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Assay and Time Course of 5-Fluorouracil Incorporation into RNA of L1210/0 Ascites Cells *in Vivo*

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SUMMARY

A method for determination of levels of incorporation of nonradiolabeled 5-fluorouracil (FUra) into RNA (F-RNA) in tissue samples is shown to be applicable to tissues *in vivo*. BDF_1 mice bearing L1210 ascites cells were injected intraperitoneally with [^{14}C]FUra, 100 mg/kg. The time course of F-RNA levels in L1210 cells was determined by following the radiolabeled drug, and by NaB^3H_4 labeling of isolated and derivatized nucleoside. RNA ribonucleotides were obtained by KOH hydrolysis of perchloric acid precipitates of cell sonicates. FUMP nucleotides were separated from remaining nucleotides by DEAE-cellulose chromatography. FUMP fractions were treated with alkaline phosphatase, and FUrd was separated from non-FUrd nucleoside contaminants by additional DEAE-cellulose chromatography. FUrd was quantitated by periodate oxidation of ribose and NaB^3H_4 reduction of the resulting nucleoside dialdehydes. Isolation of tritiated FUrd-trialcohol from remaining tissue contaminants and background radioactivity was done by silica gel thin layer chromatography. Comparison of results obtained by isolation of [^{14}C]FUrd with results of NaB^3H_4 labeling of the same samples showed parallel results with comparable biological standard deviations, although the tritium method consistently gave slightly lower values. The peak level of F-RNA at 3 hr was 1 base substitution per 174 normal nucleotides. The level of F-RNA after 3 hr declined slowly, so that at 96 hr there still remained 1 FUra base per 597 normal nucleotides. Serial determinations of RNA content showed marked decreases, on the basis of either DNA or protein level, that continued up to 96 hr after FUra administration. These biochemical effects are among the most prolonged reported for FUra, suggesting the possibility that F-RNA represents a storage compartment for release of toxic metabolites and emphasize the need for additional study of RNA effects at long time points. Our method for assay of F-RNA appears to be suitable for study of biopsy specimens of tumors and normal tissues following nonradiolabeled FUra administration.

INTRODUCTION

The synthesis of the antitumor agent FUra[3] was largely based on the rationale that, as an analog of Ura, it would inhibit thymidylate synthetase and be readily incorporated into newly synthesized RNA (1). This has been found to occur in all living organisms and tissues examined (2, 3), although the hope that cancer cells would show greater F-RNA levels than found in normal cells has not yet been shown. The presence of FUra in RNA results in profound inhibition of ribosomal maturation (4), inhibition of post-transcriptional modification of transfer RNA (5), alterations in the incorporation of radiolabeled precursors into RNA (6), and impairment of polyadenylic messenger RNA synthesis (7). Other biologic effects of FUra related to alterations in cellular RNA include nucleolar enlargement (8) and phenotypic mutation caused by incorporation of the FUra metabolite, 5-fluorocytidine, into RNA (9).

FUra is widely used systemically against a range of

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[3] The abbreviations used are: FUra, 5-fluorouracil; F-RNA, FUra incorporated into RNA; FUrd, 5-fluorouridine; FUMP, 5-fluorouridine monophosphate; IP, intraperitoneal.

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human solid cancers, and is most effective in topical application for treatment of low grade superficial malignancies. We have recently reported on the effects of intravenous FUra in causing inhibition of thymidylate synthetase activity in tumors of patients with bowel or breast cancer (10). Patients with superficial transitional cell carcinoma of the bladder respond to intravesical FUra combined with allopurinol protection, and tissues of such patients also have been studied for FUra effects (11); although evidence of significant thymidylate synthetase inhibition was found, study of F-RNA levels was not done because of clinical problems in giving radiolabeled drug. Assay of F-RNA would seem particularly desirable in the situation of topical use, since F-RNA levels are probably linearly related to FUra dose (12, 13) and duration of exposure (14, 15), and high levels of F-RNA have been related to its toxicity to normal tissues (16).

