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Pyrimidine Biosynthesis in Normal and Transformed Cells

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We have developed procedures for sensitive measurement of specific radioactivities of pyrimidine nucleosides excreted from cells in culture. The changes in the observed values reflect dilution of the added isotope through de novo biosynthesis of nonradioactive pyrimidine nucleosides or by shifting and equilibration of other nucleotide pools into the free uridine pool. It is thus possible to monitor uridine biosynthesis occurring in intact cells without destroying or disrupting the cell population. On comparing a series of normal and transformed lines, we have observed several growth-dependent patterns of change in specific activity and levels of uridine excretion and the temporal appearance of these changes.

Hamster embyro fibroblasts slows pyrimidine biosynthesis at mid-growth while the hamster cell line V79 continues to dilute the pyrimidine pool at about 7% of the rate observed during exponential growth at confluence. Both cells exhibit Urd excretion beginning at one-half maximal growth.

Passageable normal rat liver cells (IARC-20) also show a cessation of pyrimidine biosynthesis with a prior increase in uridine excretion. Two chemically transformed lines IARC-28 and IARC-19 derived from IARC-20 show different patterns. IARC-19 begins uridine excretion in early log growth and the specific activity continues to decrease at about 2% of the rate observed during exponential growth at confluence. The IARC-28 cells also begin excretion in early log growth but pyrimidine biosynthesis stops at about midlog. This method may prove to be an additional aid in recognizing and differentiating transformed cells in culture that do not exhibit the transformed phenotype.

Key words: pyrimidine biosynthesis, V79, IARC19, IARC20, IARC28, biomarker, pleiotropy, confluence, rat liver epithelial cells

We have recently discovered uridine and cytidine to be normal excretion products of cells in culture and the excretion process is regulated so that the maximal rate of excretion occurs as the cells enter G_1/G_0 [1]. The excretion of uracil and deoxynucleosides is also known to occur in fibroblast cultures, especially with mutants blocked in a utilization step [2]. It would appear that the processes leading to the normal excretion of these compounds is related to the balance of salvage and de novo synthetic pathways.

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The balance between salvage and de novo biosynthetic pathways in growing cells is regulated by several phenomena including the concentration of base or nucleoside in the medium [3, 4]. For example, the presence of Urd in culture of HTC cells can introduce a repression of Urd synthesis that requires new RNA and protein synthesis for derepression [3]. On the other hand, studies of key enzymes in the biosynthetic pathway of normal and transformed cells have shown certain key enzymes to increase in malignantly transformed cells [5]. Pyrimidine biosynthesis is one of these pleiotropic manifestations along with increased utilization of UDP and its derivatives and decreased rates of catabolism of uracil and thymine [5].

The normal presence of nucleosides in the medium provides a unique opportunity to continuously monitor biosynthesis and excretion of uridine. Using recently developed techniques for affinity chromatography and sensitive analysis of nucleosides in culture, we have examined sets of fibroblastic and epithelial cells to see if we could detect the transformation dependent pleiotropic effects on pyrimidine biosynthesis and salvage.

MATERIALS AND METHODS

Growth of Cells

Syrian hamster embryo fibroblasts obtained from Charles River Farms were grown in DMEM supplemented with 0.45% glucose and 10% FCS in 8.5% CO_2 at 37°C and 98% R.H. [1]. The cells grew synchronously in tertiary subculture when confluent secondary cells (seeded at 2×10^6 cells per 100 mm dish and grown for 72 hrs) were suspended at 200,000 per ml in the medium and 10 ml added to each 100 mm plate. The V79, IARC-20, IARC-19, and IARC-28 lines were grown in William's medium according to Montesano [6]. See the figure legends for plating data. Cells were counted with a hemocytometer or a cytofluorograph. These cell lines were routinely checked for PPLO and found negative.

Radioactive Labeling

Either 2-[^{14}C]Urd (58 μCi/μmol, Schwartz/Mann) or 6-[^{3}H]Urd (20 ci/mmol, New England Nuclear) were used as the radioactive tracers. The label was diluted in fresh medium so that 1 ml of medium contained the correct amount of tracer. This was added to the cells 7 hrs after seeding. See figure legends for the amount of radioactivity added.

All samples were counted by adding 3 ml of scintillation fluid to 0.2 ml of aqueous sample. All samples were counted in a Beckman LS250 for 10 minutes.

