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Inhibition of Friend Murine Leukemia Virus Production by Low-Ionic-Strength Medium

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The effect of medium of low ionic strength on the release of virus from Friend leukemia cells has been studied. The release of infectious Friend leukemia virus is almost completely inhibited in medium of low ionic strength, as measured by a focus-forming assay (XC assay), by endogenous RNA-dependent DNA polymerase activity of released virus particles, and by electron microscope studies of the production of C-type particles. Friend leukemia virus-transformed proerythroblasts undergo extensive morphological changes in low-ionic-strength medium. The cells are viable in this medium, but they can no longer be stimulated with dimethyl sulfoxide to produce hemoglobin and increase virus production. Infectious virus is released between 30 and 120 min of resuspension of inhibited cells in normal medium. The rate of virus release after reversal of the inhibition is much greater than the rate of virus release during normal cell growth. The morphological changes occurring after dimethyl sulfoxide stimulation of Friend leukemia cells are compared with those resulting from resuspension in normal medium of cells inhibited by low ionic strength.

The effect of low-ionic-strength cell culture media on the maturation and release of viruses that have a membranous envelope has been studied previously in the arbovirus group, the smallest and simplest viruses of this type. Electron microscope studies have shown that Sindbis virus obtains its envelope by budding through the plasma membrane or into cytoplasmic vacuoles (1, 9).

Waite and Pfefferkorn (19) originally reported that lowering the NaCl concentration so that the osmotic pressure was 60% of that in normal medium inhibited the release of Sindbis virus by more than 99%. Replacement of the inhibitory medium by normal medium caused a rapid release of virus particles. Within 20 min, as many infectious particles were released as were observed during hours of incubation of uninhibited control cultures.

Later, Waite and Pfefferkorn discovered that the effect was not due to lowered osmotic pressure but lowered ionic strength, since reversal would not occur upon restoration of normal osmotic pressure with glycerol or sucrose (20). Different salts were effective in restoring release. Divalent ions were four times as effective on a molar basis as monovalent ions. Experiments indicated that nucleocapsids were formed in low-ionic-strength media. It was concluded that inhibition involved a very late function in virus maturation prior to budding (20).

Here we report that release of a relatively complex RNA tumor virus is also blocked in low-ionic-strength medium. The release of Friend leukemia virus is inhibited when murine leukemia virus-transformed proerythroblasts (Friend leukemia cells) are cultured in media of low ionic strength. Apparently, a somewhat different mechanism is involved for this virus than was observed in the inhibited Sindbis system. In the latter, nucleocapsids accumulated under the plasma membrane (18); no analogous structures were observed in Friend leukemia cells under conditions of low ionic strength.

MATERIALS AND METHODS

Cells and growth conditions. The Friend leukemia virus-transformed proerythroblastic line, FSD-1/clone F4, was kindly provided by W. Ostertag, Max-Planck Institut für Experimentelle Medizin, Gottingen, Germany. These cells were grown in suspension culture with Eagle minimal essential medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with nonessential amino acids and 10% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.). Viable cell number was determined by exclu-

sion of erythrosin. Thymidine uptake and DNA synthesis was measured by incubating cells for 1 h in medium containing 10 μCi of [^{3}H]thymidine per ml (18.4 Ci/mmol). Cells were washed twice in unlabeled medium, after which an aliquot was counted in a Beckman liquid scintillation counter to determine total counts incorporated. The remainder of the sample was precipitated with cold 5% trichloroacetic acid, washed, collected on membrane filters (Millipore Corp., Bedford, Mass.), and counted to determine the amount of incorporation into DNA. Low-ionic-strength medium was identical to Eagle minimal essential medium, except the NaCl concentration was lowered from 0.137 to 0.083 M and sucrose (0.11 M) was added to restore osmolarity. Medium not corrected to normal osmolarity caused rapid cell death.

The Friend leukemia cells in logarithmic growth phase were stimulated to produce hemoglobin by the addition of Me_2SO (reagent grade from Merck, Darmstadt, Germany, or from Polysciences, Warrington, Pa.) to give a final concentration of 1.5%. After 24 h, cultures were diluted by the addition of one volume of fresh medium containing 1.5% Me_2SO. The cells were harvested after 96 h. Generally, 40 to 50% of the cells were positive for hemoglobin production as determined by benzidine staining (3).

