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Dual Pathways for Ribonucleic Acid Turnover in WI-38 but Not in I-Cell Human Diploid Fibroblasts

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The turnover rates of ^{3}H-labeled 18S ribosomal ribonucleic acid (RNA), 28S ribosomal RNA, transfer RNA, and total cytoplasmic RNA were very similar in growing WI-38 diploid fibroblasts. The rate of turnover was at least twofold greater when cell growth stopped due to cell confluence, ^{3}H irradiation, or treatment with 20 mM NaN_3 or 2 mM NaF. In contrast, the rate of total ^{3}H-protein turnover was the same in growing and nongrowing cells. Both RNA and protein turnovers were accelerated at least twofold in WI-38 cells deprived of serum, and this increase in turnover was inhibited by NH_4Cl. These results are consistent with two pathways for RNA turnover, one of them being nonlysosomal and the other being lysosome mediated (NH_4Cl sensitive), as has been suggested for protein turnover. Also consistent with the notion of two pathways for RNA turnover were findings with I-cells, which are deficient for many lysosomal enzymes, and in which all RNA turnover was nonlysosomal (NH_4Cl resistant).

Even in actively growing cultured cells, the net level of protein is modulated by turnover as well as synthesis; the average turnover rate is about 20 to 50% the rate of protein synthesis (5, 14, 17), and specific turnover rates of several proteins have been measured (e.g., references 10 and 12).

Because added NH_4^+ ion concentrates in lysosomes, it raises their internal pH to levels that inhibit lysosome function. Because of this effect, NH_4Cl and other lysosomotropic amines have been used to define two modes of protein degradation, one of them probably mediated through lysosomes (NH_4Cl sensitive; 2, 3, 10, 12), but the mechanisms involved are poorly understood.

Even less is known about the mechanism and regulation of ribonucleic acid (RNA) turnover. Studies to date have primarily shown only that cytoplasmic RNA can be degraded as fast as bulk protein, especially in cultures of diploid fibroblasts (1, 15, 18, 22).

Here we report the rates of bulk turnover of cytoplasmic RNA in a variety of cell growth conditions. In addition, we assessed turnover rates in cells presumably deficient in lysosomal function, derived from patients with I-cell disease (9, 16). Measurement of protein turnover was used as a control for technique and to permit comparison of RNA and protein turnover.

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MATERIALS AND METHODS

Materials. [5-^{3}H]uridine (28.5 Ci/mmol), [2-^{14}C]-thymidine (52.4 mCi/mmol), L-[4,5-^{3}H]leucine (51.6 Ci/mmol), and [*methyl*-^{3}H]methionine (11 Ci/mmol) were obtained from New England Nuclear Corp. Ready-Solv was from Beckman. Cycloheximide was from Sigma. Fetal bovine serum was from Kansas City Biologicals.

Cell culture. Primary human diploid fibroblasts, WI-38 cells at passage 14, were obtained from the American Type Culture Collection. I-cells (4, 9, 16), strain L. T., were obtained from W. S. Sly, Department of Medicine, Washington University School of Medicine. Cells were maintained as monolayers at 37°C in 75-cm^2 culture flasks with 25 ml of minimal essential medium supplemented with 100 μg of sodium pyruvate per ml and fetal calf serum (10% for WI-38 cells, 15% for I-cells). Cells were split 1:4 every 3 to 4 days. To prepare ^{3}H-arrested cells (23), cultures were prelabeled with 10 μCi of [^{3}H]uridine per ml or 20 μCi of [*methyl*-^{3}H]methionine per ml for 24 h (13, 21).

