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Incorporation of Precursors into Ribonucleic Acid, Protein, Glycoprotein, and Lipoprotein of Avian Myeloblastosis Virions

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Freshly explanted leukemic myeloblasts produce avian myeloblastosis virus (AMV) at a constant rate without any obvious cytopathic effect; therefore, subviral components are continually synthesized at a steady rate. The incorporation of various radioactive precursors into virions was monitored by determination of radioactivity in purified virus after density equilibrium sedimentation in preformed sucrose gradients. The kinetics of incorporation of ^{3}H-uridine have shown that there is an average time interval of 3 to 4 hr (half-life) between the time viral ribonucleic acid (RNA) is synthesized and the time it is released as a mature virus particle; this represents the average time interval spent by AMV-RNA in an intracellular pool. Studies with ^{14}C-phenylalanine have revealed that some protein synthesis takes place at or near the cell surface immediately prior to maturation and release of virus. ^{14}C-glucosamine also appears to be incorporated into the outer viral envelope shortly before maturation. On the other hand, there is an average lag of about 16 to 20 hr before ^{14}C-ethanolamine incorporated into intracellular lipoprotein appears in free virions; this probably reflects the kinetics of replacement of cellular surface membrane. Actinomycin D inhibits AMV-RNA within 30 min but permits the maturation of AMV to continue for at least 2 hr. AMV released in the presence of actinomycin D contains AMV-RNA synthesized before the addition of the drug.

Avian myeloblastosis virus (AMV) is a ribonucleic acid (RNA) tumor virus which causes acute myeloblastic leukemia as well as other tumors of the hematopoietic system and kidneys. The virions are spheroid particles having a diameter of approximately 100 nm, an outer lipoprotein envelope with knob-like structures, and a second inner membrane which surrounds an electron-dense RNA-containing nucleoid of 40 nm in diameter (*see* 26). The virion has a dry weight of about 7.5×10^{-16} g and a density of 1.16 g in sucrose (8, 22). AMV contains 2.2% RNA, 35% lipids (mostly phospholipids), and 63% protein; there are 1.9 moles of glucosamine per 100 moles of total amino acids (8, 20). There is less than 0.05% (the limit of assay) deoxyribonucleic acid (DNA), if any (Baluda, *unpublished data*). The RNA genome is single stranded, with a molecular weight of 12×10^{6} daltons and can, therefore, code for about 60 peptides of 200 amino acids each (22). The site of synthesis of the viral components is unknown, but AMV matures at the cell surface and is released by a budding process of the cellular membrane (11). The outer envelope of AMV contains both viral and cellular antigens; the virions also possess adenosine triphosphatase at their surface when produced by cells which contain this enzyme at their surface (11, 13).

The production of AMV is compatible with cellular proliferation; infection with AMV even induces in vitro the multiplication of target cells which otherwise would not divide (5). After transformation in vitro or in vivo, all the leukemic myeloblasts produce virus at a rate of about 40 virions per cell per hr (6, 9; Baluda, *unpublished data*). Leukemic myeloblasts can be cultured in vitro and provide a convenient system for studying the synthesis of virus in a steady state of virus production.

The production of AMV and of other RNA tumor viruses is arrested by inhibitors of DNA transcription, e.g., actinomycin D (1, 23, 25; Baluda, *unpublished data*). This finding, coupled with the fact that inhibitors of DNA synthesis prevent the initiation of virus production if added

during the first 12 hr postinfection, whereas they do not later, suggests that the synthesis of viral RNA is mediated via a DNA template (3, 16, 23).

The present study approaches the problems of synthesis of viral components and assembly of virions, and is concerned with the kinetics of incorporation of radioactive precursors of RNA, protein, and phospholipid into released virions.

MATERIALS AND METHODS

Virus and cells. The BAI strain A of AMV was used in these experiments. All studies were carried out with myeloblasts obtained from leukemic chicks. The procedures of infecting chick embryos (12 day old) with AMV and obtaining myelobalsts from leukemic chicks have been described previously (22). Only the chicks that showed a very high leukemic cell count (5×10^5 or more per mm^3) were exsanguinated. Blood from the exsanguinated chicks was treated with heparin and centrifuged to separate the myeloblasts forming the upper layer from red cells on the bottom layer. These leukemic cells were suspended in modified Eagle medium containing 5% chick serum, 5% fetal calf serum, and 10% tryptose phosphate broth (NMF). Portions (10 ml) of cell suspension on 100-mm plastic culture dishes (5 to 10×10^6 cells/ml) were incubated in a humidified CO_2 incubator at 37 C. Nutrient medium was replaced every 48 hr. These myeloblasts from leukemic chicks appear highly homogenous morphologically and nearly all are virus producers (Baluda, *unpublished data*). These cells grow in suspension and often form clumps. In the experiments reported here, myeloblasts were usually used between the 3rd day and the 10th day of in vitro culture, when more than 80% of the cells are virus producers. The leukemic cells gradually lose their ability to produce virus after prolonged maintenance in tissue culture.

Labeling of cells. The basic medium used for labeling with ^{3}H-uridine (^{3}H-UR), ^{14}C-ethanolamine (^{14}C-EA), or ^{14}C-glucosamine was modified Eagle medium containing 5% dialyzed calf serum, 5% dialyzed chicken serum, and 0.02% sodium bicarbonate, without tryptose phosphate broth. ^{3}H-UR (specific activity 27 c/mM, New England Nuclear Corp.), ^{14}C-ethanolamine (specific activity 2 mc/mM, New England Nuclear Corp.), or ^{14}C-glucosamine, (specific activity 8 mc/mM, New England Nuclear Corp.) were used. Unlabeled thymidine (final concentration 10^{-6}M) was added to the medium. For incorporation of ^{14}C-phenylalanine (^{14}C-PA; specific activity 300 mc/mM, Schwartz BioResearch Inc.), the medium used was the same as above, except that the amino acid mixture did not contain any unlabeled phenylalanine unless otherwise mentioned. In experiments involving incorporation of both ^{14}C-PA and ^{3}H-UR, nutrient medium without unlabeled phenylalanine was used. For the experiments involving incorporation of ^{14}C-EA, Eagle medium was prepared without choline.

All culture media were equilibrated to *p*H 7.4 and warmed to 37 C prior to use.

Assay of radioactivity in purified virus. After sedimenting the myeloblasts at 2,000 rev/min for 5 min, the cell culture supernatant fraction was further freed from cellular debris by centrifuging at $12,100 \times g$ for 10 min in a Sorvall centrifuge at 2 C. Samples (10 ml) of the supernatant fraction were frozen at −70 C and used for determination of radioactivity in purified virus. Two methods of virus purification were used. (i) In earlier experiments, virus including all the proteins was precipitated by adding $(NH_4)_2SO_4$ to 50% saturation. The precipitate was redissolved and analyzed by density equilibrium centrifugation in a 15 to 60% sucrose gradient (22). Trichloroacetic acid-precipitable radioactivity was determined in each fraction collected from the bottom of the gradient. (ii) The second method used most extensively was that described by Baluda (4). A 10-ml amount of the supernatant fraction was layered over two layers of sucrose (1 ml of 65% sucrose and 4 ml of 20% sucrose), and the virus was sedimented to the interface of 60 and 20% sucrose by centrifuging for 1 hr at 25,000 rev/min in an SW25.3 rotor. The virus band, collected in 1.5 ml, was diluted to 4 ml, layered over 13 ml of a preformed sucrose gradient (15 to 60%), and centrifuged for 4 hr at 25,000 rev/min in an SW25.3 rotor. Usually, 0.8-ml fractions were collected from the bottom of the tube, and trichloroacetic acid-precipitable radioactivity was determined in each fraction. Total counts in virions were determined from the sum of radioactivity in all the fractions present in the virus peak. All experiments were done at least in duplicate, and the mean deviation between duplicates was less than 10% of the total counts. A typical experiment is shown in Fig. 3.

