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ELECTRON MICROSCOPY OF LATENT PSITTACOSIS VIRUS IN McCOY CELLS

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ABSTRACT

KAJIMA, MASAHIRO (University of Notre Dame, Notre Dame, Ind.), NEHAMA SHARON, AND MORRIS POLLARD. Electron microscopy of latent psittacosis virus in McCoy cells. J. Bacteriol. **88**:709-715. 1964.—Replication of psittacosis virus in McCoy cells was observed periodically by electron microscopy, and was correlated with the appearance of inclusion bodies in infected cells stained by acridine orange fluorochrome. Three well-defined morphological stages characterized the replication cycle: (i) noninfectious initial body, (ii) noninfectious intermediate body, and (iii) infectious mature or elementary body. When replication of virus was interrupted by aminopterin, the virus failed to develop beyond the noninfective initial body stage; however, after treatment of such cultures with folinic acid, intermediate and mature virus particles appeared in the cells. Latent psittacosis virus, as described here, represented a virus in which the replication process was interrupted at a noninfective (eclipse) stage of the developmental cycle.

The morphology of psittacosis, lymphogranuloma venereum, trachoma (PLT) viruses, including their developmental cycle, has been studied by many investigators with light and electron microscopes. Electron microscopic studies with thin sections of infected tissues were carried out by Gaylord (1954); Tajima, Nomura, and Kubota (1957); Higashi, Tamura, and Iwanaga (1962); Litwin et al. (1961); Armstrong, Valentine, and Fields (1963); and Mitsui, Fujimoto, and Kajima (1964).

Higashi et al. (1962) observed the first stage of infection: virus particles were adsorbed on the surface of the host cell and then, by an invagination process, invaded the cytoplasm. Tajima et al. (1957), Armstrong et al. (1963), and Mitsui et al. (1964) described the virus matrix appearing first in the cytoplasm of host cell as a primary component from which viral particles were produced, but did not describe the first stage of infection. The first part of this report describes the developmental cycle of psittacosis virus (TT strain) in McCoy tissue cells, as examined by electron microscopy and as compared with the results obtained by fluorescence microscopy.

McCloskey and Morgan (1961) demonstrated a prolongation of the latent stage of psittacosis virus in "L"-cell tissue cultures by withholding certain vitamins and amino acids from the nutrient fluid. During this latent period, viral inclusion bodies and structures were not detected by light or electron microscopy. Pollard and Sharon (1963) showed that replication of psittacosis virus was inhibited at an early stage if aminopterin (AP) was added to the culture medium from the time of virus inoculation and thereafter. The AP inhibition could be reversed by addition of folinic acid to the culture medium, and mature, infectious virus particles appeared even after 30 days of latency. The above results were determined by acridine orange fluorescence microscopy and by biological assay methods. The second part of this report describes the stage at which viral replication was interrupted by aminopterin, and the reversal of the effect by folinic acid as observed by electron microscopy.

MATERIALS AND METHODS

Cell cultures and virus inoculation. The general procedures involving tissue cultures, virus inoculations, virus assay, and fluorescence microscopy have been described (Pollard and Starr, 1962). McCoy cells were prepared as confluent monolayer cell cultures in prescription bottles. Duplicate cell cultures were prepared on cover slips in Leighton tubes, with which the infected cells were examined by fluorescence microscopy. The nutrient fluid consisted of mixture 199 supplemented with 0.5% lactalbumin hydrolysate, 5% heat-inactivated calf serum, and 0.1 mg of streptomycin sulfate per liter. Each cell culture was infected by 1.3×10^6 TT strain of psittacosis virus particles (Boney et al., 1952), and

was then incubated at 37 C during the course of the experiment.