The method of the present report was therefore developed for assay of nonradiolabeled F-RNA in tissue samples. Our procedure relies on multistep isolation of fluoropyrimidines from normal nucleic acid constituents and conversion of FUrd to its isotopically labeled nucleoside trialcohol. Periodate oxidation of vicinal hydroxyl groups in ribose compounds excludes deoxyribose derivatives and permits NaB^3H_4 reduction of the resulting dialdehydes (17). This approach has led to methods for end group analysis of polynucleotides (18) and base composition and sequence analysis of RNA (19). In the latter, two-dimensional chromatography and film techniques (fluorography and autoradiography) have been used for separation of derivatives of nucleosides, including FUrd. For relevance to experimental chemotherapy, however, we found it necessary to develop separation methods that could allow detection of 1 FUra base substitution per 20,000 or more normal bases in RNA.

The applicability of this method to study of L1210 cells *in vivo* is demonstrated in a time course determination of F-RNA levels up to 96 hr. A surprising result was the increasingly high percentage of total FUra metabolites present as F-RNA with time, which provides evidence for the possibility that F-RNA may provide a depot storage form of FUra (20) that could contribute to the persistence of FdUMP levels noted by several investigators at long times after FUra administration (20, 21).

MATERIALS AND METHODS

Materials. All chemicals used were of the highest available grade and were used without further purification. DEAE-cellulose was purchased from Eastman Organic Chemicals; alkaline phosphatase (bacterial origin, 27 IU/mg) was from Worthington Diagnostic; FUra was from Aldrich; FUrd and FUMP were from Calbiochem; other, nonradiolabeled ribonucleosides were from Sigma; [2-^{14}C]FUra, [2-^{14}C]FUrd, and [2-^{14}C]FUMP were from Moravek Biochemicals, Brea, CA. NaB^3H_4 (6.5 Ci/mmol, lot TRK-45) was purchased from Amersham and diluted as follows: the content of a 100-mCi vial was dissolved in 1.0 ml of 0.5 N NaOH, divided in 10-μl aliquots, lyophilized in ampules, sealed under vacuum, and kept at −20°. A working solution was prepared by dissolving the content of one ampule in 2.0 ml of 0.5 N NaOH and adding to it 30 mg of unlabeled $NaBH_4$ (Aldrich) giving a specific activity of 1.3 mCi/mmol. All operations involving NaB^3H_4 were carried out in a hood. Samples were counted using RIA-Solve II cocktail in a Beckman LS 9000 liquid scintillation spectrophotometer that was equipped with software programs for separation of ^{14}C and ^{3}H radioactivity using external standard ratios.

Animals. L1210/0 lymphocytic leukemia cells that had been passaged in DBA/2 mice were transplanted in ascites form (10^6 cells) IP to female BDF_1 mice, weighing 18–20 g. Treatment with single dose FUra, 100 mg/kg IP, to such mice the day after inoculation regularly results in a 3-day or 30% increase in lifespan over control mice. On day 9 after transplantation, the mice were injected IP, with 100 mg/kg of [2-^{14}C] FUra (0.13 mCi/mmol), approximately 2 μCi/mouse. They were divided into groups of four and sacrificed at 0, 30, 60, 90, 180, and 360 min, and 12, 24, 48, 72, and 96 hr after drug administration. Ascites was collected by needle aspiration and the cells were washed once at 4° with phosphate-buffered saline prior to homogenization.

Tissue processing. Tissue homogenization was done by dilution of L1210 packed ascites cells in Tris-HCl buffer, pH 7.4, that contained 100 mM NaF, 20 mM 2-mercaptoethanol, and 15 mM cytidine-5′-monophosphate, and sonicated as previously described (22). Protein present in the cell sonicates was assayed by the Lowry procedure (23). Nucleic acids were processed by a modified Schmidt-Tannhauser procedure (24, 25), as follows. To 100 μl of sonicate were added 400 μl of distilled water plus 250 μl of 1.5 N $HClO_4$, and the mixture was vortexed and left at 4° for 10 min to complete precipitation. The mixture was centrifuged for 10 min at 3000 × *g* at 4° and the precipitate was washed twice with 0.5 ml of 0.2 N $HClO_4$. Phospholipids were removed by two extractions with 1 ml of 95% ethanol containing 2% sodium acetate at room temperature, followed by two extractions with ethanol:ether (3:1, v/v). To the phospholipid-free pellet, 0.4 ml of 0.3 N KOH and 0.6 ml of distilled water were added at 37° for 3 hr to complete RNA hydrolysis (25). The hydrolysate was cooled to 4° and 250 μl of 1.5 N $HClO_4$ was added for 10 min. This was followed by centrifugation for 10 min at 3000 × *g* at 4°; the pellet was washed with 0.75 ml of 0.2 N $HClO_4$ and centrifuged, and the supernatants containing the ribonucleotides were combined.

