ELECTRON MICROSCOPIC STUDY OF THE ATPASE ACTIVITY OF THE BAI STRAIN A (MYELOBLASTOSIS) AVIAN TUMOR VIRUS

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ABSTRACT

Thymus glands of chicks with leukemia induced by BAI strain A (myeloblastosis) virus were fixed in cold 4 per cent formaldehyde-sucrose. Frozen sections were incubated in the ATPase medium of Wachstein and Meisel and studied by light microscopy and electron microscopy. The ATPase activity of the virus is localized to the outermost membrane of the virus. The membrane of the blast-like cells of the thymus cortex from which the virus emerges, by budding, also possesses such activity. It appears likely that the outermost membrane of the virus is derived from the plasma membrane of these cells.

INTRODUCTION

The normal lymphoid cell population of the cortex of the thymus of chickens with myeloblastosis induced by the BAI strain A virus is replaced by large non-neoplastic blast-like cells, presumably primitive lymphoblasts (1, 2). Conventional cytochemical studies showed a high level of adenosine triphosphatase (ATPase) activity of the membrane of these cells (1). Electron microscopic examination revealed, further, that virus particles emerged from the blast-like cells, as buds, from the cytoplasmic membrane, and that virus particles were present in the intercellular spaces (2). It was of interest to extend the studies to examination of the ATPase reaction of the cells at the electron microscopic level, for two reasons: (*a*) to determine whether virus liberated by membrane budding of the blast-like cells possessed ATPase activity like the agent (3–8) in leukemic myeloblasts where it is apparently synthesized in cytoplasmic structures called viroplasts (9–12); and (*b*) to ascertain whether the enzyme activity was localized in the inner nucleoid or the outer membrane of the virus. Because it is a solid tissue, the thymus lends itself to application of frozen section techniques (13–19) for such electron microscopic examination of ATPase localization.

MATERIALS AND METHODS

Two lots of white Leghorn chickens were used in the experiments. They were inoculated, at 5 days of age, with BAI strain A virus as described elsewhere (1). Birds with severe leukemia could readily be selected for study by examination of blood smears for leukemic myeloblasts.

The animals were decapitated and the thymus glands rapidly removed into cold fixative. For preparation of tissue for light and electron microscopy, both formol-calcium (20) and formol-phosphate (21) were used with 5 per cent or 7.5 per cent sucrose present in both fixatives (16). Following overnight fixation at 2–4°C, the tissue was rinsed in cold 7.5 per cent sucrose and sectioned, with a *sharp knife*, on a Bausch and Lomb microtome, employing CO_2

gas to freeze the tissue. Sections, 40 μ or 60 μ, were transferred immediately into cold 7.5 per cent sucrose. In subsequent steps the frozen sections were transferred from one solution to the next with wide-mouth medicine droppers. Following rinses in sucrose, the sections were incubated for varying lengths of time in the ATPase medium of Wachstein and Meisel (22) to which sucrose was added to a final concentration of 7.5 per cent. Following incubation, the sections were rinsed in cold 7.5 per cent sucrose. One section, to be checked by light microscopy, was treated for a few minutes in dilute ammonium sulfide in sucrose, rinsed in distilled water, and mounted in Kaiser's glycerogel (21). The others were post-fixed in cold buffered osmium tetroxide-sucrose (23) for 60 minutes, dehydrated in increasing concentrations of ethanol, and embedded in Epon, following the procedure of Kushida (24). Sections were cut on a Servall Porter-Blum microtome, employing diamond knives. They were mounted on naked grids, stained in uranyl nitrate–lead hydroxide by a procedure of Feldman (25), and examined in either the RCA EM-3B or the Siemens Elmiskop I. In the controls, substrate was omitted from the incubation medium. In addition to 40 μ or 60 μ sections, 10 μ sections were cut and examined by *light microscopy*.

RESULTS

Light Microscopy

As found previously (1), the cells with high ATPase activity in or near their plasma membranes were concentrated in the cortex of the glands (Figs. 1 to 3). Relatively few were present in the medulla. Individual cells of the cortex were frequently separated from each other in these frozen sections (Figs. 2, 3). The separation probably occurred during the freezing and sectioning, since it was seen in sections that had not been incubated.

Incubation at reduced temperature (9–10°C) for short periods (2 to 4 minutes) yielded reaction product at intervals along the plasma membranes of many cortical cells. With longer incubation, the accumulation spread over all of the surfaces of these cells. In addition to smooth accumulations in the plasma membrane, as in other cells (26), there were small irregular masses (arrows, Fig. 3). Such masses were also present in the extracellular spaces.

Electron Microscopy

The appearance of thinly sectioned cells of thymus, sampled from animals with myeloblastosis, is shown in Fig. 4. Virus particles are seen on or near the surface of blast-like cells and in the spaces between them. A comprehensive description of this cell type will be found elsewhere (2). The viral particles are approximately 80 mμ in diameter. They show an inner electron-opaque nucleoid surrounded by inner and outer membranes (Fig. 4, insert).

