage II cells are. One might infer from the fact that some of the elements are left relatively unchanged in appearance that the infection has not yet disturbed some of the normal cell functions. The fact that *U bodies* are found in cells of stage II, and are absent in stage III cells, appears to link them rather specifically with the time at which virus release is the most rapid from the culture as a whole. Their size, approaching 150 mμ, makes them inappropriate for consideration as virus. Their lack of any particulate, inner structure would seem to rule them out as small virus inclusions. The only conclusion at this time would seem to be that these bodies result from infection of the cells with polio virus, but that their significance is unknown.

The nuclear changes described in stages II and III confirm the light microscope observations of others, but do not appreciably augment them. It is impossible to determine whether there is nucleolar material still present in the nucleus, since it could not be distinguished from the masses of chromatin that accumulate along the margins of a highly folded nuclear membrane. Small bodies of indistinct appearance are occasionally present in the nucleus, but when they are examined at high magnification, they do not contain particulate material of regular dimensions. We could in no way confirm the assertion of Ruska, Stuart, and Wineser (15) that a particulate nuclear inclusion in polio-infected material is to be identified as the virus.

Our failure in these experiments to find particles that could at least be presumptively identified with those of polio virus calls for some discussion of the problems of virus identification within sections of cells. The usual way in which virus-infected cells are examined by electron microscopy is as follows: a sample of tissue is taken from an infected host, and from gross observations of the entire host or, perhaps, upon the selected sample, it is reasonably well established that there has been virus proliferation. A control sample of uninfected tissue is also taken. Observations of sections of cells from the infected material show that in some of them, at least, there are particles present that are not seen in the control material. If the infecting virus is a type such that its particles are large and well characterized, as for example vaccinia, and if those seen in the sections can be deduced to be of similar size and shape, a reasonable and useful presumption is that they are the virus particles. But it is to be noted that this kind of operation does not constitute an *identification* of a virus in a strict sense. Virus identification involves the demonstration that infection can be caused by a sample of the same material that one is investigating in other ways— such as observing it in the electron microscope. In this sense, one can never "identify" particulate material seen in sections as virus particles, since any infecting capability has obviously been destroyed. But we would still like to arrive as close as possible to some reasonable certainty about virus identification within sections, particularly as we work with smaller and smaller viruses and as we speculate upon the mysteries of their structural development within cells.

The uncertainties in the identification of particles seen in sections can be reduced if we have some presumptive evidence that the particles seen may have caused infection. To arrive at any notion of this kind it is necessary, as a minimum condition, to know that there is strong likelihood that a given cell under examination is actually infected. This knowledge must be gained independently of electron microscopy; it is clearly

circular reasoning to use as an aid in viral identification the allegation that a cell is infected, if the criterion of infection is that the cell contains "virus-like" particles! The use of tissue-cultured cells as the host system is advantageous in this regard. In the experiments reported here, for example, we know by independent assay that about 90 per cent of the suspended-cell culture had been successfully inoculated, and that, therefore, only a negligible fraction of the cells examined in section could have been truly negative.

The virus-cell system must meet to a certain degree another criterion, if it is to be used to its fullest advantage as an aid in the identification of virus particles. This criterion deals with the synchrony of viral proliferation or growth from cell to cell within the population sampled. One would like to believe that all the cells that have been simultaneously and successfully inoculated are in the same physiological state as regards the infection process when sampled some hours later. But this cannot be assumed, and what little evidence exists indicates a lack of synchrony. Even in bacteriophage infections, the evidence seems to be that the rate of development and maturation of the new virus may vary by a factor of two (10). The form of the growth curve we have presented (Text-fig. 1), contrasted with those obtained from isolated cells infected with polio virus (9), suggests that one of our 4-hour infected cells, say, may be no farther along its infection route than an advanced cell taken from a 2-hour sample. A generalized assessment of the degree of synchrony of the morphological changes within the cells may be secured from cytological observations, but what we should like to know is the relative stage of each cell, measured in terms of production of new virus. If we had such an ideal system, we could begin to establish particle identification of either mature viruses, or incomplete ones, by observing correlations between the number of particles of recognizably unique morphology and the time of observation (measured by a "virus-growth" clock). We would still, however, be unable by electron microscopy alone to distinguish between virus particles of unknown morphology and particulate byproducts of the infection process that are masquerading as viruses.

In the experiments reported in this paper, the virus-cell conditions leading to a presumptive identification of virus particles were fairly good, lacking primarily only in a high degree of synchrony of infection. Despite these conditions, we found no particles suggestive of polio virus. (Parenthetically, the occurrence of the U bodies is a warning of what can happen if the morphology of the virus being sought is not independently known; without this knowledge, the temptation to conclude that the U bodies were polio virus particles in various stages of development would have been great.)

