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USE OF PERIODATE AND METHYLAMINE FOR THE QUANTITATION OF INTRACELLULAR 5-FLUORO-2′-DEOXYURIDINE-5′-MONOPHOSPHATE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

ROBERT DREYER and ED CADMAN*
Departments of Medicine and Pharmacology, Yale School of Medicine, New Haven, CT 06510 (U.S.A.)
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SUMMARY

High-performance liquid chromatography was used for the quantitation of [^{3}H]fluorouracil and metabolites in L1210 cells with or without pre-treatment with methotrexate. Ribonucleotide pools were evaluated on a chemically bonded anion-exchange column. Deoxynucleotide pools were determined following periodate and methylamine treatment which eliminated the ribonucleotides (>99.9% complete) and allowed for a rapid quantitation of the acid-soluble 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) pools. FdUMP levels as low as 10 fmol/10^6 cells were detectable by polystyrene anion-exchange chromatography. Confirmation of these FdUMP values was performed by rechromatography on a reversed-phase ion-pair column. The use of periodate and methylamine as a means for the reliable elimination of the ribonucleotides will allow accurate isolation of deoxynucleotides by high-performance liquid chromatography.

INTRODUCTION

5-Fluorouracil is a clinically useful drug for treating carcinoma of the breast and gastrointestinal tract. We have previously reported that when methotrexate (MTX) precedes fluorouracil (FUra) there is synergistic cytotoxicity of L1210 cells[1]. The therapeutic and toxic effects of FUra are presumed to be mediated from the inhibition of thymidylate synthetase (E.C. 2.7.4.6) by FdUMP and/or the effect of FUTP incorporated into RNA[2].

Possible pathways leading to FdUMP formation include the transfer to FUra of the phosphoribosyl moiety from 5-phosphoribosyl-1-pyrophosphate by orotate phosphoribosyltransferase (E.C. 2.4.2.10). The FUMP formed can then be phosphorylated to FUDP, reduced to FdUDP and finally converted into FdUMP. An alternative mechanism uses uridine (E.C. 2.4.2.3) or thymidine phosphorylase (E.C. 2.4.2.4) to transfer a ribose or deoxyribose to FUra to form the ribonucleoside or deoxyribonucleoside derivative which can be phosphorylated by uridine-cytidine kinase (E.C. 2.7.2.48) or thymidine kinase (E.C. 2.7.1.21) to FUMP or FdUMP, respectively.

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A high-performance liquid chromatographic (HPLC) method was developed specifically to evaluate FdUMP. Thin-layer[3] and column chromatography[4–6] have been used to evaluate metabolic *de novo* pyrimidine and purine pathways, but poor resolution, inadequate sensitivity and the time required to perform these separations render these methods unacceptable for rapid and repetitive determinations. A very sensitive competitive ligand-binding assay for measuring FdUMP levels in cell culture has been developed by Moran *et al.*[7] and Murinson *et al.*[8]. Garrett and Santi[9] recently reported a rapid and sensitive method for evaluating deoxynucleoside triphosphates in cell extracts following destruction of the ribonucleotides by periodate and methylamine, which was then used by Washtien and Santi[10] for the evaluation of FdUMP levels in cell extracts previously exposed to [^{3}H]FUra. The chemical degradation of ribonucleotides as a result of periodate and methylamine treatment has been elegantly described by Khym[11], Brown and Read[12] and Rammler[13], and has been utilized by others for the evaluation of ^{32}P-labelled deoxynucleotides[14,15]. Ritter and Bruce[16] did not use methylamine with the periodate-treated cell extracts, and reported variable and inconsistent resolution of deoxynucleotides because of interfering ribonucleotides.

Our study was designed to define the precise methodology by which multiple cell extracts could be prepared for rapid and reproducible analysis of both ribonucleotides and deoxynucleotides by HPLC. A second objective was to determine by several chromatographic methods that the radioactivity eluting in the deoxynucleotide region of cells treated with [^{3}H]FUra was indeed FdUMP and that the procedure could be quantitative in the fmol/10^6 cell range, which is *ca.* 1000-fold more sensitive than previous methods using HPLC and [^{3}H]FUra.

