STUDIES ON THE MECHANISM AND METABOLIC ROLE OF THYMIDYLATE SYNTHETASE

by

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ABSTRACT

STUDIES ON THE MECHANISM AND METABOLIC ROLE
OF THYMIDYLATE SYNTHETASE

by

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Thymidylate synthetase catalyzes the facile dehalogenation of 5-bromo-2'-deoxyuridylate (BrdUMP) and 5-iodo-2'-deoxyuridylate to give 2'-deoxyuridylate (dUMP), the natural substrate of the enzyme. The reaction does not require folate cofactors and stoichiometrically comsumes 2 equiv of thiol. In addition to dUMP, a minor product is formed during the debromination of BrdUMP which has been identified as a 5-alkylthio derivative formed by displacement of bromide ion by thiolate. The reaction has been found to proceed with a substantial α-secondary inverse tritium isotope effect (k_T/k_H = 1.212-1.258) with [2-^{14}C, 6-^{3}H]BrdUMP as the substrate. Similarly, an inverse tritium isotope effect of 1.18 was observed in the non-enzymatic chemical counterpart of this reaction, the cysteine-promoted dehalogenation of [2-^{14}C, 6-^{3}H]-5-bromo-2'-deoxyuridine. Previous evidence for the mechanism of action of this enzyme has rested largely on information obtained from its stoichiometric interaction with the inhibitor 5-fluoro-2'-deoxyuridylate. The magnitude of the secondary isotope effect during the enzymatic dehalogenation described here provides direct proof for nucleophilic catalysis and formation of 5,6-dihydropyrimidine intermediates in a reaction catalyzed by thymidylate synthetase.

The possibility that the enzyme DNA-uracil glycosylase might proceed via a similar mechanistic pathway was investigated. With PBS 2 DNA labelled with [2-^{14}C, 6-^{3}H]uracil as the substrate, k_T/k_H for uracil formation was not significantly different from one. This

result does not allow unambiguous interpretation of the mechanism of the reaction.

An assay for simultaneous quantitation of deoxyribonucleoside triphosphates (dNTPs) in cell extracts is described. Following destruction of ribonucleotides by treatment with periodate and methylamine, dNTPs are separated by high pressure liquid chromatography and quantitated by their ultraviolet absorbance upon elution. Destruction of ribonucleotides is complete (>99.9%), the resulting mixture is suitable for direct injection without further manipulation, and recovery of dNTPs is reproducible and near quantitative. This technique offers a simple and rapid alternative to either DNA polymerase or thin-layer chromatography assays for dNTPs in cell extracts.

The periodate-amine assay was applied to measurement of nucleoside triphosphate pools of L1210 murine leukemia cells treated with fluorouracil and methotrexate, both in combination and together with exogenously supplied nucleosides. Results from this preliminary study indicate the following major conclusions: (1) dGTP pools are intimately tied to dTTP pools in L1210 cells under a variety of conditions. (2) Inhibition of GDP reduction by dATP, as proposed from *in vitro* studies with purified ribonucleotide reductase from *E. coli*, is not apparent in this *in vivo* situation. (3) The regulation of CDP reduction is more complex than previously realized from *in vitro* studies. (4) High-dose methotrexate, an increasingly common chemotherapeutic treatment, appears to have a qualitatively different effect on dNTP pools in that it induces collapse of *all* dNTP pools, rather than just those of dTTP and dGTP. (5) Little evidence was found to support the hypothesis that inhibition of *de novo* dTMP synthesis spares intracellular pools of reduced folates during methotrexate treatment.

The effects of mutation in the genes for DNA-uracil glycosylase and dUTP phosphorylase on survival of *E. coli* cells during fluorodeoxyuridine-induced thymine starvation was investigated. It was

found that cells deficient in dUTP phosphorylase were strikingly sensitive to thymine starvation, and that this sensitivity was completely reversed by comutation of the DNA-uracil glycosylase gene. In addition, cells deficient in DNA-uracil glycosylase alone were somewhat protected from the effects of thymine deprivation. These results are in accord with a hypothesis suggesting that during thymineless states the incorporation of uracil into DNA increases dramatically, and that base excision-repair of these misincorporated residues is in part responsible for the DNA damage and resultant cell death observed.

INTRODUCTION

Thymidylate synthetase catalyzes the reductive methylation of 2'-deoxyuridylate (dUMP) to 2'-deoxythymidylate (dTMP) with the concomitant conversion of 5,10-methylenetetrahydrofolate (CH_2-H_4folate) to 7,8-dihydrofolate (H_2folate). This reaction is unique in that CH_2-H_4folate serves the dual function of both one-carbon carrier and reductant; with other enzymic reactions utilizing folate cofactors for carbon transfer H_4folate acts as a carrier and is regenerated unchanged. Since the amount of H_4folate within a cell is limited, the continuous synthesis of dTMP requires that the H_4folate formed be reduced and converted to CH_2-H_4folate. These three enzyme-catalyzed reactions, functioning in concert for the continuous production of dTMP and regeneration of CH_2-H_4folate, have been referred to as the "dTMP synthesis cycle" (Figure 1, Huennekins et al., 1963). Thymidylate synthetase is the sole de novo source of dTMP, and it is not surprising that enzymes of the dTMP synthesis cycle have been important targets of cytotoxic agents used in cancer chemotherapy. 5-Fluoro-2'-deoxyuridine (FdUMP), a metabolite of 5-fluorouracil, is a potent inhibitor of thymidylate synthetase and directly inhibits the formation of dTMP; and inhibitors of dihydrofolate reductase such as trimethoprim and

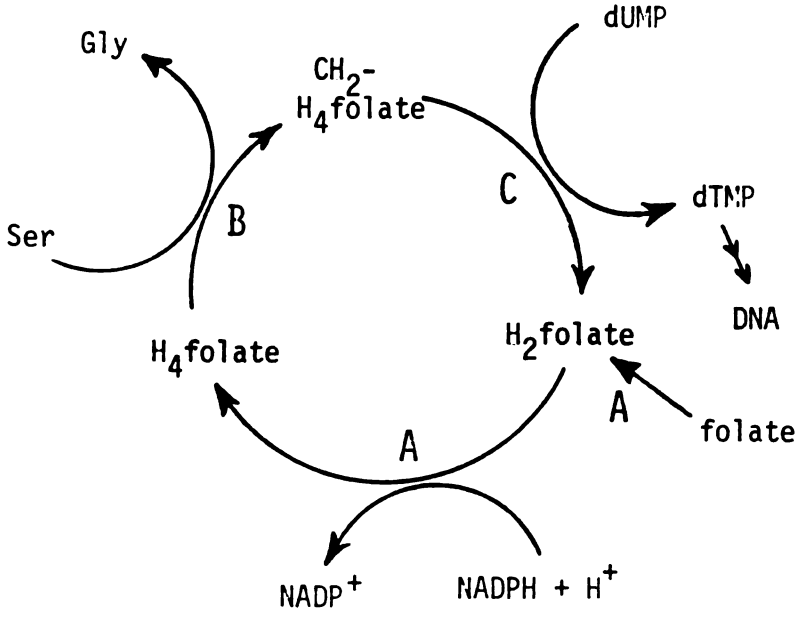

FIGURE 1: dTMP Synthesis Cycle. Enzymes involved are (A) dihydrofolate reductase, (B) serine hydroxymethyl transferase, and (C) thymidylate synthetase.

methotrexate prevent regeneration of H_4folate from H_2folate and deplete the cell of CH_2-H_4folate necessary for dTMP synthesis.