Uridine Isolation and Analysis

Two ml of medium was processed as described in [7]. The nucleoside, adsorbed to 2 ml of the affinity column were collected by elution with 30 ml acetic acid and then lyophilized. The residue was dissolved in about 1 ml of water and transferred to conical test tubes and lyophilized again. The residue was dissolved in 0.2 ml of the initial chromatographic solvent (1.5% v/v acetonitrile 0.03 M NH_4 phosphate pH 5.1). The analyses were done with a Water's C18 ODS μBondpak column at a flow rate of 0.67 ml/min at room temperature [1]. The fractions (0.22 ml) 11 to 60 were counted for radioactivity. The solvent was changed to 20% acetonitrile at 15 min to cleanse the column and returned to the original solvent at 35 min for reequilibration. Uridine eluted at 12.3 min (8.25 ml) with this system.

The amount of uridine was measured by comparison of the peak height to standards. We routinely analyze at least 250 pmoles based on absorbancy measurements and usually

1,000 pmoles so the outer range of precision is ± 10%. The error range is very close to the symbol size on the exponential plot. The sensitivity of the assay is 25 pmoles for a peak height of 0.0008 A at 254 nm. The absorbance (A) was measured continuously with an LDC monitor (1 cm light path 8 μl cell volume) using a recorder span of 0.08 or 0.02 depending on the amount of uridine in the sample.

RESULTS

Pyrimidine Metabolism in Hamster Cells

Figure 1 shows the excretion properties of synchronized III° HEF grown to confluence and then stimulated to grow again by replacement of the culture fluid with fresh medium. The specific activity of uridine in the medium decreases exponentially as it equilibrates with the endogenous pools and newly synthesized uridine. At 46 hr, the cell number is 1/2 the cell number at confluence and the specific activity of the excreted uridine becomes constant. The excretion of uridine appears to have begun earlier, between 30 and 40 hr post seeding as the cells were in G_2 phase. At 72 hr the medium was replaced and a burst of cell growth occurred accompanied by an additional decrease in uridine specific activity due to additional biosynthesis of uridine. The specific activity becomes constant after the additional growth. The number of cells increase by 41% and the specific activity decreased from the level before changing the medium. The endogenous uridine in the medium contributes less than 5% to this change. The rate of isotope dilution decreases from 4 per hr in exponential growth to zero in the G_1/G_0 state.

Figure 2 illustrates the change in uridine concentration and specific activity in cultures of V79. The excretion process begins during exponential growth approximately two generations before the cells reach a maximum number. The cells continuously take up the external uridine during early log growth until a minimum is reached at about 40 hr after seeding. The specific activity continues to decrease throughout the incubation period at a rate of 3 per hr during exponential growth to 0.5 per hr at about 1/2 maximal growth to 0.1 per hr when growth ends.

Pyrimidine Metabolism in Liver Epithelial Cells

Montesano [6] has observed these cells to be morphologically indistinguishable from the normal phenotype and will not overgrow at confluence. These IARC cells, however, show metabolic differences between the normal and transformed phenotype. IARC-20, a passageable epithelial cell line derived from normal rat liver grows to confluence with uridine excretion beginning at about 1/2 maximum cell number (Fig. 3) as with the hamster embryo fibroblasts. In contrast to the HEF, the specific activity becomes constant at a time closer to confluence. The rate of isotope dilution goes from a value of 15 in early log to zero at confluence.

IARC-19, a malignantly transformed line, shows density inhibited growth and the culture appearance is very similar to the IARC-20 line (Fig. 4). The excretion of uridine however begins about 3 generation times prior to confluence and the specific activity continues to decrease from a rate of 4 per hr during exponential growth to a value of 0.06 per hr at maximum cell number.

IARC-20 also shows early changes in excretion (about 4 generation times before confluence) (Fig. 5) but the specific activity change becomes zero at about 1/2 maximum growth. The appearance of the culture is again not strikingly different than the normal cell population.