MATERIALS AND METHODS

Chemicals

[2-^{14}C]FUMP (45 mCi/mmol), [6-^{3}H]FdUMP (25 Ci/mmol), [6-^{3}H]FUMP (20 Ci/mmol), [2-^{14}C]FdUMP (50 mCi/mmol) and [6-^{3}H]FUra (25 Ci/mmol) were obtained from Morevak Biochemicals (City of Industry, CA, U.S.A.) and greater than 95% pure by HPLC. UMP, UDP, UTP, FdUrd, UDP-Glc, FUra and FUrd were purchased from Sigma (St. Louis, MO, U.S.A.) FdUMP was purchased from Calbiochem-Behring (La Jolla, CA, U.S.A.). Aquasol Liquid Scintillant was purchased from New England Nuclear (Boston, MA, U.S.A.). Certified ACS sodium phosphate monobasic monohydrate, ammonium acetate anhydrous, sodium acetate anhydrous and glacial acetic acid were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). Phosphoric acid (85%), sodium hydroxide, and ammonium hydroxide were purchased from Mallinckrodt (St. Louis, MO, U.S.A.). ACS certified HCl, sodium periodate and methylamine ($CH_3NH_2 \cdot HCl$) were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Methanol and UV grade acetonitrile were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Tetra-*n*-butylammonium bromide, 99%, was purchased from Aldrich (Milwaukee, WI, U.S.A.).

A flow chart of the methodology for analysis of cells is shown in Fig. 1. Detailed explanation of each aspect of the analysis is given in the following paragraphs.

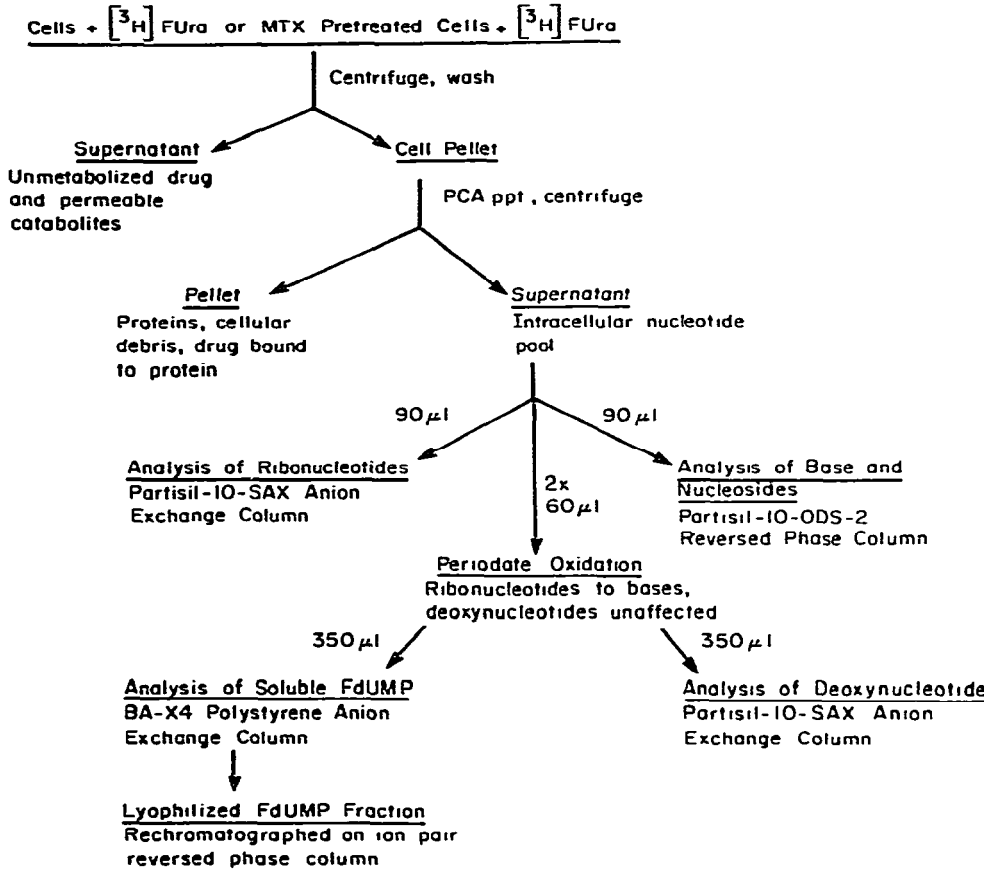

Fig. 1. Schematic representation of the preparation and evaluation of fluoropyrimidine nucleotides.