It is reasonable to make some guesses as to why the search was negative. In consideration of the numerous particles of unknown identity that are found in a cultured cell, any class of particle that is to be even tentatively considered as a virus must have one or more of three morphological characteristics: (1) The particles may be rarely found, but if they are large, and of distinctive size and shape, they may be presumptively identified from knowledge of the morphology of the virus in the purified state; (2) if the particles pack in an ordered array in demarcated regions of the cell, they need not be large nor particularly abundant to call attention to the uniqueness of their presence in the infected cells; (3) if the particles are small, are without distinctive morphology, and do not assemble in ordered arrays, they must be present in great abundance in order to be distinguished from similar appearing particles within the control cells.

It is known from previous work (16) that the particles of polio virus, at least in the extracellular form, are neither large nor of distinctive shape. From our extensive examination of sections of infected cells, we must conclude that the intracellular virus does not pack in ordered arrays, at least within some 9 hours after the start of infection. There remains the possibility that the fully formed virus particles may exist in sufficient numbers to reveal a suggestion of their presence. However, there seems to be no direct way to calculate the number of virus particles per cell that might be present at any instant (the instant of fixation). We know that at the end of 6 hours, say, the average cell has released about 80 PFU, and this might well amount to 5,000 to 10,000 polio virus particles, depending upon the sensitivity of assay. But the number of particles within a cell at any one time depends upon the difference of the rates of formation and release, integrated from zero time to the time of sampling, and the form of the integrand is unknown. However, an upper limit can be placed upon the number of particles that could be present within the cell at any time during the growth cycle; this number

should not be much greater than the number of released virus particles measured after the growth cycle is complete (6 to 8 hours). We conclude, then, that the average cell, no matter when it is sampled for electron microscopy, will not contain more than 1×10^4 mature virus particles. Assuming an average cell volume of 600 μ^3, and a section thickness of 0.05 μ, we would estimate that, if the virus particles are randomly distributed throughout the cell, we should encounter between 1 and 2 of them per μ^2 of the section. It may well be that indeed they are distributed approximately at random, since we see no sign of aggregation. If virus particles are within these sections, they are evidently quite scarce. This may be an intrinsic scarcity, or it may be that the preparative procedures have destroyed the virus particles or grossly altered their appearance. In any event their scarcity, combined with the ubiquitous presence in sectional cells of normal particles not unlike those of polio virus in dimensions, would result in failure to detect them let alone to identify them even tentatively.

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EXPLANATION OF PLATES

PLATE 167

FIG. 1. A control monkey kidney tissue culture cell (fixed in 1 per cent OsO_4 pH 7.5) $7\frac{1}{2}$ hours after being infected with polio type 1 (Brunhilde strain) *stock virus plus anti-Brunhilde serum*. No qualitative differences are noted between cells from this sample and other control cells treated only with uninfected tissue culture fluid. (The symmetrical black bodies in this, as well as in all succeeding figures, are polystyrene latex, 260 mμ in diameter, sprayed on after sections are mounted as an aid in determining magnification and focus.) N, nucleolus; M, mitochondria; L, lipide; ER, endoplasmic reticulum. $\times$ 23,000.

