We have measured the pyrimidine nucleotide contents of the culture fluid, acid-soluble fraction, and acid-insoluble fraction of cultures of hamster embryo fibroblasts (third subculture) through the final two divisions of growth in culture. The cells show a growth delay between the penultimate and ultimate division periods and a concomitant biochemical synchrony of pyrimidine metabolism.

The cells exhibit normal excretion of pyrimidine nucleosides beginning with the ultimate division cycle. This excretion results from the net breakdown of ribonucleic acid and a cell-regulated maximum for pyrimidine mononucleoside polyphosphate content. This upper limit for the pyrimidine nucleoside polyphosphate content is not a steady state phenomenon but rather an absence of both synthesis and utilization.

The hamster embryo fibroblast exhibits a directed flow of salvage uridine for ribonucleic acid synthesis. We show that de novo synthetic uridine 5'-monophosphate also can be used for ribonucleic acid synthesis without prior entry into the cytoplasmic uridine nucleoside polyphosphate pool. During attachment and first division salvage uridine does enter the cytoplasmic nucleotide pool.

The properties of the cytidine pools differ from the uridine pools in specific activity and levels of cytidine, due to turnover of the terminal C-C-A of cytoplasmic transfer ribonucleic acid and the delay in conversion of nonradioactive de novo synthetic uridine 5'-monophosphate to cytidine 5'-triphosphate.

The partial synchrony in these cultures has been used as a temporal marker of the observed events.

The periodic uptake and excretion of Urd and Cyd by cells in culture (1, 2) creates an opportunity to monitor continuously intracellular pyrimidine metabolism by measuring changes in the quantity and specific radioactivity of Urd or Cyd in the medium (3). Since the excretion product is transported by facilitated diffusion, the amount of nucleosides accumulated in the medium will depend only upon the nucleoside concentration gradient across the membrane (4). We have recently observed normal excretion of uridine, cytidine, and modified nucleosides in cell cultures (1-3) using techniques that measure picomole quantities of these nucleosides in culture fluids (5). During cell quiescence the concentration of uridine in the culture fluid can increase to 2 µM after 2 days at confluence and is not due to cell lysis (1). We observed the excretion process to be linked temporally to cells entering quiescence with concomitant RNA degradation (1).

The uptake of salvage Urd into cells has been shown to be governed by the extent of cellular phosphorylation to mono- and polyribonucleotides (6, 7). On the other hand, the relationship of the pathways for synthesis of the cytoplasmic mononucleotide pool and macromolecular RNA in most cells is less clear. Khym et al. (8) and Plagemann (9) have suggested a compartmentalization of the cytoplasmic and nuclear UXP pools such that salvage Urd preferentially enters the small nuclear pool for RNA synthesis. This hypothesis has been qualified further by Khym et al. (8) that some cells are capable of rapidly equilibrating the two intracellular mononucleotide pools into a single intracellular nucleotide pool (10, 11).

We have measured the uptake, utilization, and excretion of pyrimidine nucleosides in growing and quiescent hamster embryo fibroblasts to determine the source(s) of excreted Urd and Cyd and to establish whether Urd excretion can play a role in the equilibration of nuclear and cytoplasmic UXP pools.

**EXPERIMENTAL PROCEDURES**

**Cell Growth**—Timed pregnant (12-day) hamsters were purchased from Charles River Farms. Dulbecco's modified Eagle's medium supplemented with 0.45% glucose, and 10% fetal calf serum (heat-inactivated), 0.37% NaHCO₃, and 2 mM L-Gln was used in all experiments unless otherwise specified. Hamster embryo fibroblasts (HEF) were grown as described (1). The plating efficiency in this medium is about 60% (7). HEF (111) grew with an extended G₂ period when 11° cells, grown at 37°C, 8.5% CO₂, 98% relative humidity for 3 days, were suspended with trypsin and then seeded from dilute suspension in complete medium. The cells were suspended at 2 x 10⁵/ml and 10 ml was added to 100 mm Falcon plastic dishes, or alternatively, 3.5 ml was added to the 60 mm culture dishes (1). Confluence has been typically reached between 50 and 60 h in the 12 experiments we have conducted. The basis for synchronization appears to be hormone-dependent as Moses et al. (12) have observed mouse embryo fibroblast growth in 10% fetal bovine serum to be limited by the culture fluid hormone content. The characterization of the biochemical synchrony is documented under "Results." In recognition of the several changes in pyrimidine metabolism as the culture goes through three divisions, we have referred to these culture-age periods as early (0 to 3).
Mechanism of Pyrimidine Excretion