Information acquired in previous experiments indicated that addition of aminopterin to cell cultures at the onset of TT infection resulted in interruption of virus replication (Pollard and Starr, 1962). Therefore, bottle cultures of McCoy cells were inoculated with TT suspended in nutrient fluid containing 25 μg of AP and 100 μg of thymidine per ml. The nutrient fluid was replaced every 4 days. Cultures of AP-interrupted preparations were maintained for 7 days; then, one set of cultures was lysed by two cycles of freezing and thawing, and was assayed for viable virus. In another set of interrupted cultures, the nutrient fluid was replaced with fresh nutrient fluid containing folinic acid (1 μg/ml). A third set of cultures served as control. All cultures were examined by electron and ultraviolet microscopy, and viable virus was assayed on fresh tissue culture cells (Tanami et al., 1960).

Preparation for electron microscopy. TT-infected cells were prepared for electron microscopy from 10 min to 71 hr after onset of infection at intervals of 3 hr. AP-interrupted cultures were prepared for electron microscopy after 7 days of incubation, to permit the virus to reach the latest stage of replication. The AP-treated cultures which received folinic acid were prepared for examination 24 hr after the addition of the latter drug. Uninfected cells processed from the same lot served as controls. Cells, detached from the glass wall by vigorous agitation with syringe and needle, were sedimented by centrifugation at 1,500 rev/min for 5 to 10 min. The packed cells were fixed with 1% osmium tetroxide solution with 0.045 g of sucrose per ml, buffered in Veronal acetate at pH 7.4, according to Caulfield (1957), and embedded in Epon, as described by Luft (1961). Ultrathin sections of embedded tissues were prepared with a Porter-Blum microtome by use of a glass knife. The sections were transferred to naked copper grids, stained for 1 to 3 hr with a saturated solution of uranyl acetate (Watson, 1958), and examined with an RCA EMU-3C electron microscope, operating at 50 kv. The photographs were taken on Kodak projector slide plates, and were subsequently enlarged as positive prints.

RESULTS

Normal cycle of replication. In the course of 48 hr after onset of infection, TT virus was adsorbed to the cell membrane. It penetrated into the cytoplasm by an invagination process (Fig. 1), and proceeded to replicate through a well-defined sequence of three morphological stages. (i) In the first stage of TT replication (12th hr), the virus particle disintegrated in the cytoplasm into fibrils (Fig. 1b), and was assembled as a viral matrix (Fig. 2) around which a limiting membrane appears about 1,000 mμ in diameter. The matrix constricted away from the outer membrane, thereby creating an inclusion cavity (Fig. 3). The large body was referred to as the initial body, and corresponded to the ribonucleic acid (RNA)-staining matrix as observed by acridine orange fluorescence microscopy. (ii) Each of the large initial bodies (1,000 to 800 mμ in diameter) divided repeatedly into two smaller initial bodies by constriction of the outer limiting membrane (Fig. 4a, b). The small initial bodies (300 to 500 mμ in diameter) showed marginal arrangement of granular material and a mesh in the center made of fibrous material. An electron-dense spot was formed by coagulation of the mesh in the center of a fibrous electron-lucid area (Fig. 4a, c). This small initial body with the electron-dense spot in the center is referred to as an intermediate body, and corresponded to the transitional viral inclusion which appeared yellow-orange by acridine orange fluorescence microscopy. Viral particles at stages i and ii were not infectious. (iii) The electron-dense center spot of the intermediate body combined with the peripheral granular material, and from this was evolved mature virus particles or elementary bodies (250 to 300 mμ), each consisting of a dense nucleoid which was separated by a space from a distinct virus membrane (Fig. 5a, b), and corresponded to the green deoxyribonucleic acid (DNA)-staining virus material as observed by acridine orange fluorescence microscopy. The virus particles increased in number during the next 48 hr and were infectious.