Measurement of RNA and protein turnover. Cells were usually incubated for 24 h in 20 ml of medium containing either 2 μCi of [^{3}H]leucine per ml or 0.3 μCi of [^{3}H]uridine per ml or 20 μCi of [*methyl*-^{3}H]methionine per ml, plus nucleoside bases and formate (21), to prelabel protein, RNA, or both. [2-^{14}C]thymidine was also added at 0.006 μCi/ml to label deoxyribonucleic acid for normalization of the cellular content of labeled protein and RNA in all of the cultures studied. Monolayers were then rinsed with warm buffer (137 mM NaCl, 2.7 mM KCl, 16 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). The cells were trypsinized and plated at 3×10^3 cells per cm^2 in 21-cm^2 dishes in medium containing 15% serum, 0.4 mM thymidine, and either 5 mM uridine–1 mM cytidine (for the measurement of RNA turnover) or 2 mM

methionine (for the turnover of RNA labeled with [*methyl*-^{3}H]methionine) or 2 mM leucine (for protein turnover). Two days later, the medium was aspirated from each dish, and cultures were rinsed once with 3 ml of the same medium (with or without serum; see text). Another 3 ml of medium with or without additives (e.g., NaN_3, NaF) was added ("time zero").

RNA turnover was measured using two approaches ("long-term" and "short-term" experiments). Long-term measurements of turnover were made by measuring the decline of acid-insoluble [^{3}H]uridine counts per minute in prelabeled cells relative to [^{14}C]thymidine in deoxyribonucleic acid (see above) over a period of 6 to 10 days (21). On the indicated days, cells from two plates were trypsinized into 3 ml of 0.25 M sucrose. A portion was precipitated with ice-cold 5% CCl_3COOH for 15 min, then collected on a filter and counted in 10 ml of Ready-Solv (Beckman) to estimate the remaining [^{3}H]uridine and [^{14}C]thymidine. Values for the decay rates of RNA were corrected for cell loss (usually <1% per day) by plotting all values as $^3H/^{14}C$.

To examine the rates of turnover of individual RNA species, RNA labeled with [^{3}H]uridine or [*methyl*-^{3}H]methionine was extracted with phenol and fractionated in agarose-acrylamide gels as described previously (23). After electrophoresis, the gels were frozen and sliced. The slices were dissolved in Ready-Solv and counted to measure the counts in each RNA species.

Identical rates of turnover were seen when we used [^{3}H]uridine, which can be reincorporated after its release from RNA, and ^{3}H-methylated bases, which cannot (see text and Fig. 1). Thus chasing with unlabeled uridine must prevent most reincorporation, so [^{3}H]uridine turnover provides a reliable measure of RNA decay in this case as in others (1, 15, 23).

The efficient chase of [^{3}H]uridine permitted equally accurate measurements of RNA turnover by short-term assays, in which the acid-soluble radioactivity released into the medium during the chase period was measured. These assays are equivalent to a standard assay for protein turnover (7). Because there is no radioactivity in the medium at time zero, the decay of 1% of the total labeled RNA is easily detected, and turnover rates can be measured in 6 to 30 h from repeated samplings of the medium of a single petri dish.

To determine the amount of [^{3}H]uridine or [^{3}H]leucine released into the medium in acid-soluble form, 1 ml of 6% CCl_3COOH was added to 0.2 ml of medium for 15 min on ice; when serum-free medium was used, 400 μg of bovine serum albumin was added as a carrier. The precipitated solids were removed by sedimentation at 8,000 *g* for 10 min. Portions of the supernatant were counted in 10 ml of Ready-Solv. Each value is an average of samples from duplicate plates.

^{3}H radioactivity released from cells into the medium is expressed as a percentage of the total acid-precipitable counts from the same plate (see Fig. 3 to 7). The total RNA or protein counts were measured by trypsinizing the cells into 3 ml of buffer and counting 0.2 ml of the suspension in 10 ml of Ready-Solv. To adjust the counting efficiency to that of the acid-soluble samples from the medium, 20 μl of 50% CCl_3COOH was added to each vial.

RESULTS

Turnover rates during the cell growth cycle. Figure 1 shows sample data from long-term experiments for RNA turnover in nearly confluent cultures of WI-38 cells labeled with [*methyl*-^{3}H]methionine (Fig. 1A) or [^{3}H]uridine (Fig. 1B). The radioactivity of total RNA and of species fractionated by gel electrophoresis was normalized per microgram of RNA and per cellular content of [^{14}C]thymidine. Similar half-lives of about 3 days were observed with each label for 4S RNA, 18S and 28S rRNA, and total RNA. Results were highly reproducible, as indicated by the overlap of sample data from two separate experiments in Fig. 1A.