Asynchronously Growing Cells—This experiment illustrates the long term excretion propensity of the HEF in culture, the cessation of isotope dilution of excreted uridine, the biochemical synchrony, the complementary changes in RNA-Urd content accompanying the excretion and that these phenomena do not require cell synchrony (Fig. 1). Culture dishes (100 mm) containing 9 ml of medium were seeded with 1 ml of medium containing 2 × 10^6 cells. After 17 h of incubation the cultures were made 10 μM in [2,14C]Urd (58 μCi/μmol). Samples were collected beginning at 24 h. The remaining dishes were then washed two times with fresh medium to remove nonabsorbed [2,14C]Urd and then left to grow in 10 ml of fresh medium. The culture fluid was collected at the indicated times and processed for Ur analysis (see “Experimental Procedures”). The cells were released from the culture dishes with trypsin and counted in a hemacytometer. The remaining cells were precipitated with acid and the pellet

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**FIG. 1.** Changes in the distribution and specific activity of Urd in asynchronously grown hamster embryo fibroblasts. The growth of these cells using heat-inactivated fetal bovine serum is not stimulated further by replenishing the culture with fresh medium (arrows). Cell number (closed squares) and DNA content (closed circles) were monitored as functions of time. (—) = — = specific activity of Urd, ▲ = ▲ = RNA-Urd per cell. The bar on the abscissa describes the interval when the cells were exposed to radioactive Urd.
was dissolved and counted. The plating efficiency of this system is about 60% and the cells are confluent at 60 h. Nineteen-nanomoles of Urd were incorporated into the cells in each culture during the 7-h exposure to 10 μM [2-14C]Urd. More than 95% of the radioactive label is recoverable as nucleoside and nucleotides in the culture. After 10 days, 90 nmol of Urd has been excreted. The specific activity of extracellular Urd changes from 53 μCi/μmol at 24 h postseeding to 3.6 μCi/μmol at 46 h due to isotopic dilution by Urd derived from de novo synthesis. The specific activity then remains constant after 46 h, even with additional medium changes at 72, 140, and 190 h, due to the cessation of de novo synthesis of UMP. The long term accumulation of uridine in the medium also begins at 46 h, then increases exponentially between 46 and 55 h and then increases in a linear fashion to a maximum excretion at about 137 h. The RNA-Urd content per cell is constant from 48 h in these asynchronously grown cultures until about 53 h. At this time, the level drops about 1% and then continues to decrease in a manner complementary to the increased excretion. The cell number is maximal at approximately 60 h and remains constant throughout the experiment. The average excretion rate for Urd between 55 and 137 h is 36 pmol/min/10^6 cells.

**Biochemically Synchronous Cells**—These short term cultures illustrate the metabolic independence of the pyrimidine nucleoside polyphosphate and RNA pools and how this phenomenon contributes to the initiation of excretion of nucleosides generated from RNA. The partial synchrony is used as a tool for temporal correlation of the several events from one experiment to the next but is inadequate to provide any mechanistic information. The several stages of the cell cycle, as described under “Experimental Procedures” were observed to distribute themselves as follows in Fig. 2. S phase has been identified by the increasing amount of DNA between 27 and 35 h. Since DNA synthesis and cell division are ending, the 27- to 35-h period is characterized as a combination of M, S, and G2. Between 35 and 46 h, the DNA content per cell is double its G1 content and is characterized as G2 or G2/M. Near 46 h the cells leave G2 and enter mitosis (M). Between 46 and 60 h the cells are dividing, synthesizing RNA and protein and this time period is identified as a combination of G2, M, and early G1. After 60 h the cell number reaches a maximum, then decreases exponentially between 60 and 180 h.