Virus isolation and assay. The XC assay was performed as described by Hackett and Sylvester (6). Briefly, nonconfluent UC1-B cell monolayers in Falcon flasks were treated with 0.5 ml of DEAE-dextran (25 μg/ml in phosphate-buffered saline) for 1 h, washed with phosphate-buffered saline (137 mM NaCl, 2.68 mM KCl, 4.29 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.2), and then infected with serial dilutions of virus-containing media in Hanks balanced salt solution. After 1 h of absorption, 5 ml of Eagle minimal essential medium was added to each flask. On day 4 the medium was changed and 1×10^6 XC cells were added per flask. On day 6 syncytia were counted after staining the monolayers with Giemsa. The cells were provided through the courtesy of A. J. Hackett.

Purification and assay of Friend leukemia virus RNA-dependent DNA polymerase activity was performed by the method of Syrewicz et al. (16) for purification of Rauscher leukemia virus. Briefly, medium was clarified by low-speed centrifugation and made 6% (wt/vol) in 20 M polyethylene glycol (Schwarz/Mann, Orangeburg, N.Y.). The precipitate formed overnight at 4 C and was collected by centrifugation and resuspended by homogenization in 0.01 M Tris-hydrochloride (pH 7.5), 0.1 M NaCl, and 0.001 M EDTA (TNE buffer solution). This virus was further purified by sedimentation equilibrium centrifugation on a 15 to 50% sucrose gradient in TNE or by pelleting through a 20% sucrose cushion. The virus was concentrated at 1.17 g/cm^3 after sedimentation equilibrium centrifugation. No exogenous templates were added for assay of the RNA-dependent DNA polymerase. The assay was performed in 0.1 ml containing 0.2 mM $MnCl_2$; 6 mM KCl; 3.9 mM Tris-hydrochloride (pH 8.1); 0.05 mM dCTP, dATP, and dGTP; 10 mCi (21 Ci/mol) of [^{3}H]TTP; 5 mM dithioerythritol; 0.02% Triton X-100 (Beckman, Fullerton, Calif.); and 60 μg of virus protein. The reaction was incubated 60 min at 37 C and chilled, and 0.1 ml of 0.1 M sodium pyrophosphate was added. The mixture was precipitated with 1 ml of 5% trichloroacetic acid, washed, collected on membrane filters (Millipore), and counted in a Beckman liquid scintillation counter.

Electron microscopy. All samples of cells were collected and washed three times by centrifugation for 5 min at low speed in a clinical centrifuge. Medium was replaced with either normal-ionic-strength or low-ionic-strength phosphate-buffered saline restored to normal osmolarity by the addition of sucrose. The pellets were fixed for 2 h at 4 C in a solution containing 2% glutaraldehyde and 25 mM sodium cacodylate (pH 7.2) to which NaCl was added to give a final concentration of 76 mM for the low-ionic-strength samples (ionic strength = 0.101) or 130 mM NaCl for the normal-ionic-strength samples (ionic strength = 0.155). The pellets were then washed in 50 mM cacodylate, pH 7.2, and postfixed for 2 h at 4 C in 25 mM cacodylate to which 2% (wt/vol) osmium tetroxide had been added. Then they were washed sequentially, first in the 50 mM cacodylate solution and then in distilled water. The pellets were stained en bloc in 0.5% uranyl acetate for 24 h in the cold, washed again in distilled water, and dehydrated through an ethanol series (25, 50, 75, 95, and 100%) and then with 100% acetone. They were infiltrated and embedded in an Epon-Araldite mixture, plastic I mixture (8), and cured at 65 C for 1 to 4 days.

Thin sections were cut with a Porter-Blum MT-1 ultramicrotome (Ivan Sorvall, Inc., Newton, Conn.), mounted on uncoated 200-mesh copper grids, and stained in 0.25% uranyl acetate in water followed by lead citrate (12). The sections were studied and photographed in a Hitachi HU-11E electron microscope (Perkin Elmer, Los Alamitos, Calif.) operated at 50 kV. Calibration of the microscope was performed using a carbon grating replica (54,864 lines/inch, Fullam, Schenectady, N.Y.).

RESULTS

Morphology and virus production. Friend leukemia cells (FSD-1/clone F4) grown in medium of normal ionic strength (Fig. 1) appeared as a population of round cells with very few cytoplasmic processes and a limited number of microvilli. A large majority of the cells had single nuclei of central or slightly paracentral location. Most of the nuclei had irregular outlines, and some showed deep invaginations of the nuclear membrane and nuclear pseudoinclusions. The chromatin was in the form of small clumps of heterochromatin uniformly distributed inside the nuclei and around the inner surface of the nuclear envelope. Large, single or multiple nucleoli were seen in most cells.