Essentially similar findings were made in frozen sections of tissues fixed in formol-calcium and formol-phosphate.

The ATPase incubation time found to be most useful for electron microscopic study was 4 minutes at room temperature. In most frozen sections studied, the cortical cells were frequently separated

FIGURE 1

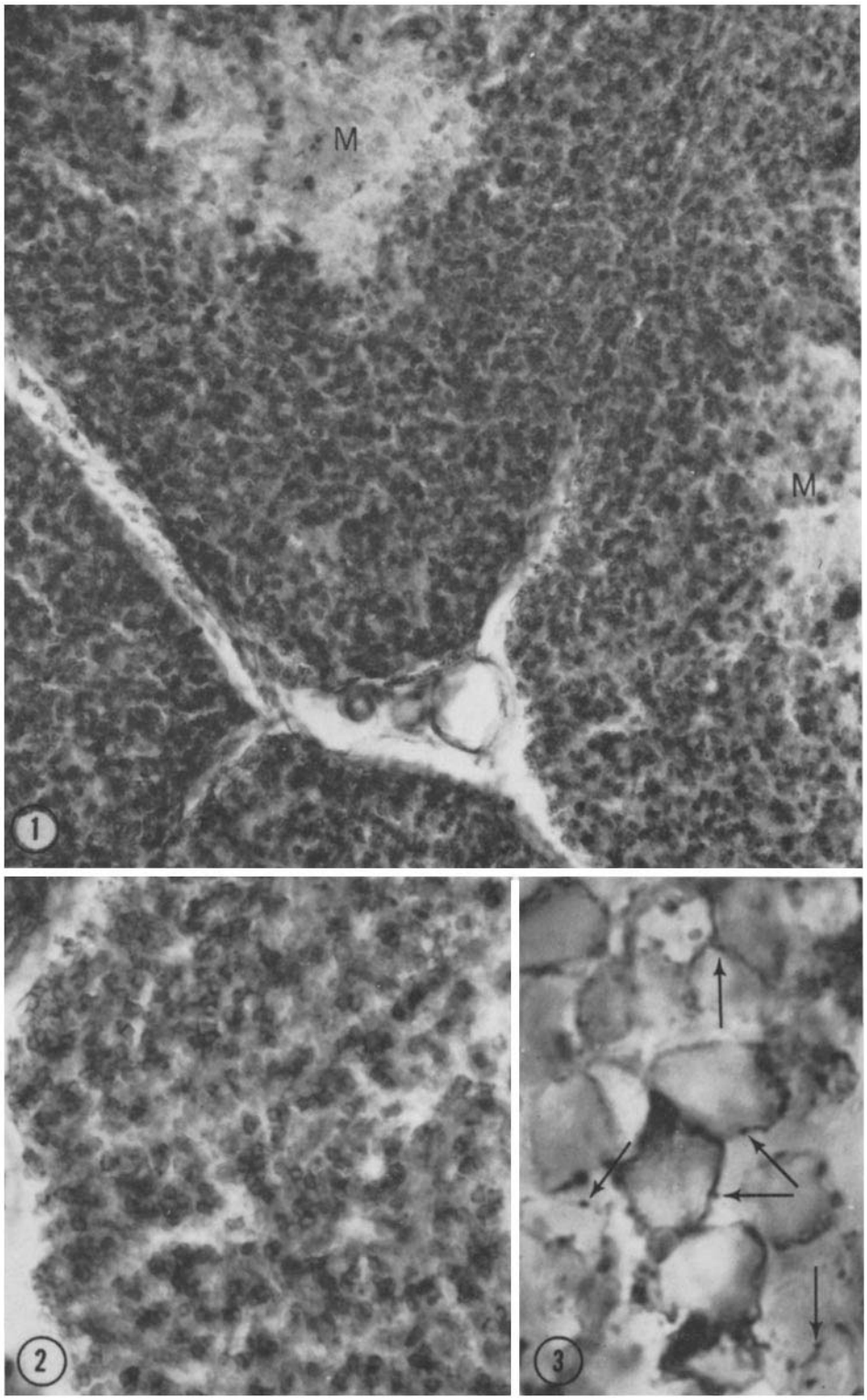
Thymus of infected bird. Tissue fixed overnight in cold formol calcium; 60 μ frozen section incubated for 4 minutes at room temperature (*ca.* 25°C) in the ATPase medium of Wachstein and Meisel. Portions of four lobules are visible. The upper one shows the extent to which ATPase-rich cortical cells have displaced the non-reactive medullary cells (*M*). The black deposits of reaction product in the cell membranes may be seen in some cells; they are shown more clearly in the enlargement (Fig. 2). × 170.