One ml of 1.5 N $HClO_4$ was added to the remaining pellet for incubation for 15 min at 70° and cooled to 4° for 10 min, and the tubes were centrifuged 10 min at 3000 × *g*. This treatment was repeated twice with fresh $HClO_4$; the supernatants were combined and kept for DNA assay by a modified Burton procedure (26). Less than 2% of the RNA fraction was present in these supernatants by orcinol reaction. The remaining material was discarded.

A 0.5-ml aliquot of the supernatants containing the ribonucleotides was used for assay of total RNA by orcinol reaction (27). DNA and protein were undetectable in these samples. The remaining supernatant was neutralized at 4° by dropwise addition of 0.3 N KOH, and centrifuged at 3000 × *g* for 10 min at 4°, and the precipitated salts were discarded. The supernatant was then diluted to 4.0 ml with distilled water, in order to dilute the remaining salts to a conductivity of less than 15 mmho, prior to application on DEAE-cellulose.

Separation and enzymatic hydrolysis of FUMP. FUMP (an equal mixture of 2′ and 3′ compounds) was separated from other nucleotides by use of DEAE-cellulose minicolumns, NH_4HCO_3 form, prepared as described (28). Each column was washed with 10 ml of distilled water before application of the neutralized, acid-soluble nucleotides of the KOH hydrolysate. The columns were eluted with 5 ml of distilled water, followed by 15 ml of 100 mM and 10 ml of 300 mM NH_4HCO_3, pH 8.0, and collected into 1-ml fractions. The 300 mM NH_4HCO_3 fractions were combined, lyophilized to dryness, reconstituted in 2 ml of water, and reapplied to a second DEAE-cellulose column for repeat collection of the 300 mM NH_4HCO_3 fractions.

To the pooled, lyophilized fractions containing FUMP were added 0.5 ml of H_2O, 5 μl of 0.5 M $NaHCO_3$, pH 9.6, and 3.8 units of alkaline phosphatase (dialyzed against water). The mixture was incubated for 1 hr at 37° and cooled to 4° for 10 min, and 100 μl of 4.4 M $HClO_4$ was added. The mixture was kept at 4° for an additional 10 min and centrifuged at 4000 × *g* for 20 min; the pH of the supernatant was adjusted with 1 N KOH to pH 6–7, and the mixture was centrifuged at 3000 × *g*. FUrd was separated from alkaline phosphatase and contam-

inating nucleosides by application on a third DEAE-cellulose minicolumn, washing with 10 ml of water, followed by elution of the nucleoside with 15 ml of 100 mM NH_4HCO_3 buffer, pH 8, and collection of 1-ml fractions. Fractions 6–25 that contained the FUrd were pooled and lyophilized.

Preparation and separation of FUrd-trialcohol. The FUrd fractions were reconstituted in 100 μl of 50 mM $NaIO_4$, pH 5–6, and the samples were left at 23° in the dark for 30 min. Then 10 μl of NaB^3H_4 working solution (approximately 6×10^6 dpm/assay) was added to the tubes, which were left overnight at 23° in the dark to complete the reduction. Of this mixture, 10 μl was applied to a silica gel 60 F_{254} analytical TLC plate (Merck) and chromatographed using a 4:1 mixture of ethyl acetate and ethyl alcohol containing 5% diethylhydroxylamine, which gives improved separation over standard systems (29). Fluoropyrimidines migrated much faster than normal pyrimidines in this system: the R_F values of FUra, FUrd, and FUrd-trialcohol were 0.78, 0.68, and 0.56; the R_F values of normal nucleosides and their trialcohol derivatives were 0.30 and 0.24 (Urd), 0.19 and 0.06 (Ado), 0.00 and 0.02 (Cyd), and 0.00 and 0.00 (Gua), respectively. Authentic nonradioactive nucleoside trialcohols were prepared as described by Rajbhandary (18). The region containing the FUrd-trialcohol was located by UV visualization of cold carrier compound, cut out, and counted for tritium radioactivity.