This thesis is concerned with several aspects of thymidylate metabolism. Firstly, experiments are described in which the catalytic mechanism of thymidylate synthetase itself has been investigated using the dUMP analogs 5-bromo- and 5-iodo-2'-deoxyuridylate. These two compounds are enzymatically dehalogenated to dUMP in the absence of folate cofactors, and the simplicity of the reaction has allowed a detailed investigation of the catalytic process involved. Secondly, the effects of 5-fluorouracil and methotrexate inhibition of dTMP synthesis on intracellular nucleotide metabolism are explored, with emphasis of the effects of these drugs in combination and in the presence of exogenously supplied nucleosides. Finally, a series of experiments is described in which the possible role of DNA-uracil during thymineless states is investigated. The goal of these studies is a better understanding of the exact enzymatic mechanism of thymidylate synthetase and its crucial role in intracellular nucleotide metabolism.

CHAPTER 1: THE MECHANISM OF THYMIDYLATE SYNTHETASE: BACKGROUND

It is generally accepted that an understanding of the catalytic mechanism of an enzyme requires knowledge of the basic mechanics of model (non-enzymic) reactions which bear resemblance to the one catalyzed by the enzyme. In the case of thymidylate synthetase, comprehensive model studies have been performed and have resulted in the proposed mechanism depicted in Figure 2 (Pogolotti and Santi, 1974). Model studies of electrophilic substitution at the 5-position of dUMP indicate that the reaction is initiated by attack of a nucleophile to the 6-position of the pyrimidine heterocycle giving the reactive enolate $\underline{\underline{1}}$. The initial condensation product $\underline{\underline{2}}$ formed between dUMP and CH_2-H_4folate is covalently bound to the enzyme and saturated across the 5,6-double bond of dUMP. Proton abstraction yields the enolate $\underline{\underline{3}}$ which should readily undergo a β-elimination to produce the highly reactive exocyclic methylene intermediate $\underline{\underline{4}}$ and H_4folate, bound to the enzyme in close proximity. Intermolecular hydride transfer from H_4folate to $\underline{\underline{4}}$, followed by β-elimination yields dTMP, H_2folate and native enzyme. The relevant point for this discussion is that model reactions of both electrophilic substitution at the 5-position of dUMP (i.e., alkylation by CH_2-H_4folate) and nucleophilic substitution at the 5-position of

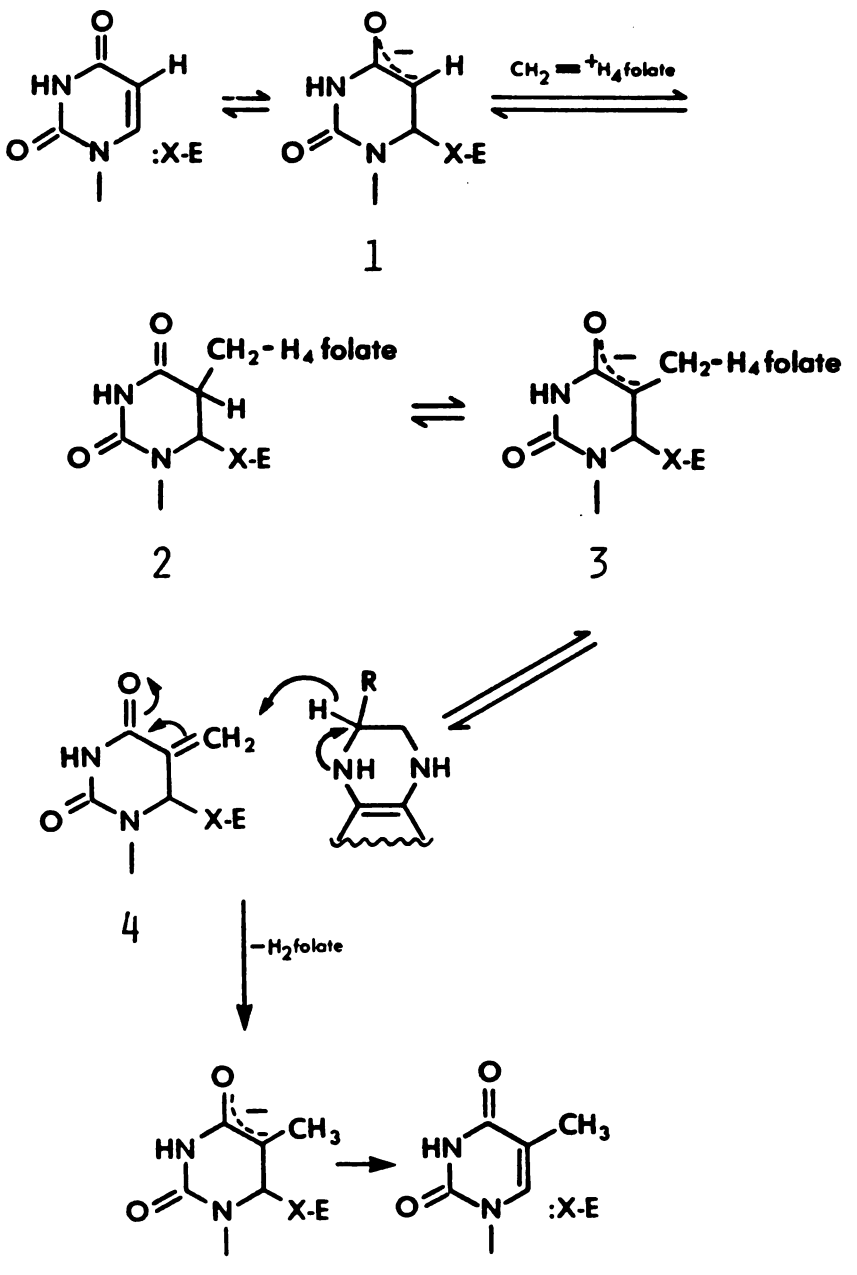

FIGURE 2: Suggested Sequence for the Thymidylate Synthetase Reaction Based on Model Studies.

thyminyl derivatives (i.e., hydride transfer from C-6 of H_4folate to the incipient methyl group of dTMP) are greatly facilitated by addition of a nucleophile to the 6-position of the pyrimidine heterocycle.

Of course, the validity of conclusions derived from chemical studies depends on the demonstration that intermediates and conversions proposed do in fact occur in the enzymatic reaction. Direct enzymic support for major aspects of the proposed mechanism of thymidylate synthetase was first derived from studies of its interaction with FdUMP. It has been known for some time (Heidelberger et al., 1960; Blakley, 1969) that this nucleotide is an extremely potent inhibitor of thymidylate synthetase, but the nature and mechanism of this inhibition was a topic of considerable controversy. In 1972 it was demonstrated (Santi and McHenry, 1972) that the dimeric enzyme from Lactobacillus casei interacts with 2 equivalents of FdUMP in a reaction that is dependent on the presence of CH_2-H_4folate. The complex formed was stable toward protein denaturants and showed a complete loss of absorbance at 269 nm, the absorbance maximum of FdUMP. Based on these observations and the model studies previously discussed, it was proposed that a covalent bond is formed between the enzyme and the 6-position of FdUMP. These results were independently confirmed by Langenbach et al., who further suggested that the 10-nitrogen of the cofactor is attached to the 5-position of FdUMP via a methylene bridge.