FIGURE 2

Enlargement of part of the lobule to the right of the vessels in Fig. 1. The localization of ATPase reaction product in the cell membranes is evident. × 300.

FIGURE 3

Thymus of same infected bird as in Fig. 1, incubated for 2 minutes at 9–10°C. Note ATPase reaction product in cell membranes and in small masses indicated by arrows. × 2400.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from each other (Figs. 5, 6, 9, 10). Two types of cells are readily discerned: one, of the lymphoid series, has relatively little cytoplasm and no significant ATPase reaction product on its surface (Fig. 6); the other, the blast-like cell (2), has dense deposits of lead phosphate on its plasma membrane (Fig. 5). Where the blast-like cells are separated from their neighbors, the precipitate continues usually for only about 100 to 300 mμ. Where two such cells are in contact with each other, the deposit is in the form of a continuous layer (Fig. 7).

Many viral particles are readily identified in the frozen sections. The nucleoid and outer membrane appear the same in sections incubated without substrate (controls) and with ATP. However, in sections incubated with ATP, a layer of dense reaction product lies close to the outer membrane. This reaction product, as in the case of the cell membrane, may be continuous, thus virtually enclosing the virus particle (Figs. 5, 8, 9, 11), or discontinuous (Figs. 8, 10). Relatively large areas with reaction product may be seen between cells. Within these areas viral particles are distinguishable, many with reaction product on their surfaces (Fig. 8).

DISCUSSION

Since electron microscopic studies of sites of enzyme reaction product are relatively recent, it is well to deal briefly with basic problems regarding localization of phosphatases, such as ATPase, in frozen sections.

The central assumption in our studies is that, in frozen sections of tissues, the site of reaction product (lead phosphate) accurately reflects the locus of the phosphatase hydrolyzing ATP. Support for this view comes from electron microscopic studies of such sections of kidney and liver incubated with adenosine triphosphate (15, 27) and inosine diphosphate (17), Morris 5123 and Reuber H-35 hepatomas incubated with inosine diphosphate and thiamine pyrophosphate (17, 18), liver incubated with inosine diphosphate (17), and a variety of tissues incubated with thiamine pyrophosphate (17, 28). In all instances, lead phosphate accumulation is restricted to specific membranes. The kidney tubule cell provides the most dramatic instance of such specificity. In many places β-cytomembranes may be less than 0.03 μ from endoplasmic reticulum membranes. With adenosine triphosphate as substrate, only β-cytomembranes show lead phosphate deposits, whereas with inosine diphosphate only the membranes of the endoplasmic reticulum show such deposits (29). Clearly, such results cannot be due to adsorption of lead phosphate produced by enzyme elsewhere.

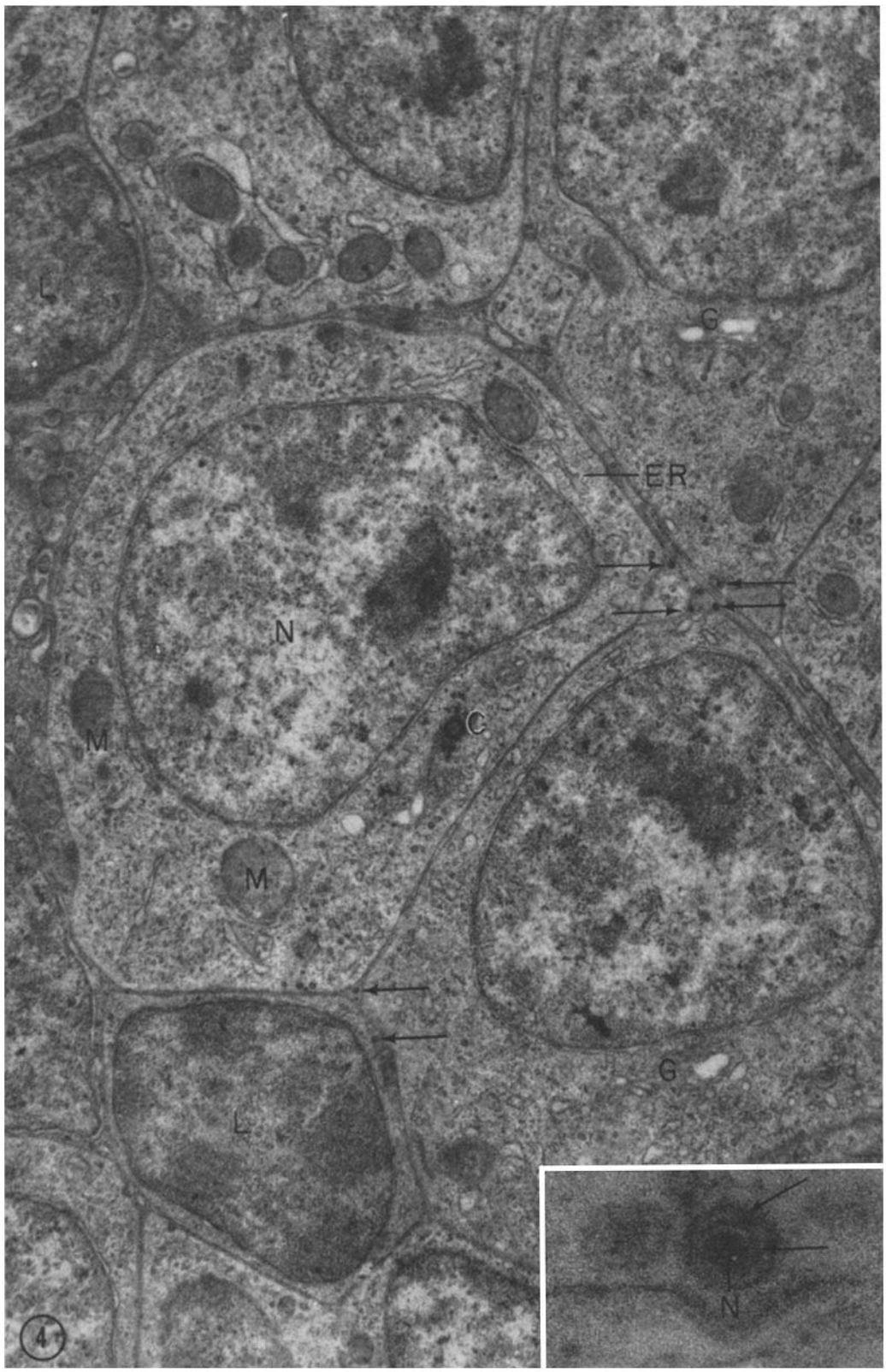
Another important question concerns possible artifact due to limitation of penetration of reagents such as the substrate or the lead ions used to trap the phosphate liberated from the substrate. As discussed elsewhere (30), this may be a serious matter when such small blocks of tissue are incubated for phosphatase activity. However, the present studies were done with frozen sections, under conditions where light microscopy on a very large number of tissues and electron microscopy on a considerable number have not revealed signs of significant penetration problems. Thus, we deduce from the absence of precipitate that the interior of the viral particles lacks ATPase activity.