FIG. 2. Another monkey kidney cell fixed at zero time, after being treated with poliomyelitis type 1 (Brunhilde strain). Time zero was approximately 50 minutes after the vrius and cells were first mixed for adsorption. The cell presents characteristics normal to other control cells. The edge of the nucleus is seen along the upper left hand margin of the cell. An unusual juxtaposition between mitochondria (M) and lipide (L) is evident. Clear vacuoles (CV) penetrate deeply into the cytoplasm. $\times$ 35,000.
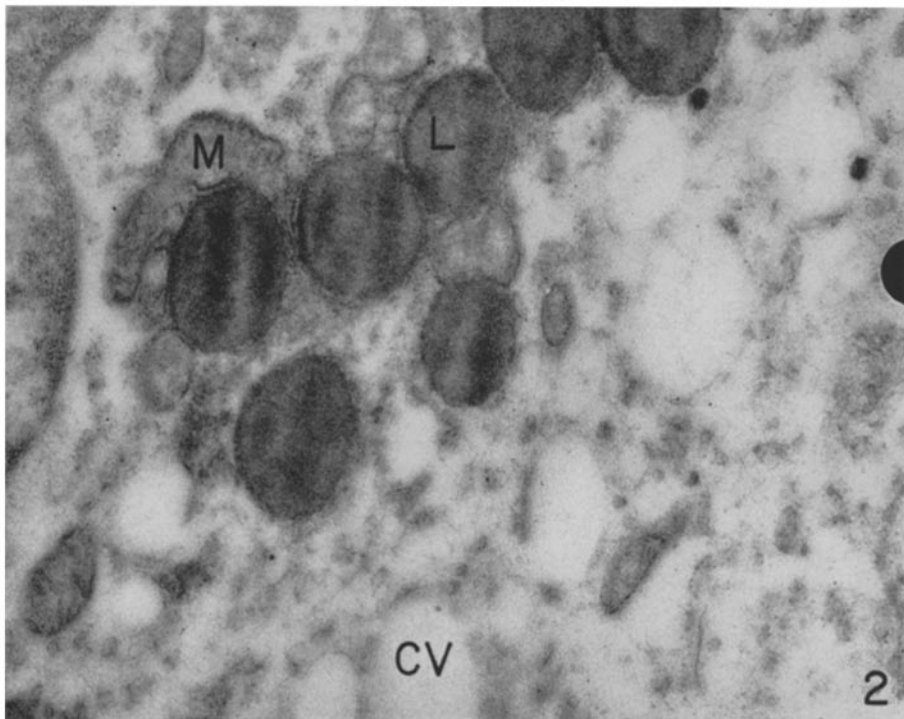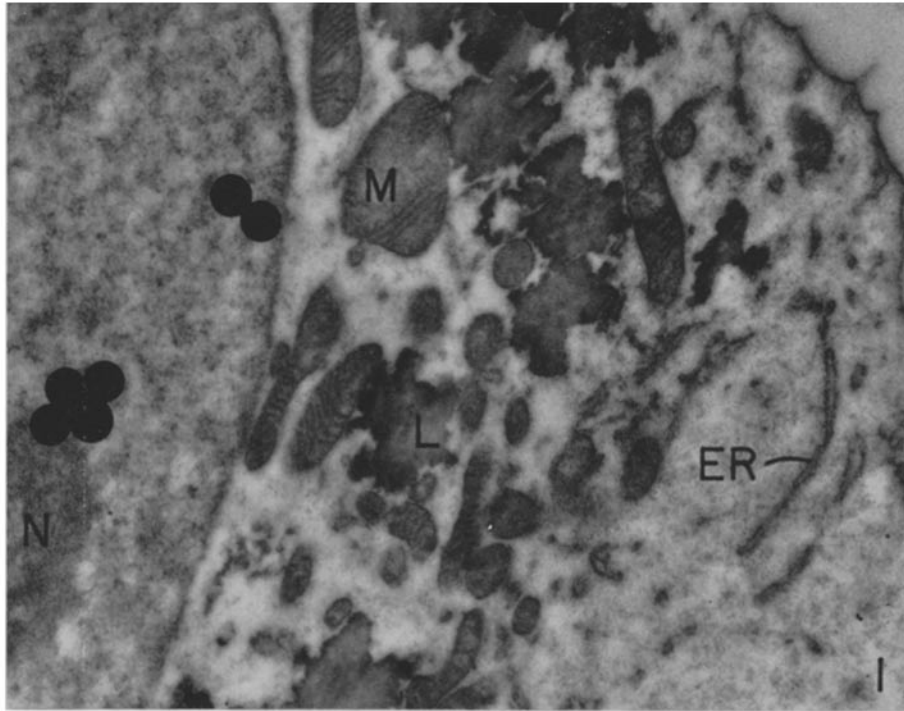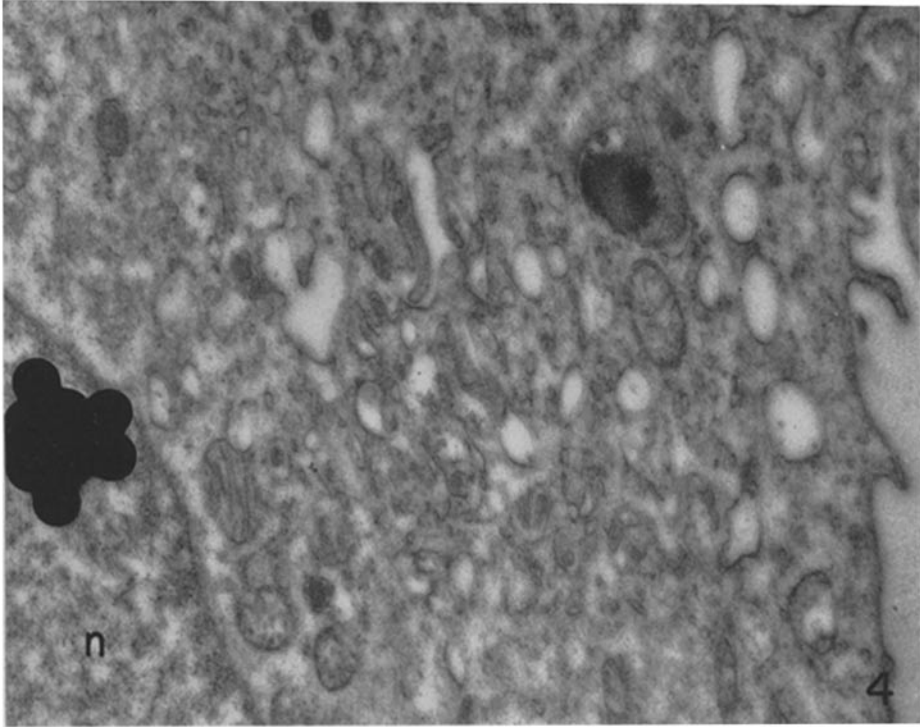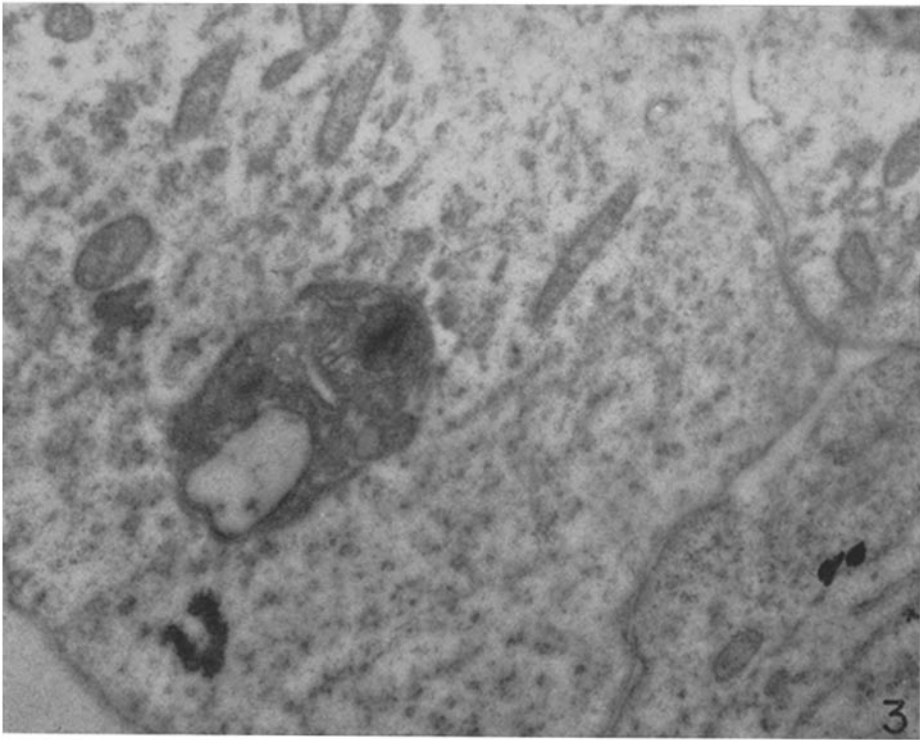
(Kallman *et al.*: Cytology of polio-infected cells)