Shortly thereafter, several lines of evidence were reported which conclusively demonstrated that a reversible covalent bond is formed between FdUMP and thymidylate synthetase within the complex. (1) From studies of the relative rates of association and dissociation of FdUMP in the enzyme-CH_2-H_4folate binary complex, the dissociation constant of the nucleotide was calculated to be ca. 10^{-13} M (Santi et al., 1974). (2) The enzyme-FdUMP-CH_2-H_4folate complex can be treated with a number of protein denaturants (urea, guanidine hydrochloride, etc.) without apparent dissociation of protein-bound ligands (Danenberg et al., 1974; Santi and McHenry, 1972; Santi et al., 1974). With few exceptions such treatment is sufficient to disrupt noncovalent interactions between low molecular weight ligands and their protein receptors. (3) Although denaturation of the enzyme yields stable protein-bound ligands, ligands bound to the native complex slowly dissociate in an unchanged form (Lam et al., 1976; Santi et al., 1974). Thus, bonds formed between ligands and protein are reversible, and their dissociation requires the integrity of catalytic groups of the enzyme. (4) Upon formation of the ternary complex, there is a decrease of absorbance at 269 nm which corresponds to stoichiometric loss of the pyrimidine chromophore of FdUMP (Danenberg et al., 1974; Santi et al., 1974). This result strongly suggests that the 5,6-double bond of the pyrimidine is saturated in the bound complex. (5) Dissociation of [6-^{3}H]FdUMP from the complex shows a secondary tritium isotope effect (k_T/k_H)

of 1.23 (Santi et al., 1974). This would correspond to k_D/k_H = 1.15 and clearly demonstrates that the 6-carbon of the heterocycle undergoes sp^3 to sp^2 rehybridization during the process, as required if the 5,6-double bond of FdUMP is saturated in the complex. (6) Proteolytic digestion of the complex yields a peptide that is covalently bound to FdUMP and CH_2-H_4folate (Santi et al., 1974). Sommer and Santi, 1974), and the ^{19}F nuclear magnetic resonance spectrum of this fragment is consistent with the C-F bond being flanked by CH and CH_2groups (James et al., 1976).

From these lines of evidence, the structure of the enzyme-FdUMP-CH_2-H_4folate complex is currently believed to be that depicted in Figure 3. Here, a nucleophile of the enzyme has added to the 6-position of FdUMP and the 5-position of the pyrimidine is coupled to the 5-position of H_4folate via the methylene group of the cofactor. Thus, FdUMP serves to "trap" a ternary complex of the enzyme which resembles to a remarkable degree the intermediate proposed from studies of model counterpart reactions.

Further confirmation that nucleophilic attack at the 6-position of dUMP is a fundamental process in the enzymic reaction was obtained upon a demonstration that thymidylate synthetase catalyzes the exchange of the 5-H of dUMP for protons of water (Lomax and Greenberg, 1967; Pogolotti et al., 1979). This reaction does not require the presence of folate cofactors, and the important possibility of displacement by electrophiles other than solvent protons was eliminated. Although the exchange reaction is slow, it does not occur when the

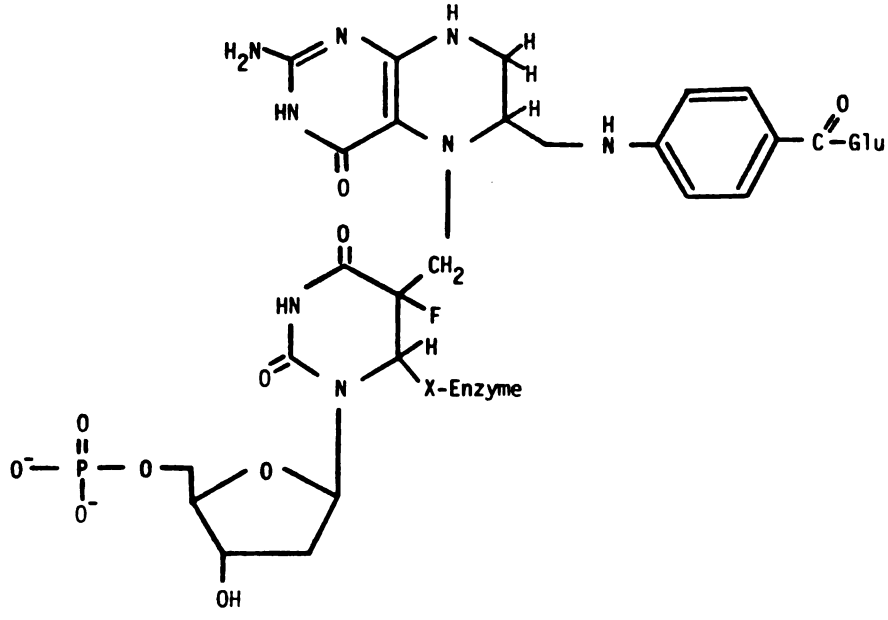

FIGURE 3: Structure of the FdUMP--CH_2-H_4Folate--Thymidylate Synthetase Covalent Complex.

enzyme is ommitted or treated with specific inhibitors. The presence of folate does not affect V_{max} of the reaction, but K_m is decreased by about 10-fold. Thus, it appears that folate increases the affinity of the enzyme for dUMP but has no effect on catalytic events leading to the exchange.

From a chemical standpoint, direct abstraction of the 5-H of dUMP is untenable since its pK_a may be estimated to exceed 50 (Cram, 1965). Numerous chemical studies (for a review see Pogolotti and Santi, 1977) have substantiated that 5-H exchange of 1-substituted uracils proceeds *via* a mechanism analogous to what Becker *et al.* (1977) have termed "nucleophilic addition-elimination". That is, a nucleophile attacks the 6-position of the heterocycle to provide a reactive enolate (structure $\underline{\underline{1}}$ in Figure 2) which upon protonation provides a 5,6-dihydropyrimidine intermediate analogous to $\underline{\underline{2}}$; reversal of these steps results in exchange of the 5-H for protons of water. Thus, catalysis of 5-H exchange by thymidylate synthetase provides convincing evidence for the existence of the dihydro-dUMP intermediate $\underline{\underline{2}}$ (Figure 2) and the precursor enolate $\underline{\underline{1}}$. The enolate $\underline{\underline{1}}$ is the nucleophilic form of dUMP which is believed to react with the cofactor in the pathway leading to dTMP, and its inferred existence provides further support for the notion that nucleophilic attack at the 6-position of dUMP is a requisite feature of reactions catalyzed by thymidylate synthetase.

In Chapter 2 yet a third reaction of thymidylate synthetase, the deahlogenation of BrdUMP and IdUMP, is described. The basic

mechanistic features of the reaction are clear and provide direct proof for the existence of 5,6-dihydropyrimidine intermediates in this thymidylate synthetase-catalyzed reaction.

A most important reason for obtaining a detailed understanding of the mechanism of thymidylate synthetase is the possibility that it may serve as a paradigm for a large number of enzymes. We believe that nucleophilic attack at the 6-position of the uracil or cytosine heterocycle may be a common mechanistic feature utilized by many enzymes to enhance the reactivity at various sites of the heterocycle. From what is known thus far, this is the most reasonable mechanism for a variety of enzyme-catalyzed electrophilic substitution reactions occuring at the 5-position of the uracil heterocycle. These would include the dUMP and dCMP hydroxymethylases, the pyrimidine methylases of RNA and DNA (the latter including certain restriction enzymes), pseudouridylate synthetase, and the large number of yet uncharacterized enzymes that alkylate the 5-position of minor bases found in tRNA. In addition, although not extensively studied, it appears that compared to the unaltered base, 5,6-dihydrouracil and cytosine derivatives are chemically more reactive toward nucleophilic substitution at the 4-position of the heterocycle and to glycosidic bond cleavage, and it is not unreasonable to suggest that at least some of the enzymes which catalyze such processes might also operate <u>via</u> nucleophilic attack at the 6-position of the heterocycle to achieve saturation of the 5,6-double bond.

CHAPTER 2: THYMIDYLATE SYNTHETASE; CATALYSIS OF DEHALOGENATION OF 5-BROMO- AND 5-IODO-2'-DEOXYURIDYLATE[1]

In a companion paper (Pogolotti et al., 1979) we reported that thymidylate synthetase catalyzes the exchange of the 5-H of dUMP for protons of water in the absence of the cofactor CH_2-H_4-folate. As this reaction requires addition of a nucleophile to the 6-position of the pyrimidine heterocycle, it represents direct evidence that a reaction catalyzed by thymidylate synthetase proceeds via nucleophilic catalysis as originally proposed (Santi and Brewer, 1968; Pogolotti and Santi, 1974.) According to this mechanism, initial attack by an enzyme nucleophile at the 6-position of dUMP serves to "activate" the neighboring 5-position for subsequent conversions. Here we describe the facile dehalogenation of BrdUMP[2] and IdUMP by thymidylate synthetase. The reaction is accompanied by a large inverse α-secondary tritium isotope effect at C-6 of the heterocycle which, together with extensively

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[1]Certain experimental portions of this work, including some kinetic measurments, the cysteine-promoted, non-enzymatic secondary isotope effect, and the effect of folate analogs, were performed by Dr. Yusuke Wataya.

