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EVIDENCE THAT INTRACELLULAR SYNTHESIS OF 5-FLUOROURIDINE-5'-PHOSPHATE FROM 5-FLUOROURACIL AND 5-FLUOROURIDINE IS COMPARTMENTALIZED

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SUMMARY: L1210 cells were exposed to equitoxic concentrations of [^{14}C]5-fluorouracil and [^{3}H]5-fluorouridine for 4 hours. The RNA from these cells was separated into cytosolic and nuclear fractions, and then further fractionated by chromatography on poly-U Sepharose, Sephadex G-200 and DEAE-cellulose. The ratio of tritium to carbon-14 incorporated into various species of RNA differed by as much as 6-fold, indicating that the respective 5-fluorouridine-5'-monophosphates synthesized from the two precursors are localized in separate pools that do not mix rapidly.

INTRODUCTION: Incorporation of the fluorinated pyrimidines FUra and FUrd into RNA with consequent effects on the processing and functioning of some species of RNA is thought to be one of the primary mechanisms of cytotoxicity and anti-tumor activity of these compounds (1). In an earlier study, we found that Urd even at the appreciable level of 10^{-4}M did not protect L1210 mouse leukemic cells from the toxic effects of FUra (2). This result is at first glance not consistent with an RNA-directed mechanism of toxicity for FUra, because nucleotides generated from Urd would be expected to compete effectively with the analogous fluorinated nucleotides generated from FUra for the active sites of enzymes involved in RNA synthesis. However, further experiments showed that the incorporation into RNA of [^{3}H]FUra was not prevented or even decreased by a 100-fold greater concentration of Urd (2), suggesting thereby that FUMP generated from FUra and UMP generated from Urd do not compete for the same enzyme molecules during RNA synthesis. Compartmentalization of nucleotide pools could account for such a phenomenon. It has been previously proposed that Urd nucleotides are located in at least two distinct pools, one of which is rapidly utilized for

Abbreviations: FUra, 5-fluorouracil; FUrd, 5-fluorouridine; Urd, uridine; FUMP, 5-fluorouridine-5'-monophosphate; UMP, uridine-5'-monophosphate; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

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RNA synthesis, while the other, larger pool, is only slowly utilized, and moreover is fed directly by exogenously administered Urd (3). Thus, since FUra and FUrd are activated by different enzymes, it is possible that their respective ribose monophosphates are preferentially generated in separate cellular compartments, the contents of which do not equilibrate rapidly.

In order to test this possibility, we exposed L1210 cells simultaneously to equitoxic levels of [^{14}C]FUra and [^{3}H]FUrd, and then performed various fractionations of the labeled RNA species of these cells. The principle behind this study is that if the [^{14}C]FUMP generated from the [^{14}C]FUra and the [^{3}H]FUMP generated from the [^{3}H]FUrd rapidly enter the same precursor pool and equilibrate, the isotope ratio will be the same throughout the fractionation profiles of the RNA, whereas if compartmentalization of the two nucleotides occurs, the incorporation of the two isotopes into all species of RNA will not be equal. The data show substantial differences in isotope ratios among different RNA species, and therefore support the idea that nucleotides derived from FUra and FUrd are compartmentalized.

MATERIALS AND METHODS: All chemicals were of the highest grade available. Poly-U-Sepharose was purchased from Sigma Chemical Co., St. Louis, Missouri. [6-^{3}H]FUrd (18 Ci/mmol) and [^{14}C]FUra (58 mCi/mmol) were obtained from Moravek Biochemicals, Brea, California. Fetal calf serum and RPMI-1640 medium were obtained from GIBCO.

Cell Growth and Treatment - The L1210 mouse leukemia cell line is routinely maintained in our laboratory as previously described (4). The cells were suspended in RPMI-1640 medium containing 10% dialyzed fetal calf serum and incubated in roller bottles for 4-7 days until the culture contained 10^6 cells per ml. For each experiment, 10^8 cells were harvested by centrifugation at 800 rpm, and resuspended in 5 ml of fresh medium containing 0.3 μM [^{3}H]FUrd and 20 μM [^{14}C]FUra for 4 hr at 37°C. Incorporation of both radiolabels into cellular macromolecules was assayed by filtering aliquots of the cell suspension through type E glass filter disks (Gelman), washing the disks with 2 x 1 ml of PBS followed by 6 x 1 ml of 10% trichloroacetic acid.