Isolated viral particles lying in the widened intercellular spaces, removed by considerable distances from the cell membranes, showed the same layer of reaction product as those in contact with the cell membrane. This fact demonstrates that the viral particle generated its own phosphate from the adenosine triphosphate rather than being covered by that generated by the cell membrane. This is in keeping with the unequivocal biochemical evidence (3–8) that free virus particles in the

FIGURE 4

Cells in the cortex of a thymus of an infected bird. Although lymphocytes constitute the major component of the cortex in normal uninfected birds, in leukemic birds there are few such cells (*L*). Most are blast-like cells, presumably primitive lymphoblasts. Each shows a distinct nucleus (*N*), a few mitochondria (*M*), a poorly developed endoplasmic reticulum (*ER*), and a characteristic Golgi apparatus (*G*), often associated with a centriole (*C*). Arrows indicate viral particles in the intercellular spaces. × 11,000.

Insert: A virus particle near a portion of cell membrane. Note the central nucleoid (*N*) and inner and outer membranes (arrows). × 200,000.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blood, formed by myeloblasts, possess ATPase activity, and provides striking evidence that the same enzyme activity is found in virus particles emerging, as buds, from the blast-like cells.

Finally, the appearance of the lead phosphate deposits requires comment. Experience in our laboratory has consistently shown that, with brief incubation of sections, precipitate appears at intervals along the membrane. Only with longer incubation is all of the membrane covered by precipitate. This may reflect localized concentrations of enzyme. On the other hand, if "precipitation foci" were present which determine the first sites of lead phosphate accumulations, intermittent accumulations could occur despite a uniform enzyme distribution in or near the membrane. The lead phosphate accumulations on the outer viral membrane and on the plasma membrane of the blast-like cell are alike, not only with regard to this variability but also with respect to thickness.

Thus, we consider our results to indicate that ATPase activity is present in both the outermost membrane of the virus and the plasma membrane of the blast-like cells.

There is no definitive evidence that the BAI strain A virus separates from the leukemic myeloblast by a budding process. In the myeloblasts, in circulating blood and in tissue, only two buds have been encountered in years of study (9–12). When grown in tissue culture, these leukemic myeloblasts show intracytoplasmic grey bodies (10) or viroplasts (12) which have high ATPase activity and in which viral particles appear to be produced. In contrast, virus of typical morphology emerge, as buds, from the plasma membranes of the blast-like cells of the thymus (2). The findings reported here provide further support for the view that the viral particles replicated in the blast-like cells are coated with portions of the cell membrane.