Comparison of F-RNA assays by internal ^{14}C labeling versus NaB^3H_4 derivatization. Following the FUrd separation step, the fractions containing FUrd were divided into equal halves for ^{14}C counting and for periodate oxidation plus NaB^3H_4 preparation of radiolabeled FUrd-trialcohol. Results of ^{14}C counting were also determined on aliquots of the crude tissue sonicates for assay of total [^{14}C]FUra metabolites, and on the perchloric acid supernatants of KOH hydrolysates as a check against loss of radioactivity by DEAE chromatography. In addition, the purity of the FUrd fractions was checked by high performance liquid chromatography. In parallel experiments, entire FUrd samples derived from single mice were reconstituted in 100 μl of H_2O and injected onto a Zorbax-ODS reversed phase column (4.6 mm × 25 cm). Elution was done with 1% methanol in 5 mM KH_2PO_4 buffer, pH 5.3, at 40 lb/m^2 and a rate of 1 ml/min. Retention times (min) of authentic nucleosides were FUrd, 3.11; Urd, 8.8; Cyd, 17.5; and Guo, 20.7. Using the FUrd fractions of the extracts from cells treated at 1 and 3 hr after FUra, only single peaks corresponding to FUrd were found.

RESULTS

Separation of fluoropyrimidines. Model elution studies with a mixture of nonlabeled AMP, UMP, CMP, and GMP (1 μmol each) plus 1 nmol of [^{14}C]FUMP applied to DEAE-NH_4HCO_3 form minicolumns showed that 15 ml of 100 mM NH_4HCO_3 removed at least 95 ± 2% (SD) of the normal nucleotides and less than 1% of the FUMP. The 300 mM wash removed over 98 ± 1% of the FUMP on the column. Thus, use of two consecutive columns reduced the concentrations of normal nucleotides relative to FUMP approximately 400-fold. The third DEAE minicolumn, following alkaline phosphatase hydrolysis of FUMP to FUrd, required weaker electrolyte conditions for separation of normal nucleosides from FUrd. It was therefore necessary to remove the ammonium sulfate present in commercial preparations of alkaline phosphatase by dialysis. The normal nucleosides eluted completely (99%) with 10 ml of water, which did not remove any FUrd (Fig. 1), and at least 98% of FUrd was eluted by 15 ml of 100 mM NH_4HCO_3 (not shown). This step also separated FUrd from the alkaline phosphatase, which remained on the column. The minor nucleosides dihydrouridine, pseudouridine, and 5-hydroxymethylcytosine, eluted ahead of Gua (Fig. 1).

Taken together, the three DEAE-cellulose separations

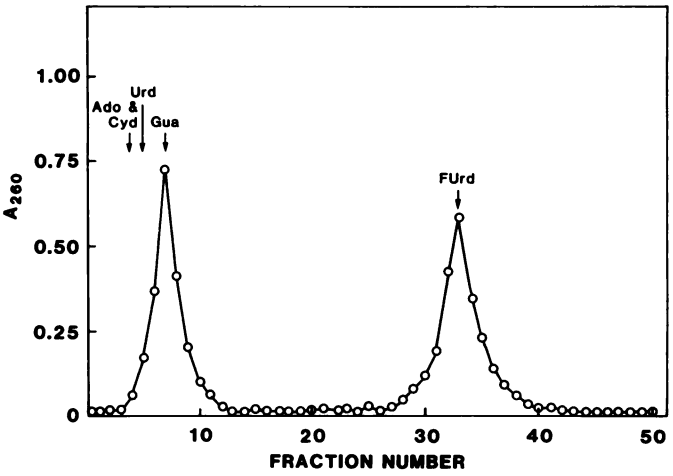

FIG. 1. *Separation of normal ribonucleosides from FUrd by water elution (1-ml fractions) of a NH_4HCO_3 form DEAE-cellulose minicolumn*