HPLC mobile phase preparation

Solutions (1 mol/l) of sodium phosphate and sodium acetate were screened for minimum ultraviolet (UV) absorbance at 254 nm (1-cm path length). Acceptable lots of phosphate and acetate had an absorbance of less than 0.02 and 0.005 O.D, respectively. All buffers were made up daily in deionized distilled water and filtered through a 0.2-µm metrical filter (Gelman GA-8; Gelman, Ann Arbor, MI, U.S.A.) and degassed by sonication prior to use.

Tissue culture

L1210 cells were grown as suspension cultures in Fischer's medium supplemented with 10% horse serum. A 50-ml volume of the cell suspension at logarithmic growth (*ca.* 2×10^5 cells/ml) were exposed for 3 h to 10 µ*M* MTX, while another group of cells served as controls. Following the MTX exposure the cells were exposed to 1 µ*M* [6-^{3}H]FUra (25 Ci/mmol) for 1, 3 and 6 h. A second group of cells was concentrated to a 5-ml suspension by centrifugation (1000 *g* for 4 min) before adding [6-^{3}H]FUra for 1 h to reduce the amount of radiolabel used. After the exposure to

[6-^{3}H]FUra, cell pellets were prepared by centrifugation and cellular extracts obtained. The concentration of L1210 cells for 1 h has previously been shown to have no effect on cellular metabolism as determined by viability studies performed in soft agar[17].

Preparation of cell extract

The cell suspensions were centrifuged at 2000 rpm in a Sorval RC-5B centrifuge (DuPont Instruments, Newtown, CT, U.S.A.) at 4°C. The cell pellet was then washed with ice-cold PBS (4°C) and centrifuged as before. The supernatant was decanted before lysing the cells by adding ice cold 0.5 *M* $HClO_4$ (500 μl). The $HClO_4$ precipitate was removed by centrifugation at 2000 rpm for 2 min, leaving a clear supernatant which contained the acid-soluble nucleotides. The pellet of cellular debris was then digested (1 *N* KOH) and the radioactivity determined. Only 10% of the total intracellular radioactivity was present in this acid-precipitable cell fraction; 90% was associated with the intracellular supernatant.

Periodate oxidation

A 60-μl volume of the KOH-neutralized $HClO_4$ L1210 cell extract was transferred to a 400-μl polypropylene test-tube (Sarstedt, G.F.R.). A 150-μl volume of 12 m*M* sodium periodate, which was prepared daily[18], was mixed with the 60-μl sample aliquot and incubated at 37°C for 30 min. A 60-μl volume of 0.4 *M* $CH_3NH_2 \cdot HCl$, pH 7.5, and 120 μl of 0.01 *M* NaOH were then added to the periodate-treated sample, and the mixture was incubated at 37°C for 15 min to complete base cleavage. A total of five reaction tubes were prepared per sample. Two reaction mixtures were analyzed by HPLC for deoxynucleotides on a Partisil 10 SAX and BAX4 column. A third mixture was used for rechromatographic analysis as described below. The final two samples were used for determining recoveries of radioisotopes from the two analytical columns. A 10-μl volume of unlabelled FdUMP at 1 m*M* was added to one reaction mixture being analyzed on the BAX4 column; 10 μl of FdUMP, UMP, UDP-Glc, UDP and UTP at 1 m*M* were added to the other reaction mixture being analyzed on the Partisil 10 SAX column. The slight separation between FUMP and FdUMP on the BAX4 column served as an internal check on the effectivenees of the periodate oxidation. All samples were kept on an ice bath prior to HPLC analysis; 350-μl aliquots (52.5 μl of actual sample) were injected onto each of the analytical columns.

Special equipment

All gradient chromatographic analyses were performed on an Altex Model 332 liquid chromatograph using two Altex Model 153 UV detectors connected in tandem to monitor 254 and 280 nm. Isocratic chromatographic analyses were run on a modularly constructed liquid chromatograph comprised of the following components: a dual-piston reciprocating pump (Model 100, Altex, Berkeley, CA, U.S.A.); a manual loop injector (Model 7120, Rheodyne, Berkeley, CA, U.S.A.); two fixed-wavelength UV detectors set for 254 and 280 nm, and a dual-pen chart recorder (Model 385, Linear Instruments, Wheaton, MD, U.S.A.). Temperature control was achieved with a circulating water bath (Blue M Electric, Blue Island, IL, U.S.A.) and a glass water jacket (Rainin Instruments, Woburn, MA, U.S.A.).