Effect of AP on replication of TT virus. The fate of AP-treated TT virus in McCoy cells followed two pathways, depending on the amount of virus in the inoculum. When 1.6×10^6 infectious virus particles were used as the inoculum, the developmental cycle of TT was interrupted completely. Only small cytoplasmic inclusions which contained few initial bodies (Fig. 7) were observed 7 days after inoculation. The structure of each initial body simulated the structures observed after 18 hr of propagation in an un-

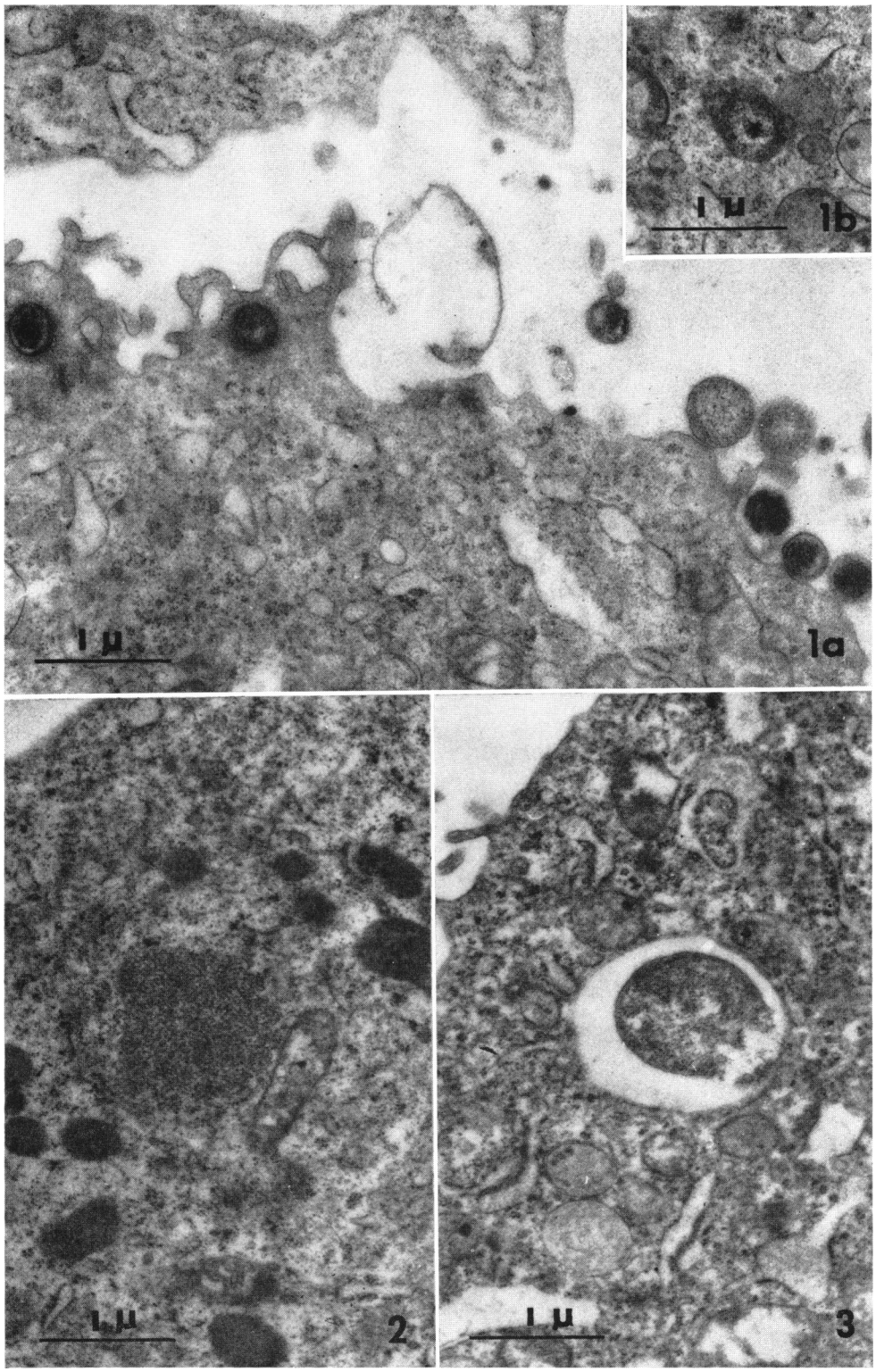

FIG. 1. *Adsorption and invagination of psittacosis virus into McCoy tissue cell;* × *20,000. Insert shows onset of virus disintegration;* × *20,000.*