RNA and protein both turned over throughout the growth cycle of WI-38 fibroblasts (Fig. 2). Labeled protein was degraded at a very similar rate in rapidly growing and confluent cultures, but the rate of RNA turnover increased sharply, from a half-life of 6.5 to one of 4 days, as cells approached confluence (Fig. 2A; note

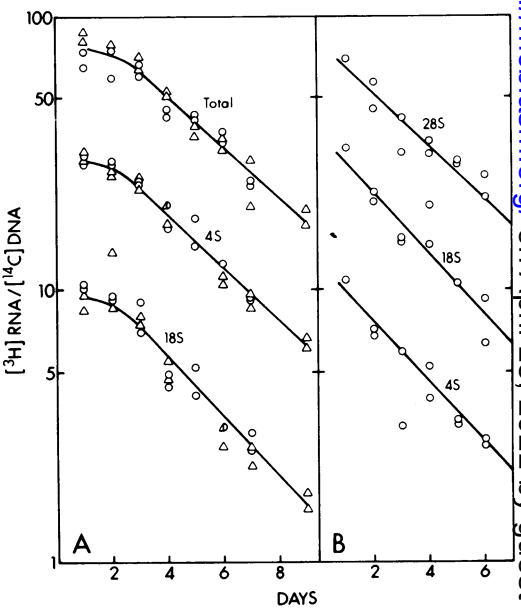

FIG. 1. *Turnover of RNA species labeled with [^{3}H]uridine or [methyl-^{3}H]methionine in WI-38 cells. Cells at culture passage 22 were prelabeled with 1 μCi of [^{3}H]uridine per ml (A) or 20 μCi of [methyl-^{3}H]methionine per ml (B) and 0.006 μCi of [2-^{14}C]thymidine per ml for 24 h. They were then trypsinized, plated at 5 × 10^4 cells per cm^2, and chased in fresh medium (see the text). Each day, RNA was extracted from cells in replicate pairs of plates and fractionated by gel electrophoresis. The specific activity of gel peaks was then determined per microgram of RNA and normalized to ^{14}C-deoxyribonucleic acid content, as in reference 23. Data points from two separate experiments are shown* (○ *and* △).