Measurement of radioactive effluent

Effluent from the Partisil 10 SAX and BAX4 polystyrene anion-exchange columns was collected in 0.9- and 1.0-ml fractions, respectively. After 0.9 ml of deionized-distilled water was combined with each fraction from the Partisil 10 SAX column, 10 ml of Aquasol were added. A similar amount of Aquasol was added directly to each fraction from the BAX4 column. Effluent from the LiChrosorb C_{18} and the Partisil ODS-2 column was collected in 0.5- and 1.0-ml fractions, respectively, and 5 ml of Aquasol were added to each fraction.

The vials were shaken vigorously to produce a clear homogenous solution before determining the radioactive content. The counting efficiencies were 25 ± 5% for ^{3}H and 75 ± 5% for ^{14}C and remained constant throughout the various gradient profiles. The recovery of radioisotope from the analytical column was determined by placing an identical volume of chromatographed sample in a tube of column effluent generated by the fraction collector.

The radiolabelled fractions were counted on a Beckman LS7000 liquid scintillation counter (Irvine, CA, U.S.A.) interfaced to a 48K Horizon I North Star minicomputer (Berkeley, CA, U.S.A.). This enabled data to be printed on a Model 43 teletype and stored on a mini floppy disk to be plotted later and integrated using software written in BASIC.

Rechromatography of soluble FdUMP on ion-pair reversed-phase chromatography

In order to characterize the FdUMP peak from the BAX4 polystyrene anion-exchange column, rechromatography of this region on a 250 × 4.6 mm I.D. LiChrosorb C_{18} 5 μm column maintained at 50°C was employed. A solution of 5 m*M* sodium phosphate, monobasic, 5 m*M* tetra-*n*-butylammonium bromide and 10% methanol was titrated to pH 6.8 with 1.0 *N* NaOH and used to elute the column at 1.0 ml/min.

The FdUMP region from the BAX4 column was collected into a 25-ml centrifuge tube and lyophilized twice. For the second lyophilization, 5.0 ml of deionized distilled water were added. The sides of the tube were rinsed with 2.0 ml of methanol and blown to dryness with nitrogen. The residue was finally dissolved in 100 μl of mobile phase and injected onto the column.

RESULTS

Limits of detection

The limit of detection for each of the chromatographic systems was based on radioactivity in three vials. With a maximum background equal to 50 cpm, 300 cpm was readily detectable and was arbitrarily chosen to represent detectable radioactivity. The limits of FdUMP detection for each of the chromatographic systems was 10–15 fmol/10^6 cells.

Periodate oxidation

In examining optimal conditions for periodate oxidation, 1 m*M* solutions of UMP, UDP and UTP in KOH neutralized $HClO_4$ L1210 cells extracts, representing *ca.* 10^6 cells/ml, were degraded under several concentrations of periodate, methylamine and sodium hydroxide. The optimal pH was 7.5. The addition of sodium hydroxide was necessary to maintain the pH at 7.5 during the reaction and therefore

effect a quantitative conversion of ribonucleotides. Reaction times were also optimized to produce a quantitative chemical degradation of ribonucleotides in the presence of deoxyribonucleotides in the cell extract. Ultimate conditions showed cleavage of