resulted in at least an overall 40,000-fold decrease in the ratio of normal nucleosides to FUrd. The separation of FUrd-trialcohol from the trialcohol derivatives of normal ribonucleosides by silica gel TLC resulted in an additional, approximately 100-fold decrease in the relative concentrations of the normal base derivatives, making it theoretically possible to detect base substitution rates of FUra in RNA as low as 1 part per million normal bases.

Preparation of isotopic ribonucleoside trialcohols. The present procedure for preparation of the radiolabeled nucleoside trialcohols resulted in a standard curve that was linear up to 0.5 μmol of FUrd/assay, with a slope of 70 dpm/nmol of FUrd (Fig. 2). Since only 9% of radiolabeled trialcohol material was transferred to TLC, the potential sensitivity of the assay is 770 dpm/nmol of FUrd. Background radioactivity of blank tubes in the FUrd-trialcohol region of the silica gel TLC averaged 0.2 ± 0.015% applied radioactivity per plate, about 1100 dpm. This means of removal of excess NaB^3H_4 was as effective as 1 N HCl at 60° for 30 min followed by methanol washing and lyophilization. Use of HCl for destruction of NaB^3H_4 was not done, however, because of partial acid-catalyzed hydrolysis of the β-glycosidic bond of FUrd-trialcohol (18), confirmed by silica gel TLC. Removal of excess radioactivity by addition of acetaldehyde was not useful because of relatively high backgrounds. Removal of excess periodate in the standard tubes by addition of ethylene glycol or rhamnose after the borohydride reaction also did not improve the backgrounds or sensitivity of the assay.

Assay of F-RNA levels in vivo. The time course of levels of F-RNA present in L1210/0 leukemia cells *in vivo* after treatment with IP. FUra, 100 mg/kg, is shown in Fig. 3. The average frequency of base substitution by FUra in RNA, calculated from experiments using [^{14}C]FUra, is given in Table 1. Results obtained by directly counting the radioactivity present in the KOH hydrolysates of perchloric acid precipitates of cell sonicates were indistinguishable from those obtained by counting the ^{14}C radioactivity present in the FUrd fractions after the third DEAE-cellulose separation.

TABLE 1

Nucleic acid and F-RNA levels in L1210/0 ascites cells in vivo after IP [^{14}C]FUra, 100 mg/kg

Nucleic acids were extracted from cell sonicates by a Schmidt-Tannhauser procedure, and assayed for DNA and RNA by use of the diphenylamine (26) and orcinol (27) reagents; F-RNA levels were determined by scintillation counting of [^{14}C]FUrd obtained from alkaline phosphatase treatment of nucleotides released by KOH digestion of perchloric acid precipitates, for comparison with total [^{14}C]FUra metabolites present in cell sonicates.

Time after FUra	Nucleic acids			F-RNA base substitution[b]	Total [^{14}C] FUra metabolites[c]	([^{14}C]F-RNA/ total ^{14}C) × 100[e]
	DNA[a]	RNA[a]	RNA/DNA			
hr	*mg/g*	*mg/g*		*%*	*μmol/g*	*%*
0	133 ± 3[d]	282 ± 2	2.16 ± 0.01			
0.5	132 ± 6	280 ± 7	2.09 ± 0.10	0.14	8.25 ± 1.20	15.8
1	142 ± 8	271 ± 4	1.99 ± 0.13	0.29	8.75 ± 0.68	27.2
1.5	134 ± 6	270 ± 15	2.00 ± 0.12	0.46	9.37 ± 1.22	37.8
3	123 ± 9	249 ± 23	1.99 ± 0.13	0.57	8.58 ± 1.18	49.6
6	123 ± 25	260 ± 15	2.11 ± 0.12	0.56	7.35 ± 1.07	54.1
12	123 ± 14	254 ± 3	2.05 ± 0.10	0.43	5.88 ± 0.64	57.4
24	125 ± 12	209 ± 18	1.69 ± 0.21	0.32	3.60 ± 0.75	56.3
48	131 ± 9	215 ± 24	1.64 ± 0.11	0.22	1.86 ± 0.47	76.2
72	124 ± 9	196 ± 11	1.59 ± 0.11	0.19	1.31 ± 0.26	84.0
96	120 ± 8	160 ± 15	1.34 ± 0.10	0.17	0.89 ± 0.19	91.2