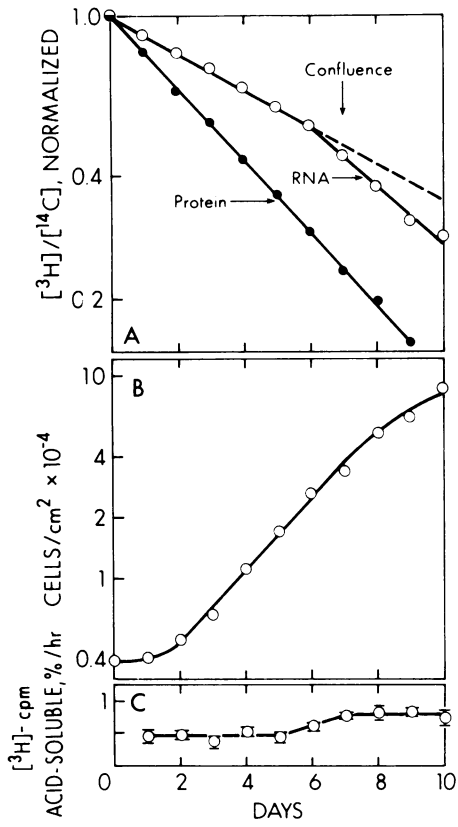

FIG. 2. *Turnover of [^{3}H]RNA and protein during the culture cycle of WI-38 cells. Cells at culture passage 35 were prelabeled with 0.3 μCi of [^{3}H]uridine per ml (○) or 2 μCi of [^{3}H]leucine per ml (●) with 0.006 μCi of [2-^{14}C]thymidine per ml for 24 h. They were then trypsinized, plated in 75-cm^2 culture flasks at 4 × 10^3 cells per cm^2, and chased in medium containing 5 mM uridine, 1 mM cytidine (or 5 mM leucine, for labeled protein), and 0.4 mM thymidine. Each day medium from duplicate flasks was aspirated off and cultures were rinsed once with chase medium. Then 6 ml of fresh chase medium was added to each flask. After 4, 6, and 8 h, 0.2 ml of medium was sampled, and the cold acid-soluble radioactivity was measured (see the text). Monolayers were trypsinized, cells were enumerated (B), and the total and acid-insoluble radioactivities were measured (see the text). All values are the average of duplicates. The values in (A) are plotted as a ratio of acid-insoluble ^{3}H/^{14}C counts, to normalize for any cell loss (< 0.003% per day). The ratio at time zero is set equal to 1 (A). For (C), values are plotted as the percentage of total counts released in a flask each hour to the acid-soluble phase. The range in duplicate flasks each day is shown.*

also the initial slower rate in preconfluent cells in Fig. 1A). RNA also turned over faster when cell division was arrested by ^{3}H irradiation, by colchicine, or in senescent cells (13, 21; M. Sameshima, manuscript in preparation).

Figure 2B shows data from short-term assays of RNA turnover. The rate of turnover measured by these assays is expressed as the percentage of turnover per hour. The half-life of RNA can be calculated from these data since the amount of radioactivity in RNA at any time, N_t, is related to the amount at time zero, N_0, by $N_t = N_0(1 - a)^t$, where a is the percentage (× 1/100) of labeled RNA degraded per hour (by the law for exponential decay). At $t = t_{1/2}$ (the half-life), $N_t/N_0 = 1/2$ and $t_{1/2} = \ln 0.5/\ln (1 - a)$, so the degradation of 0.45% RNA per hour in the preconfluent culture of Fig. 2 and 0.75% in the confluent culture corresponds to half-lives of 6.5 and 4 days, the same values obtained in the long-term assays.

Since the rates of turnover of 4S, 18S, and 28S RNA (>90% of total cellular RNA) are all about the same as that of total RNA, short-term assays, which measure only bulk RNA turnover, were used extensively to permit rapid assays of the effects of a number of parameters on turnover.

Turnover in WI-38 cultures with or without serum and NH_4Cl. Although the rate of protein turnover does not change when WI-38 cells become confluent, protein turnover does increase in mouse fibroblasts at confluence (11). The rate of protein turnover also increases both in WI-38 fibroblasts and in all other cell types studied when cells are incubated in serum-free growth medium. Since this increase in turnover ("autophagic response", 2, 3, 6, 8, 10, 12, 17, 20, 21) is inhibited by treatment with weak bases and NH_4Cl (2, 19), it is thought to be mediated partially through lysosome action. Figure 3

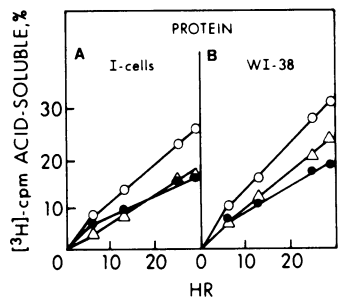

FIG. 3. *Effect of serum starvation and NH_4Cl addition on protein turnover in I-cells and WI-38 fibroblasts. Protein was prelabeled with [^{3}H]leucine for 24 h and then chased in the presence of an excess of unlabeled precursors for 2 days (as described in the text). At time zero, the medium in each of a series of petri dishes was replaced with 3 ml of fresh chase medium containing (△) 15% fetal bovine serum, (○) no serum, or (●) 20 mM NH_4Cl with no serum. The release of label from prelabeled protein to acid-soluble form was then measured as a percentage of total incorporated label as described in the text.*

shows the effects of NH_4Cl treatment on protein turnover in short-term experiments. Similar results were found with WI-38 fibroblasts and with I-cells.