The ATPase activity in the cell membrane of the blast-like cell constitutes an additonal means of providing evidence that the outermost membrane of some viruses derives from the plasma membrane as they leave the host cell. Other parameters have been ultrastructural appearances—in herpes virus (31) and a variety of animal tumor viruses (32–37)—and lipid or protein analyses, in Myxoviruses (38–40; but see 41). It may prove that one of the antigens of BAI strain A virus, immunologically like that of normal chicken tissue (42), is also localized in the outermost membrane of the virus.

We wish to thank Dr. Edward Essner for easing the electron microscopic studies in unnumerable ways.

FIGURE 5

Cells in thymus cortex of infected bird; 40 μ section of formol-phosphate-fixed tissue incubated as for Fig. 1, but not treated with ammonium sulfide.

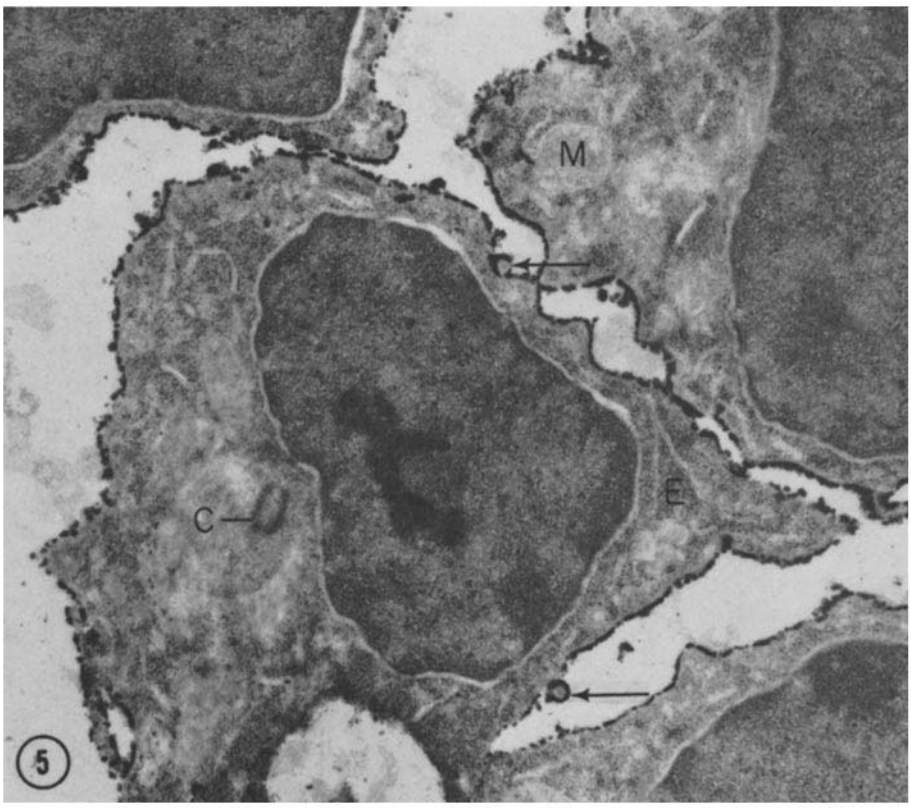
This blast-like cell has a large nucleus and abundant cytoplasm (*cf.* with large cell in Fig. 4). In this material the cytomembranes remained largely unstained when the sections were treated with the uranium and lead solutions. Note the "negative images" of mitochondria (*M*), the ribosomes of the ergastoplasm (*E*), and a centriole (*C*).

The dense material is lead phosphate, the precipitated reaction product of ATPase activity. Note that it extends around the cell membrane in fairly continuous fashion. It is somewhat more discontinuous in the left part of the cell. Arrows indicate two virus particles. Note the lead phosphate deposit on their surfaces (see Figs. 9 to 11). × 18,000.

FIGURE 6

Cell in thymus cortex, from same section as in Fig. 5. This cell, of the lymphoid series, has a large nucleus and little cytoplasm. Compare it with the lymphoid cell (*L*) in Fig. 4. There are a few mitochondria (*M*) in the cytoplasm. The ribosomes are abundant but show little sign of organized pattern.