[2]Abbreviations other than those recomended by IUPAC-IUB are as follows: XdUrd and XdUMP, where X = Br, Cl, F, or I, indicate 5-halogenated-2'-deoxyuridines and deoxyuridylates. CysdUrd is S-[5-(2'-deoxyuridyl)]cysteine, and CysdUMP is its 5'-monophosphate.

studied chemical counterparts, provides conclusive evidence that this enzyme-catalyzed reaction also proceeds *via* nucleophilic attack at the 6-position of the heterocycle and transient formation of 5,6-dihydropyrimidines.

EXPERIMENTAL PROCEDURES

Materials. BrdUMP, IdUMP, folate, H_2folate, 5-CH_3folate, 5-CHO-H_4folate, and aminopterin were purchased from Sigma Chemical Co. dUMP, dTMP, BrdUrd, and CF_3dUrd were obtained from P. L. Bio-Chemicals and FdUMP from Terra-Marine Bioresearch. Pteroic acid, homofolate, methotrexate, dichloromethotrexate, and 10-deazafolate were gifts of the Drug Research and Development Division of the National Cancer Institute. N^{10}-methylfolate was a gift from Lederle Laboratories and N^{10}-methyl-5,8-deazafolate from Parke, Davis and Company. [6-^{3}H]BrdUrd and [2-^{14}C]BrdUrd were purchased from New England Nuclear. *dl*-L-H_4folate was prepared from folate by the procedure of Hatefi *et al.* (1960), and CH_2-H_4folate was formed by the addition of a 15- to 30-fold excess of H_2CO. Other materials were of reagent grade or better quality from commercial sources.

Chromatographic Techniques. Chromatography on Aminex A-27 was as described by Garrett *et al.* (1977; Appendix 1), except that equipment capable of operating at 2000-3000 psi was used. The buffer systems used with Aminex A-27 are as follows: System A was 0.75 *M* ammonium acetate, pH 4.4, containing 8% *n*-propanol;

system B contained 0.5 mol ammonium bicarbonate and sufficient NH_4OH to give a final pH of 9.0 in a total volume of 1 liter; system C contained 0.35 mol ammonium bicarbonate, NH_4OH to give pH 9.0, and 80 ml n-propanol in a total volume of 1 liter. Separations were performed at ambient temperature except when using system B, in which case a temperature of 85° was maintained with a water-jacketed column. Chromatography on Lichrosorb C_{18} (4.6 x 250 mm column; 10 μ particle size) was performed at ambient temperature using an eluting buffer (pH 7.0) containing 5 mM tetrabutylammonium hydrogensulfate, 5 mM potassium phosphate, and 1% acetonitrile. Two-dimensional paper chromatography of nucleotides was performed on Whatman 3MM paper using isopropanol:28% NH_4OH:H_2O (7:1:2) in the first direction and isobutyric acid:0.5 N NH_4OH (5:3) in the second; two-dimensional thin layer chromatography of nucleosides was performed on 20 x 20 cm cellulose plates (Merck, 0.10 mm) using n-butanol:H_2O (86:14) in the first direction and n-butanol:acetic acid:H_2O (2:1:1) in the second.

CysdUrd and CysdUMP. CysdUrd was prepared by incubating a solution (0.5 ml) containing 5 mg (16 μmol) BrdUrd and 0.25 M cysteine at pH 9.0 for 2.5 hr at 37° (Wataya et al., 1973.) The product was purified on a 9 x 250 mm Aminex A-27 column using system B at 55°. CysdUMP was prepared from BrdUMP (5 mg, 12 μmol) in an identical manner; the product was well separated from small amounts of contaminating dUrd and CysdUrd. Its identity was confirmed by the following criteria: (a) it had uv spectra identical to that

of CysdUrd in both acidic and alkaline solution, and (b) upon treatment with E. coli alkaline phosphatase it was converted to a compound indistinguishable from CysdUrd by chromatography on Aminex A-27 systems B and C, and on Lichrosorb C_{18}.

Thymidylate Synthetase. Thymidylate synthetase was obtained from a methotrexate-resistant strain of Lactobacillus casei (Crusberg et al., 1970) and purified as previously described (Wataya and Santi, 1977.) Protein concentration was determined using A_{278} = 1.07 x 10^5 $M^{-1}cm^{-1}$ (Santi et al., 1974.) FdUMP binding sites were titrated spectrophotometrically as follows: One of two previously balanced cuvettes contained in 1.0 ml ca. 5 μM thymidylate synthetase, 50 mM N-methylmorpholine-HCl (pH 7.4), 6.5 mM dithiothreitol, 25 mM $MgCl_2$, and 1 mM ethylenediamine tetraacetate. Enzyme was omitted in the reference cuvette, and the uv absorbance of the enzyme at 278 nm was recorded. CH_2-H_4folate was then added to both cuvettes to give a final concentration of 0.20 mM, the baseline was recorded, and successive additions (10 μl) of an 0.12 mM solution of FdUMP were made to each cuvette until no further increase of uv absorbance at 330 nm was observed. The concentration of titratable enzyme binding sites was then calculated using $\Delta\varepsilon_{330}$ = 17,700 $M^{-1}cm^{-1}$ for formation of the enzyme-CH_2-H_4folate-FdUMP complex (Santi et al., 1976.) The molar amount of titratable enzyme was calculated assuming two binding sites per 70,000 dalton dimer and corresponded to 85% of the amount of enzyme calculated from the A_{278}.

Standard Assay Conditions. Unless otherwise noted, dehalogen-

ation reactions were performed at 30° in a buffer containing 50 mM N-methylmorpholine-HCl (pH 7.4), 25 mM $MgCl_2$, 1 mM ethylenediamine tetraacetate, and 75 mM β-mercaptoethanol. Kinetic assays were performed by monitoring the decrease in absorbance at 285 nm for BrdUMP ($\Delta\varepsilon$ = 5320) and at 290 nm for IdUMP ($\Delta\varepsilon$ = 6520); reference cuvettes lacked enzyme.

Calculation of Secondary Tritium Isotope Effects. Unless otherwise specified, reactants and products were separated by high-performance liquid chromatography as described; care was taken to collect each radioactive peak in its entirety to avoid isotope separation. The radioactivity in each peak was counted 3-4 times and a minimum of 2 x 10^5 ^{14}C counts collected; standard errors (S. E.) for determination of $^3H/^{14}C$ ratios and ^{14}C dpm's were ca. 0.25% and 0.5%, respectively. In the case of determinations on CysdUMP, fewer total counts were collected; S. E.'s of isotopic ratios and ^{14}C dpm's were 0.5-2.0%. Isotope effects were calculated from the isotopic ratios of either product or reactant as described by Melander (1960.) All statistical estimates are presented as mean ± S.E.

Miscellaneous. [6-^3H, 2-^{14}C]BrdUMP was prepared from [6-^3H, 2-^{14}C]BrdUrd by phosphorylation with thymidine kinase and purification on diethylaminoethyl cellulose (Wataya and Santi, 1977.) The product was further purified on Aminex A-27 (system A) to remove contaminating AMP. Radiochemical purity was greater than 99.9% as judged by chromatography on Aminex A-27 using systems A or B. CF_3dUMP

was prepared similarly, except that final purification was performed by ascending paper chromatography (Whatman No. 1 paper; n-butanol: acetic acid:H_2O, 2:1:1; R_f = 0.47.)