**Mechanism of Pyrimidine Excretion**

### Figure 2. Results of a typical experiment to measure changes in the accumulation and specific activities of pyrimidine nucleosides in each of the following pools: culture fluid, cellular nucleotides, RNA and DNA. A. The accumulation of pyrimidine nucleosides in the culture and the changes in specific activity of Urd isolated from the culture fluid and the hydrolysis products of the cellular nucleotide pools and RNA: Urd was isolated as described under “Experimental Procedures.” HEF (7 × 10^6) were seeded in 60-mm dishes and were exposed to 1 μM [2-14C]Urd from 7 to 72 h postseeding. The intracellular and the extracellular Urd pools had equilibrated prior to the 24-h time point. As the cells begin their penultimate division in the culture the two pools are diluted at different rates by nonradioactive Urd until 46 h, when the UXP pool reaches its lowest value, and 48 h when the medium Urd attains its lowest value. The cells are shown to grow to confluence at about 60 h in a partially synchronous manner on the basis of repeated experiments where data were collected at those time intervals. The accumulation of Urd and Cyd in the total culture is 90% complete at 48 h. The letters and bars at the top of the figure indicate the overlapping stages of the cell cycles present at the designated time period. Cell number × 10^6, O—O; Urd specific activity in the culture fluid, V—V; Urd specific activity from the cellular nucleotide fraction, A—A; total pyrimidines in the culture, nanomoles, [—]. B, changes in Urd concentration in the medium and the specific activity and total synthesis of DNA in synchronous culture. These are the same cells as in A. The accumulation of Urd in the medium reaches a minimum at 46 h and then increased accumulation (excretion) begins after that time. The specific activity of DNA-Thd is constant from 35 to 72 h. The total accumulation of radioactivity in DNA is 99% complete at 35 h and does not reinitiate exponential accumulation in the culture even though the cell number doubles. Cell number × 10^6, O—O; Urd concentration in culture fluid (micromolar), [—]. C, changes in the accumulation of pyrimidine nucleosides in RNA and the specific activity of RNA-Urd. These data are also from the analyses of the experiment described in A. RNA pyrimidines reach a maximum in the culture at 48 h with a 30% increase occurring between 46 and 48 h. Of the total drop in RNA-Urd specific activity between 35 and 72 h, 30% occurs between 35 and 46 h, 50% between 46 and 48 h, and the final 20% between 48 and 72 h. The curves are drawn to reflect the accumulated experience from three experiments. Cell count × 10^6, O—O; RNA-Urd specific activity, [—]; and total RNA pyrimidines (nanomoles), [—].
RNA synthesis ends, and there is no reinitiation of DNA synthesis. The quiescent cells are thus in an extended G₀ or G₀ state. The distinction between an extended G₂ and G₂₀ is operationally difficult to prove and so this region is identified as the G₀G₂₀ state (15, 16). The cell number doubles from the G₂₀ period to the G₀G₂₀ period. This empirical characterization of the biochemical cell growth pattern has been useful as a tool to emphasize the biochemical differences between the penultimate and ultimate growth cycles.

Changes in Specific Activity in Monomeric Pyrimidines—In Fig. 2A, the continuous presence of labeled Urd in both the cell and culture fluid, beginning 7 h postseeding time, shows changes in specific activity due to dilution of both the culture fluid and acid-soluble Urd pools by de novo synthetic UMP. These two pools are diluted at different rates during the penultimate cell cycle. At the G₂M/G₁ boundary, the specific activity of each of the two pools reaches a minimum (medium at 48 h, acid-soluble at 48 h) and remains constant thereafter. At 48 h, the accumulation of Urd and Cyd in the whole culture is 80% complete. The continuing differences in specific activity between the culture fluid and acid-soluble pools in the presence of a potential further 10% dilution by de novo synthetic UMP indicates that the utilization of the de novo synthetic UMP must be linked to some intermediate step before entering either the culture medium or cellular pool of Urd.