Separation of Nuclear RNA and Cytosolic RNA - The cells were lysed and the cytosolic and nuclear RNA's isolated essentially according to the procedure described by Lee et al. (5). About 1 mg of cytoplasmic RNA was obtained from 10^8 cells.

Poly-U Sepharose Chromatography - The column material (1.5 g) was swollen in 1.0 M sodium chloride, pH 7.5, for 5 min, then washed with 150 ml of 0.1 M sodium chloride, pH 7.5. After the material was packed into a column, it was further washed with 150 ml of eluting buffer (10 mM potassium phosphate, pH 7.5, 10 mM EDTA, 0.2% SDS, in 90% formamide). The column was then equilibrated with 150 ml concentrated salt buffer (0.7 M sodium chloride, 50 mM Tris, pH 7.5, 10 mM EDTA, in 25% formamide). The samples of RNA to be applied were dissolved in a solution containing 1% SDS and 30 μM EDTA, and then diluted 5-fold with the concentrated salt buffer. Elution was carried out with 25 ml of the concentrated salt buffer, followed by 25 ml of the elution buffer.

Sephadex G-200 Gel Filtration - The gel was swollen according to the manufacturer's instructions and packed into a 1.5 x 70 cm glass column. The column was equilibrated with 170 ml of a buffer containing 20 mM potassium phosphate, pH 7.5, 20

mM EDTA, and 0.1 M sodium chloride. Elution was carried out with this same buffer at a flow rate of 20 ml per hr, collecting 5 fractions of 5.4 ml followed by 75 fractions of 1.8 ml each.

DEAE-Cellulose Chromatography - The DEAE-cellulose was prepared according to Rustum and Schwartz (6) and packed into 0.9 x 3.5 cm plastic columns. Elution was carried out with a linear gradient of 0.03 M to 1 M sodium chloride in 300 ml of Tris buffer, pH 7.6.

Counting of the Radioactivity - Samples of 1.0 ml were diluted with 9 ml of RIA-Solve II (Research Products International) and counted in a Beckman LS 9000 liquid scintillation counter.

RESULTS: Table 1 shows the specific incorporation of [^{14}C]FUra and [^{3}H]FUrd into cytosolic and nuclear RNA and the relative isotope ratios after a 4 hr incubation of L1210 cells with equitoxic levels of both drugs simultaneously. FUra was incorporated to about the same extent into both types of RNA, whereas the incorporation of FUrd was 1.3-fold greater into nuclear RNA than into cytosolic RNA. The molar incorporation of FUra was 45-fold and 62-fold greater than that of FUrd into nuclear RNA and cytosolic RNA, respectively.

Poly-U-Sepharose Fractionation - Poly-U Sepharose separates poly-A RNA from RNA's not having poly-A sequences, among which are included rRNA and tRNA (6). Fig. 1A shows that cytosolic poly-A RNA had an average isotope dpm ratio (^{3}H/^{14}C) of 3.5, whereas the isotope ratio in the non-poly-A RNA varied from 2.5 to 6, indicating non-homogeneous incorporation into the multiple RNA species located in this fraction. The poly-A RNA fraction of nuclear RNA had an isotope ratio of 2.0, whereas the non-poly-A RNA had an isotope ratio of 4.0 (Fig. 2A).

Sephadex G-200 Fractionation - The Sephadex fractionation profile of cytosolic RNA shown in Fig. 1B indicated three distinct classes of RNA's with regard to isotope ratios. The high molecular weight species (fractions 4-14) had an isotope ratio varying

Table 1

Incorporation of [^{14}C]FUra and [^{3}H]FUrd into total nuclear and total cytosolic RNA of L1210 cells

	[^{3}H]FUrd (dpm/A_{260})	[^{14}C]FUra (dpm/A_{260})	^{3}H/^{14}C ratio
nuclear RNA	34,409	4885	7.04
cytosolic RNA	26,622	5117	5.20