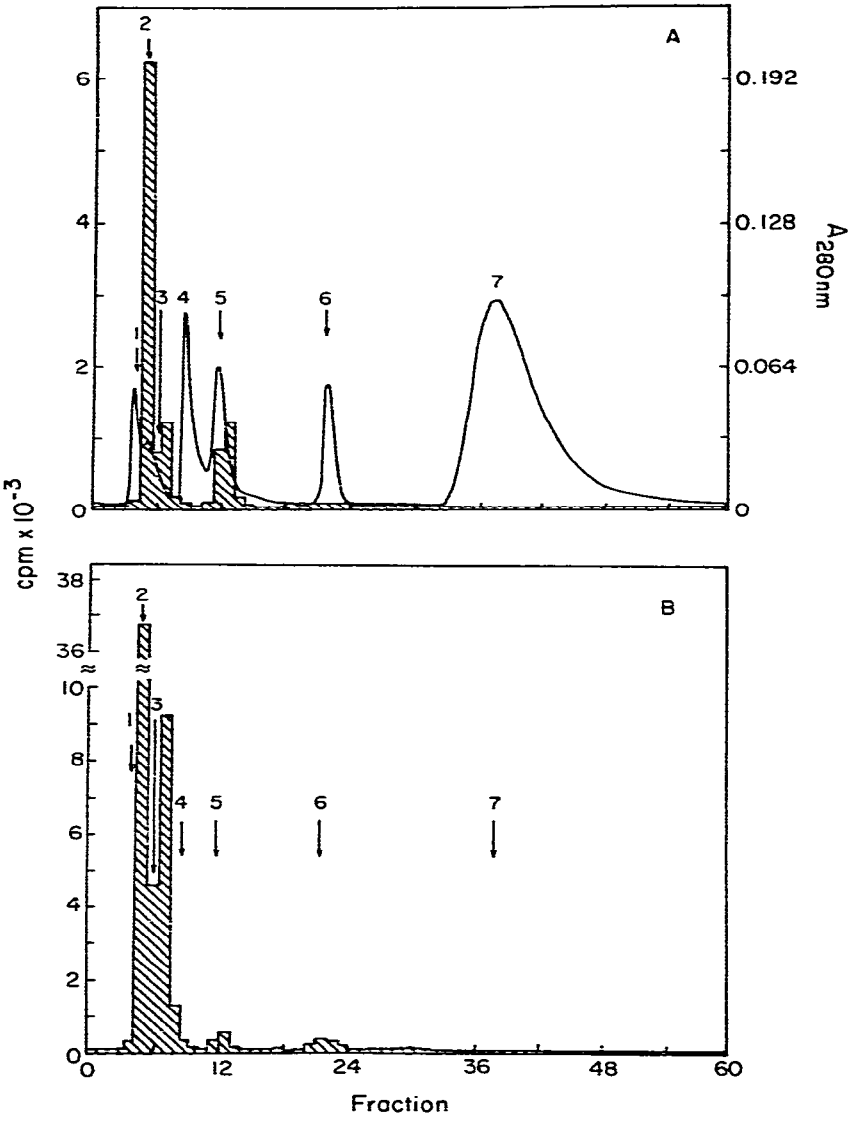

Fig. 2. Separation of nucleosides and bases was performed using a Whatman 250 × 4.6 mm I.D. Partisil ODS-2 10 μm reversed-phase column. KOH-neutralized $HClO_4$ cell extract (90 μl) was injected onto this column and eluted isocratically at ambient temperature at a flow-rate of 0.7 ml/min with 0.15 *M* sodium acetate, pH 5.5. (A) L1210 cells treated for 1 h with 1 μ*M* [6-^{3}H]FUra (20 Ci/mmol). (B) Cell extracts of MTX (10 μ*M*) pre-treated cells followed by 1 h of [6-^{3}H]FUra treatment. Cold markers of UTP, UDP-Glc, UDP, FUMP, FdUMP, FUra, FUrd and FdUrd (represented by peaks 1–7, respectively) were coinjected with cell extracts, and radiochromatograms were plotted. Endogenous cellular components are not visible at this wavelength and sensitivity.

ribonucleotide [2-^{14}C]FUMP (45 mCi/mmol) was always at least 99.9% quantitative. Similar results were achieved with [6-^{3}H]FUMP, (20 Ci/mmol). The final conditions agree closely with those outlined by Rammler[13]. Appropriate controls were carried out to ensure that under the reaction conditions employed, the chemical integrity of the deoxynucleotide species was maintained. [6-^{3}H]FdUMP (20 mCi/mmol) was less than 0.1% degraded. Similar results were obtained with [2-^{14}C]FdUMP (50 mCi/mmol). Non-radiolabelled dUDP and dUTP were not affected with periodate,while UDP and UTP were completely degraded as monitored by UV at 254 and 280 nm (0.04 a.u.f.s.).

Lyophilization

Lyophilization of [6-^{3}H]FdUMP (20 Ci/mmol) as a control demonstrated that 90% was recovered with no radioactivity appearing in any region except that marked by FdUMP.