[a] Nucleic acids in milligrams/g of protein (approximately 1.5 × 10^{10} cells).
[b] Represents substitution rate by FUra per number of normal bases in RNA times 100; data were calculated from results of [^{14}C]FUra study.
[c] Total [^{14}C]FUra metabolites per g of protein in cell sonicates; includes [^{14}C]F-RNA.
[d] Mean ± standard deviation of results in 4 mice per time point.
[e] Gives percentage of total [^{14}C]FUra metabolites present as [^{14}C]F-RNA in cell sonicates.

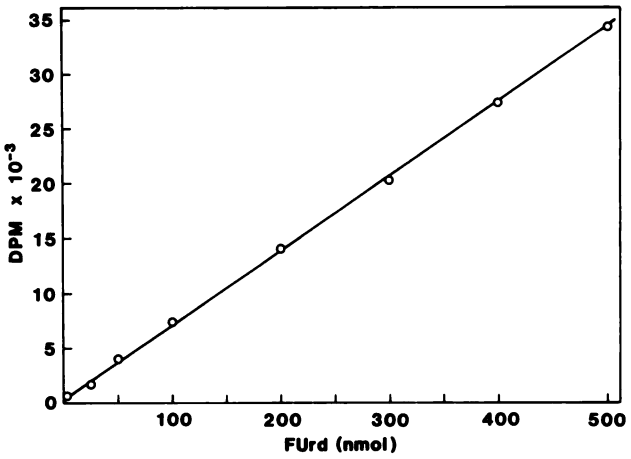

FIG. 2. *Standard curve for assay of FUrd by NaB^{3}H$_4$ derivatization and silica gel TLC isolation of [^{3}H]FUrd-trialcohol*

Standard deviations of quadruplicates are approximately the size of the points shown.

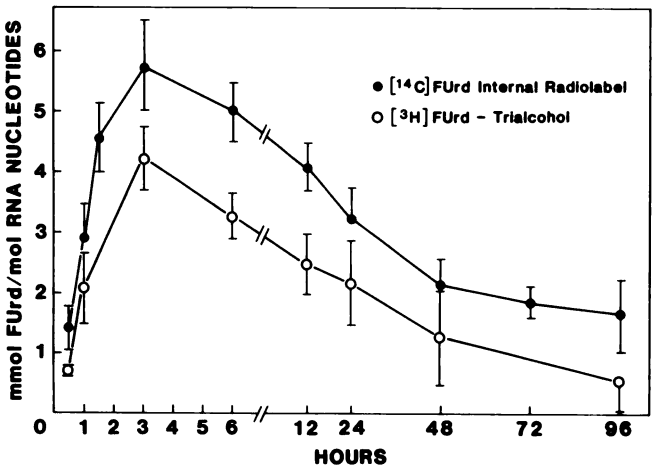

FIG. 3. *Levels of F-RNA in L1210/0 ascites cells as a function of time after [^{14}C]FUra treatment, 100 mg/kg I.P.*

Assay of F-RNA was done by isolation and NaB^{3}H$_4$ derivatization of FUrd present in F-RNA (○), for comparison with results of counting ^{14}C radioactivity (●) in paired samples. Values are means ± standard deviation of tumors of four animals per time point.