Note the absence of ATPase reaction product from the cell surface. The meaning of reaction product in the vacuole-like structures within the cell (arrows) is not known. The dense areas over part of the nucleus are stain deposited when the section was stained with uranium and lead solutions. × 26,000.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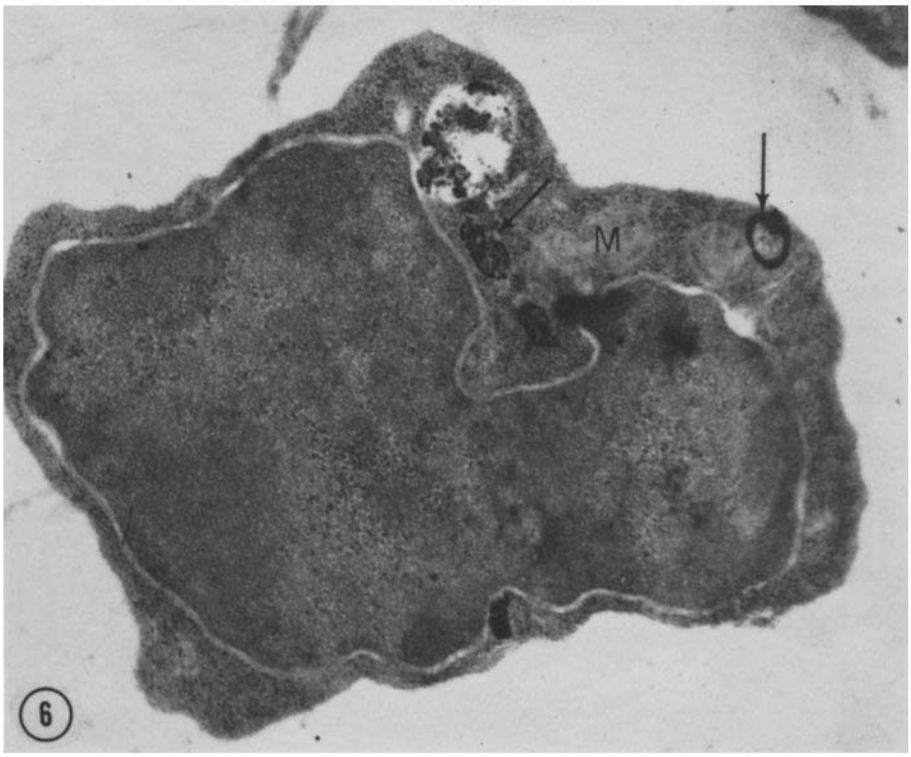
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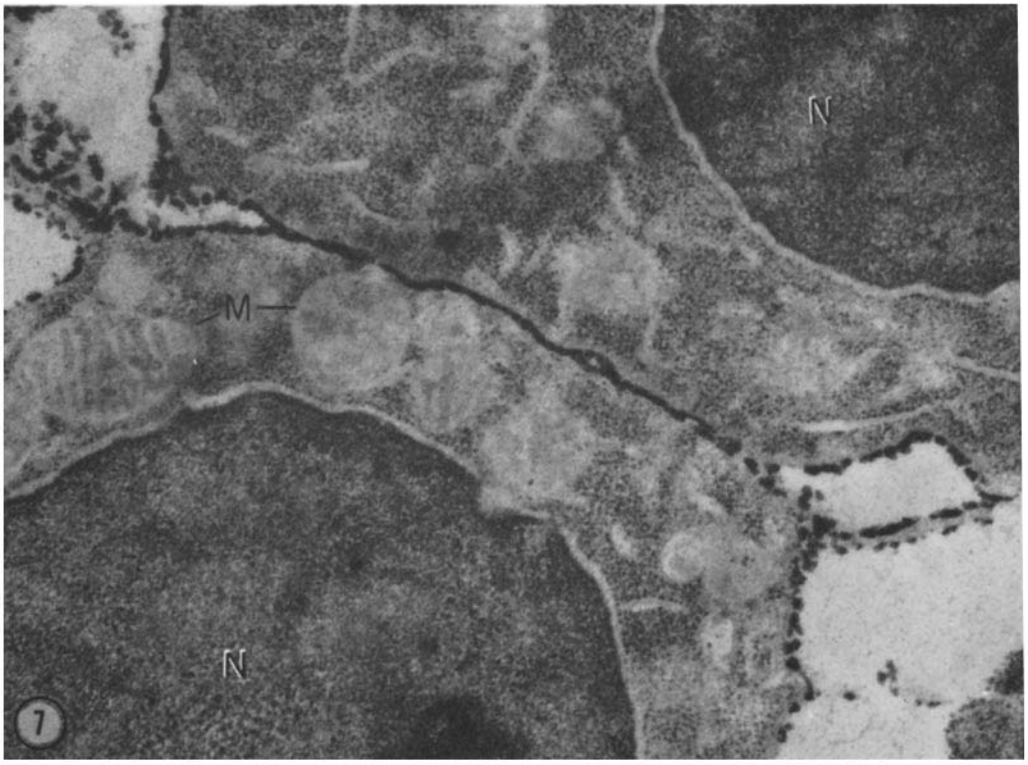
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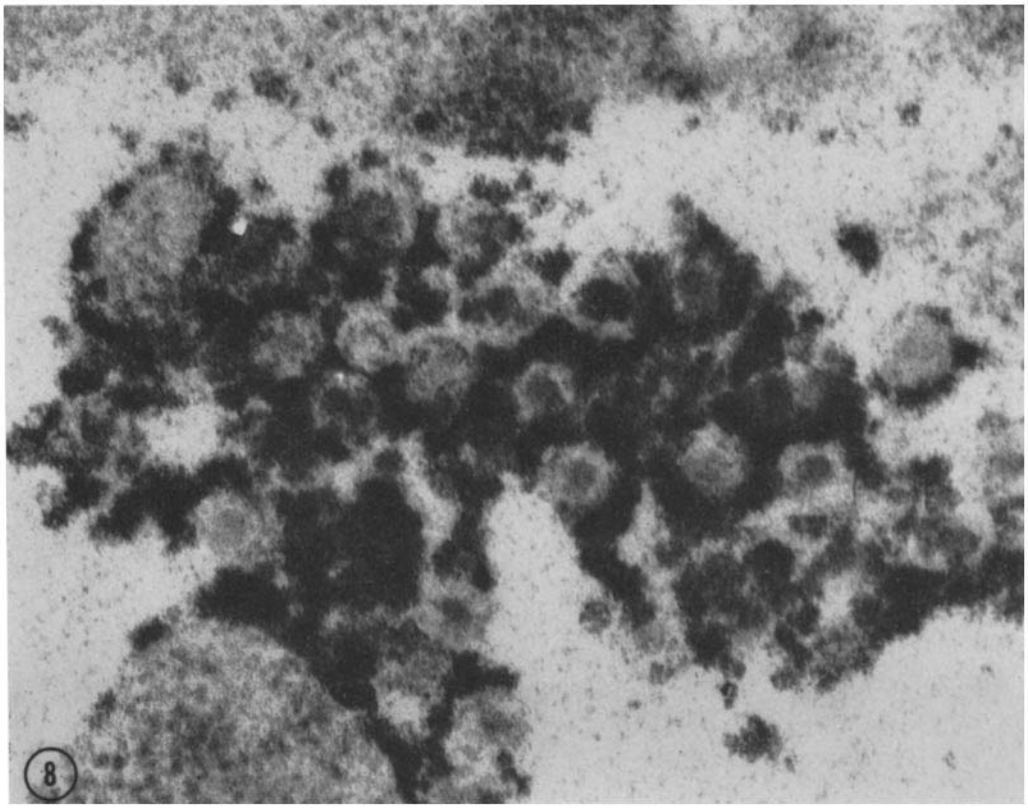
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Miss Phyllis Iaciofano prepared the frozen sections, and Mr. Jack Godrich aided in the photography.