RESULTS

Kinetics of incorporation of ^{3}H-uridine in virions. Leukemic myeloblasts produce AMV at a constant rate without any apparent interference of cellular activity. Therefore, at any time viral genomes are synthesized at a constant rate inside the cell. By following the kinetics of incorporation of ^{3}H-UR into virions, it should be possible to determine how long a viral RNA molecule remains inside the cell before being released in a mature virion.

In the first experiments designed to answer this question, myeloblasts were exposed to a 1-hr pulse of ^{3}H-UR, washed with phosphate-buffered saline (PBS), *p*H 7.4, and incubated in NMF. Supernatant fluids were harvested at 8-hr intervals for 48 hr from samples of myeloblast cultures. AMV was purified, and trichloroacetic acid-precipitable radioactivity in virus was determined. The results (Fig. 1) show that 53% of the ^{3}H-UR appeared during the first 8 hr. Thus, ^{3}H-UR appeared in mature AMV within less than 8 hr after myeloblasts were exposed to this label. Similar results were obtained when only two cultures of myeloblasts were used and their supernatant fluids were harvested and replaced with fresh medium every 8 hr. The appearance of ^{3}H-UR in virions was then studied at shorter time inter-

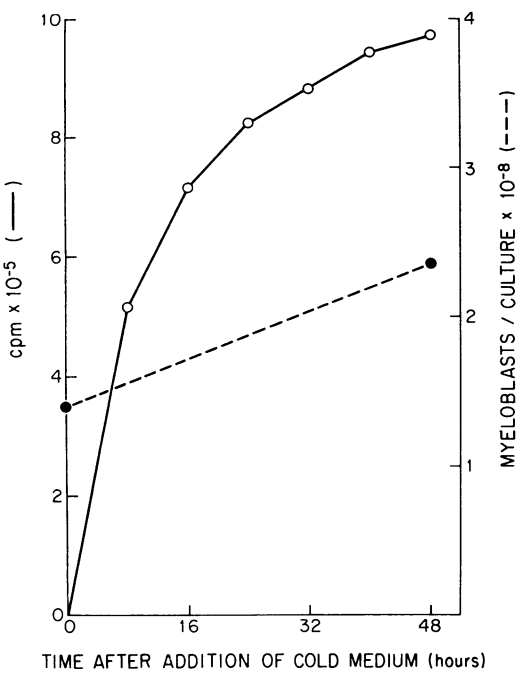

FIG. 1. *Pulse and chase of ^{3}H-UR into virions. Approximately 10^9 myeloblasts were exposed to ^{3}H-UR (500 μc) for 1 hr, washed, and incubated at 1.4×10^8 cells per dish in 10 ml of NMF. Supernatant fluids were harvested at 8-hr intervals from each of two dishes, AMV was purified, and trichloroacetic acid-precipitable ^{3}H radioactivity in virus was measured (solid line). The cells were counted in a hemocytometer at 0 and 48 hr (dashed line). Each point is the average of two determinations.*

vals by purifying AMV from the supernatant fluids of myeloblasts cultured continuously in the presence of ^{3}H-UR. Few virus particles containing ^{3}H-UR (5.5% of maximum at 6 hr) were released during the first 2 hr of labeling, and only after 4 hr of labeling was a large number of ^{3}H-UR virions (65% of maximum at 6 hr) released (Fig. 2).

Thus, there seems to be a lag of 2 to 4 hr before ^{3}H-UR appears in virions. Part of the delay needed for the appearance of ^{3}H-UR in free virions might be due to the rate of entry of ^{3}H-UR into the intracellular uridine pool and to the rate of turnover of this uridine pool. To avoid the influence of the rate of entry of ^{3}H-UR on the labeling of viral RNA, a quasi pulse-chase experiment was done. It has already been shown that in animal cells complete pulse and chase experiments cannot be done because of the large size of the uridine pool (17). However, the external source of ^{3}H-UR can be removed and replaced by unlabeled uridine, thus preventing any further entry of external ^{3}H-UR into the intracellular pool. Accordingly, in a number of experiments cells were labeled with ^{3}H-UR for 15 min, washed, and incubated in medium containing unlabeled uridine (10^{-6}M). At various time intervals thereafter, the amount of ^{3}H-labeled virus which had been released in the medium was determined. The results of one such experiment (Fig. 3 and 4) show that labeled virus was barely detectable 1 hr after the cells were resuspended in NMF (1.5 hr after the pulse) and that most of the virus containing labeled RNA was released between 3 and 6 hr after addition of unlabeled medium. This indicates that the delay in the appearance of ^{3}H-UR into mature virions (Fig. 2 and 4) was not due to a delay in the entry of external ^{3}H-UR into the intracellular pool. This lag could, then, mean that there was either (i) a lag of 3 to 4 hr between the time of synthesis of viral RNA and its maturation as virion at the cell membrane, or (ii) slow

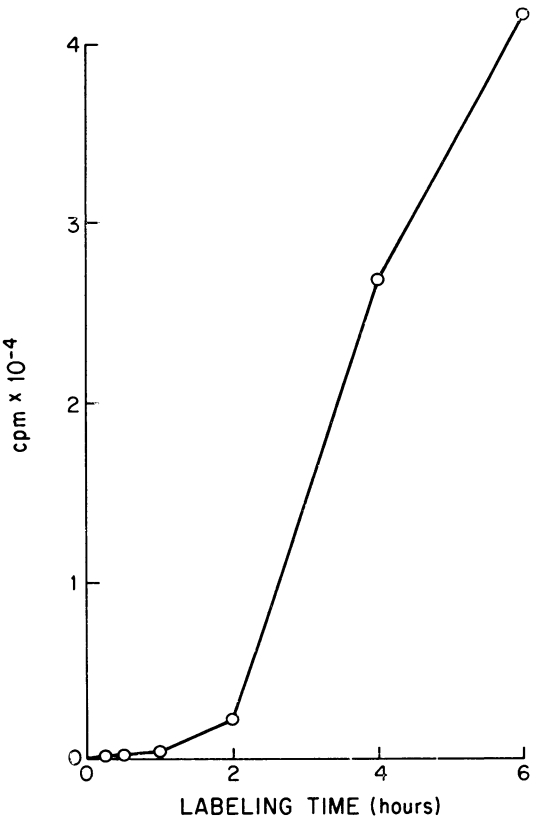

FIG. 2. *Continuous labeling of virions with ^{3}H-UR. Myeloblasts were incubated at 10^8 cells per dish in 10 ml of medium containing ^{3}H-UR (3.3 μc/ml). Supernatant fluids were harvested from duplicate cultures at various time intervals, and acid-precipitable radioactivity in purified virions was determined as in Fig. 1.*

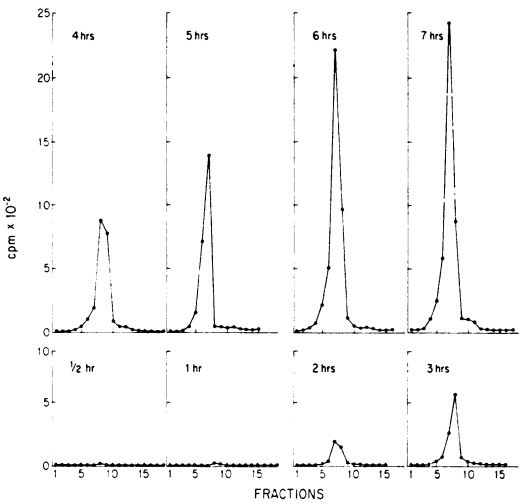

FIG. 3. *Incorporation of ^{3}H-UR into purified virions after a pulse of 30 min. Approximately 2 × 10^9 myeloblasts were washed twice with warm PBS and incubated for 15 min in 10 ml of warm medium containing ^{3}H-UR (50 μc/ml). The cells were then washed three times with 40 ml of warm NMF containing unlabeled uridine (10^{-5} M) and suspended in NMF containing uridine (10^{-6} M) at 10^8 cells per 11 ml per dish. About 30 min elapsed between the end of the pulse and resuspension in NMF. After incubation at 37 C in a 5% CO$_2$ atmosphere for various time intervals, duplicate supernatant fractions were harvested and freed from cellular debris by centrifugation in a Sorvall RC-2B centrifuge at 12,100 × g for 10 min. The supernatant fluids (10 ml) were kept frozen at −70 C until used for AMV purication (see second method, Materials and Methods). Fractions were collected from the bottom of the sucrose density gradients, precipitated with trichloroacetic acid (5% final concentration), and filtered. ^{3}H radioactivity was measured in a Picker Nuclear liquid scintillation counter. The time shown for each gradient represents the time of incubation in NMF.*

incorporation of ^{3}H-UR from the intracellular pool into viral RNA. To rule out the second possibility, the kinetics of cellular RNA synthesis were determined by using ^{3}H-UR as precursor, since we can assume that viral RNA and cellular RNA use the same nucleotide pool and probably draw nucleotides from the pool at the same rate.