Measurements of free thiol concentration were made by the method Ellman (1959) using ε_{412} = 14,100 for the thiophenolate product (Collier, 1973.) E. coli alkaline phosphatase treatment of CysdUMP was performed by adding 0.9 units of an enzyme suspension (Worthington BAPF, 32.3 U/mg) to 0.03-0.75 μmol of the nucleotide in 0.3 ml 0.1 M $(NH_4)_2CO_3$ buffer, pH 9.0, and incubating overnight at 37°. Spectrophotometric determinations were made using a Cary 118 recording spectrophotometer. Radioactive isotopes were measured by counting in a fluid containing 0.4% Omnifluor in xylene:Triton X-114 (3:1) or in Aquasol-2 (New England Nuclear) using a Nuclear Chicago Isocap 300 liquid scintillation spectrometer. Counting efficiencies were determined by the external standards ratio method and dpm calculations were aided by a tape-fed Hewlett-Packard computer.

RESULTS

Reaction of BrdUMP and IdUMP with Thymidylate Synthetase and Identification of dUMP as the Major Product. Figure 4 shows the ultraviolet spectral changes which occur upon treatment of BrdUMP or IdUMP with thymidylate synthetase; in these reactions dithiothreitol was used in place of β-mercaptoethanol to minimize end absorbtion due to atmospheric oxidation of the thiol and side product formation (see below.) In both cases there is a time-dependent change of the

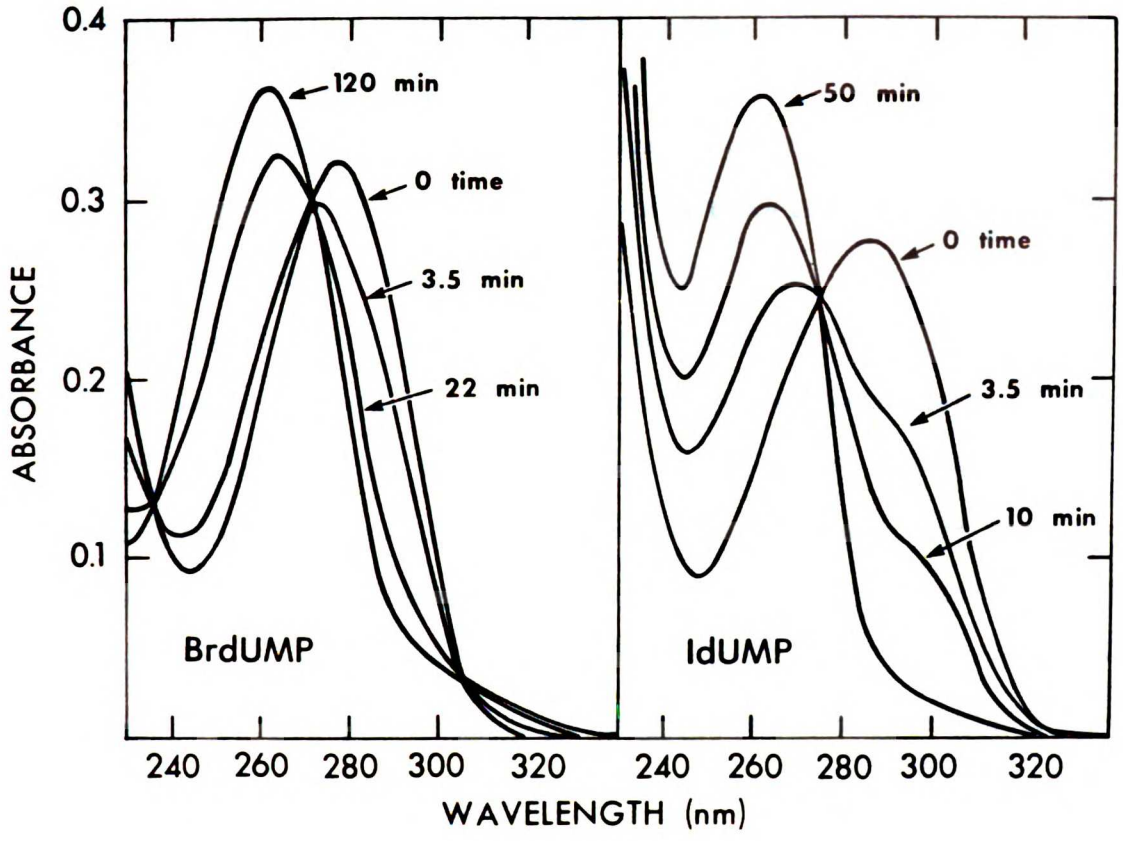

FIGURE 4: Ultraviolet spectral changes of (A) 40 μM BrdUMP and (B) 38 μM IdUMP when reacted with 0.6 μM thymidylate synthetase in the presence of 6.5 mM dithiothreitol. Other components of the reaction mixture are as described in Experimental Procedures.

absorption maximum of the halogenated substrates to 262 nm. No spectral changes were observed when CldUMP or FdUMP were treated similarly, and inhibition of the enzyme (0.8 μM) by pre-treatment with CF_3dUMP (3.7 μM) for 30 min at room temperature decreased the rate of reaction of IdUMP by at least 95%.

The major product of the reaction was ascertained to be dUMP by the following criteria: (i) The products of the reaction of BrdUMP and thymidylate synthetase were analyzed by two-dimensional paper chromatography and by chromatography on Aminex A-27 (systems A, B, and C.) In all cases the major uv-absorbing product had a mobility identical to that of dUMP. Elution of the major spot obtained from the paper chromatography yielded a product which had an ultraviolet spectrum identical to that of dUMP in acidic and alkaline pH regions. (ii) After completion (2 hr) of the reactions depicted in Figure 4, excess CH_2-H_4folate was added to the reaction mixture to give a final concentration of 0.18 mM and the increase in absorbance at 340 nm (characteristic of the formation of dTMP and H_2folate from dUMP and CH_2-H_4folate) was monitored. From $\Delta\varepsilon_{340}$ = 6400 (Whaba and Friedkin, 1962) the products obtained from BrdUMP and IdUMP were ascertained to be 89 and 100% dUMP, respectively. The dehalogenation of IdUMP is accompanied by a corresponding increase in absorbance at 230-260 nm (Figure 4) which is attributable to end absorption of I^-; Br^- does not absorb in this region, and the conversion of BrdUMP to dUMP shows a well-defined isosbestic point at 236 nm. Using ε_{240} = 330 for I^-, ε_{240} = 4920

for dUMP, and ε_{240} = 330 for oxidized thiol (see Discussion) it can be calculated that I^- and dUMP are formed in stoichiometrically equivalent amounts during the deiodination reaction.

In an attempt to measure the reversibility of the reaction, 50 μM BrdUMP, 1.2 μM enzyme, and [6-^{3}H]dUMP (1.1 μCi/ml, 21 Ci/mmol) were incubated with other components of the standard reaction mixture. After approximately 50% completion of dehalogenation of BrdUMP, a 100 μl aliquot was separated on Aminex A-27, system C, and fractions counted for tritium content. We detected no formation of BrdUMP from dUMP ($\leq$ 0.4%) by this method even when 100 mM NaBr was included in the reaction mixture.

Kinetics of Dehalogenation. Under the standard conditions described in Experimental Procedures, the initial reaction velocity is linearly proportional to enzyme concentration up to 6 μM enzyme with 75 μM BrdUMP as the substrate, and up to at least 8 μM using 75 μM IdUMP. Kinetic parameters of the thymidylate synthetase-catalyzed dehalogenation are shown in Table 1; values for dTMP synthesis from dUMP and CH_2-H_4folate are also given for comparison. Both dTMP and dUMP are competitive inhibitors of the dehalogenation of BrdUMP (Figure 5), with K_i values of 8.7 and 2.3 μM, respectively; dTMP and dUMP also inhibit the deiodination of IdUMP.