The results of F-RNA assay by NaB^{3}H$_4$ preparation of tritiated FUrd-trialcohol showed a satisfactory parallelism in the time course of F-RNA levels with the direct method using [^{14}C]FUra (Fig. 3). However, results of NaB^{3}H$_4$ assay were consistently 60–70% of values by ^{14}C counting. By either assay, the most rapid rate of rise in F-RNA was observed in the 1st hr after FUra administration, and peak F-RNA levels were at 3 hr. Values at 48 and 96 hr were surprisingly high, averaging 37.6 and 29.2%, and 30.7 and 12.7% of peak F-RNA levels by the ^{14}C and tritium methods, respectively.

As a percentage of total [^{14}C]FUra metabolites present in cell sonicates, the levels of F-RNA progressively increased over the entire 96-hr time course. By 12 hr, approximately one-half of all metabolites of FUra were present as F-RNA, and by 96 hr, this exceeded 80% (Table 1).

Uptake of [^{14}C]FUra into L1210/0 ascites cells was rapid, with 88% of maximal total ^{14}C-metabolite radioactivity (at 90 min) being reached at the earliest time point studied, 30 min. The rate of disappearance of non-[^{14}C]F-RNA radioactivity (total ^{14}C radioactivity minus [^{14}C]F-RNA in cell sonicates, equivalent to "acid-soluble" FUra metabolites) was significantly faster than the rate of decrease in [^{14}C]F-RNA levels. By 48 and 96 hr after drug administration, total FUra metabolites were only 20.0 and 9.5% of peak values, with most of this present as F-RNA, as indicated.

Assays of nucleic acids present in the cells as a function of protein content showed no significant change in DNA, although a 10% decrease was apparent by 96 hr. However, on the basis of either protein or DNA content, RNA levels in the cells underwent striking changes, with a 20–25% reduction occurring by 24 hr and an approximate 40% overall loss by 96 hr (Fig. 4 and Table 1).

DISCUSSION

The objective of this study was to demonstrate that levels of F-RNA *in vivo* following a therapeutic dose of FUra could be determined in tissues exposed to the nonradiolabeled drug. This is shown by the results of our study of L1210/0 ascites cells treated with [^{14}C]FUra, and then assayed for F-RNA by preparation of the isolated, radiolabeled FUrd-trialcohol derivative of FUrd present in F-RNA, with comparison made with the results of counting ^{14}C radioactivity (Fig. 3).

Because the method for radioisotopic derivatization may be expected to radiolabel virtually all saturated compounds with vicinal hydroxyl groups (17–19), the specificity of our assay for F-RNA results from the multistep separation sequence combined with the lack of radiolabeling of deoxyribose compounds. In the DEAE-cellulose separations of FUMP from normal nucleotides (28) and FUrd from normal nucleosides (Fig. 1), the fluorinated compound elutes last; in the silica gel separation of nucleoside trialcohols, the FUrd derivative moves well ahead of normal pyrimidine derivatives. Earlier attempts by us to use an additional DEAE-cellulose separation, instead of silica gel TLC, on the tritiated nucleoside trialcohols had resulted in unacceptably high blank values, suggesting the presence of contaminating compounds. However, use of silica gel TLC for separation of the trialcohol compounds resulted in tissue blanks that were no higher than water blanks, and in estimates of F-RNA levels that were actually slightly and consistently lower than results by following the internal ^{14}C radiolabel (Fig. 3). The cause for the slightly lower F-RNA levels indicated by NaB^{3}H$_4$ radiolabeling is unknown, but may be due to the presence of 5-fluorodeoxyuridine and other, nonidentified tissue contaminants that could react with the nucleoside dialdehydes in the assay prior to silica gel chromatography.

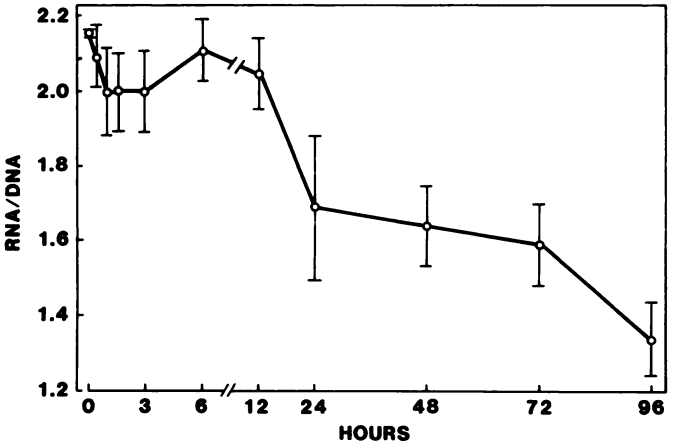

FIG. 4. *Time course of change in whole cellular RNA as a function of DNA content in advanced L1210/0 ascites cells treated with FUra, 100 mg/kg, IP.*