Excretion of Uridine and DNA Synthesis—The temporal changes in DNA accumulation and Urd excretion are shown in Fig. 2B. Uridine is taken up by the cultures from the time of its addition to the cell culture fluid. In the period prior to 24 h, Urd enters both the acid-soluble and acid-insoluble pyrimidine pools (see Fig. 2 A and C). Between 40 and 46 h, the concentration of extracellular Urd reaches a minimum indicating an end to the net salvage of this nucleoside by the cells. At 46 h, a net efflux (excretion) of Urd begins and continues to the end of the experiment.

DNA synthesis, as measured by alkalai stable, acid-insoluble macromolecules, occurs during the penultimate cell cycle (24 to 46 h) from a single pool of Thd that has a constant specific activity from 35 to 72 h. The accumulation of DNA ends between 35 and 46 h and does not reinitiate even though the cells progress through their ultimate division between 46 and 60 h which is consistent with entering a G₀G₂₀ state.

Changes in RNA-Urd—Fig. 2C illustrates the change in RNA-Urd specific activity accompanying the accumulation of RNA during cell growth. The maximum accumulation of Urd plus Cyd in RNA occurs at 48 h after an approximate doubling of RNA content from the 35-h value. The specific activity of the RNA-Urd during this period, however, decreases by 2.4-fold. This isotope dilution of RNA-Urd is accompanied by an increase in excreted Urd into the culture fluid (Table I) (1). Urd is found only in intact RNA and is not made at the mononucleotide level so that the increased excretion of Urd can result only from RNA breakdown. Thus, during the ultimate cycle, entry into early G₁ is accompanied by a doubling of cell number, a doubling of RNA, increased de novo synthesis of UMP and turnover of a fraction of RNA synthesized earlier. This results in a significant lowering of the RNA-Urd specific activity due to incorporation of nonradioactive UMP. As the cells progress from early G₁ to G₂M, there is an increasing accumulation of pyrimidine nucleosides in the medium due to continuing net breakdown of RNA since the UXP are not catabolized in this stage of progression to G₂M (Table I). The complementary decrease of 50% in RNA-Urd content that begins with the initiation of excretion and continues for 8 days, further supports the idea that RNA degradation continually contributes to the excretion of nucleosides. Since ribosomal RNA is almost 80% of the cellular RNA it must contribute to the excreted nucleosides. This is also consistent with observations of ribosomal RNA degradation by hamster embryo fibroblasts entering quiescence (17).

Excretion Mechanism—Excretion begins at the ultimate division cycle a time when there are several changes in the properties of the UXP pool. The total UXP in the cells doubles in content between 35 and 46 h, then reaches a maximum at some time prior to 46 h and then remains constant through the late culture-age period (Fig. 2B, Table I). This cellular nucleotide pool must then be maintained either by a steady-state flow from precursors and into RNA products or maintained by metabolic isolation with no significant influx or efflux of nucleotides. In either case, the influx of Urd into the nucleotide pool from net RNA breakdown cannot be retained, otherwise the total cellular UXP would increase. The transport mechanism for pyrimidine nucleosides is diffusion-controlled (4) so the nucleoside concentration gradient across the membrane determines both the net flow and the direction of flow (1).