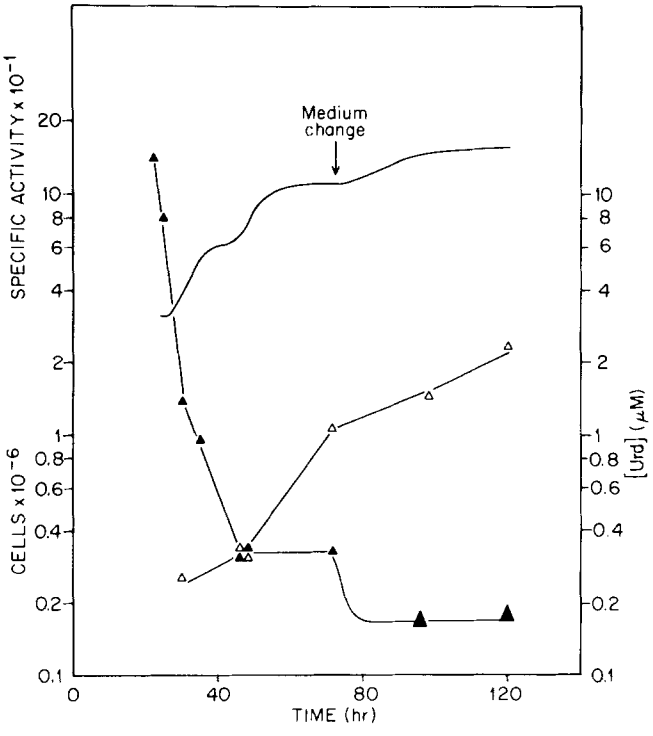

Fig. 1. Metabolism of uridine in synchronous growing hamster embryo fibroblasts. The cells were plated at 200,000 per ml from dilute suspension of 3-day-old, confluent, 11° hamster embryo fibroblasts. After 7 hr the medium was replaced with fresh medium, 0.02 μCi/ml Urd was added to 0.4 μM and left for continuous labeling. At the times indicated duplicate cell counts were made in the cytofluorograph; the medium was collected and analyzed for uridine concentration and specific activity. Cell counts ——; uridine concentration — △ —; uridine specific activity --- ▲ —. The medium was changed at 72 hr.

DISCUSSION

We have presented two examples of phenotypic variation of pyrimidine biosynthesis and excretion when comparing morphologically similar normal and transformed cell lines of fibroblasts and epithelial cells that do not exhibit overgrowth. There was no predictable form to the variation except that there was a difference in one or both properties when comparing the normal and transformed lines. V79 is a rapidly growing fibroblastic line obtained from normal hamster lung that can produce tumors in nude mice [E. Huberman, personal communication]. The putative normal counterpart is the 3rd subculture of hamster embryo fibroblasts. Of the parameters measured, only the specific activity change differs between these fibroblastic cells and that continues to decrease in the V79 cells. Considering the net synthesis of RNA that occurred, the time period, and the rate of isotope dilution in the culture, the continued decrease can arise only from a continued de novo synthesis of uridine.