The cytoplasm was densely packed with uniformly distributed free ribosomes and some polysomes. Rough-surfaced endoplasmic reticulum was characterized by a small number of

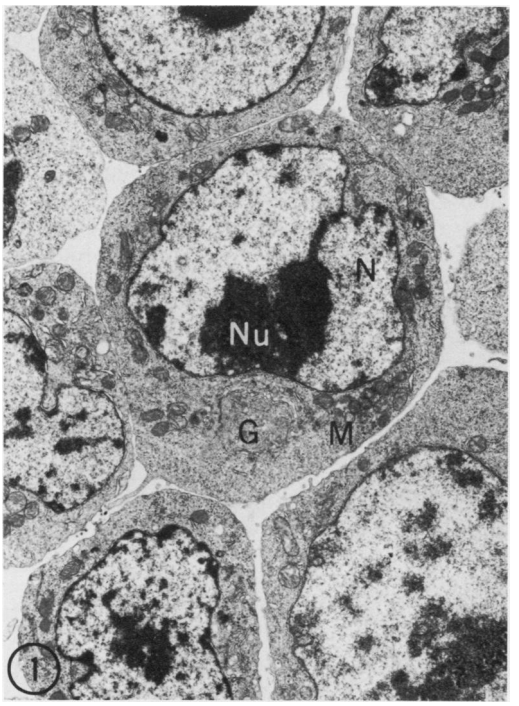

FIG. 1. *Unstimulated FSD-1/clone F4 cells pelleted from medium of normal ionic strength. N, Nucleus; Nu, nucleolus; G, Golgi apparatus; M, mitochondria.* ×4,200.

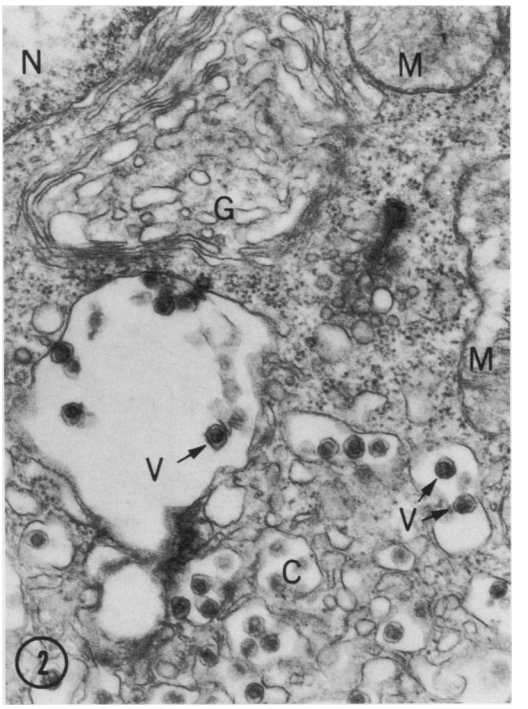

FIG. 2. *C-type virus particles (V) in dilated cisternae (C) in the cytoplasm of a FSD-1/clone F4 cell. Note proximity of the cisternae to the Golgi apparatus (G). N, Nucleus; M, mitochondria.* ×28,600.

short cisternae. The Golgi apparatus was well developed and present in most of the cells as a juxtanuclear system of closely apposed cisternae and small vesicles. The mitochondria were variable in shape, oval outlines being the most common. Most of the mitochondria showed irregular cristae and moderately dense matrices. In some of the cells, myelin-like bodies were seen in the cytoplasm or inside membrane-bound, lysosome-like bodies.

Some of the cells grown in normal medium (less than 10%) showed dilated, smooth-surfaced cisternae and vesicles with irregular outlines that contained C-type virus particles (Fig. 2). These cisternae were in many cases closely associated with the Golgi apparatus. Virus particles, with diameters of 85 to 105 nm, were characterized by a membrane envelope and a dense, centrally located and indistinct nucleoid. Very few particles were seen budding from the plasma membrane. These particles showed the typical dense, crescent-shaped nucleoid under the plasma membrane and were morphologically similar to those observed in cells stimulated by addition of Me_2SO (Fig. 3). All of the extracellular virus particles observed were of the C-type and similar to the ones found intracellularly.

Friend leukemia cells were found to release significant amounts of virus during normal growth. Virus release was measured by focus formation in the XC assay (Fig. 4A). The Friend leukemia virus complex has both spleen focus-forming components (SFFV-F) and a lymphatic leukemia helper virus (LLV-F) (15). Virus release as measured by the XC assay most probably reflects the concentration of the LLV-F virus and has no direct relation to the concentration of SFFV-F in the media (10). The maximal concentration of infectious virus released into the culture medium reached about 2×10^4 focus-forming units (FFU)/ml when the cell concentration was approximately 1×10^6 viable cells/ml. Thus, Friend leukemia cells constantly release infectious virus during logarithmic cell growth in the absence of any inducing medium, albeit at a fairly slow rate per cell.