The pH optimum of the debromination reaction is ca. 7.4, with one-half maximal rates at pH 6.6 and 8.0. The reaction rate was affected by the solvent buffer concentration, increasing more than three-fold between 25 and 200 mM N-methylmorpholine. Figure 6 shows

TABLE 1: Kinetic Parameters for the Thymidylate Synthetase-Catalyzed Dehalogenation of BrdUMP and IdUMP[a]

Substrate	K_m (μM)	k_{cat} (min^{-1})[b]
BrdUMP	5.7	5.9
IdUMP	1.4	2.1
dUMP (dTMP formation)[c]	3.0	384

[a]The assay mixture contained 0.21 μM enzyme and other components as described in Experimental Procedures. Kinetic parameters were estimated by non-linear curve fitting techniques. [b]Values are per 70,000 dalton dimer. [c]The reaction mixture contained 0.2 mM CH_2-H_4folate

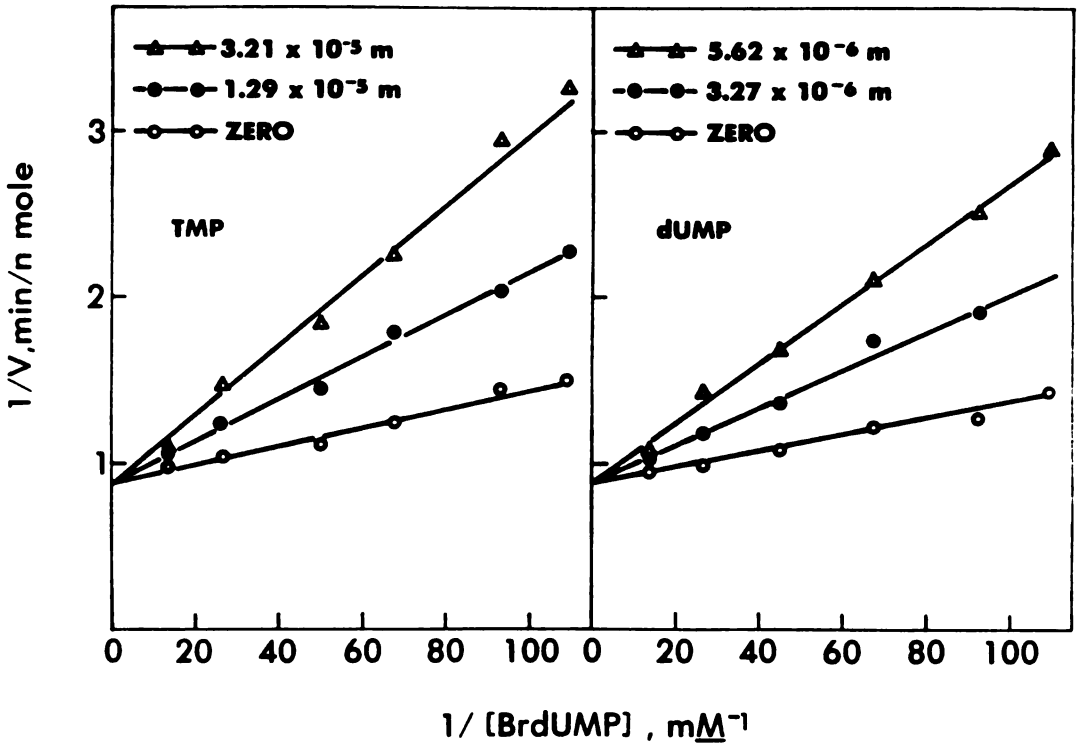

FIGURE 5: Double reciprocal plots of the inhibition of enzymatic debromination by dUMP and dTMP. Reaction mixtures were as described in Experimental Procedures; enzyme concentration was 0.25 μM.

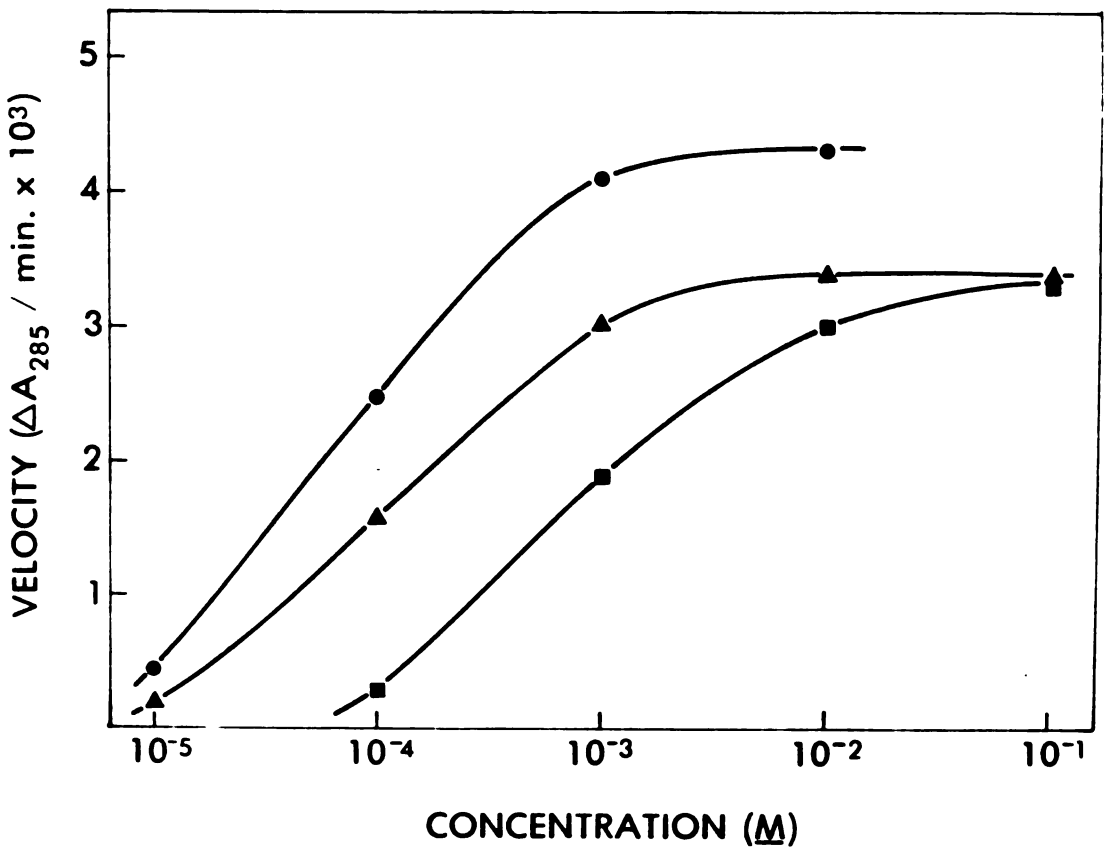

FIGURE 6: Effect of thiols on the thymidylate synthetase-catalyzed debromination of BrdUMP. The standard reaction mixture contained 50 μM BrdUMP, 0.29 μM enzyme, and thiol reagents as indicated: ●, dithiothreitol; ▲, β-mercaptoethanol; ■, cysteine. Incubations were at 25°.

the rate dependence of debromination of the concentration of dithiothreitol, β-mercaptoethanol, and cysteine. Glutathione also supported the debromination reaction; over the concentration range 0.5-10 mM it was nearly as effective as cysteine. In the absence of added thiols the reaction proceeded extremely slowly; however, at high enzyme concentrations (5 μM) some reaction was noted, perhaps due to participation of enzyme thiol groups as reductants in the reaction.