The sensitivity of the present assay of F-RNA could be greatly increased, if necessary, by use of NaB^{3}H$_4$ at higher specific activities (19) and by use of silica gel column chromatography or high performance liquid chromatography for separation of the trialcohols. For example, if sufficient molar quantities of NaB^{3}H$_4$ at 6.5 Ci/mmol are used for radiolabeling, and the entire [^{3}H] FUrd-trialcohol sample is isolated by silica gel column chromatography or high performance liquid chromatography for counting, low picomole levels of FUrd extracted from F-RNA should be detectable. This would make assay of F-RNA feasible for levels as low as 1 part per 30,000 normal bases in a 100-mg-size tissue sample containing 1 mg of RNA.

The levels of F-RNA in the L1210/0 ascites cells were relatively high, perhaps a result of the favorable pharmacokinetic situation of giving 100 mg/kg of FUra IP. The maximum level of F-RNA at 3 hr was less than 1 FUra substitution/200 normal nucleotides, which is approximately three times the level we found in subcutaneous colon adenocarcinomas of mice given 80 mg/kg FUra IP. (22). Both of our studies have shown that levels of F-RNA decline slowly after reaching peak values, so that significant F-RNA levels persist for several days following FUra administration in tumors *in vivo*.

Surprisingly, there appear to be no other publications that report the kinetics of F-RNA levels in tumors over long time intervals after FUra. There are several reports of the kinetics of acid-soluble metabolites of FUra (20, 21, 30), with the general finding that most of these decrease at rates significantly faster than the F-RNA levels of our studies, except for FdUMP (the metabolite that inhibits thymidylate synthetase) which persists for longer periods of time (20, 21). The persistence of FdUMP may be related to its slow release from thymidylate synthetase, although our present results lend support to the postulate that F-RNA may represent a depot storage form of FUra because of the relatively slow turnover of RNA, which could provide FUrd substrate for activation to the deoxynucleotide (20). Persistent levels of F-RNA could also be a source of the prolonged basal plasma levels of FUra occasionally noted (31, 32).

An interesting finding of the present study was the progressive decrease in cellular RNA content following bolus FUra on the basis of protein content (Table 1) or DNA content (Fig. 4). FUra treatment is traditionally held to result in the "unbalanced growth" or thymineless death of cells, whereby intracellular RNA and protein accumulate concomitant with a cessation of DNA synthesis (33). Although there is some histologic evidence for this phenomenon (34), direct assays of RNA, DNA, and protein have not yet confirmed such an effect (12, 35). After an initially small decline at 1 hr, the RNA content of the L1210/0 cells in our study dropped dramatically at 24 and 96 hr. This effect may be of the same order of magnitude as reported for FUra-mediated inhibition of ribosomal maturation (2, 4), although the kinetics of ribosomal effects at longer time points appear not to have been studied. The sigmoidal oscillation in

the RNA/DNA ratio is suggestive of phase-specific effects of FUra during cell cycle traverse. Progression through the cell cycle may be necessary for expression of toxicities associated with both thymidylate synthetase inhibition (S-phase) and RNA effects (G_1-phase) (35). The overall loss of cellular RNA is striking, and suggests the biologic importance of RNA effects in FUra therapy. The kinetic observations of Chadwick and Chang (21) that FUra at a dose of 100 mg/kg given to non-tumor-bearing mice results in a reversible, 10% decrease in body weight over several days, parallels the kinetics of F-RNA levels that we find and would be consistent with the thesis (16) that dose-dependent toxicity effects of FUra may be related to F-RNA levels.

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