Chromatograpy

Several investigators have used reversed-phase chromatography to examine nucleoside profiles in biological fluids[20–22]. In this study a modification of these reversed-phase techniques was used to elute nucleosides, nucleotides and deoxynucleosides of FUra isocratically. Resolution between drug and nucleoside metabolites was achieved after optimizing flow-rate, pH and ionic strength on the Partisil ODS-2 column (Fig. 2). All nucleotide metabolites eluted within the first 9 min and were well resolved from drugs and nucleosides.

Chromatographic systems for nucleotide separation using gradient elution on anion-exchange columns are also available[23,24]. Baseline separation was achieved between mono-, di- and triphosphate nucleotides of FUra on the Partisil 10 SAX column (Fig. 3). All nucleosides and drugs eluted in the void volume. Again FdUMP and FUMP remained unresolved.

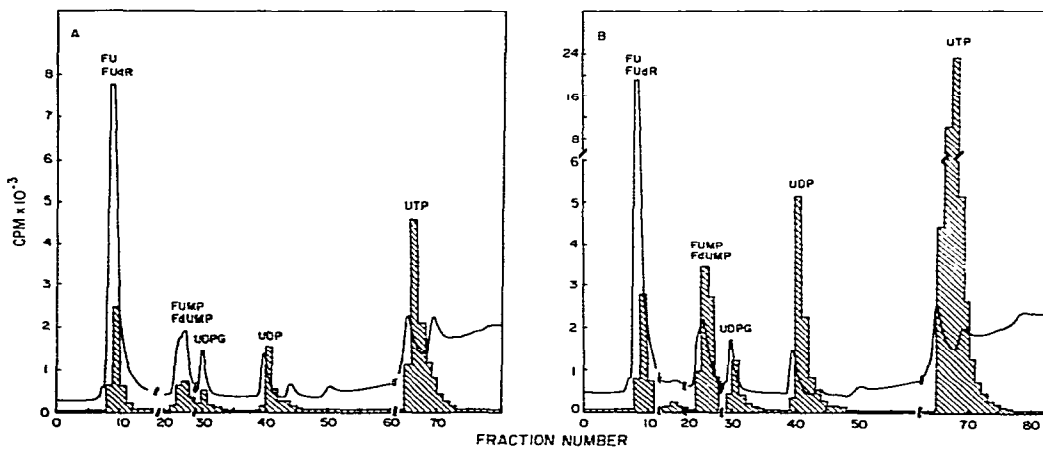

Fig. 3. KOH-neutralized cell extract (90 μl) was mixed with 10 μl of 1 m*M* UMP, FdUMP, UDP, UDP-Glc and UTP as cold markers and injected onto a Whatman 10 SAX 250 × 4.6 mm I.D. anion-exchange column. A linear gradient profile was run from 0.02 to 0.75 *M* sodium phosphate, pH 3.3, over 40 min. Ambient column temperatures were used with a flow-rate of 1.8 ml/min. A and B show the separation of control and MTX pre-treated cells each exposed for 1 h to FUra. The radioactivity co-eluting with UDPG, UDP and UTP represent FUDPG, FUDP and FUTP.

A BAX4 polystyrene anion-exchange column at elevated pH and temperature was used to obtain the desired resolution between FdUMP pools and the void volume radioactivity which was associated with chemically degraded ribonucleotides (Fig. 4).