Formation of Minor Nucleotide Products. During the course of the enzymatic dehalogenation of BrdUMP, but not IdUMP, a distinct increase in uv absorbance at 310-320 nm occurs (Figure 4.) This observation, together with the lower yield of dUMP as compared to that obtained during the dehalogenation of IdUMP, suggested the formation of a minor product in the debromination reaction. When [2-^{14}C]BrdUMP (50 μM) was allowed to react with enzyme (1.1 μM) until all spectral changes had stopped, and the reaction mixture was separated on Aminex A-27, a second radioactive peak corresponding to a product other than dUMP was found. Both the amount of this product and its chromatographic properties were determined by the thiol reagent in the reaction. After separation on Aminex A-27 with the indicated buffer systems, the following amounts of minor product were formed, expressed as a percentage of total product: With 75 mM and 0.2 mM β-mercaptoethanol (system C), 30 and 32%, respectively; with 10 mM cysteine (system B), 9%; with 6.5 mM dithiothreitol (system C), 7%. In each case the product elutes be-

tween dUMP and BrdUMP, a position characteristic of 5-substituted deoxyuridylates (Garrett et al., 1977.) With β-mercaptoethanol and cysteine, the indicated chromatographic system allows full separation of the second product from both dUMP and BrdUMP, and it was seen that the minor product is formed in constant proportion to dUMP throughout the course of the reaction; thus it is not an intermediate in the conversion of dUMP to BrdUMP. No indication of side product formation was found with IdUMP as the substrate.

Identification of CysdUMP as the Minor Reaction Product Formed in the Presence of Cysteine. [2-^{14}C]BrdUMP (50 μM, 23 Ci/mol) was reacted with thymidylate synthetase (1.1 μM) in the presence of 10 mM cysteine; after 25 min at 30° the reaction was 97% complete. (In a parallel control reaction lacking enzyme ca. 1% dehalogenation was observed.) CysdUMP was added to a portion of the reaction mixture as a chromatographic marker, and the mixture was separated on Aminex A-27 using system B. In addition to the radioactive peaks corresponding to dUMP and BrdUMP, a third peak corresponding to the minor product was found intermediate between them and at the exact position of CysdUMP. The product was isolated from the remaining reaction solution and converted to the nucleoside with E. coli alkaline phosphatase; after this treatment it was indistinguishable from CysdUrd by chromatographic analysis on Aminex A-27 systems B and C, on Lichrosorb C_{18}, and on the four paper chromatographic systems used for the identification of CysdUrd by Wataya et al. (1973.)

Stoichiometry of Thiol Utilization. A reaction mixture, containing 100 μM BrdUMP, 1.2 μM enzyme, and an initial concentration of 200 μM β-mercaptoethanol, was incubated at room temperature (21°) in an N_2-purged chamber. Progress of the reaction was monitored spectrophotometrically and aliquots were removed for determination of thiol concentration; over the time period of the experiment (1.5 to 2.0 hrs) control reactions lacking enzyme or BrdUMP showed no significant changes in thiol concentration.

The results of three such experiments demonstrate that the disappearance of thiol groups is proportional to the amount of BrdUMP dehalogenated up to at least 50% completion of the reaction, and that under these conditions 1.8 ± 0.1 (n = 12) equivalents of thiol are oxidized per mole BrdUMP debrominated. By analysis on Aminex A-27 (system C) of an identical reaction mixture in which the substrate was radioactively labelled, it was determined that the product distribution was 68% dUMP and 32% another nucleotide, presumably 5-β-hydroxyethylthio-dUMP. Assuming that one equivalent of thiol is used in the formation of each mole of side product (see Discussion) it may be calculated that 2.1 moles thiol are consumed per mole dUMP formed. Similar experiments were attempted using cysteine in place of β-mercaptoethanol, but rapid oxidation of cysteine in the control reaction prevented accurate determination of the stiochiometry of its consumption.

Inverse Secondary Isotope Effect upon Enzymatic Debromination of BrdUMP. As shown in Figure 7, when [6-^{3}H, 2-^{14}C]BrdUMP is allow-

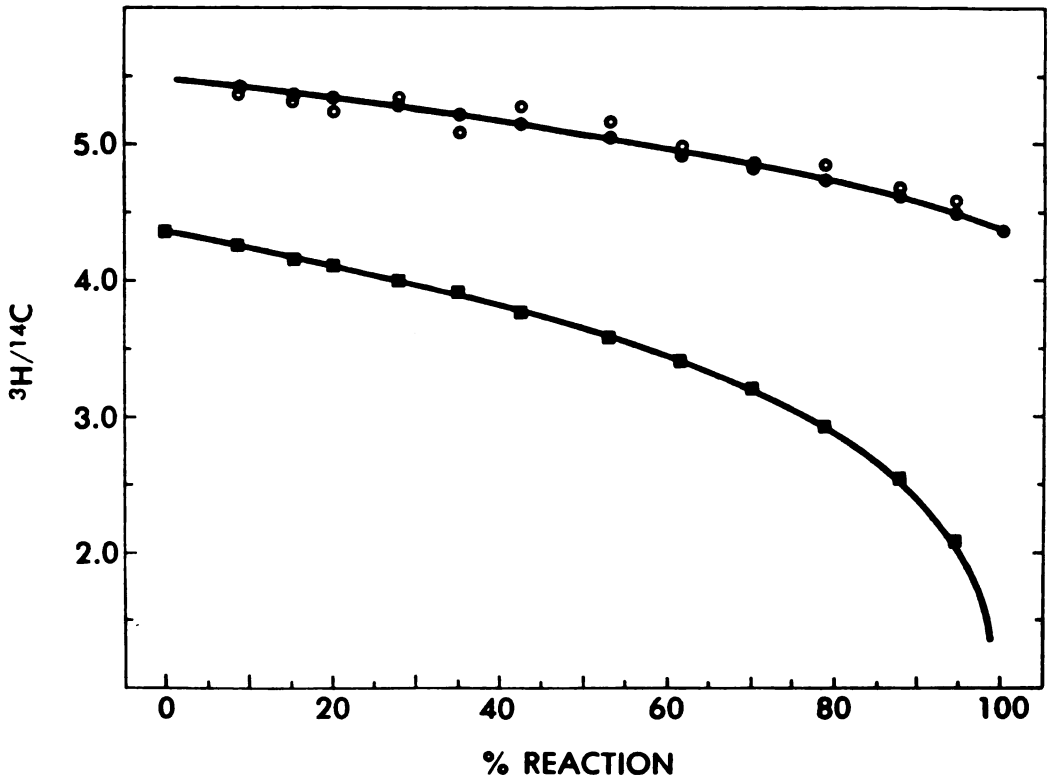

FIGURE 7: Inverse secondary isotope effect in the thymidylate synthetase-catalyzed debromination of [6-^{3}H, 2-^{14}C]BrdUMP. The standard reaction mixture (3.5 ml) contained 50 μM BrdUMP (5.21 Ci/mol of ^{14}C, $^3H/^{14}C$ = 4.360), 10 mM cysteine, and 1.1 μM enzyme. Aliquots (0.15 ml) were withdrawn, dUMP and BrdUMP were added as chromatographic markers (ca. 1 mM each), and the aliquots were kept on ice until separation of reactant (BrdUMP) and products (dUMP and CysdUMP) on Aminex A-27 system B. Data points represent the $^3H/^{14}C$ ratios of dUMP (●), CysdUMP (○), and BrdUMP (■); also depicted are the theoretically expected changes in isotopic ratios of product (upper line) and reactant (lower line) for a reaction with k_T/k_H = 1.258 (Melander, 1960).

ed to react with thymidylate synthetase in the presence of 10 mM cysteine, the $^{3}H/^{14}C$ ratios of the reactant (BrdUMP) and the products (dUMP and CysdUMP) change in a manner indicating a more rapid dehalogenation of the 6-tritiated compound. Dehalogenation in a parallel rection lacking enzyme was negligible. The inverse secondary tritium isotope effects (k_T/k_H) are 1.253 $\pm$ 0.003 and 1.31 $\pm$ 0.03 calculated from the $^{3}H/^{14}C$ ratios of dUMP and CysdUMP, respectively (n = 12.) If, for each determination during the course of the reaction we combine the isotopic content of each product, the mean isotope effect for product formation computed in this way is 1.256; this agrees well with the value of 1.260 $\pm$ 0.003 (n = 12) calculated from the decreasing isotopic ratio of the substrate BrdUMP. These and other data collected in similar experiments in which the nature or concentration of thiol reagent were changed are summarized in Table 2.