Friend cells grown in the presence of Me_2SO undergo a partial differentiation sequence, which has been shown to include transcription of globin messenger RNA, increased production of Friend leukemia virus, and hemoglobin synthesis (13, 14). Upon addition of 1.5% Me_2SO, cells were stimulated to release large amounts of

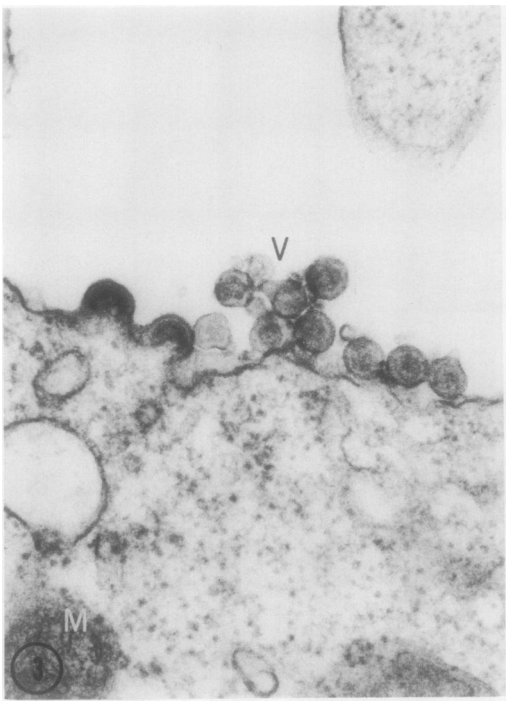

FIG. 3. *Extracellular virus (V) and virus budding from the plasma membrane after 5 days of Me_2SO stimulation. M, Mitochondrion.* ×52,800.

virus on day 4 after stimulation as measured in the RNA-dependent DNA polymerase assay. Since both components of the Friend leukemia complex, SFFV-F and LLV-F, would require an RNA-dependent DNA polymerase for replication of their genome, this assay does not distinguish between the transforming virus and its helper but reflects the total amount of both present in the medium. The peak of activity recovered on day 4 after Me_2SO addition was 30-fold higher than that observed in the absence of Me_2SO. On day 4 after Me_2SO stimulation, the concentration of infectious virus in the medium was 5×10^7 FFU/ml, as measured by the XC assay (unpublished data). This is more than 1,000 times the number of infectious virus released by unstimulated cells (Fig. 4A). On day 4 after the addition of Me_2SO to the nutrient, hemoglobin was detectable by the benzidine test (3). The red color of hemoglobin was seen in packed cells of older stimulated cultures.

Cells grown in a medium of normal ionic strength containing Me_2SO showed several morphological changes. The surfaces of most of the cells became irregular, and many cells showed long cytoplasmic processes. The total number of ribosomes decreased and more polysomal patterns were observed than in the unstimulated cells. A greater percentage of cells (39% after 5 days of treatment with Me_2SO) showed bodies composed of multiple vacuoles containing large numbers of C-type virus particles (Fig. 5). The vacuoles were delimited by smooth membranes. Some of the particles were also seen inside dilated cisternae similar to the ones found in unstimulated cells (Fig. 2). The number of virus particles budding through the plasma membrane was very low and similar to the numbers obtained from cells grown in the absence of Me_2SO. Most of the vacuolar bodies were located under the plasma membrane; they extended beyond the surface of the cell and were separated from the extracellular space by extremely thin layers of cytoplasm.

Effect of low ionic strength. The release of Friend murine leukemia virus was rapidly inhibited by low ionic strength (Fig. 4B). The cells