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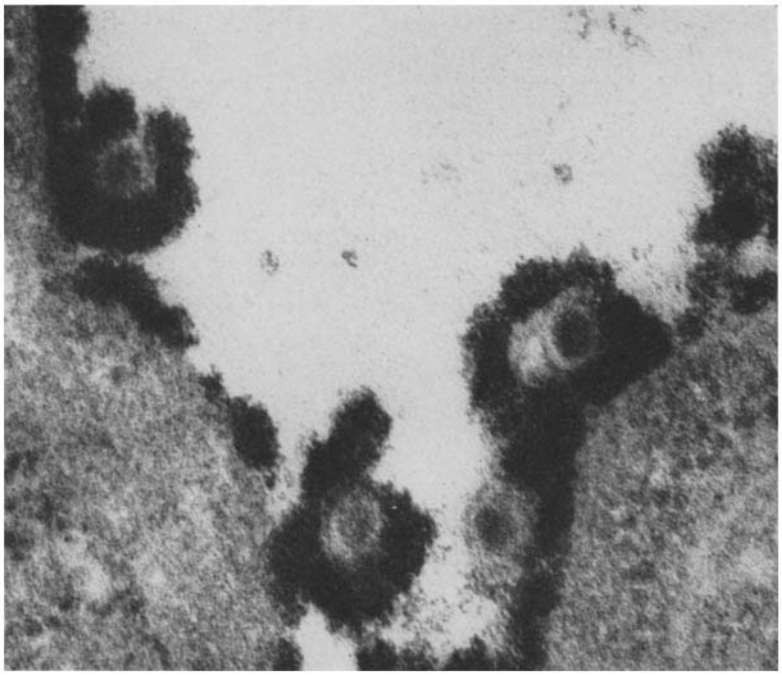

FIGURE 9

Portions of surfaces of two blast-like cells. Four viral particles are seen near the surface of the cells. The density of the nucleoids is similar to that in control sections incubated without substrate. ATPase reaction product is seen over the surface of three of the viruses. × 70,000.

FIGURE 7

Two blast-like cells. The lead phosphate precipitate, resulting from ATPase activity, is continuous where the two cells make contact with each other. Note nuclei (*N*) and mitochondria (*M*). × 31,000.

FIGURE 8

Portion of intercellular space between two blast-like cells. Dense deposits of ATPase reaction product are seen on the surfaces of the viral particles. × 100,000.