Myeloblasts were labeled with ^{3}H-UR continuously, and samples of cells were taken at various time intervals. Trichloroacetic acid-precipitable and trichloroacetic acid-soluble radioactivity were determined as described above. Three separate experiments gave identical results; the results from one of them are presented in Fig. 5. Radioactivity in the intracellular pool increases for up to 3 to 4 hr and remains constant thereafter up to 8 hr. The trichloroacetic acid-soluble pool in these experiments represents the total radioactivity present in uridine and in products formed from uridine, such as uridine monophosphate, uridine diphosphate, uridine triphosphate, or uracil or other ribonucleic acid precursors, e.g., cytidine monophosphate. Incorporation of ^{3}H-UR into newly synthesized RNA, determined by trichloroacetic acid-precipitable radioactivity, starts without any apparent delay and continues linearly for 4 hr, at which time it slows down and follows a slower linear rate of synthesis. This indicates that at least two major classes of RNA are synthesized in these cells. Up to 4 hr, the cellular RNA synthesized has a high turnover rate and probably represents the synthesis of messenger RNA, which has a half-life of about 4 hr (*see also* Fig. 13). RNA synthesized after 4 hr represents predominantly stable cellular RNA (e.g., ribosomal RNA) which is synthesized at a slower rate.

In another experiment, myeloblasts were again

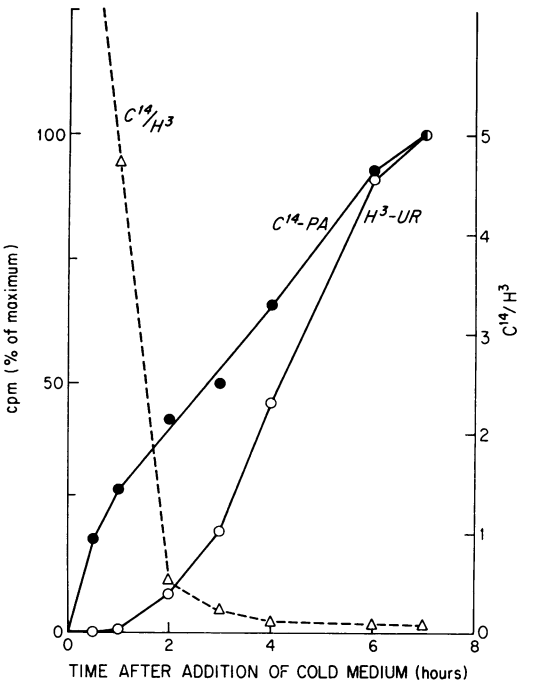

FIG. 4. *Pulse and chase of ^{3}H-UR and ^{14}C-PA into virions. This experiment was carried out as in Fig. 3, except that the cells were exposed for 15 min to ^{3}H-UR (50 μc/ml) and ^{14}C-PA (5 μc/ml) in medium without unlabeled uridine and phenylalanine. The average of duplicate determinations of ^{3}H (○) and ^{14}C (●) radioactivity in purified virions are plotted as percentage of maximum incorporation after 7 hr of incubation (43,687 counts/min for ^{3}H-UR and 4,027 counts/min for ^{14}C-PA). ^{14}C/^{3}H ratio (△).*

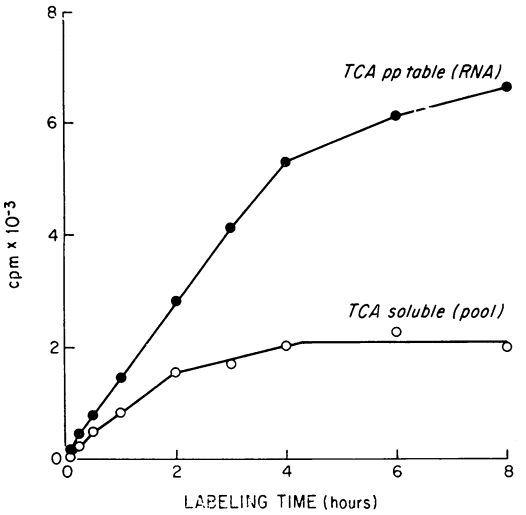

FIG. 5. *Incorporation of ^{3}H-UR into the acid-soluble pool and into RNA in leukemic myeloblasts. The cells (2.28 × 10^{6}/ml) were incubated in 50 ml of ^{3}H-UR medium (0.5 μc/ml) in a 200-ml Erlenmeyer flask fitted with a loose aluminum cap. At various time intervals, duplicate 1-ml samples were removed and mixed with 10 ml of ice-cold PBS. Total (washed with PBS only) and acid-precipitable counts were determined at each point. The acid-soluble radioactivity was obtained by subtraction. The myeloblasts were counted at 0, 1, 4, and 8 hr and remained constant in number.*

continuously labeled, but the trichloroacetic acid-soluble counts/minute and trichloroacetic acid-precipitable counts/minute were determined at shorter time intervals to analyze the two curves during the first 60 min of labeling. It was found (Fig. 6) that the trichloroacetic acid-soluble pool increased linearly and that there was no apparent delay in the synthesis of trichloroacetic acid-precipitable RNA. By extrapolation of the early linear part of the RNA curve, it can be seen that the time lag between the entrance of ^{3}H-UR into the intracellular pool and its incorporation into RNA is no longer than 2 min. Another minor shift in the rate of incorporation of ^{3}H-UR into RNA was also detected between 30 and 60 min. From the continuous labeling experiments it can be concluded that (i) there is no lag in the entrance of ^{3}H-UR into the intracellular uridine pool, which becomes saturated within 3 hr with a half-life of 1.25 hr, and (ii) there is a maximum delay of only 2 min in the incorporation of ^{3}H-UR into RNA. Therefore, the delay of 2 hr in the appearance of ^{3}H-UR into mature virions and the great burst of ^{3}H-UR into virions between 2 and 4 hr in continuous labeling experiments (Fig. 2) and in pulse-chase experiments (Fig. 4) was not caused by the low specific radioactivity of ^{3}H-UR in the intracellular uridine pool nor by the slow incorporation of ^{3}H-UR from the pool into RNA. These experiments indicate that the time lag between the addition of ^{3}H-UR and its appearance in mature virions reflects a time lag between the synthesis of viral RNA and its maturation as virion at the cell membrane.

Incorporation of ^{14}C-PA into virions. To study the kinetics of incorporation of proteins into virions, myeloblasts were labeled continuously with ^{14}C-PA and ^{3}H-UR. AMV was purified from the supernatant fraction at various time intervals and analyzed for trichloroacetic acid-precipitable ^{14}C and ^{3}H counts/minute. The results (Fig. 7) show that ^{14}C-PA was incorporated into virions about 1 hr earlier than ^{3}H-UR and at a faster rate for at least 2 hr. At 15 min (the first point), 3.2% of the amount of ^{14}C-PA that could be incorporated into virions at 4 hr was already found in mature virus, whereas it took 1.25 hr for 3.2% of ^{3}H-UR to be incorporated. There was progressively more incorporation of ^{3}H-UR compared to ^{14}C-PA from 2 hr on.