Like protein turnover, RNA turnover increased in cells incubated in serum-free medium. However, RNA turnover also increased when cell was arrested. Two modes of RNA turnover could be clearly distinguished. Turnover which occurred both in growing cells and in cells ^3H-arrested in the presence of serum was unchanged by the addition of 5, 10, or 20 mM NH_4Cl to the growth medium (Fig. 4a and c). Another mode of turnover, which occurred in cells incubated in the absence of serum, was inhibited by added NH_4Cl (Fig. 4b and d). In seven experiments, 20 mM NH_4Cl decreased the rate of turnover in serum-free culture to nearly the same rate observed in cells incubated with serum.

These results are consistent with the notion that "autophagic" turnover of protein and RNA is mediated by lysosomes (NH_4Cl sensitive), since nonlysosomal RNA turnover occurs at the same rate whether or not NH_4Cl is added.

Loss of one mode of RNA turnover in I-cells. An independent approach to removing lysosome function independent of NH_4Cl is provided by I-cells, which are generally deficient in a number of lysosomal enzymes (4, 13). As mentioned above, however, the rate of protein turnover was comparable in I-cells and WI-38 cells during growth in media with and without serum and in the presence of NH_4Cl (Fig. 3). The results with RNA turnover were rather different. In the presence of serum, the rate of RNA turnover was higher in I-cells than in WI-38 cells (Fig. 5). Although the rate of turnover increased in both cells when serum was removed from the medium, the rate of RNA turnover in I-cells was not reduced at all by added NH_4Cl (Fig. 5). Thus, the regulation of RNA turnover was altered in I-cells, with one mode of RNA turnover (lysosomal) absent.

Response of turnover to blockage of adenosine 5'-triphosphate or protein synthesis. RNA and protein turnover responded very differently to agents customarily used to block energy generation. Figure 6 shows results from short-term experiments, reported as percent of labeled RNA or protein rendered acid soluble per hour. Protein turnover was moderately inhibited by added NaF or NaN_3 (Fig. 6B), as previously reported (12, 17). Similar inhibition was seen with cells incubated in the presence or the absence of serum. RNA turnover, on the other hand, increased when NaF or NaN_3 was added to growing cells (Fig. 6A) or to ^3H-arrested cultures (Fig. 7), but these reagents had little or no effect on the high rate of RNA

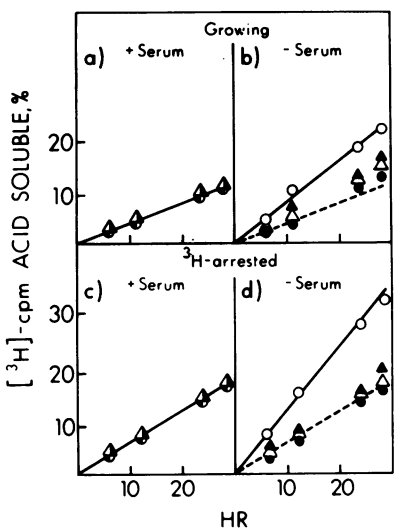

FIG. 4. *RNA turnover in growing or ^3H-arrested WI-38 cells incubated in presence of serum (a, c) or in its absence (b, d). Cells were labeled for 24 h with [^3H]uridine as described in Fig. 1 (a, b) or with 10 μCi/ml to arrest their growth (c, d). After 2 days of chase, the release of label into acid-soluble form was measured as described in the text and Fig. 2. (a, c) Release of ^3H label in cells incubated in the absence (○) or in the presence of 5 (▲), 10 (△), or 20 mM (●) NH_4Cl (all four points at each time point were essentially superimposable). (b, d) Release of ^3H label in the absence (○) or in the presence of 5 (▲), 10 (△), or 20 mM (●) NH_4Cl. In (b) and (d), the lines of panels (a) and (c) are reproduced as dashed lines.*