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FIG. 3. The cytoplasmic body seen in the center of the figure represents an apparently normal constituent sometimes seen in uninfected monkey kidney cells. This may be similar to the "dense bodies" described by Clark (3) in developing kidney. × 26,000.

FIG. 4. A 1-hour infected monkey kidney cell that does not fit the classification scheme. Extensive vacuolization on a very fine scale is evident to the right of the nucleus (n). It is suggested that this represents a non-specific, physiological effect of the virus that may be quite transitory, since it is not seen in a majority of cells. × 25,000.

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FIG. 5. A 3-hour infected monkey kidney cell, classified as stage I. The nucleus (*n*) is distorted and displaced toward the side of the cell. The density of the cytoplasm is increased centrally and decreased somewhat peripherally. × 23,000.

FIG. 6. A cytoplasmic area of relatively homogeneous content is seen at *V* in a 3-hour infected cell (stage I). Elements of the endoplasmic reticulum (*ER*) surround the area. Since this body is seen in a small proportion of stage I cells, its relevance to infection is unknown. × 25,000.
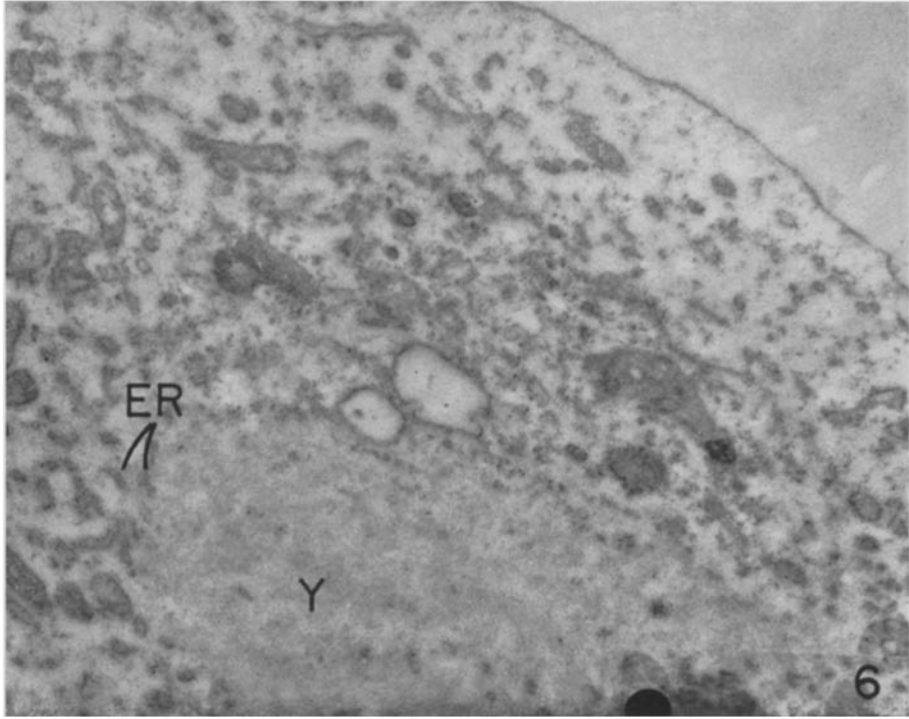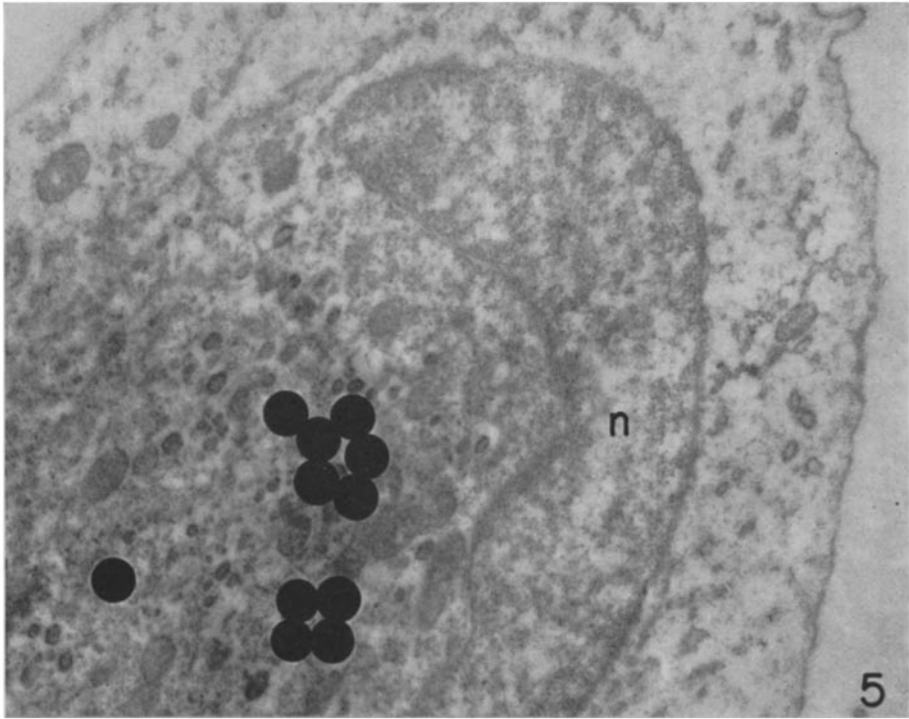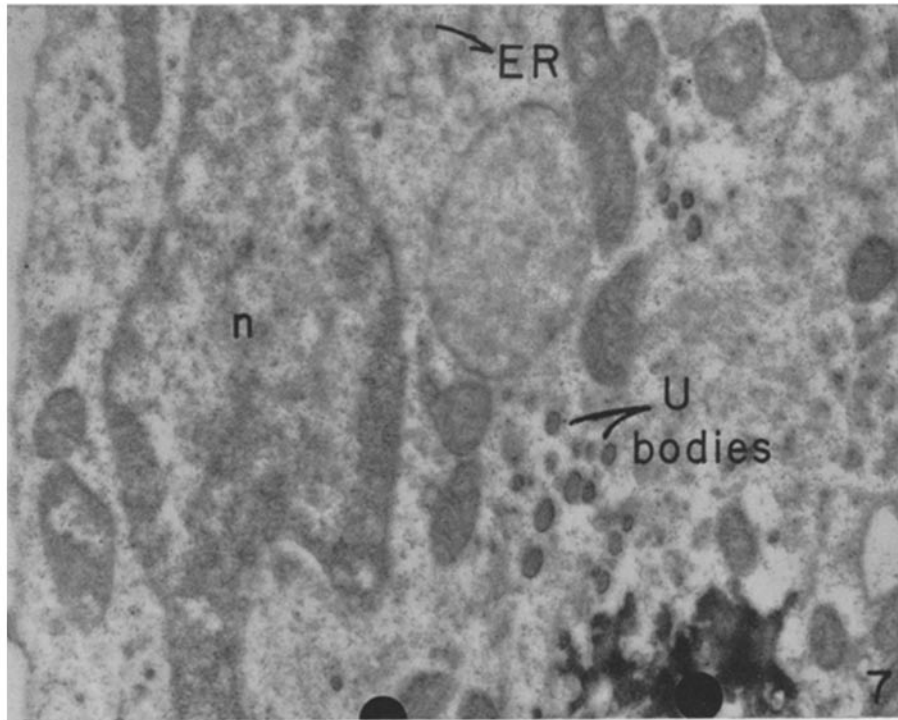
(Kallman *et al.*: Cytology of polio-infected cells)