To obviate the effect of the rate of entry of these precursors into their intracellular pool, and of saturation of the pool, on their incorporation into trichloroacetic acid-precipitable radioactivity, myeloblasts were labeled with ^{3}H-UR and ^{14}C-PA for only 15 min, at which time the cells were washed and resuspended in NMF. After various time intervals, AMV was purified from the supernatant fraction and analyzed for trichloroacetic acid-precipitable ^{3}H-UR and ^{14}C-PA. Figure 4 shows that 19% (of maximum) of ^{14}C-PA was incorporated into virus as early as 30 min (the first assay point) after resuspending the cells in cold medium, whereas it took 3 hr for 20% of ^{3}H-UR to appear in virions. The

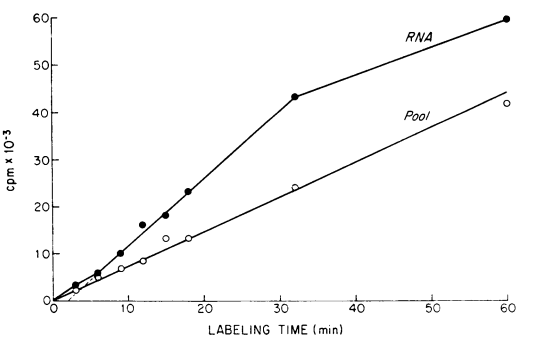

FIG. 6. *Incorporation of ^{3}H-UR into the acid-soluble pool and into RNA in leukemic myeloblasts. The procedure was the same as that of Fig. 5, except that samples were taken at shorter time intervals during the first 20 min of labeling.*

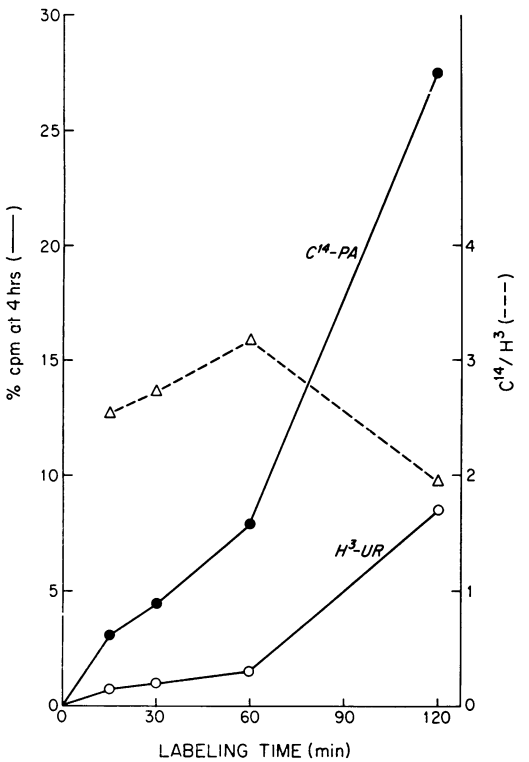

FIG. 7. *Continuous labeling of virions with ^{3}H-UR and ^{14}C-PA. Myeloblasts were incubated at 10^{8} cells per 10 ml per dish in medium containing ^{3}H-UR (3.3 μc/ml) and ^{14}C-PA (0.33 μc/ml). Supernatant fluids from duplicate cultures were harvested at various time intervals and analyzed for ^{3}H (○) and ^{14}C (●) radioactivity in purified virions as usual. Each point is the average of duplicate analyses and represents the percentage of maximum radioactivity incorporated after 4 hr of incubation (26,761 counts/min for ^{3}H-UR and 16,143 counts/min for ^{14}C-PA). The dashed line represents the $^{14}C/^{3}H$ ratio.*

ratio of $^{14}C/^{3}H$ also shows that ^{14}C-PA appeared earlier than ^{3}H-UR in released virions. This indicates that amino acids are incorporated into virions at a faster rate than uridine because of (i) faster entry into cells and incorporation into viral protein or (ii) incorporation into the late stage of maturation of the virions, or both i and ii. Therefore, experiments were done to determine the size of the pool of phenylalanine and the rate of incorporation of phenylalanine into protein by continuously labeling myeloblasts and monitoring the total and trichloroacetic acid-precipitable radioactivity. There is no intracellular pool of phenylalanine in these cells, and ^{14}C-PA is almost directly incorporated into acid-precipitable protein (Fig. 8). Therefore, the early appearance of labeled protein into virions is partly due to a fast incorporation of ^{14}C-PA into trichloroacetic acid-precipitable protein. The early entry of ^{14}C-PA into released virions also removes the possibility that the delay in ^{3}H-UR appearance in free virus was caused by sticking or readsorption of released virions at the cell surface.

Incorporation of ^{14}C-glucosamine into virions. Glucosamine is a precursor of mucoproteins of cell membranes and possibly, therefore, of the viral envelope. To show the kinetics of incorporation of glucosamine into virions, myeloblasts were labeled with ^{3}H-UR and ^{14}C-glucosamine for 30 min, washed, and placed in NMF. At different time intervals, AMV was purified from supernatant fluids and analyzed for radioactivity as before. When compared with uridine incorporation, glucosamine appeared earlier in virions and followed the same patterns as that of phenylalanine (Fig. 9). This suggests that glucosamine

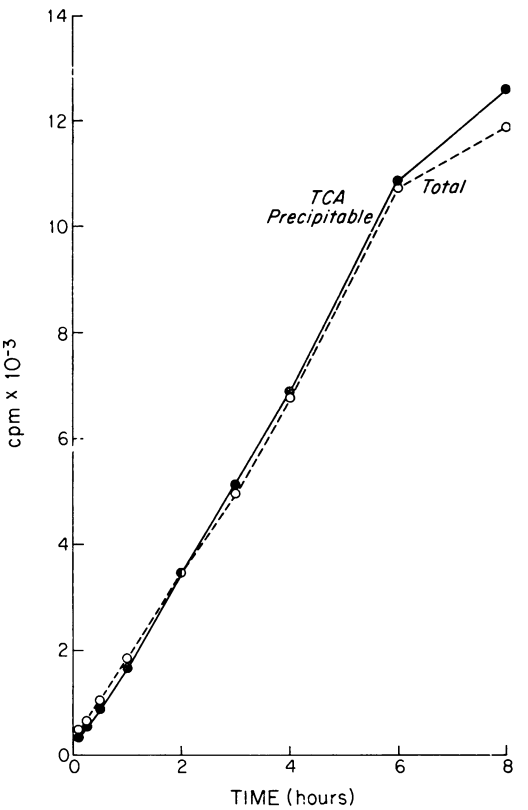

FIG. 8. *Incorporation of ^{14}C-PA in total and acid-precipitable radioactivity in leukemic myeloblasts. Leukemic cells were incubated continuously in ^{14}C-PA medium (0.05 μc/ml) and treated as in Fig. 5. Total radioactivity (○); acid-precipitable radioactivity (●).*

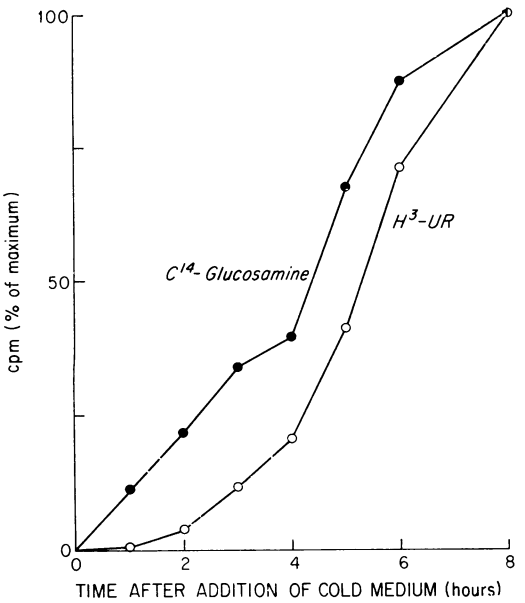

FIG. 9. *Pulse and chase of ^{14}C-glucosamine and ^{3}H-UR into virions. Approximately 2.5 × 10^9 myeloblasts in 20 ml of medium were exposed to ^{14}C-glucosamine (10 μc/ml) and to ^{3}H-UR (10 μc/ml) for 30 min. After three washings with NMF containing unlabeled uridine (10^{-4} M), the cells were suspended in NMF at 10^8 cells per 11 ml per dish and treated as in Fig. 3. ^{14}C (●) and ^{3}H (○) radioactivity are presented as percentage of maximum incorporation after 8 hr of incubation (5,050 counts/min for ^{14}C-glucosamine and 18,000 counts/min for ^{3}H-UR).*

is incorporated into a late stage of virus maturation, e.g., formation of the outer envelope.