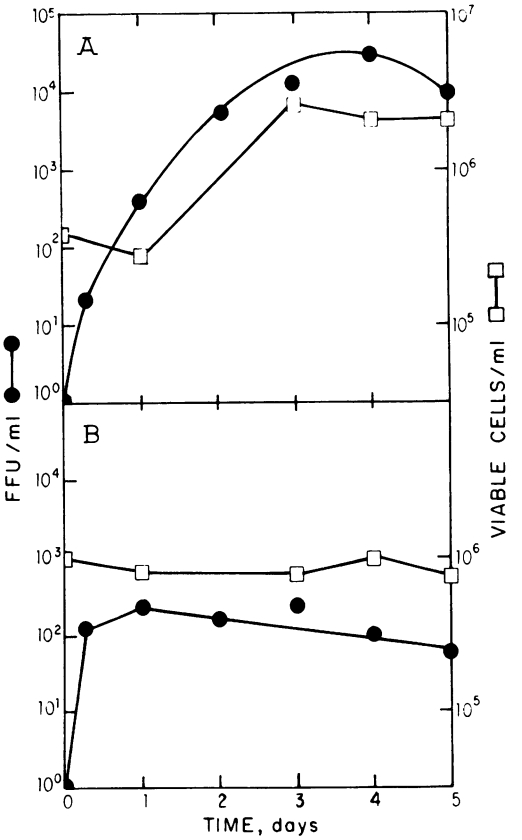

FIG. 4. *Friend leukemia virus production in normal medium (A) and low-ionic-strength medium (B). Virus release was measured by the number of FFU per milliliter in the XC assay (●). The number of viable cells per milliliter is given for comparison (□).*

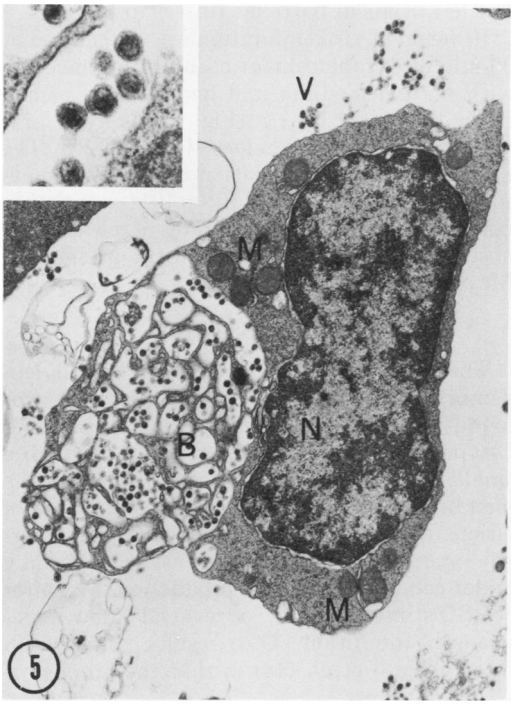

FIG. 5. *Cell 7 days after stimulation with Me_2SO. Large numbers of extracellular virus (V) are apparent, as well as virus-containing vacuolar bodies (B). N, Nucleus; M, mitochondria. ×8,700. Insert: Vacuolar body containing C-type virus particles. ×40,100.*

ceased dividing but remained viable for several days in low-ionic-strength medium containing sucrose. DNA synthesis, as measured by incorporation of [^{3}H]thymidine, was inhibited to 40 to 60% of the level observed in stationary-phase control cultures upon addition of low-ionic-strength medium (Fig. 6B). This inhibition was complete by 2 h. The total uptake of [^{3}H]thymidine was increased twofold as compared with cells in normal medium. Protein synthesis (as measured by [^{3}H]leucine incorporation) and RNA synthesis (as measured by [^{3}H]uridine incorporation) were also initially inhibited by low-ionic-strength medium but recovered to normal levels of synthesis within 8 h (unpublished data). The amount of virus released by cells under low-ionic-strength conditions was reduced by at least 2 orders of magnitude from the amount released by cells in normal medium (no Me_2SO added) as measured by the XC assay (Fig. 4B).

Cells grown in low-ionic-strength medium showed very few virus particles budding from the plasma membrane and almost no virus-containing vacuolar bodies (Fig. 7). The cytoplasm of these cells (Fig. 8), however, showed membrane-bound vacuoles with relatively clear contents and small, electron-dense particles with diameters ranging from 50 to 85 nm (insert to Fig. 8). The cisternae of the granular endoplasmic reticulum, as well as the nuclear membrane, were extremely dilated. Also, the mitochondria were swollen. They contained few cristae and very sparse and clear matrices. The Golgi apparatus region was characterized by large number of dilated vesicles and an unusually high proportion of small coated vesicles, with an approximate diameter of 80 nm (5).

Cells treated with Me_2SO in normal medium experienced a slowed growth rate but maintained a high percentage of viable cells. However, cells treated with both low ionic strength and Me_2SO were rapidly destroyed; therefore, any investigations of hemoglobin production and virus production under these circumstances were precluded. Cells treated with low-ionic-