FIG. 2. *Reticulated matrix of psittacosis virus in cytoplasm of McCoy tissue cell.* × *20,000.*

FIG. 3. *Formation of initial body of psittacosis virus in cytoplasm of McCoy tissue cell.* × *20,000.*

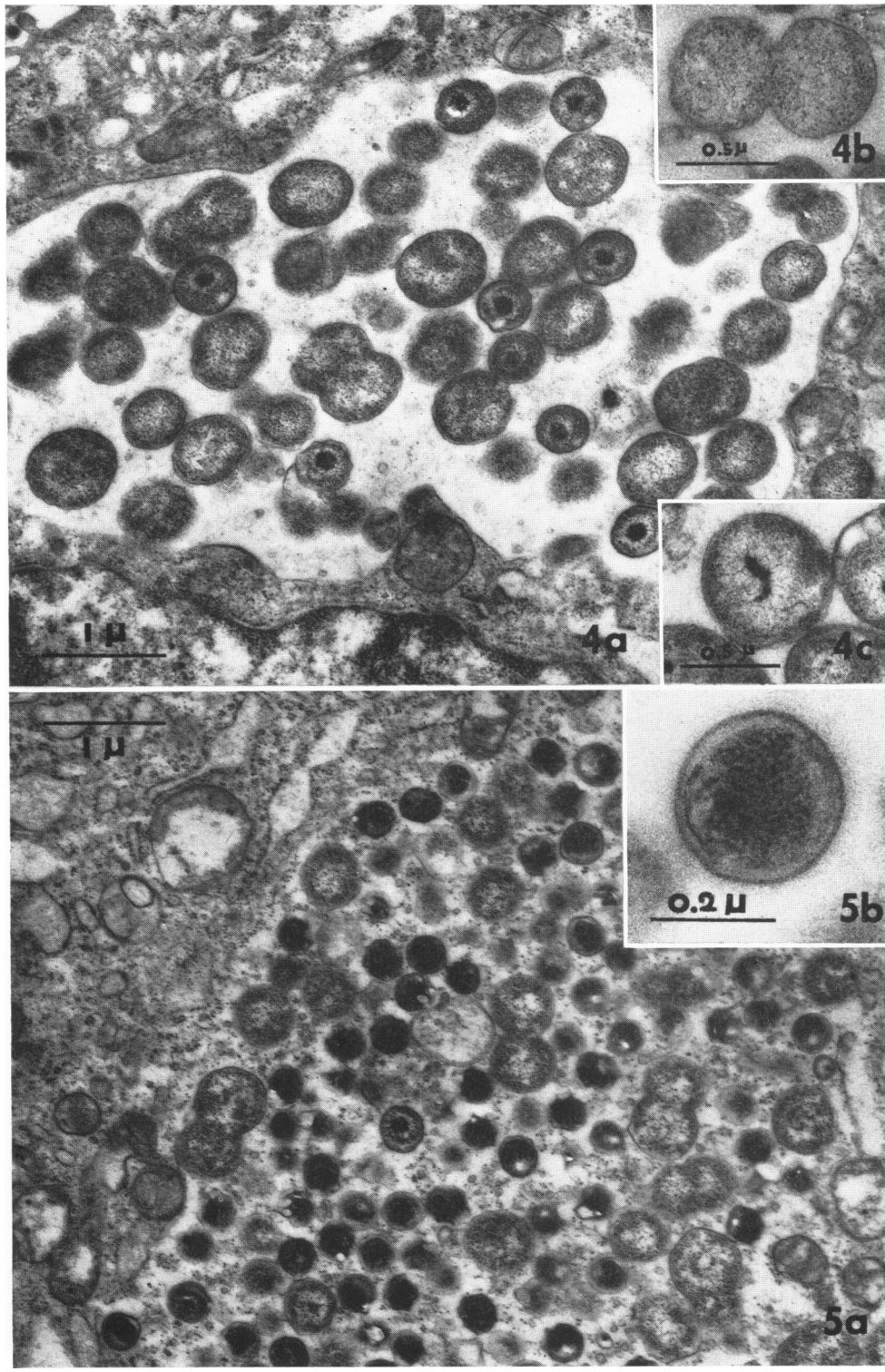

FIG. 4. *Development of smaller initial and intermediate bodies of psittacosis virus. Note electron-dense spot in center of the intermediate body. 4a: × 18,000; 4b, 4c: × 30,000.*