Incorporation of ^{14}C-EA into virions. AMV contains lipids in both envelopes. ^{14}C-EA was used to study the incorporation of a phospholipid precursor into virions. Myeloblasts were labeled with ^{14}C-EA for 30 min, washed, and incubated in NMF. The time elapsed between the addition of ^{14}C-EA and the addition of NMF was 52 min. AMV in the supernatant fraction was purified and analyzed for trichloroacetic acid-precipitable ^{14}C radioactivity at various time intervals over a period of 48 hr. Only 7% of the ^{14}C label appeared in virions 8 hr after removal of ^{14}C-EA (Fig. 10), whereas 62% was incorporated during the next 16 hr. To determine more accurately the time lag required by ^{14}C-EA before it appears in virions, the previous experiment was repeated with shorter time intervals (4 hr) between 8 and 20 hr after a 1-hr pulse of ^{14}C-EA. After the pulse, the cells were washed and resuspended in NMF (90 min after the addition of the label). Very little ^{14}C-EA is incorporated into virions before 8 hr, and the biggest incorporation occurs between 16 and 20 hr (Fig. 11).

To determine whether these results were caused by the slow entrance of ^{14}C-EA into an intracellular pool or the slow incorporation of ethanolamine into lipoprotein, the following experiment was carried out. Myeloblasts were labeled continuously with ^{14}C-EA and trichloroacetic acid-precipitable and trichloroacetic acid-soluble radioactivity were determined. The presence of ^{14}C-EA in lipids was checked by its solubility in chloroform-methanol (2:1). There was a relatively large intracellular pool of ethanolamine which became saturated within approximately 2 hr (Fig. 12). The pool size per cell remained constant over a testing period of 24 hr. Also, ^{14}C-EA entered without apparent delay into a trichloroacetic acid-precipitable state which was over 99% soluble in chloroform-methanol (2:1). Even when tested after 32 hr of labeling, the trichloroacetic acid-insoluble ^{14}C-EA was 96% soluble in the lipid solvent, indicating that it was still in a lipidic state. Thus, ^{14}C-EA appears to be incorporated without delay into phospholipids which become associated in a stable lipoprotein complex almost immediately. Therefore, the time lag required for ^{14}C-EA to appear in

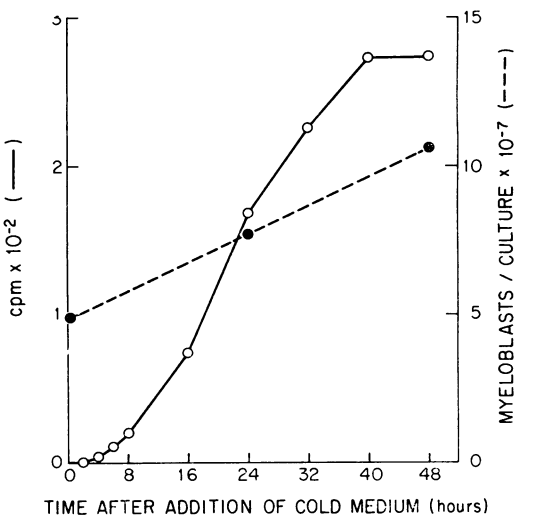

FIG. 10. *Pulse and chase of ^{14}C-EA into virions. Approximately 10^9 leukemic cells were washed with PBS, incubated for 30 min in 10 ml of medium without choline, and exposed to 25 μc of ^{14}C-EA for 30 min. After three washings with PBS, the cells were suspended in NMF and plated at 5 × 10^7 cells per 10 ml per dish. After various time intervals, supernatant fluids were harvested and treated as in Fig. 3. The cells were counted at 0, 24, and 48 hr (●). Acid-precipitable ^{14}C radioactivity (○) in purified virions is plotted as a function of time of incubation in NMF.*

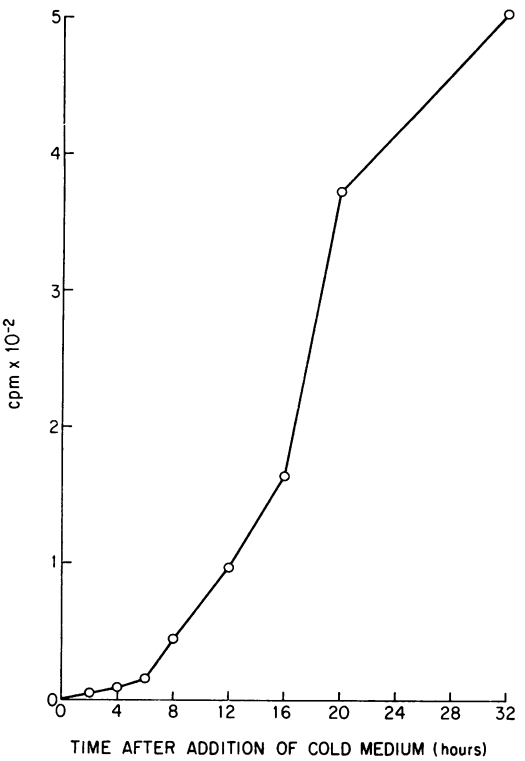

FIG. 11. *Pulse and chase of ^{14}C-EA into virions. Approximately 3 × 10^9 leukemic myeloblasts in 20 ml of medium were exposed to 100 μc of ^{14}C-EA for 1 hr, as in Fig. 10, and plated at 1.5 × 10^8 cells in 11 ml of NMF per dish. Supernatant fluids from duplicate cultures were harvested at 4-hr intervals and analyzed for ^{14}C-EA in purified virions as before. The curve represents the average of duplicate analyses.*

free virions probably represents the time interval required by newly synthesized membrane lipids to reach the cell surface where AMV matures. These results also throw light on the biosynthesis of the cellular membrane.

Effect of actinomycin D on virus replication. Actinomycin D inhibits the production of AMV in infected fibroblasts (2) and in leukemic myeloblasts (Baluda, *unpublished data*). Although actinomycin D is well known for its inhibition of DNA transcription, it can also inhibit phospholipid synthesis more than it inhibits protein synthesis (18) and is rapidly cytotoxic. Experiments were therefore carried out to determine whether the inhibitory effect of this drug acts upon the synthesis of viral RNA or upon viral assembly and maturation, i.e., budding and release. It is possible to distinguish between these two phenomena, since there is a delay of several hours between the synthesis of viral RNA and its incorporation into virions and since ^{14}C-PA is incorporated without delay. Two types of experiments were done. (i) It was demonstrated that ^{3}H-UR does not get incorporated into virions when added after actinomycin D, whereas