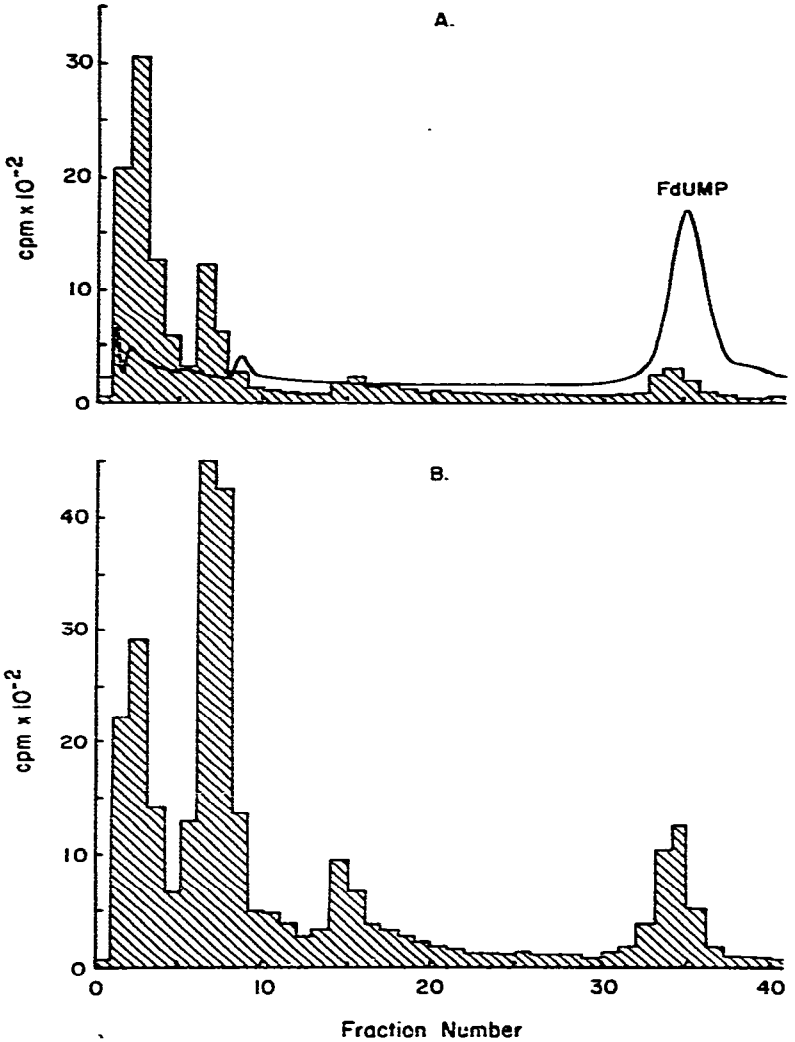

Fig. 4. Separation on the BAX4 polystyrene column of the periodate- and methylamine-treated acid-soluble extract of L1210 cell suspensions, unexposed (A) and exposed (B) to 10 μM MTX for 3 h before adding 1 μM [6-^{3}H]FUra (25 Ci/mmol) for 1 h. Cold FdUMP was used as an unlabelled marker. The analytical column was prepared by slurry-packing a 316 stainless-steel tube (10 cm × 4.6 mm I.D.; Analabs, North Haven, CT, U.S.A.) with 3 g of 4% cross-linked polystyrene anion-exchange resin, *ca.* 10 μm particle size, BAX4 (James B. Benson, Reno, NV, U.S.A.) suspended in 15.0 ml of 0.4 *M* ammonium acetate, pH 7.0. A flow-rate of 1.0 ml/min was maintained during the packing process with pressures not exceeding 1000 p.s.i. Conditions for elution of the periodate oxidized sample were as follows: temperature, 50°C; flow-rate, 1.0 ml/min; 0.4 *M* ammonium acetate buffer, pH 7.0, run isocratically for 6.8 min followed by a linear gradient from 0.4 *M* to 0.6 *M* ammonium acetate, pH 7.0, for 25.0 min. Initial composition was resumed over a 2.0 min period with 15 min re-equilibration prior to the next injection. Regeneration became necessary usually if back-pressure exceeded 1000 p.s.i. for the above chromatographic conditions and was performed by a previously described procedure[19].

This idea is an extension of the chromatography developed by Khym and Cohn[5]. The separation of the FdUMP region from the void volume was much improved compared with analysis on a Partisil 10 SAX column.

Ion-pair reversed-phase chromatography with the LiChrosorb C_{18} 10 μm column provided further evidence for the existence of FdUMP. Washtien and Santi[10] employed a LiChrosorb C_{18} 5-μm column at ambient temperatures to achieve separation between FUMP and FdUMP. By elevating the temperature of the column to 50°C and using 10% methanol in the mobile phase, the column efficiency improved, yielding reasonable retention times and effectively separating FdUMP from FUMP. This chromatographic method clearly demonstrated that the collected radioactive peak in the FdUMP region from the BAX4 column was quantitatively associated with FdUMP. Table I summarizes the results of these chromatographic systems.