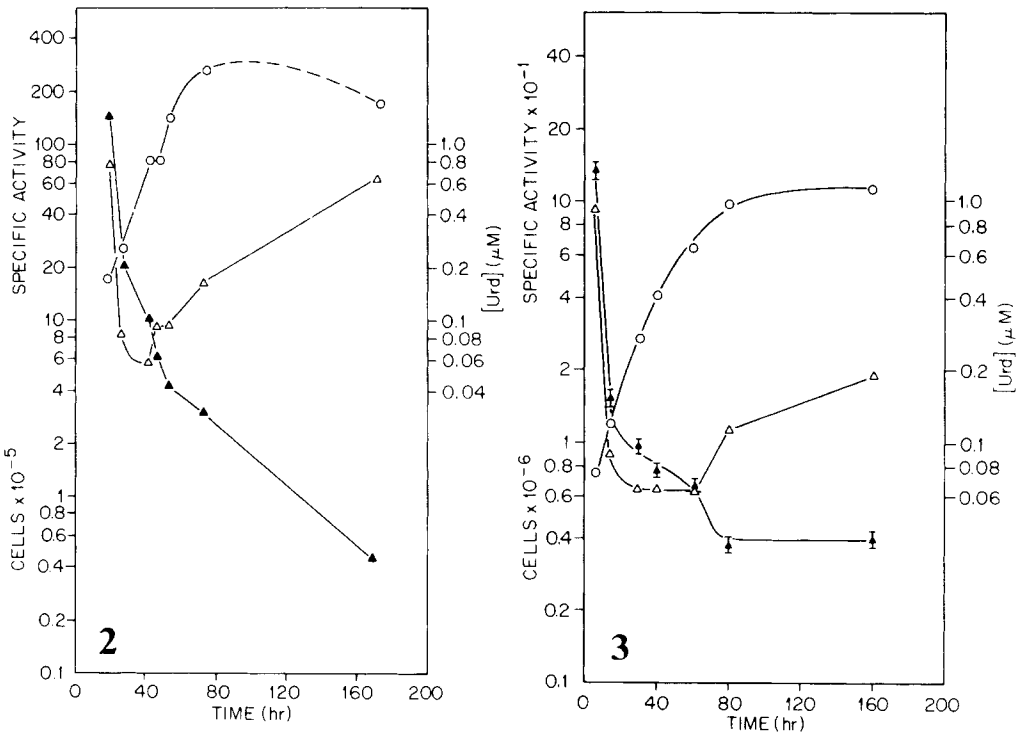

Fig. 2. Pyrimidine metabolism in V79 cells. Freshly confluent cells were collected with trypsin and seeded at 2×10^6 cells per 100 mm dish. After 7 hr the medium was replaced with fresh medium containing 0.1 μCi/ml of 0.1 μM uridine. At the indicated times the medium was collected and the cells counted. The culture fluid was analyzed for uridine concentration and specific activity. Uridine concentration — △ – –; uridine specific activity —— ▲ ——; and cell count — ○ —.

Fig. 3. Pyrimidine metabolism in IARC-20 cells. Freshly confluent cells were collected with trypsin and seeded at 1×10^5 cells per 100 mm dish. After 7 hr the medium was replaced with fresh medium containing 0.1 μCi/ml of 0.1 μM uridine. At the indicated times the medium was collected and the cells counted. The culture fluid was analyzed for uridine concentration and specific activity. Uridine concentration — △ —; uridine specific activity —— ▲ ——; and cell count —— ○ ——.

The malignantly transformed IARC lines show a more complex set of changes. IARC-19 differs from the normal IARC-20 cells in two ways: continued de novo synthesis at confluence while the control specific activity becomes constant and, a much earlier appearance of the excretion process; 20% of maximum cell growth compared to 70% of maximum cell growth in IARC-20. IARC-28 shows a difference only in the early appearance of uridine excretion at 10% maximum cell growth. At confluence de novo synthesis ends as with IARC-20. Because of potential phenotype variations it is probably better to compare cells from a common parental line. For example, the liver-derived normal epithelial line ends biosynthesis near confluence rather than at 1/2 maximum growth.

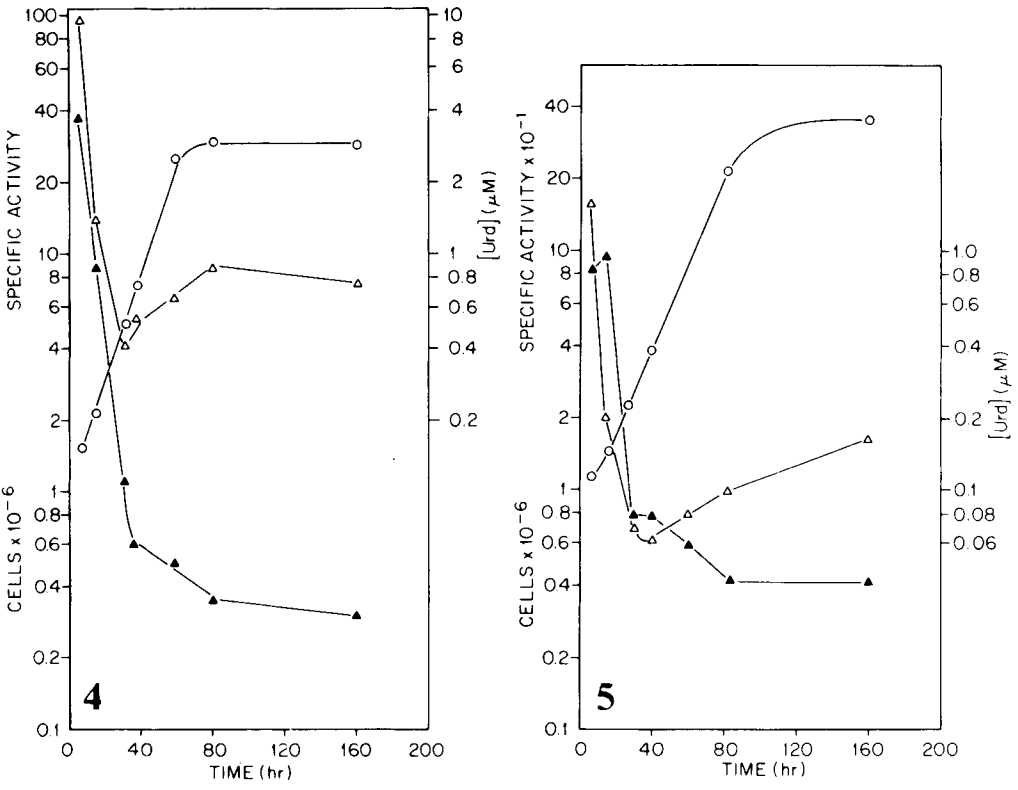

Fig. 4. Pyrimidine metabolism in IARC-19 cells. Freshly confluent cells were collected with trypsin and seeded at 1×10^5 cells per 100 mm dish. After 7 hr the medium was replaced with fresh medium containing 0.1 μCi/ml of 0.1 μM uridine. At the indicated times the medium was collected and the cells counted. The culture fluid was analyzed for uridine concentration and specific activity. Uridine concentration — △ ---; uridine specific activity —— ▲ ——; and cell count — · · · —.