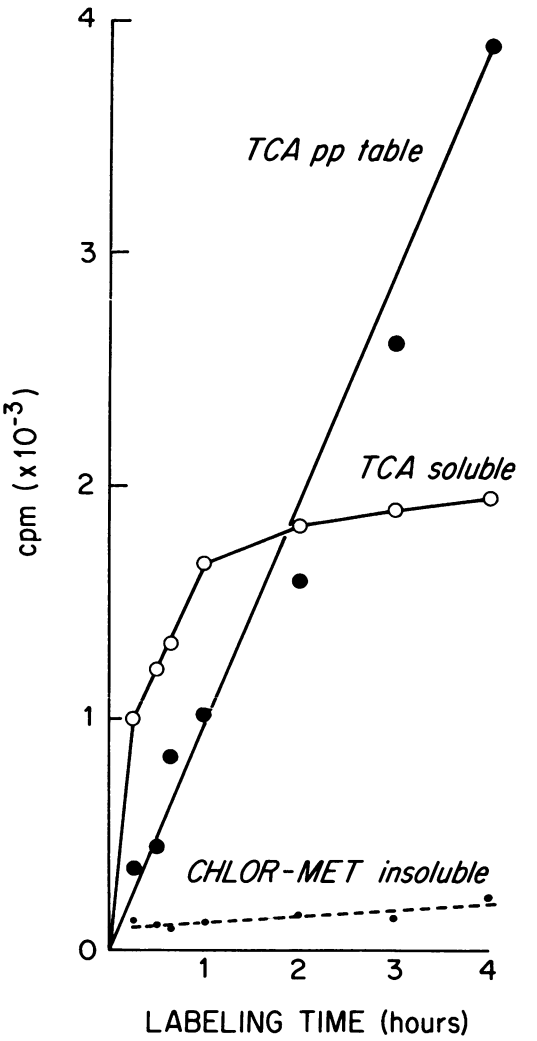

FIG. 12. *Incorporation of ^{14}C-EA into the trichloroacetic acid-soluble pool and into lipoproteins in leukemic myeloblasts. Leukemic cells (8.4 × 10^6 per ml) were incubated in 60 ml of ^{14}C-EA medium (8.3 μc per ml) in a flask with a loose metal cap. At various time intervals, six portions of 1 ml each were removed and mixed with 5 ml of cold PBS; the cells were trapped on membrane (Millipore Corp.) filters. All six samples were washed with 50 ml of PBS each, four were then washed with 50 ml of 5% trichloroacetic acid, and two of these four were additionally washed with 25 ml of chloroform-methanol (2:1). The ^{14}C radioactivity in each filter was then counted. The acid-soluble radioactivity (pool) was obtained by subtracting the acid-precipitable counts per minute from the counts per minute in filters washed with PBS only.*

^{14}C-PA does for a while, and (ii) ^{3}H-UR polymerized into RNA before the addition of actinomycin D is incorporated into virions after addition of the drug.

In the first type of experiment, myeloblasts were treated with actinomycin D (2 μg/ml), and then ^{3}H-UR (10 μc/ml) and ^{14}C-PA (0.2 μc/ml) were added together at 0, 0.5, 2, or 4 hr after addition of the drug. Control cells were treated similarly, but actinomycin D was omitted. After 3 hr of incubation in presence of ^{3}H-UR and ^{14}C-PA, the supernatant fluids were collected and AMV was purified and analyzed for ^{3}H and ^{14}C radioactivity. The incorporation of ^{3}H-UR into AMV is inhibited by 98%, even when it is added at the same time as actinomycin D (Table 1). Analysis of the RNA isolated from the purified virions revealed that all the ^{3}H was present in a low-molecular-weight RNA (4*S*) species. Thus, ^{3}H-labeled high-molecular-weight RNA (71*S*) characteristic of the AMV genome was either not made or, if made, not incorporated into virions when ^{3}H-UR was added simultaneously with, or after, actinomycin D. On the other hand, ^{14}C-PA was incorporated into virions to the extent of 23% of control cells when added simultaneously with actinomycin D. When ^{14}C-PA was added 30 min after the drug, the virions contained 20% of ^{14}C incorporated normally. Even when ^{14}C-PA was added 2 hr after actinomycin D, the virions released in the next 3 hr contained 17% of the ^{14}C radioactivity normally released. Therefore, maturation of AMV containing newly synthesized protein continues for at least 2 hr in the presence of actinomycin D.

TABLE 1. *Effect of actinomycin D upon AMV maturation*[a]

Time between actinomycin D and ^{3}H-UR + ^{14}C-PA	Counts/min in virions		^{14}C/^{3}H
	^{3}H	^{14}C	
Control (no actinomycin D)	8,951 (100%)	2,009 (100%)	0.224
0	204 (2.3%)	463 (23.0%)	2.270
30 min	220 (2.5%)	408 (20.3%)	1.855
2 hr	406 (4.5%)	331 (16.5%)	0.815
4 hr	418 (4.7%)	130 (6.5%)	0.311

[a] Myeloblasts were washed and suspended in medium containing dialyzed calf serum (5%), dialyzed chicken serum (5%), and PA 1/6-medium [medium containing one-tenth the amount of phenylalanine (PA) present in normal medium]; they were then distributed in 10 plates (6×10^7 cells in 9 ml). After 15 min of incubation at 37 C, groups of two plates each were treated as follows: (i) For control, add 1 ml of PA 1/6 medium, and 1 ml of PA 1/6 medium containing ^{14}C-PA (10 μc/ml) plus ^{3}H-UR (100 μc/ml) immediately after; (ii) add 1 ml of PA 1/6 medium containing actinomycin D (20 μg/ml) and 1 ml of ^{14}C-PA plus ^{3}H-UR medium immediately after (0 hr); (iii) add actinomycin D and then ^{14}C-PA plus ^{3}H-UR 30 min later; (iv) add actinomycin D and then ^{14}C-PA plus ^{3}H-UR 2 hr later; (v) add actinomycin D and then ^{14}C-PA plus ^{3}H-UR 4 hr later. After 3 hr of incubation in presence of ^{3}H-UR and ^{14}C-PA, AMV was purified from the supernanant fluids and analyzed for ^{3}H and ^{14}C radioactivity.

In the second type of experiments, myeloblasts were cultured in presence of ^{3}H-UR (10 μc or more/ml) for at least 20 hr, washed with PBS, treated with trypsin to remove newly made and budding AMV, washed again, and suspended in medium with or without actinomycin D (2 μg/ml). AMV produced during a 30-min period immediately after addition of actinomycin D was purified, 1.5 or 3.5 hr later, from the supernatant fluids and analyzed for ^{3}H content. It was found that ^{3}H-UR continued to be released in virions at a normal rate for 2 hr after addition of actinomycin D, and at a decreased rate for at least 4 hr. Additional experiments, in which the amount of virus synthesized after addition of actinomycin D was monitored by incorporation of ^{14}C-PA, yielded similar results.

To determine whether the virions released after actinomycin D treatment contained high-molecular-weight RNA, the following experiment was done. Myeloblasts were labeled for 20 hr with ^{3}H-UR (1 μc/ml), washed with PBS, trypsinized to remove any virions attached to the cell, suspended in phenylalanine 1/10 medium with actinomycin D (2 μg/ml), and incubated. ^{14}C-PA was added at different time intervals and incubated for 4.5 hr. Controls were run without actinomycin D. The supernatant fraction was harvested, the cells were trypsinized, and the trypsin supernatant fraction was mixed with the medium. Virus was purified from the total supernatant fraction and analyzed for ^{3}H and ^{14}C counts. The incorporation of ^{14}C in AMV was similar to that of Table 1. RNA was then extracted from purified virus and analyzed in sucrose velocity gradients. After 4.5 hr of incubation in the presence of actinomycin D, the newly synthesized virus contained the same amount of ^{3}H-UR as AMV produced in the absence of the drug. However, AMV produced in presence of actinomycin D contained only 22% of the amount of 71*S* ^{3}H-RNA found in control virus (14% versus 63% of the total RNA). The rest of the ^{3}H label was in a 4*S* RNA species. Other similar experiments have shown that normal AMV is produced for about 2 hr after the addition of actinomycin D, and then virions containing less 71*S* RNA and more 4*S* RNA are made for at least the next 2 hr. There is also the appearance of 28*S* and 18*S* cellular RNA in the RNA isolated from AMV cultured in the presence of actinomycin D. However, it is not known

whether ribosomal and 4S RNA are present inside virions or in contaminating cellular debris caused by the cytopathic effect of actinomycin D which increases with time. It appears, therefore, that AMV maturation continues for at least 4 hr in presence of actinomycin D and that there is synthesis of viral protein.