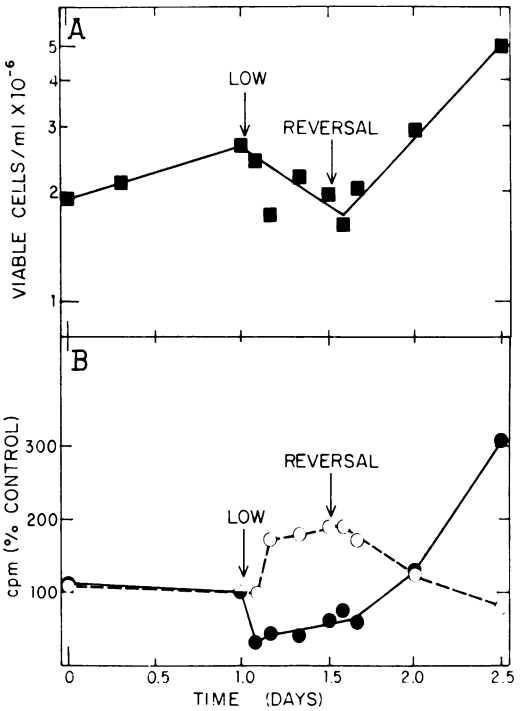

FIG. 6. *Cell viability (■, A) and [^{3}H]thymidine incorporation (B) in cells suspended in medium of low ionic strength (indicated by "LOW") for 12 h and then resuspended in normal medium (indicated by "REVERSAL"). Control values (stationary cells in normal medium) for incorporation of total [^{3}H]thymidine (○) were 100% = 466,792 counts/min per 10^6 cells and for cold trichloroacetic acid-precipitable counts (●) were 100% = 12,728 counts/min per 10^6 cells. Values after 1.5 days were corrected for 1:5 dilution upon resuspension in normal medium.*

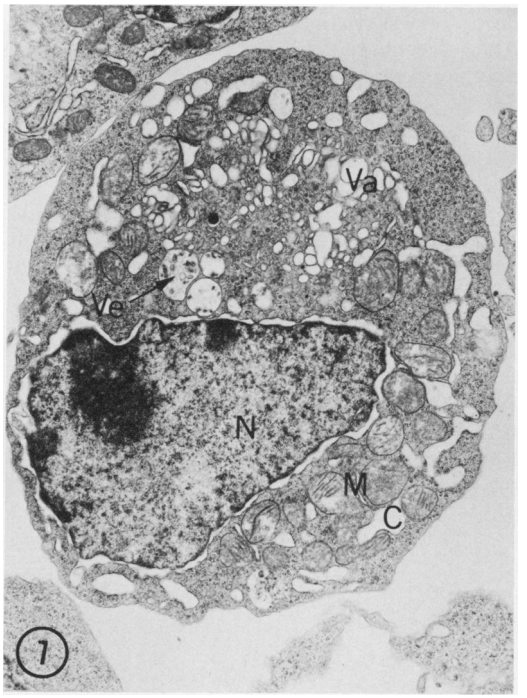

FIG. 7. *Cell incubated for 8 h in the presence of low-ionic-strength medium. Dilated cisternae (C), extensive vacuoles (Va), and small dilated vesicles (Ve) are apparent in the cytoplasm. N, Nucleus; M, mitochondria.* ×6,000.

strength medium for 6 h and Me_2SO for 1 h showed extensive vacuolation and destruction of mitochondrial organization (not shown).

Restoration of normal ionic strength. After the low-ionic-strength medium was replaced by normal medium, the cells morphologically resembled those grown in normal medium alone (Fig. 9). Within 4 h after restoration of normal ionic strength, cells were dividing exponentially (generation time, approximately 16 h, Fig. 6A) and evidenced rapid DNA synthesis (Fig. 6B), as well as protein and RNA synthesis (unpublished data). Numerous mitotic figures were observed in electron micrographs. Dilation of granular endoplasmic reticulum, nuclear membrane, and mitochondria was not evident. No changes as compared with normal medium were noticed in the number of virus particles budding from the surface. The main morphological change as compared with low-ionic-strength medium was the replacement of vacuoles containing atypical dense particles (insert to Fig. 8) by complex vacuolar bodies (Fig. 10) similar to those seen in cells stimulated with Me_2SO (Fig. 6). These bodies contained many C-type virus particles and some membranous components but few atypical particles (insert to Fig. 10).

Reversal of virus inhibition was detectable by 30 min after replacement of inhibitory medium with normal medium and had reached a relatively high level by 2 h (Fig. 11). A very large amount of virus was released within 2 h. The maximal virus concentration obtained as measured by either assay was 10^3 to 10^4 times that in normal media. Comparable rates of release were observed only in cells grown in the presence of Me_2SO for 4 days.