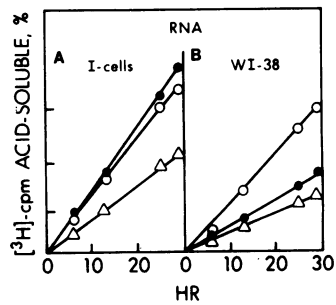

FIG. 5. *Effect of serum starvation and NH_4Cl addition on RNA turnover in I-cells and WI-38 fibroblasts. RNA was prelabeled with [^3H]uridine for 24 h and then chased in the presence of an excess of unlabeled precursors for 2 days (see the text). At time zero, the chase medium was replaced in a series of pertri dishes as described in Fig. 3, in order to measure the rate of release of label from protein in fresh medium (△), medium with no serum (○), or medium with no serum and 20 mM NH_4Cl (●). The released label was measured as a percentage of total incorporated label as described in the text.*

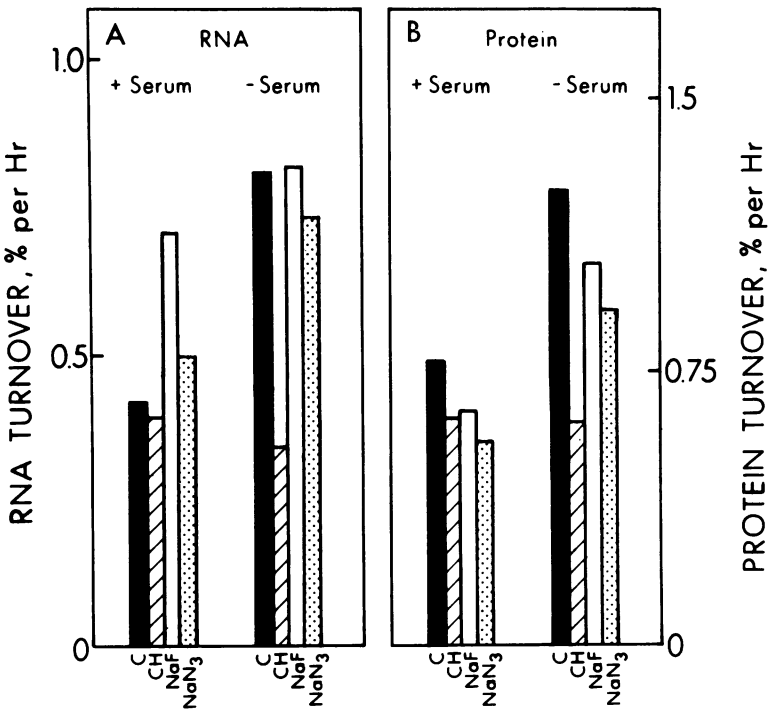

FIG. 6. *RNA and protein turnover in WI-38 cells incubated in presence or absence of serum or metabolic inhibitors. Cells were prelabeled with [^{3}H]uridine or [^{3}H]leucine as described in Fig. 1. The rate of release of acid-soluble radioactivity (as a percentage of the total incorporated) was then measured over a 36-h period for control cultures in the presence or absence of serum (C; solid bars) or parallel cultures in the presence of 20 μg of cycloheximide per ml (CH), 2 mM NaF, or 20 mM NaN_3.*

turnover in cells in serum-free medium (Fig. 6B and 7).

RNA and protein (8, 12) turnover were both affected when protein synthesis was blocked by cycloheximide. In this case, however, both protein and RNA turnover were slightly inhibited in cells growing in the presence of serum. Cycloheximide also blocked the augmentation in the rate of RNA and protein turnover seen in cells cultured in serum-free medium (Fig. 6).

A number of other agents which induce lysosomal enzyme activity in macrophages (asbestos, zymosan A, and dextran sulfate [6]) were tested, but none had any detectable effect of the rate of RNA turnover.

DISCUSSION

These results suggest that there are two pathways of RNA turnover. One pathway, apparently nonlysosomal, is increased when WI-38 cells grow slowly at confluence or stop growing (for example, after treatment by a high dose of ^{3}H). This effect of confluence has been reported for many cultured cells (e.g., references 1, 15, 17), The pathway is not inhibited by the addition of NH_4^+ ions. A second RNA turnover pathway is activated in WI-38 cells in media lacking serum and is inhibited by ammonium chloride. Analogously, the turnover of total protein (2, 19) and individual proteins (10, 12) increases in the absence of serum and is inhibited by lysosomotropic amines; this NH_4Cl-sensitive pathway has been inferred to be lysosome mediated (2, 3, 6, 8, 10, 12, 17, 20, 21).