Having shown that no viral RNA synthesized after the addition of actinomycin D is incorporated into newly formed virions, we next investigated whether viral RNA is synthesized in the presence of actinomycin D. First, the incorporation of ^{3}H-UR and of ^{14}C-PA into RNA and protein of myeloblasts was studied in the presence or absence of actinomycin D (2 μg/ml). Myeloblasts were washed and suspended (5×10^6 cells/ml/tube) in PA 1/10 medium (medium containing one-tenth the amount of phenylalanine present in normal medium) containing ^{3}H-UR (1 μc/ml) and ^{14}C-PA (0.1 μc/ml) with or without actinomycin D. After incubation at 37 C for 1, 2, 4, or 6 hr, duplicate samples of cells were washed three times with cold PBS and trichloroacetic acid precipitated. The cells were then analyzed for acid-precipitable ^{3}H and ^{14}C radioactivity. RNA synthesis was inhibited immediately by actinomycin D, whereas protein synthesis continued normally for 4 hr (Fig. 13). Analysis by velocity sedimentation ultracentrifugation in sucrose gradients of the RNA synthesized in presence of actinomycin D revealed it to be 4S RNA.

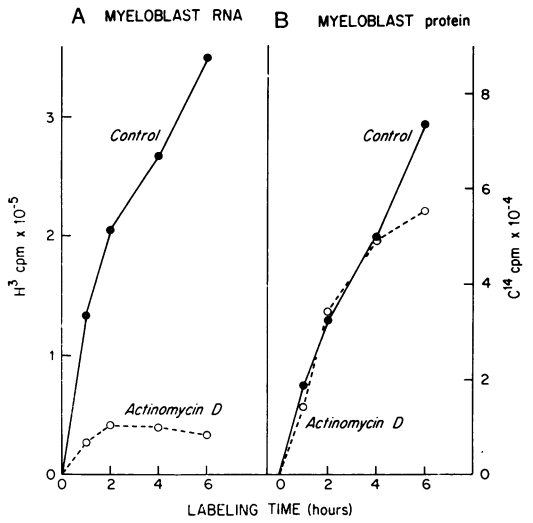

FIG. 13. *RNA and protein synthesis in myeloblasts in the presence or absence of actinomycin D (2 μg/ml). Myeloblasts were washed with PBS, suspended in phenylalanine 1/10 medium, and distributed into small tubes (5×10^6 cells per ml per tube). One half of the tubes received ^{3}H-UR (1 μc/ml) and ^{14}C-PA (0.1 μc/ml) plus actinomycin D. The other half received ^{3}H-UR and ^{14}C-PA only. After various time intervals of incubation with frequent shaking at 37 C in a CO_2 incubator, two tubes from each group were removed and received 9 ml of ice-cold PBS. The cells were washed three times with PBS and suspended in 1 ml of PBS; trichloroacetic acid was added to a final concentration of 5%. Acid-precipitable ^{3}H (A) and ^{14}C (B) radioactivity was determined in each sample.*

To check further on the absence of viral RNA synthesis after treatment with actinomycin D, the rate of RNA synthesis in myeloblasts treated with actinomycin D (2 μg/ml) was compared with that in noninfected fibroblasts. The rate of protein synthesis was also studied in the same cells. Myeloblasts (5×10^6/ml/tube) and chick embryo fibroblasts (60-mm confluent plates) were incubated in medium with or without actinomycin D. After 1, 2, 4, 6, or 8 hr of incubation, one portion of each cell culture was exposed for 30 min to ^{3}H-UR (3 μc/ml) and to ^{14}C-PA (0.3 μc/ml). After exposure to the radioactive labels, the cells were trypsinized and washed, and the amount of acid-precipitable ^{3}H and ^{14}C radioactivity was determined. The effect of actinomycin D upon the rates of protein and RNA synthesis was similar in myeloblasts and in noninfected fibroblasts (Fig. 14).

Similar results were obtained, i.e., absence of synthesis of AMV-specific RNA, when the syn-

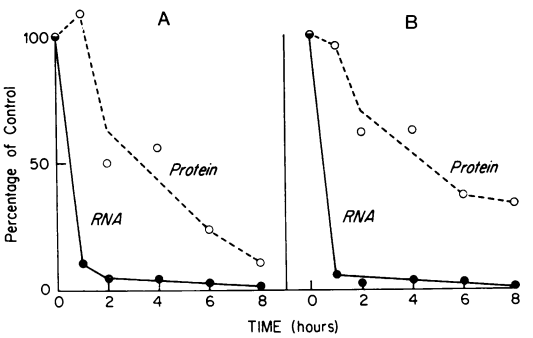

FIG. 14. *Rate of synthesis of RNA and protein in presence of actinomycin D (2 μg/ml) in leukemic myeloblasts (A) and in uninfected chick embryo fibroblasts (B). Myeloblasts were incubated in small tubes (5×10^6 per ml per tube) in either presence or absence of actinomycin D. After various periods of incubation, two tubes each in the actinomycin D group and in the control group were exposed to ^{3}H-UR (3 μc) and ^{14}C-PA (0.3 μc) for 30 min. Acid-precipitable ^{3}H and ^{14}C radioactivity in each sample was then determined as in Fig. 13. For the chick embryo fibroblasts, confluent monolayers in 60-mm culture dishes were used, and the cells were trypsinized just prior to washing in PBS and acid precipitation. The results are expressed as percentage of radioactivity in the control group (no actinomycin D) at each time interval tested. (●) ^{3}H radioactivity; (○) ^{14}C radioactivity.*

thesis of RNA and the nature of the RNA synthesized in the presence of actinomycin D were studied in chick embryo fibroblasts either noninfected or infected with AMV. These fibroblasts had been checked for the absence of leukosis viruses prior to infection with AMV by testing their susceptibility to infection by Rous sarcoma virus.

It appears, therefore, that RNA synthesis is inhibited to the same extent by actinomycin D in both normal and AMV-producing cells. Furthermore, viral RNA synthesis is inhibited by more than 98% within 30 min after addition of actinomycin D, whereas virus maturation and release continues normally for at least 2 hr.

DISCUSSION

The successive stages of AMV maturation and release at the cell surface, i.e., budding, have been well documented by electron microscopy (11). Electron microscopy and light microscopy with fluorescent antibodies against viral surface antigens have also revealed that virions are confined to the cell surface and are only rarely seen intracellularly (27). Treatment of intact cells with trypsin and anti-AMV antibody removed or neutralized nearly all cell-associated virus (27). Release and maturation are therefore simultaneous processes for AMV. Rapid radioactive labeling of protein in particles which are released by trypsin suggested that some protein synthesis occurs at the time of release (2).

The present study supports the previously proposed model for the maturation of AMV and indicates that there is a pool of viral genomes in leukemic cells which are producing virus continuously at a steady rate. The RNA might be naked, but is probably in a ribonucleoprotein complex. The single-stranded RNA viral genome has a molecular weight of 12×10^6 daltons; by using the rate of synthesis of poliovirus RNA upon its RNA template, the synthesis of one molecule of AMV-RNA would require about 3 min (15). The present study has also shown that it takes only 2 min, at most, for ^{3}H-UR to be polymerized into cellular RNA. In addition, numerous studies on the synthesis of various viral RNA types have never shown a lag in the polymerization of ^{3}H-UR from its intracellular pool. Therefore, the delay of about 4 hr between the time ^{3}H-UR is added to cells until 50% of the maximal incorporable ^{3}H-UR is released in virions represents mostly the median time that a viral genome spends in an intracellular state (pool).