Fig. 5. Pyrimidine metabolism in IARC-28 cells. Freshly confluent cells were collected with trypsin and seeded at 1×10^5 cells per 100 mm dish. After 7 hr the medium was replaced with fresh medium containing 0.1 μCi/ml of 0.1 μM uridine. At the indicated times the medium was collected and the cells counted. The culture fluid was analyzed for uridine concentration and specific activity. Uridine concentration — △ —; uridine specific activity — ▲ —; and cell count — ○ —.

The above comparisons are useful at this time primarily to illustrate that different metabolic phenotypes can be observed independent of morphological growth characteristics. The value of this system as a marker for malignant transformation will depend on many more examples where the normal and transformed cells are derived from the same cell population also under well-controlled conditions of growth.

The prospects for this type of data to become a biomarker for carcinogenesis are based on Weber's extensive studies of malignancy-linked biochemical processes [5]. Pyrimidine biosynthesis is one of several metabolic systems that expresses a class I biochemical imbalance, ie, malignancy linked. The pleiotropic mainfestations of the biochemical imbalance include increased rates of de novo synthesis of UMP and deoxynucleotides coupled with a decreased level of catabolic enzymes. This environment in the malignant cell would be expected to minimize loss of Urd from the cell and increase the biosynthetic capacity to form pyrimidine nucleotides. Our observations are consistent with this model

however, we have observed the changes in concentration and time of appearance of uridine in the medium not to be predictable based on the normal or transformed phenotype. The fibroblasts excrete almost 10 times more uridine than the liver epithelial cells at comparable growth states (see uridine concentration at confluence Figs. 1–5) while the level of excretion from the transformed cells can be the same or different than the normal counterpart (Figs. 3–5). The time when excretion begins can vary from 10% to 50% of maximum cell number. The absence of predictable change reflects the complexity of the overall metabolic balance for each cell type and the lack of specific knowledge concerning the events regulating excretion.

The excretion and specific activity changes reflect different aspects of the metabolic pathways described in Fig. 6. The amount of Urd in the medium will depend on several kinetic processes: 1) the balance of influx and efflux; 2) the rate of intracellular Urd formation; and/or 3) the appearance of new enzyme systems that would favor release of Urd from the cell. Thus, compartmentalization could contribute to the rate of Urd excretion, and to the observed specific activity. Whether or not the excretion process per se can alter observed specific activities will depend on both the size and specific activity of the presumed compartment. The fact that the different cell populations show a variable time for appearance of the excretion products would suggest that the excretion process and de novo synthesis are not necessarily coordinately linked.