DISCUSSION

The maturation and release of Friend leukemia virus from FSD-1/clone F4 cells was found to differ significantly from most other C-type virus systems studied (17). Although a small amount of budding from the plasma membrane was noted, the primary source of virions was from budding into enlarged cisternae and complex vacuolar bodies especially under conditions of rapid production, i.e., after Me_2SO stimulation (14) or reversal of low-ionic-strength inhibition. Our results differ from those of Sato et al. (14) in that the number of

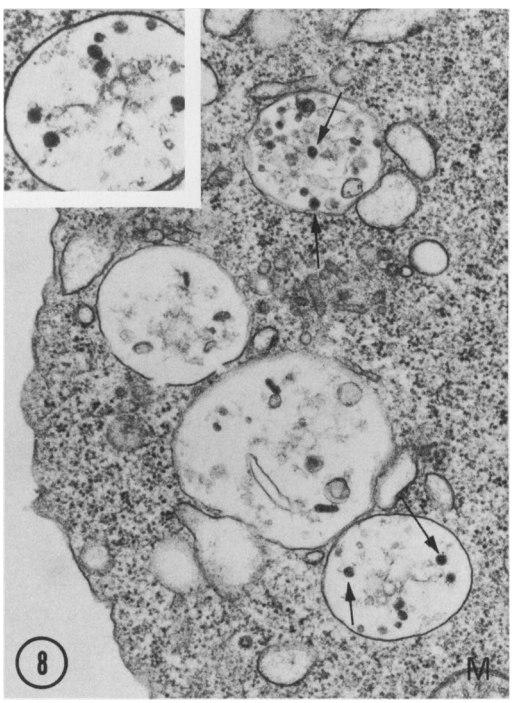

FIG. 8. *Small, electron-dense, atypical particles (indicated by arrows) in vacuoles of a cell incubated for 8 h in the presence of low-ionic-strength medium. M, mitochondrion.* ×22,800. *Insert: Vacuole with atypical particles.* ×33,600.

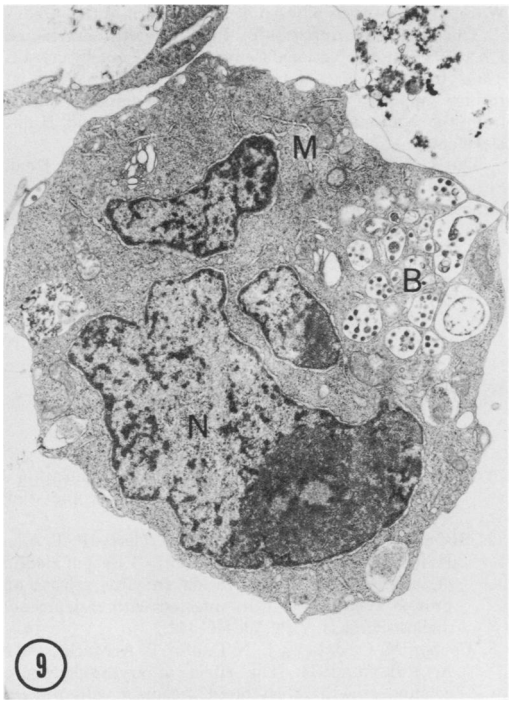

FIG. 9. *Cell preincubated for 8 h in medium of low ionic strength and then resuspended in normal-ionic-strength medium for 30 min. C-type virus particles are evident in vacuolar bodies (B). N, Nucleus; M, mitochondrion.* ×7,700.

viruses budding at the plasma membrane did not increase with increased virus production and we were not able to find any intra- or extracellular virus particles of the enveloped A-type (particles characterized by two concentric shells surrounding an electron-lucent core).

The observation of virus-containing vacuolar bodies in cells returned to normal media after incubation in low-ionic-strength media as well as in Me_2SO-stimulated cells suggests that these bodies are not artifacts resulting from secondary effects of Me_2SO. These vacuolar structures were often closely associated with the plasma membrane, suggesting the possibility of fusion and release of virus into the extracellular environment. Studies on marker enzymes in these bodies should help elucidate their origin and eventual fate in the process of viral production (4). The inhibition of cell growth and macromolecular synthesis observed under low-ionic-strength conditions was reversed within 2 h of resuspension in normal medium. The ability of these cells to divide rapidly indicated that little permanent damage was caused by incubation in low-ionic-strength medium. Virus production was inhibited within 1 h after treatment of cells with low-ionic-strength media. However, in contrast to the arbovirus system (18, 20), an accumulation of viral "cores" or other possible precursor structures was not discernible in the cytoplasmic matrix. Corelike bodies were observed within the vacuoles of cells incubated in low-ionic-strength medium (Fig. 8). Particles with the same buoyant density as that found for Friend leukemia virus cores (7) have been observed in other murine leukemia virus-transformed cells (11) and in Me_2SO-stimulated Friend cells (21). No identification of these particles as cores or indication of their possible function in viral morphogenesis has been reported to date. The large vacuoles formed in the cytoplasm of cells exposed to low-ionic-strength media could correspond to digestive vacuoles. In this case the small electron-dense particles found inside would represent destroyed cell organelles and substances captured by the autophagic vacuoles. The presence of multivesicular bodies, the relative cellular disorder, and the extensive vacuolation could also be an indication of cytopathic effects