The ATP/ADP ratios for all cell extracts analyzed were 5:1, indicating little hydrolysis at the time of analysis and a good energy balance (data not shown). Although there was a five-fold increase in FUTP levels compared to controls observed for cells pre-treated with MTX, periodate oxidation of these samples followed by analysis of the reaction mixture on the BAX4 polystyrene anion-exchange chromatography system revealed there was no radioactivity remaining in the triphosphate region. There was, however, an approximately 5-fold increase in FdUMP levels of MTX pre-treated samples (114 fmol/10^6 cells) over controls (22 fmol/10^6 cells). The effect of MTX on FUra metabolism has been reported in detail elsewhere[25].

DISCUSSION

The destruction and elimination of ribonucleotides by periodate oxidation is a method that will allow the subsequent separation and quantification of deoxyribonucleotides by HPLC, specifically FdUMP from the fluoropyrimidine ribonucleotides. This method may be applicable for the investigation of any intracellular deoxynucleotide pool or other drugs which could be metabolized to deoxyribonucleotide derivatives. An example of the use of this method was demonstrated in L1210 cells pre-treated with MTX in which a 5-fold increase in FdUMP was documented (22 fmol/10^6 cells *vs.* 114 fmol/10^6 cells). Washtien and Santi[10] found 80 fmol of the complex of FdUMP–5,10-methylenetetrahydrofolate–thymidylate synthetase per 10^6 cells (L1210) after incubating with 0.25 μM [6-^{3}H]FUra for 22 h. These investigators were able to detect only intracellular levels of free FdUMP after heat treatment of the sample, which dissociated this enzyme complex. A stable ternary complex has been demonstrated[26,27] between FdUMP, thymidylate synthetase and 5,10-methylenetetrahydrofolate when prepared in acid as in our studies. The formation of this covalent complex offers a tenable explanation for differences in FdUMP levels found in MTX pre-treated cells and control cells. MTX, which inhibits dihydrofolate reductase, would result in low levels of the co-factor 5,10-methylenetetrahydrofolate, which could therefore reduce the amount of the stable covalent complex formed. Hence cells pre-treated with MTX would show high levels of unbound intracellular FdUMP. The control samples, which would have higher levels of 5,10-methylenetetrahydrofolate, might be expected to form a stable protein complex with FdUMP, which could conceivably precipitate during $HClO_4$ extraction and therefore result in less free intracellular FdUMP. We are currently using high-performance gel filtration chro-

TABLE I

RETENTION DATA FOR HPLC

Values in minutes.

Column	*Chromatographic system*	*FUra*	*FUrd*	*FdUrd*	*FdUMP*	*FUMP*	*UDP-Glc*	*UDP*	*UTP*
Partisil SAX	Chemically bonded anion-exchange	4.8	4.8	4.8	11.0	–	18.0	23.0	33.0
BAX4	Polystyrene anion-exchange	7.2	6.5	8.6	32.2	36	24.2	–	–
LiChrosorb C_{18}	Ion-pair reversed-phase	4.8	–	–	40.6	33.7	–	–	–
Partisil ODS-2	Reversed-phase	11.2	22.6	37.8	8.7	6.0	4.0	4.2	4.0

matography for isolating this ternary complex in control and MTX-treated cells to evaluate this possibility[28].

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ABBREVIATIONS

FUra	= 5-fluorouracil
Furd	= 5-fluorouridine
FdUrd	= 5- fluoro-2′-deoxyuridine
FdUMP	= 5-fluoro-2′-deoxyuridine-5′-monophosphate
FUMP	= 5-fluorouridine-5′-monophosphate
FUDP	= 5-fluorouridine-5′-diphosphate
FUTP	= 5-fluorouridine-5′-triphosphate
UMP	= uridine-5′-monophosphate
UDP	= uridine-5′-diphosphate
UTP	= uridine-5′-triphosphate
HPLC	= high-performance liquid chromatography
MTX	= methotrexate
dUDP	= 2′-deoxyuridine-5′-diphosphate
dUTP	= 2′-deoxyuridine-5′-triphosphate
FdUDP	= 5-fluoro-2′-deoxyuridine-5′-diphosphate
FdUTP	= 5-fluoro-2′-deoxyuridine-5′-triphosphate
FUDP-Glc	= 5-fluoro-uridine-5′-diphosphoglucose
UDP-Glc	= uridine-5′-diphosphoglucose

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