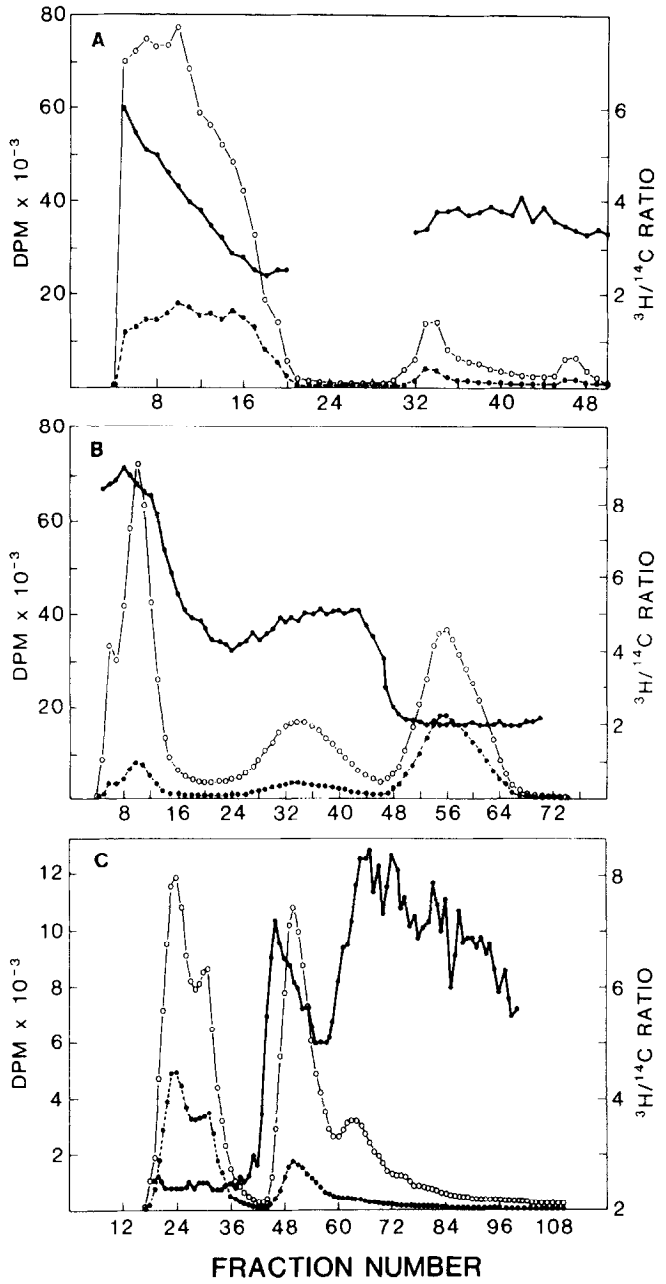

Figure 1. Fractionation of cytosolic RNA containing incorporated [^{14}C]FUra and [^{3}H]FUrd by chromatography on columns of a) poly-U Sepharose, B) Sephadex G-200, C) DEAE-cellulose: ^{3}H(—○—); ^{14}C(···●···); ratio of ^{3}H/^{14}C(—●—).

between 8 and 9, the intermediate peak (fractions 26-42) had an isotope ratio of 5, whereas the third peak had a very constant isotope ratio of 2. The fractionation profile of nuclear RNA (Fig. 2B) was less defined than that of the cytosolic RNA, but