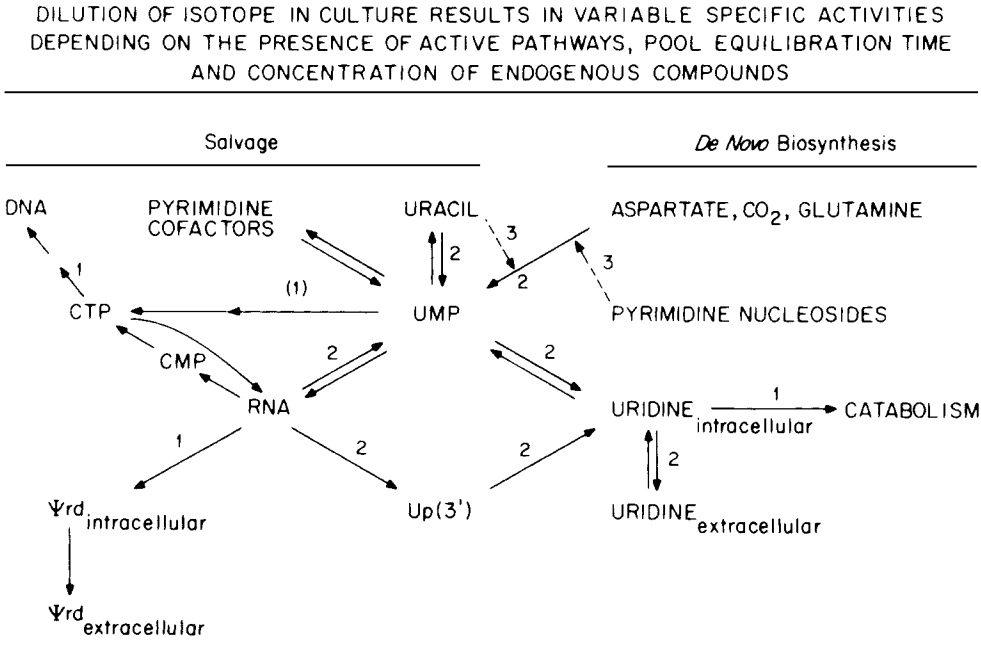

Fig. 6. Summary of uridine metabolism. The several sources of salvage and the biosynthetic pathways are illustrated. The dotted lines indicate regulatory blocks and the putative inducer. The numbers refer to the utilization of uridine. 1. Irreversible loss of uridine through conversion. The parenthesis indicates the process may not be complete. 2. Sources of metabolism dependent isotope dilution. 3. Regulation of biosynthesis.

The specific activity measurement is a function of the several variables illustrated in Fig. 7. These are kinetic factors, reflecting the rate of equilibration of the various pools, and quantitative limits, determined by the total amount of the endogenous pools (which are generally low due to overall cell growth of a factor of 10 or more), the amount of catabolism to non-Urd derivatives and the increase in Urd by de novo synthesis. The initial specific activity will be determined by the input label plus any endogenous Urd (Fig. 7). The subsequent changes will reflect equilibration with RNA breakdown products, uridylate derivatives, and endogenous uracil derived from ribo- and deoxy-ribonucleosides. The ultimate value for the specific activity of uridine will then be determined by the size of the endogenous pool, compartmentalization, the amount of de novo synthesis and irreversible losses of Urd through modification (eg, to pseudo-uridine) and other metabolic end products if these processes are not continuous through the cell cycle. The specific activity can thus become constant under one of the two conditions, compensating input of a high specific activity compartment and de novo synthesis – an unlikely prospect considering the time factors – or a cessation of de novo biosynthesis. However, in those times when the specific activity is changing there is little doubt that compartment shifts are occurring as well as dilution by de novo synthesis. The latter is known to occur in the hamster embryo fibroblasts (Uziel and Selkirk, unpublished results), in addition, the amount of the enzymes for de novo biosynthesis of pyrimidines has been observed to reach a maximum during S and then falls to a very low values at mitosis in 3T3 and HTC cells [4] which is consistent with the observed end to de novo synthesis at confluence in the hamster embryo fibroblasts and the liver cell lines.

Several parameters associated with uridine excretion have been shown to vary on comparaing normal and transformed cell populations. These include the changes in concentration of uridine in the medium, the specific activity changes and the temporal relationship of these measurements to the cell cycle and growth stage. The correlation of these events to the appearance of a new homeostatis in the transformed cells is still speculative; however, de novo biosynthesis can be monitored by measuring the specific activity of extracellular uridine. The prediction of the direction and extent of change in specific activity is not yet possible without prior specific knowledge on the levels key enzymes. On the other hand, with the use of selected substrates such as orotic acid, the empirical observations may be used to describe the intracellular state of some of the key enzymes. This latter approach may provide a basis for nondestructive (cell) assessment of transformation-dependent change in homeostatis.

$$\text{SPECIFIC ACTIVITY:} \quad \frac{\text{Ci (URIDINE)}}{\left[\text{Moles } (\text{Urd}_{\text{LABEL}} + \text{Urd}_{\text{ENDOGENOUS (MEDIUM)}} + \text{Urd}_{\text{(SALVAGE)}} + \text{Urd}_{\text{(DE NOVO BIOSYNTHETIC)}}\right]}$$

VARIABLES: A) RATE OF BIOSYNTHESIS; RATE OF RNA TURNOVER
B) CATABOLISM WILL BECOME A VARIABLE IF THE BREAKDOWN IS COMPARTMENTALIZED.

Fig. 7. Specific activity measurements. The calculation of specific activity depends on four major compartments. Two are invariant, the original label and endogenous uridine. One is variant with a fixed limit, the pool of uridine derivatives initially present in the cells. And one compartment is variant depending upon the regulatory processes operating on de novo biosynthesis.

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