FIG. 5. *Psittacosis inclusion body with initial, intermediate, and mature stages of virus; × 17,000. Insert is a higher magnification of the mature infectious virus particle; × 90,000.*

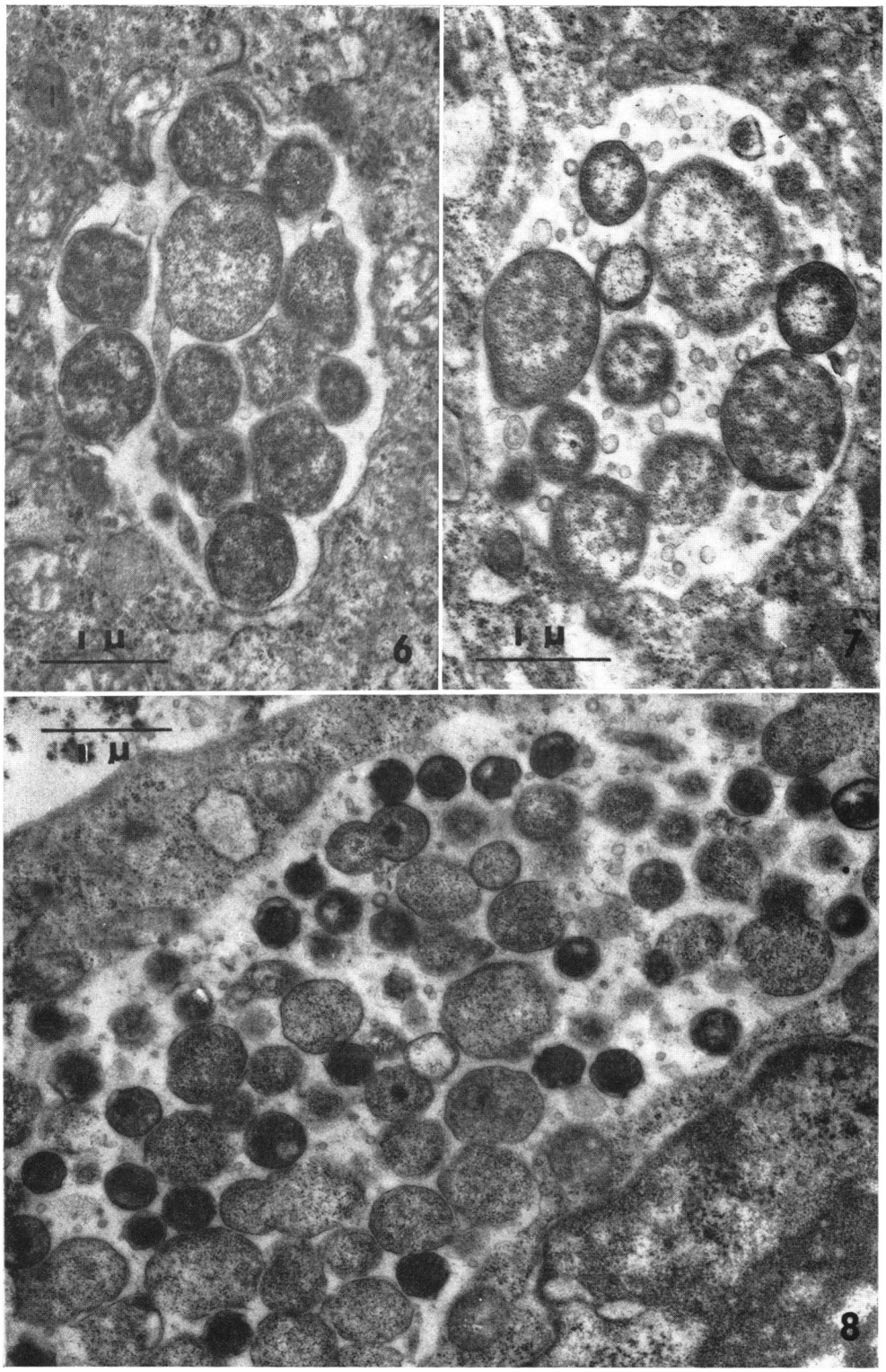

FIG. 6. *Initial body of psittacosis virus at 18 hr after inoculation into McCoy tissue cell. Virus control for Fig. 7.* × *19,000.*

FIG. 7. *Psittacosis virus interrupted by aminopterin at the initial body stage of replication.* × *19,000.*

FIG. 8. *Aminopterin-inhibited psittacosis virus with the three stages of replication at 36 hr after addition of folinic acid to the culture medium.* × *19,000.*

TABLE 1. *Virus assay after one cycle of replication*

Drug treatment	No. of particles
Control*	1.3×10^6
Medium + AP†	Negative
Medium + AP + FOA‡	7.4×10^5

* Assay result for virus particles per milliliter.
† Aminopterin, 25 μg/ml.
‡ Folinic acid, 1 μg/ml.

interrupted virus replication (Fig. 6). But, when 1.3×10^7 infectious particles were present in the inoculum, some of the viral inclusions contained intermediate and elementary bodies.

Effect of folinic acid on AP-interrupted TT virus. Addition of folinic acid to tissue cultures containing AP-interrupted TT virus resulted in the appearance of mature, infectious virus in the cultures within 24 hr. The virus inclusion body enlarged and showed many intermediate and elementary bodies which were the same size and structure as in untreated infected cultures (Fig. 8). The reversal of the effect of AP on TT virus by addition of folinic acid was confirmed by acridine orange fluorescence microscopy and by virus assay. DNA-staining particles appeared in the cytoplasm of stained cells, and infective virus was demonstrated in cell cultures which had been assayed in fresh cultures of McCoy cells. A comparison of virus production, as influenced by drug treatment, is indicated in Table 1.

DISCUSSION