The results with I-cells support the notion of two modes of turnover, one of them lysosomal. Those cells are depleted of many lysosomal enzymes (13) and accumulate some products which are usually degraded in lysosomes in normal cells. One might therefore expect that macromolecular turnover, which ordinarily occurs in lysosomes, might be deficient in I-cells.

In the presence of serum, I-cells showed a higher rate of turnover than WI-38 cells and tended to become growth-arrested at a lower cell density, but serum starvation still further increased the rate of turnover. The increase was not affected by NH_4Cl. Ammonium chloride must have entered I-cells as it did WI-38 cells, since it inhibited protein turnover to a comparable extent in both cell types (Fig. 3). These findings suggest that lysosomal RNA turnover

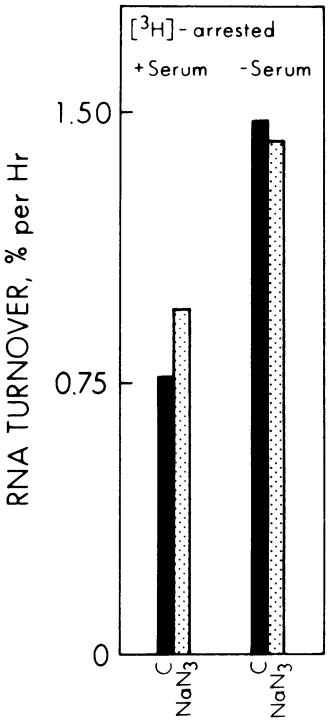

FIG. 7. *RNA turnover in ^{3}H-arrested WI-38 cells incubated in the presence or absence of serum or metabolic inhibitors. Conditions were as described for Fig. 6.*

in I-cells is more impaired than is lysosomal protein turnover. Not all lysosomal enzymes are deficient in I-cells, and it seems possible that lysosomal cathepsins involved in turnover are at normal levels, but lysosomal ribonuclease is deficient. Recent evidence suggests that in I-cells a defect in the phosphorylation of a mannose "recognition marker" (4, 9) leads to the secretion of some enzymes rather than their incorporation into lysosomes. It may be that proteases involved in lysosomal protein turnover do not require phosphorylated mannose for their uptake into the organelle.

The response of RNA turnover to the blockage of ATP formation was much greater than that of protein turnover. This effect may somehow be related to the relative sensitivity of the two processes to growth arrest, but the bases for energy requirements and for the process of turnover are probably much more complex. For example, uptake of ribosomes or proteins into lysosomal vesicles may itself require energy, and NaF or NaN_3 addition might affect intracellular transport as well as degradative processes. Also, the requirements for the degradation of "stable" RNA (ribonucleic and transfer RNAs) may be very different from the requirements for the gross degradation of "average" proteins (8) or messenger RNA.

Nevertheless, the results suggest that several regulatory features are the same for both RNA and protein turnover. For both, dual pathways are inferred, one of them lysosome mediated. For both, turnover is accelerated in serum-starved cells. And for both, the augmentation of turnover in serum-starved cells is sharply inhibited by cycloheximide (Fig. 4; also cf. references 8 and 12 for protein turnover). It remains to be determined whether de novo protein synthesis is required for the augmentation of turnover rates in serum-free medium.

These experiments measure only the final step in RNA degradation, the production of acid-soluble nucleotides. They do not distinguish any of the intermediate steps that may be required to initiate or complete the breakdown process. However, the similarity of the turnover rates of ribosomal and transfer RNAs suggests that the rate-limiting steps in turnover may be the same for all cytoplasmic RNA except messenger RNA. For lysosome-mediated turnover, uptake into the organelle could be such a step, but there is as yet no hint of where or how nonlysosomal turnover occurs.

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