Although the site of synthesis of AMV-RNA is not established, it might be the nucleus. This is possible, especially since there is immunofluorescent staining of the nucleus in infected cells by an antibody against the avian leukosis group-specific antigen which may be the viral ribonucleoprotein (19). If AMV-RNA is made in the nucleus, it would require a longer time to appear in virions than if it were made in the cytoplasm. Cellular messenger RNA and ribosomal RNA (28*S*) require 30 to 45 min to mature from precursors and to be transported from nucleus to cytoplasm (24; E. C. Henshaw, Annu. Symp. Fundamental Cancer Res. 22nd Abstr., p. 14–17, 1968). Thus, part of the delay may be accounted for in this manner.

In the steady state of virus replication investigated here, RNA molecules enter the pool at the same rate at which they leave it to enter virions. The pool appears to turn over with a half-life of 3 to 4 hr. It is difficult to determine its exact rate of turnover because of the large intracellular uridine pool which itself has a half-life of 2 hr. AMV genomes are removed from their pool in a random fashion, i.e., maturation occurs regardless of time spent in the pool. This is demonstrated by the release of some ^{3}H-labeled virions within 30 min after exposure of the cells to ^{3}H-UR, although the highest specific activity in ^{3}H-UR-labeled virions does not appear until after 3 or 4 hr. This accounts for the dependence of the lag period, between exposure of cells to ^{3}H-UR and release of ^{3}H-labeled virions, upon the concentration of ^{3}H-UR added to the nutrient medium. This early appearance of ^{3}H-UR-labeled RNA into some virions also eliminates the possibility that the viral RNA undergoes a long maturation process which requires several hours. The existence of a pool of viral genomes is further demonstrated by the actinomycin D experiments. This drug inhibits viral RNA synthesis almost immediately but permits the maturation of already formed genomes for at least 2 hr.

It should, therefore, be possible to demonstrate the intracellular presence of high-molecular-weight (71*S*) viral RNA in virus-producing cells. However, such attempts have not been successful; it has been repeatedly found that leukemic myeloblasts and noninfected chick embryonic cells have the same RNA profiles after analysis by sucrose gradient ultracentrifugation, and by agarose or cellulose chromatography (Baluda and Nayak, *unpublished data*). Similar negative results have also been obtained with transformed cells which were producing Rous sarcoma virus (RSV), another leukosis virus closely related to AMV (Duesberg, *personal communication*). A possible explanation for the absence of 71*S* AMV-RNA in leukemic cells is that this RNA may be present as an RNA

species with a smaller sedimentation coefficient of 36S. This 36S RNA could represent either a smaller molecule of 3×10^6 daltons or a molecule of different configuration. It has been shown that 71S RNA isolated from AMV (14) or from RSV (P. H. Duesberg, *personal communication*) is changed to 36S RNA by agents such as heat or dimethyl sulfoxide which destroy hydrogen bonds. In either case, if most of the AMV-RNA is present as 36S RNA in the intracellular pool, and if 71S RNA is found only after maturation into the virion, it would be difficult to demonstrate this RNA species as specific for myeloblasts, since the normal cellular RNA profile contains a wide spectrum of RNA species, including 36S RNA. Assuming that a myeloblast produces 40 virions per cell per hr, less than 0.5% of the intracellular RNA would be virus specific.

There has been a report of the accumulation of 71S RNA in cells producing Moloney murine sarcoma-leukemia virus by arresting virus maturation with the inhibitor of protein synthesis, cycloheximide (7). An attempt by us to accumulate 71S AMV-RNA in myeloblasts treated with cycloheximide has not been successful. A similar attempt in RSV-transformed cells has also failed (12). It is possible that the two virus systems are affected differently by cycloheximide; however, the possibility also exists that the 71S RNA observed in the murine sarcoma-leukemia virus was isolated from virions attached to the cell surface.

From the present study it appears that newly synthesized proteins are incorporated rapidly into free virions, e.g., within 30 min after a pulse of labeled phenylalanine. ^{14}C-amino acids may even appear in virion proteins within 10 min (2). This indicates that there is no intracellular pool of at least some proteins present in virions and that there is synthesis and incorporation of some protein during the maturation of virus at the cell membrane. It is tempting to assume that it is the envelope protein which becomes the most rapidly labeled, since it is the last one to be incorporated into virion. The experiments with glucosamine, which is a precursor of membrane glycoproteins, support this hypothesis. The kinetics of labeling of the different protein components of virions should be determined to resolve this question.

There is a lag of several hours before a pulse of ^{14}C-EA is incorporated into virions, and the highest specific activity of ^{14}C per virion is reached between 16 and 20 hr after the pulse. It is evident from Fig. 12 that this delay is not due to a lag in the rate of conversion of acid-soluble ethanolamine into acid-precipitable phospholipid in the cell. The incorporation of ^{14}C-EA into trichloroacetic acid-precipitable lipoproteins, like the synthesis of RNA, takes place without any appreciable delay and continues linearly for at least 8 hr. The morphogenesis in the cell cytoplasm of vaccinia virus membrane within 3.5 hr postinfection also shows that a viral membrane can be synthesized in a relatively short time (10). Since AMV picks up its outer envelope at the cell surface via the cell membrane, it appears that there is a lag of several hours in the ^{14}C-EA labeling of the cell membrane which appears to turn over with a half-life of about 22 hr. There are no available data on the lipid composition of the surface membrane of myeloblasts, but the lipid composition of whole myeloblasts is qualitatively similar to that of AMV (21). However, there are quantitative differences, e.g., AMV contains more cholesterol (34 versus 12%), more phosphatidyl ethanolamine (21 versus 15%), fewer neutral lipids (5 versus 33%), and less lecithin (11 versus 20%) (21). Nevertheless, there is no reason to assume that the mechanism of synthesis of the viral membrane differs from that of the cellular membrane. These experiments, therefore, support the hypothesis that the cellular membrane is synthesized at a centralized factory, e.g., Golgi apparatus, and moves out in a conveyor belt fashion to the cell surface, via the endoplasmic reticulum, to replace sections lost during normal cell functions or budding of AMV, or to increase the surface area during cell growth and division. It is also evident that additional antigens or proteins can be incorporated in the lipoprotein matrix of the cell membrane without disrupting the stability of the membrane. The virion spicules might be added at this time since, as shown in a vaccinia virus study (10), spicules are essential for the structure of the viral envelope and for the morphogenesis of the virion. Interestingly, treatment with actinomycin D prevents the formation of spicules and causes the formation of aberrant vaccinia viral membranes which are flexible and round up into micelles. This may explain the effect, observed in this study, of actinomycin D upon AMV production. The drug may inhibit protein synthesis and stop the formation of spicules for the viral envelope, thereby arresting the formation of new virions. Such affected cell membranes would in turn collapse into micelles, accounting for the increased release of viruslike particles which contain adenosine triphosphatase and cellular RNA. This may explain the rise in adenosine triphosphatase activity of the surrounding medium which has been often observed in presence of actinomycin D.

The experiments with actinomycin D have shown that the major inhibitory effect of this

drug is upon AMV-RNA synthesis, which is arrested within 30 min at most. Only after several hours is virus maturation inhibited, and this inhibition parallels the decrease in the rate of cellular protein synthesis. These results, therefore, rule out the possibility that actinomycin D inhibits AMV production by arresting viral maturation and release.

In conclusion, it appears that, in leukemic myeloblasts, AMV-RNA is synthesized via an actinomycin D-sensitive mechanism and accumulates somewhere in the cell, but apparently not as 71*S* RNA. There is an intracellular pool of viral genomes which turns over with a half-life of approximately 4 hr. The RNA genomes are removed at random from the pool and mature within 30 min by a budding process which requires protein synthesis or glycoprotein synthesis (or both). The phospholipids of the outer virion membrane follow the slow pathway used by cellular lipoproteins which become incorporated into the cell surface membrane. These phospholipids in the surface membrane turn over with a half-life of approximately 22 hr, although we are again dealing with a random process, and some labeled precursor may appear earlier. Since avian leukosis viruses have a latent period of 14 to 20 hr, and if there are virus-specific lipoproteins, the synthesis of viral lipoproteins might be the limiting step in the latent period.

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