Flow of Pyrimidine during Culture of HEF—The pathway of maintenance of the constant size of the cellular UXP pool appears to be through a metabolic isolation of the cytoplasmic UXP pool. Equation 1 illustrates the steady state flow of nucleosides (tides) through the UXP pool and into RNA accumulation or, in reverse, to nucleoside excretion. This flow of pyrimidines predicts continuous dilution of all nucleotide pools by de novo synthesis of UMP. However all specific activity

\[
\text{Urd} \rightarrow \text{UMP} \leftarrow \text{UXP} \rightarrow \text{RNA}
\]

values should converge when de novo synthesis of UMP ends. In fact, the extracellular Urd pool and intracellular UXP maintain constant and different specific activities while the de novo synthetic UMP content of the culture doubles in value (Fig. 2, A and C). The Urd pool is the only component whose specific activity decreases (Fig. 2C). Thus, we observe the de novo synthetic UMP can bypass the cytoplasmic UXP pool and be incorporated directly into RNA. A similar argument

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* The time after seeding (in hours) (within 10 min).
* A range of symbols are given to reflect the presence of cells in different phases of the cycle.
* Cell number × 10⁶ in a 60-mm dish (± 3%).
* The total nanomoles found in Fraction I (± 3%) (see “Experimental Procedures”).
* The specific activity (Sp. Act.) given in Curies per mol (± 3%).
* Net change in nanomoles of Urd excreted (±0.05 nmol) per culture.
RNA during early culture age. The specific activity of extracellular Urd also for the flow of extracellular Urd into the several nucleoside pools. One branch is for the flow of Urd into nuclear UXP and into RNA during the early culture-age period and the second branch is for the differential flow, during the middle culture-age, of de novo synthetic UMP into either RNA or into cytoplasmic UXP. This latter pathway to UXP is closed during the late stages of culture growth by the metabolic isolation of the cytoplasmic UXP pool. The size of the UXP pool remains constant since there are no further pressures to deplete it through conversion to deoxynucleotides or through conversion to RNA.

Properties of Cytidine Nucleotides—The properties of the Cyd pools are similar to those of Urd in these cultures with two significant differences. First, the specific activity of the Cyd derivatives is always higher due to prior conversion of UTP to CTP with continuing dilution of the UTP pools by de novo synthetic UMP during the early and middle culture-age periods. During the metabolic isolation of the UXP pool, a continued decrease of CXP specific activity due to the conversion of UTP to CTP was not seen. The CXP also acts like the UXP in the period through 48 h and does not change its level or specific activity until the period 48 to 72 h when there is a small but significant increase in CXP specific activity and a drop in the quantity of CXP (Table I). These changes are consistent with a partial equilibration of CXP with the higher specific activity cytoplasmic RNA and by a loss of CXP through dephosphorylation and excretion or by conversion to RNA. The cytoplasmic saturation of the tRNA.C-C-A terminus is well known (18) and cytoplasmic CTP does equilibrate with tRNA (19).

FIG. 3. The flow of pyrimidines through hamster embryo fibroblasts during growth and quiescence. This diagram illustrates the partial separation of the several intracellular nucleotide pools based on our and others (8, 9) observations. The flow of Urd into or out of the cell is governed by the concentration gradient across the plasma membrane. During the early culture age, this Urd can enter both the UXP pool (---) or RNA. During this period and during the penultimate division cycle, DNA synthesis occurs, de novo synthetic UMP can go to either the cytoplasmic UXP pool, the extracellular Urd or RNA. The parentheses around direct conversion of UMP to RNA indicates an uncertainty whether UMP goes directly to nuclear UXP or by way of Urd. During the ultimate division leading to quiescence, 1) UMP can go directly to RNA without entry into the cytoplasmic UXP; 2) RNA turnover (nuclear or cytoplasmic) released intermediate nucleotides (or UMP) that are converted to Urd can diffuse into the medium but do not enter the cytoplasmic UXP; and 3) there may be some equilibration of cytoplasmic CXP with cytoplasmic RNA (---). During quiescence (through 72 h), the extracellular Urd and the cytoplasmic UXP pool do not mix with each other, there is no DNA synthesis and there is net RNA hydrolysis giving rise to excretion of Urd and Cyd.