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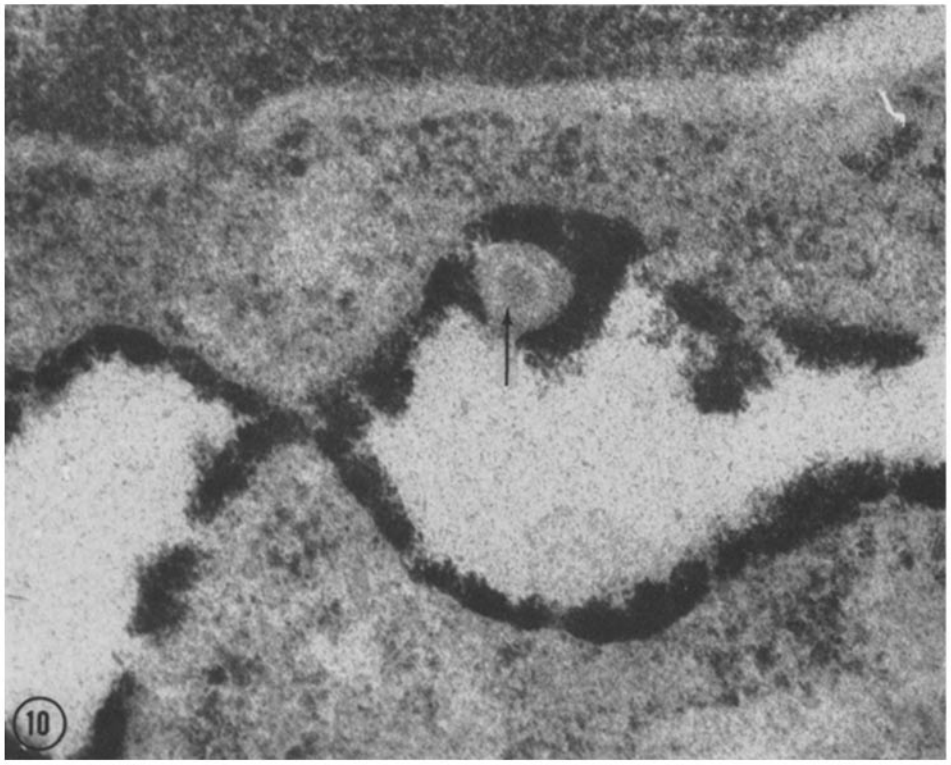
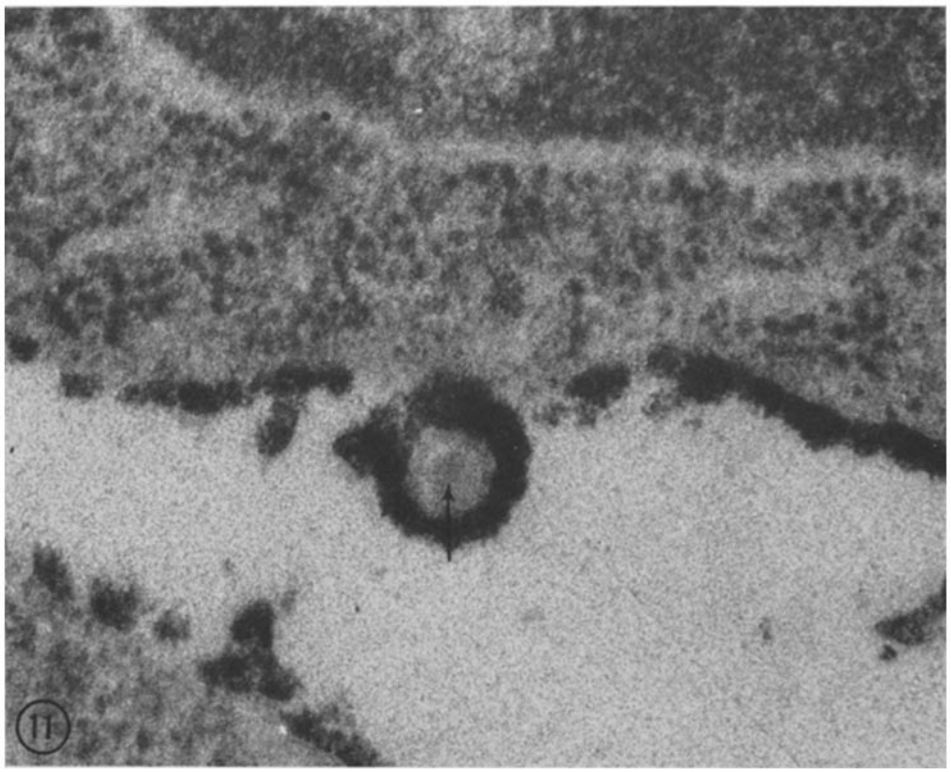
FIGURES 10 AND 11

Viral particles of Fig. 5, enlarged. Note the ATPase reaction product on their surfaces, and the nucleoids within (arrows). × 135,000.





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