The well-delineated morphological sequence through which TT virus replicated in cells was based on a synchronous type of propagation of virus in tissue culture cells, and on correlation of the results with the extensively studied acridine orange fluorochrome microscopy and biological assay procedures (Pollard and Starr, 1962). Three replication stages were described: initial, intermediate, and mature virus particles. Only the last stage was infectious. It is likely that the initial body is made up of DNA fibrils and RNA granules. Binary fission of the mature virus particle was not observed. In AP-treated cultures, TT virus growth cycle was interrupted at the noninfectious initial body stage, as observed by electron microscopy. This stage corresponded to the noninfectious RNA-staining stage as observed in acridine orange-stained preparations and described by Pollard and Starr (1963). TT virus was thus visualized in the eclipse or noninfective stage. After treatment of AP-inhibited cultures with folinic acid, TT virus resumed replication through the intermediate to the mature stage. Since the roles of AP and of folinic acid in DNA synthesis are known (Friedkin and Roberts, 1956), and since the AP-interrupted stage (the initial body) preceded viral DNA formation, it may be assumed that the intermediate and mature stages contain newly synthesized viral DNA, whereas the initial body does not.

Interruption of TT replication by AP in relation to inoculum concentration may indicate that the large amount of virus may contain (i) folinic acid in sufficient concentration to reverse progressively the effect of AP on immature particles, (ii) AP-resistant virus particles in quantity sufficient to be detected by the electron microscope, or (iii) particles of different replication speed. Since citrovorum factor (folinic acid) has been detected in purified preparations of psittacosis virus (Colón, 1961), and in TT-infected cultures at the last stages of the replication cycle (Pollard et al., 1961; Pollard and Tanami, 1962), it is likely that the first alternative is the logical explanation.

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