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FIG. 7. A stage II monkey kidney cell infected $7\frac{1}{2}$ hours, which exhibits one form of distribution of the U *bodies*. The highly distorted nucleus (*n*) typical of stage II cells is present at the left hand side of the picture. U *bodies* are seen distributed in small clumps. Elements of endoplasmic reticulum (*ER*) are not appreciably altered from the normal condition. × 25,000.

FIG. 8. A stage II cell infected $7\frac{1}{2}$ hours. The nucleus (*n*) is seen to be highly distorted in outline, while dense accumulations of chromatin (*C*) have developed along its margins. In the center of the cell U *bodies* are abundantly present. In their midst are found normal cytoplasmic constituents, mitochondria and lipide. The U *bodies* appear not to be grouped in membranous envelopes. × 25,000.
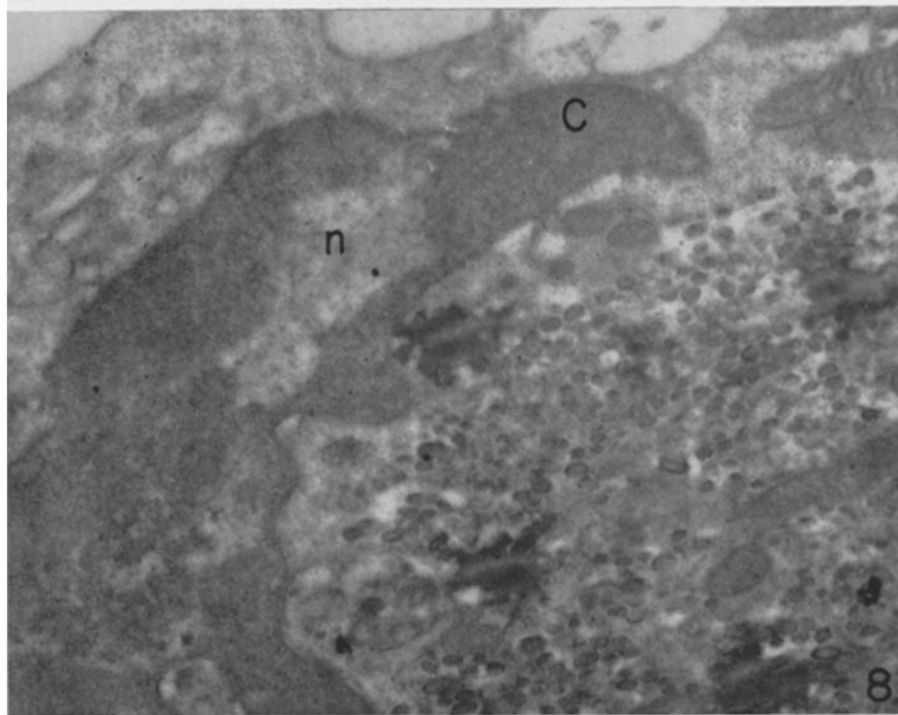
(Kallman *et al.*: Cytology of polio-infected cells)