Several parameters have been shown to govern the size and turnover of the cytoplasmic pyrimidine nucleoside polyphosphate pool and the RNA-pyrimidine pool. These parameters include a cell determined maximum pyrimidine mononucleotide content (Table I) that results in the excretion of the excess nucleoside (Fig. 2B) generated during net RNA breakdown (1). In addition, there is a cell culture-age dependence on the entry of exogenous Urd into the cytoplasmic UXP pool and the metabolic isolation of the cytoplasmic UXP pool. A clear temporal separation of these events is not present in all cells since the excretion of Urd and the change in rate of isotope dilution of extracellular Urd have been observed to occur in both the early and late stages of several cell lines in culture such as V-79, hamster lung fibroblasts, or rat liver normal and chemically transformed epithelial cells (3).

Because of temporal differences, the observed flow of radioactive Urd into the several nucleotide pools and into RNA (8, 9, 11, 20-23) may also reflect the effects of specific experimental design, rather than nucleotide pool utilization. Our observations support the concept of compartmentation of RNA precursors (7-9) and also show how excretion and metabolic isolation of the cytoplasmic UXP pool (CXP) can create a barrier that separates the compartments in the cells we have studied (Fig. 3).

Goody and Elem (23) observed the absence of continued conversion of cytoplasmic UTP to RNA in 6C3HED cells during the pulse-chase period and concluded that only a part of the cytoplasmic UTP is functional during RNA synthesis. However, Plagemann (9) found tracer quantities of isotopic UDP to preferentially enter RNA, bypassing the cytoplasmic UTP pool. On the basis of these observations, and, since de novo synthesis of UMP is of cytoplasmic origin, one might speculate that these cells can only utilize salvage Urd for RNA synthesis. We have now shown that de novo synthetic UMP can also bypass the cytoplasmic UTP pool and be incorporated into RNA. This occurs partially during the middle culture-age period and completely during the late culture-age (Fig. 2). Thus, the metabolic isolation of the cytoplasmic UXP pool creates a barrier to utilization of that cytoplasmic pool while RNA synthesis continues to utilize either or both salvage Urd or de novo synthetic UMP. Additional support for this idea is found in Kihm et al. (8) where acid extracts of HeLa cell cultures exposed to labeled Urd show UTP and UXP specific activities to be at least 4-fold larger than the specific activity of UMP.

While this study has been primarily concerned with Urd utilization, the properties of the Cyd nucleotides appear to follow the same overall pathway with the differences discussed under "Results" that include cytoplasmic RNA synthesis (18). This complex utilization of CTP for both nuclear and cytoplasmic RNA synthesis may compromise the use of the UMP/CMP ratio (16, 17) as a criterion of number of cellular nucleotide pools. In view of the complex feedback regulation of deoxy- and ribonucleotides on NTP synthesis in growing and quiescent cultures (16, 24-29), study of the cytoplasmic pool replenishment may give further insight into the process of dNTP synthesis and initiation of DNA synthesis in intact cells.
In conclusion, we have observed that the cytoplasmic pyrimidine nucleotide polyphosphates became metabolically isolated in HEF cells during late cell-culture age but that the cytoplasmic de novo synthetic UMP was not a part of the isolated nucleotides. Thus, both salvage Urd and de novo synthetic UMP were not a part of the isolated nucleotides. Thus, both salvage Urd and de novo synthetic UMP can bypass the cytoplasmic UXP pool and be utilized for RNA synthesis. We have also shown excretion of pyrimidine nucleosides results from concurrent metabolic isolation of the cytoplasmic pyrimidine nucleoside polyphosphates, with a maximum cellular mononucleotide content, and a net breakdown of RNA to pyrimidine nucleosides.

The generalized compartmentalization of RNA synthesis in eukaryotic cells (8), now also observed in HEF, appears to result from concurrent metabolic isolation of the cytoplasmic nucleoside polyphosphates and RNA synthesis. Further studies on the excretion and turnover of the cytoplasmic nucleoside polyphosphates are needed to establish how salvage Urd enters the UXP pool in early culture-age. It would be interesting to know whether those cells that can equilibrate nuclear and cytoplasmic nucleotide pools (8,10,11) use RNA turnover and excretion as the equilibration pathway.

Acknowledgments—We appreciate the excellent technical assistance of A. J. Bandy and K. Dearstone.

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