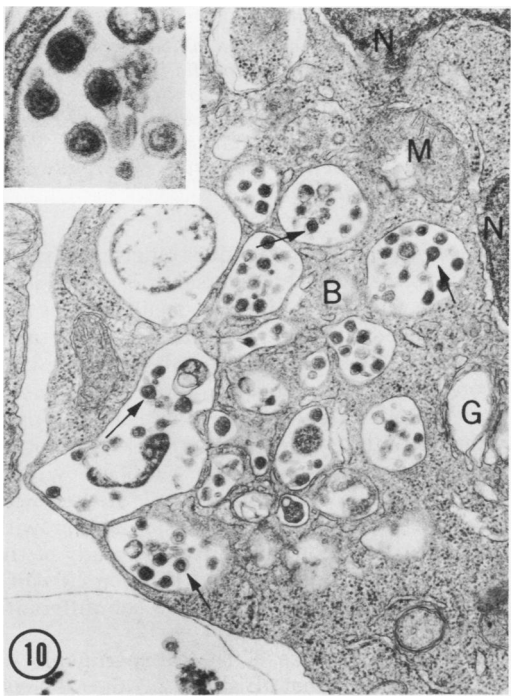

FIG. 10. *Higher-magnification micrograph of the cell shown in Fig. 9. The complex vacuolar body (B) consisted of vacuoles with smooth membranes, clear contents, some amorphous material, and virus-like particles (arrows). M, Mitochondrion; N, nucleus; G, Golgi apparatus.* ×19,400. *Insert: Vacuole with C-type virus particles.* ×53,900.

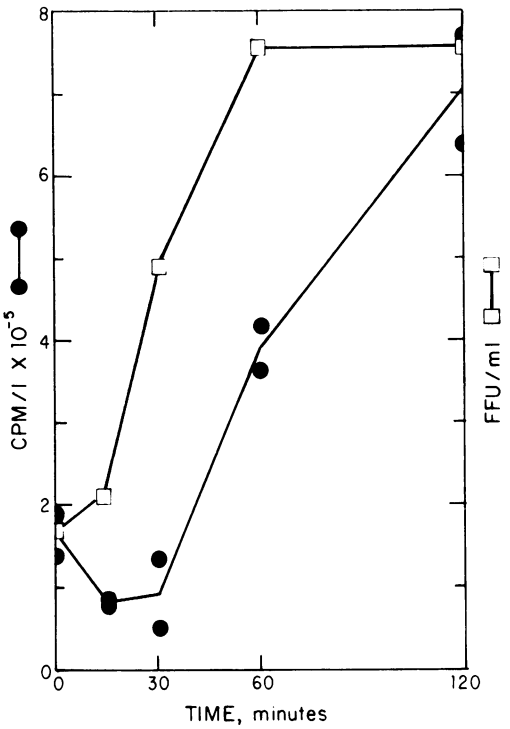

FIG. 11. *Reversal of virus inhibition. Cells incubated in low-ionic-strength media for 8 h were resuspended in normal media at zero time. Virus production was measured by the production of FFU per milliliter in the XC assay* (□) *and endogenous RNA-dependent DNA polymerase activity of partially purified virus particles* (●).

(5) caused by the low-ionic-strength media.

Virus release after reversal to normal ionic strength was very rapid. This result implies that the blocked step is very late in viral morphogenesis. However, the absence of viral "core" structures beneath the plasma membrane implies a different mechanism for type-C virus assembly than that proposed for other RNA-containing viruses, e.g., the arboviruses (18, 20). The low viability of cells treated with low-ionic-strength media in conjunction with Me_2SO stimulation precluded the study of the effect of low ionic strength upon Me_2SO-induced virus production and/or cell differentiation.

During the course of these experiments we became aware that Bishop et al. (2) have independently shown that avian reticuloenditheliosis virus release is inhibited by low-ionic-strength treatment and that this inhibition is reversible.

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ADDENDUM IN PROOF

Although the Friend cell line used in this study was obtained from W. Ostertag, it now differs from the original FSD-1/clone F4 in the amount of virus release (cf. reference 10). This was recently confirmed by W. Ostertag (personal communication).

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