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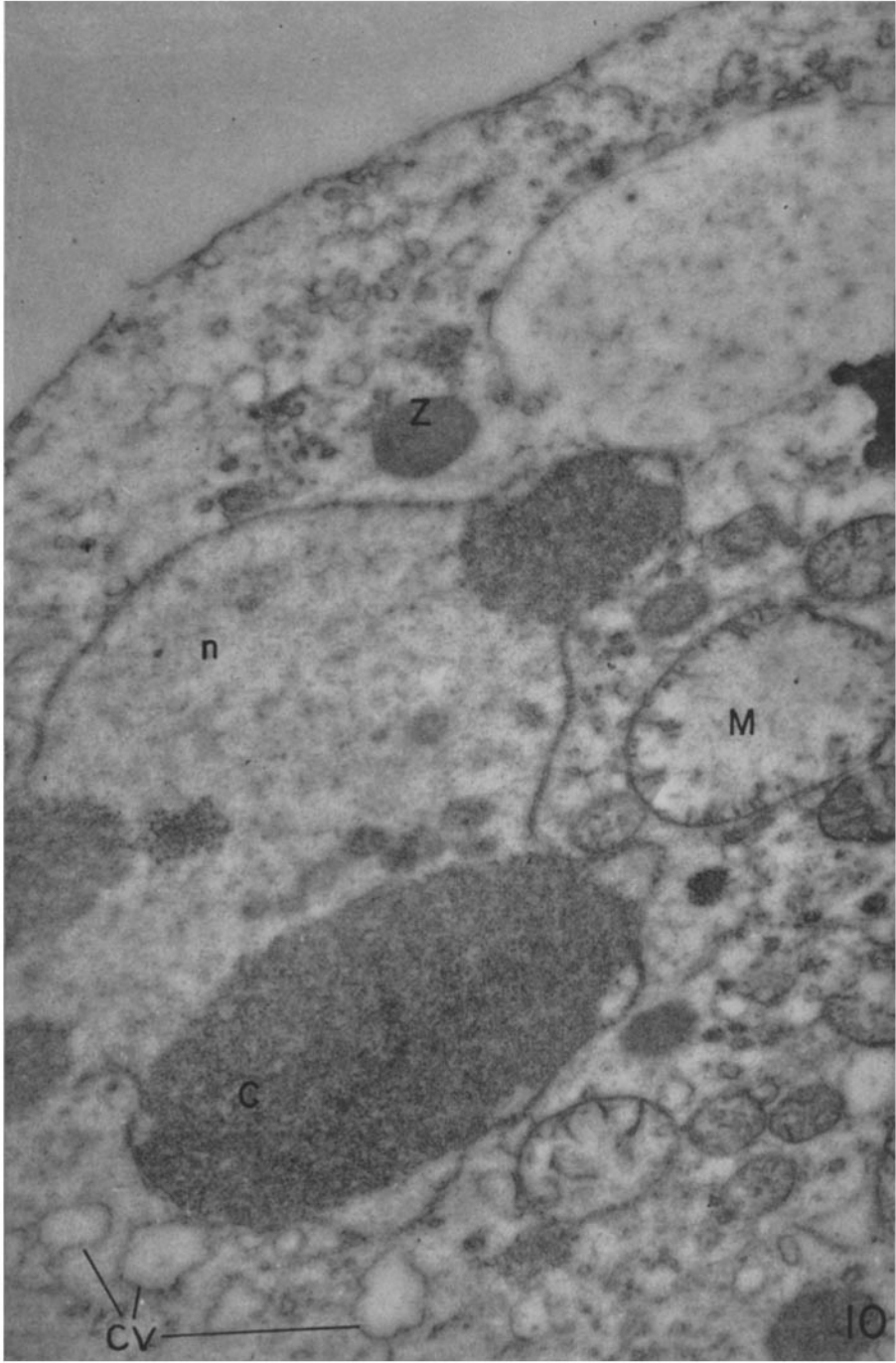FIG. 9. A higher magnification micrograph of a 7½-hour infected cell (stage II). Part of the cell boundary is shown in the upper left hand part of the picture. *U bodies* are sometimes seen to be surrounded by membranes (indicated by arrow). X 61,000.
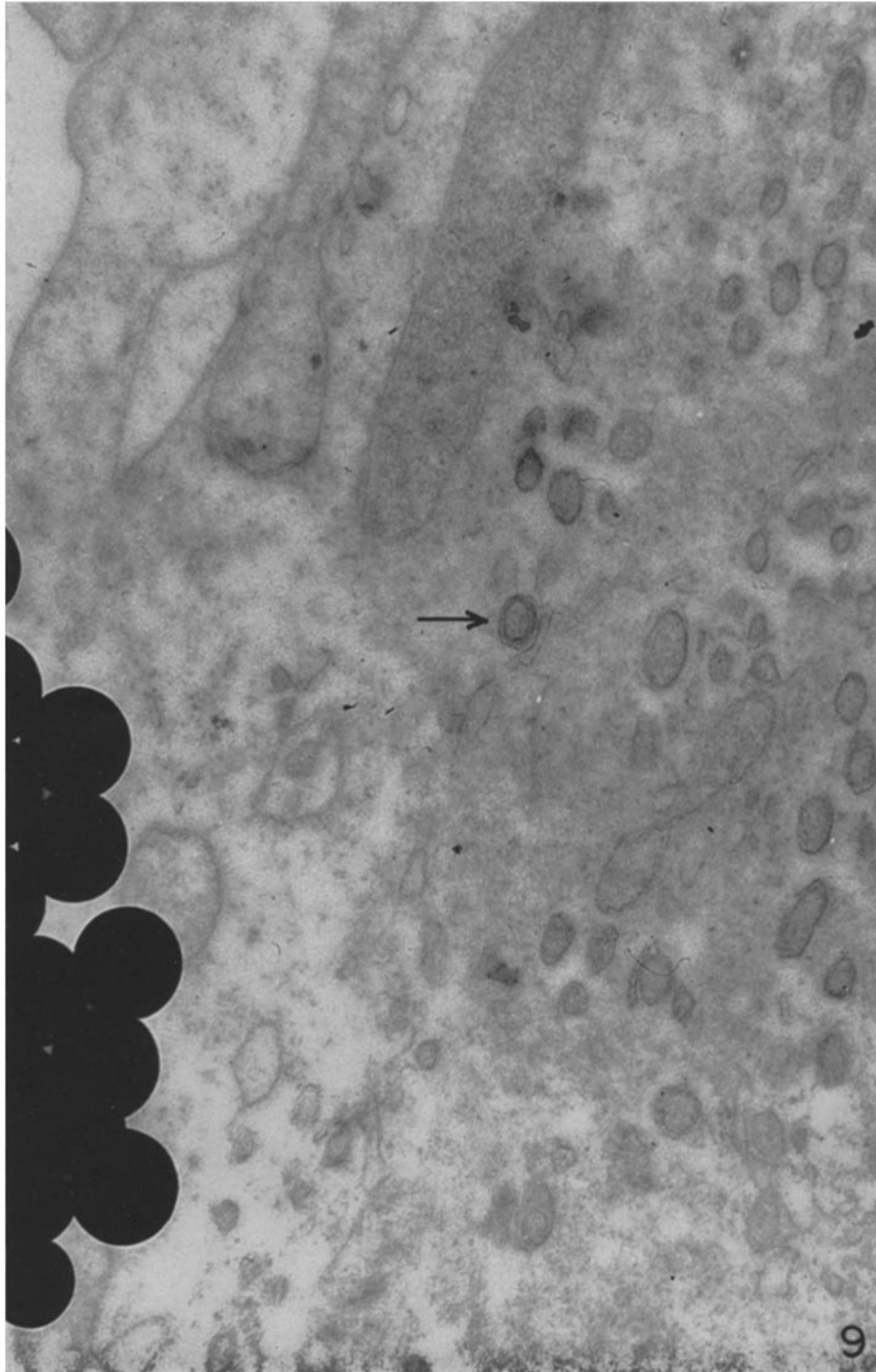
(Kallman *et al.*: Cytology of polio-infected cells)

PLATE 172

FIG. 10. A 9-hour infected cell that exhibits stage III characteristics. The nucleus (n) exhibits changes which are probably classical pyknotic degeneration. Dense aggregations, chromatin (C), bulge the nuclear membrane. Round bodies with a diffuse, dense content (Z) are widely dispersed through the cytoplasm. Mitochondria (M) are highly swollen. The endoplasmic reticulum is coarser and less distinct in its form than in normal cells. CV identifies clear vacuoles. × 25,000.

(Kallman *et al.*: Cytology of polio-infected cells)