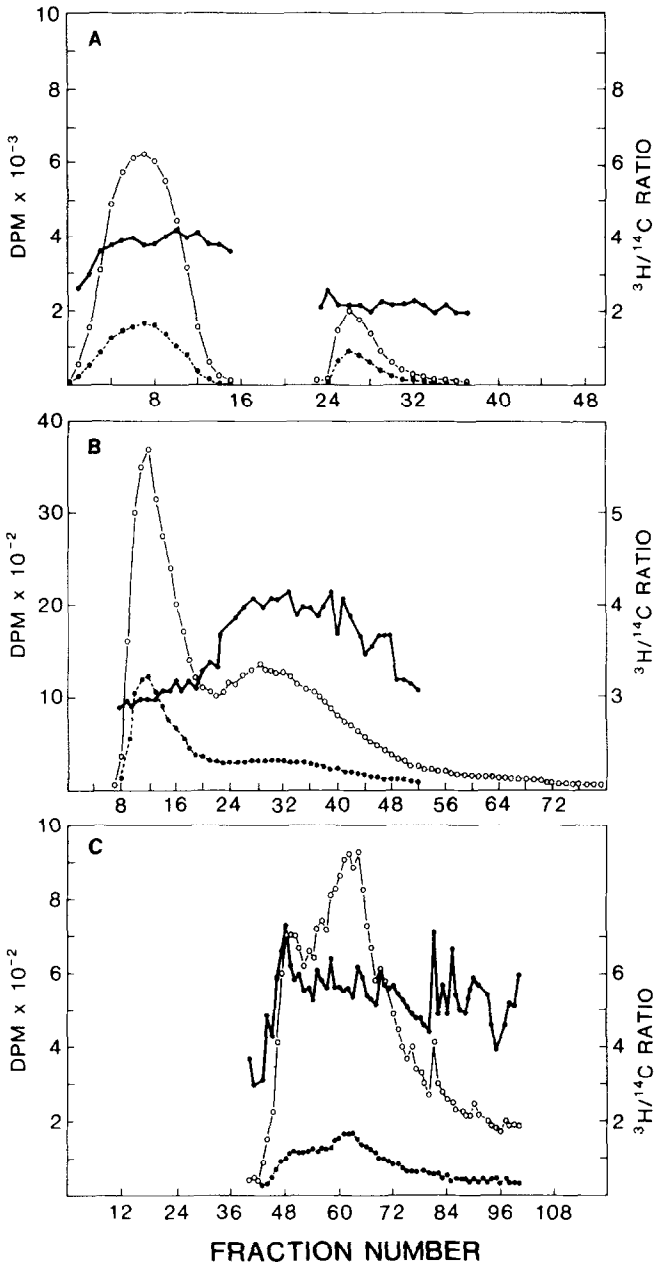

Figure 2. Fractionation of nuclear RNA containing incorporated [^{14}C]FUra and [^{3}H]FUrd by chromatography on columns of A) poly-U Sepharose, B) Sephadex G-200, C) DEAE-cellulose: ^{3}H(—○—); ^{14}C(···●···); ratio of ^{3}H/^{14}C(—●—).

nevertheless appeared to show two distinct isotope ratios of 3 in the high molecular weight peak and about 4 in the second peak.

DEAE-Cellulose Fractionation - Chromatography of the cytosolic RNA on DEAE-cellulose resulted in four distinct peaks (Fig. 1C). The first two peaks had an isotope

ratio of 2.5, the third peak had an isotope ratio varying between 5 and 6.5, whereas the isotope ratio in the fourth peak varied between 7 and 8. The apparent variability of the isotope ratio in the latter peak is probably attributable to the low number of carbon-14 counts. In contrast to the cytosolic RNA, DEAE-cellulose chromatography of the nuclear RNA did not produce any distinct separations, and thus the isotope ratio is about the same throughout the profile (Fig. 2C).

DISCUSSION: The variability in the ratio of tritium to carbon-14 found among the various species of RNA, up to 6-fold in some cases, shows that FUMP generated from FUra is not incorporated into RNA identically to FUMP generated from FUrd. This observation supports the hypothesis that two distinct pools of FUMP are created which do not mix rapidly and retain their distinctiveness over long periods of time as RNA is being synthesized. We believe that these results cannot be explained simply on the basis of rate phenomena. Even if the isotope ratio of the FUMP pool were constantly changing because of different rates of synthesis of FUMP from the two precursors, a rapid mixing of the two labeled pools of FUMP would at any given time point result in the same isotope ratio being incorporated into all types of RNA, regardless of the rate of synthesis of the particular RNA species.

A probable cause of functional compartmentalization of nucleotide pools in cells is the existence of multi-enzyme complexes that are capable of directly channeling substrates from one enzyme to another in a sequential metabolic pathway (7). Since efficient functioning of such an apparatus requires that the metabolites not be able to diffuse away readily from their point of generation, it follows that exogenous compounds would not be able to penetrate easily into these compartments. The differential incorporation of FUra and FUrd that we report here is consistent with this hypothesis. It has been known for some time that a number of enzymes of the de novo pyrimidine nucleotide biosynthetic pathway are aggregated in multi-enzyme complexes (8), among which is orotate phosphoribosyl transferase, the enzyme that converts FUra to FUMP (1). FUrd, however is converted to FUMP by uridine kinase (1), which is a salvage enzyme not known to be associated with the multi-enzyme complexes of de novo synthesis.

The question is raised as to how the compartmentalization of nucleotide metabolism would affect studies of the mechanisms of action of the fluoropyrimidines. Among the consequences of this phenomenon might be that FUra and FUrd exert RNA-directed cytotoxic effects at different sites in the cell's RNA. It also seems likely that compartmentalization would have to be taken into account in rescue and protection studies, which are often designed and interpreted on the basis of a priori assumptions about the site of competition between the rescuing agent and a drug metabolite.

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