As pointed out by Kirsch (1977), small deterministic errors may far outweigh the statistically determined standard errors in any single experiment, and we do not consider the relatively small differences in kinetic isotope effects observed in separate experiments reported here to be significant.

Inverse Secondary Isotope Effect upon Cysteine-Catalyzed Debromination of BrdUrd. 10 mM [6-^{3}H, 2-^{14}C]BrdUrd was allowed to react with 0.25 M cysteine (pH 7.3) at 37°. The extent of debromination was monitored spectrophotometrically, and aliquots were removed at intervals. The reactant (BrdUrd) and products (dUrd

TABLE 2: Inverse Secondary Isotope Effect in the Thymidylate Synthetase-Catalyzed Debromination of [6-^{3}H]BrdUMP.

Thiol Additive	Isotope Effect[a]
10 m$\underline{M}$ Cysteine	1.258 ± 0.002 (n = 24)[b]
6.5 m$\underline{M}$ Dithiothreitol	1.243 ± 0.002 (n = 5)[c]
0.01 m$\underline{M}$ Dithiothreitol	1.229 ± 0.002 (n = 5)[c]
75 m$\underline{M}$ β-Mercaptoethanol	1.212 ± 0.012 (n = 20)[b]

[a]Isotope effects were determined as described in the text. In the case of dithiothreitol and β-mercaptoethanol separations of product and reactant were performed using Aminex A-27 system C. [b]Average of all determinations as calculated from products and reactant. [c]Calculated from dUMP at $\leq$ 30% reaction.

and CysdUrd) were separated by two-dimensional thin layer chromatography, and the isotopic ratio of each was determined after elution with water.

Values of k_T/k_H calculated for formation of dUrd and CysdUrd were 1.187 $\pm$ 0.006 and 1.156 $\pm$ 0.007, respectively; k_T/k_H from dehalogenation of BrdUrd was 1.174 $\pm$ 0.005 (n = 9.) Upon completion of the reaction CysdUrd constituted 8% of the product.

Effect of Folate Analogs on Dehalogenation. The effect of a variety of folate analogs is shown in Table 3. Most of the compounds tested had either no effect or were inhibitory to the reaction. Methotrexate and aminopterin are notable exceptions, showing marked stimulatory effects on the reaction rate. Folic acid was observed to demonstrate intersecting hyperbolic non-competitive inhibition (Segal, 1975) of the debromination reaction (data not shown.)

DISCUSSION

Treatment of BrdUMP and IdUMP with catalytic amounts of thymidylate synthetase in the presence of thiol reagents results in their facile conversion to dUMP, the natural substrate of the enzyme. By monitoring the loss of free thiol groups from the reaction mixture and, in the case of deiodination of IdUMP, the formation of halide anion, we have determined the stoichiometry of the reaction to be as follows:

$$\text{BrdUMP(IdUMP)} + 2\text{RSH} \longrightarrow \text{dUMP} + (\text{RS})_2 + \text{HBr(HI)}$$

TABLE 3: Effect of Various Folate Analogs on the Rate of the Thymidylate Synthetase-Catalyzed Dehalogenation of IdUMP and BrdUMP.[a]

Analog	Relative Rate	
	IdUMP	BrdUMP
None (control)	1.00	1.00
H_4Folate	1.00	0.57
$\underline{N}^5$-CHO-H_4Folate	0.67	0.39
$\underline{N}^5$-CH_3-H_4Folate	0.90	0.89
Folic acid	0.93	0.54
$\underline{N}^{10}$-CH_3-Folic acid	0.58	0.31
Pteroic acid	0.89	0.93
Homofolic acid	0.84	0.56
Aminopterin	1.55	3.21
Methotrexate	2.08	3.25
Dichloromethotrexate	0.95	0.97
10-Deazafolic acid	0.86	0.50
5,8-Deaza-$\underline{N}^{10}$-CH_3-Folic acid	0.19	0.05

[a]The standard reaction mixture contained 40 μM substrate and 0.17 μM enzyme. All analogs were used at a concentration of 21 μM. Rates (initial velocities) were determined as ΔA_{290} per unit time.

Neither CldUMP nor FdUMP serve as substrates for the reaction, and we could detect no reversal of the reaction.

The optimal pH for the reaction under the standard conditions described here using N-methylmorpholine buffer is pH 7.4. Since the rate of reaction increased at higher buffer concentrations, the pH optimum might vary depending on the buffer used. The rate of the reaction is also dependent upon the concentration of thiol reagents in the reaction mixture. With dithiothreitol or β-mercaptoethanol, approximately 0.01 M concentrations are sufficient for maximal activity; with cysteine a ten-fold higher concentration is required.[3]

Unlike the normal enzymatic reaction, dehalogenation does not require the presence of CH_2-H_4folate.[4] Folate was observed to de-

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[3]The glutathione concentration in mammalian tissues is 0.5-10 mM (Jocelyn, 1972.) This level of glutathione is sufficient to support the dehalogenation reaction described here, and it appears possible that an important pathway for biological catabolism of 5-bromo- and 5-iodouracil derivatives may involve their dehalogenation at the deoxyribonucleotide level by thymidylate synthetase. With CH_2-H_4folate the overall reaction involves conversion of these halogenated nucleotides to dTMP and represents a route by which they may be utilized as precursors of DNA. This fact warrants consideration in experiments involving *in vivo* incorporation of 5-bromo- and 5-iodouracil into DNA.

[4]A standard assay for thymidylate synthetase activity involves monitoring the increase in uv absorbance at 340 nm which is characteristic of the formation of dTMP and H_2folate from dUMP and CH_2-H_4folate (Wahba and Friedkin, 1962.) The accuracy of this assay is dependent upon the purity of the highly unstable cofactor and for this reason often suffers from day-to-day variations using the same enzyme sample. Since the dehalogenation reaction has no requirement for folate cofactors it may serve as a convenient assay for the enzyme in situations where the greater sensitivity of the conventional method is not necessary.

monstrate intersecting hyperbolic non-competitive inhibition kinetics (Segal, 1975), and although the complexity of the system did not permit an exact evaluation of kinetic constants it is clear that within the enzyme-folate-BrdUMP complex dehalogenation occurs, albeit at a slower rate than in the enzyme-BrdUMP complex. In contrast, methotrexate and aminopterin enhance the rate of dehalogenation of BrdUMP, demonstrating that the complex formed with these analogs is more reactive than the binary enzyme-BrdUMP complex. Whether these effects result from subtle catalytic differences when the folate analogs are bound to the same site of the enzyme as the cofactor or from different binding characteristics is currently unknown.

The K_m values of BrdUMP (5.7 μM) and IdUMP (1.4 μM) are similar to that of dUMP (3.0 μM) in the normal enzymic reaction, but V_{max} for dehalogenation is some 100-fold slower than it is for dTMP synthesis. Both BrdUMP and IdUMP are competitive inhibitors with respect to dUMP in the normal enzymic reaction, having K_i values of 1.4 μM and 1.6 μM, respectively (Wataya et al., 1977.) Likewise, dUMP (K_i = 2.3 μM) and dTMP (K_i = 8.7 μM) are competitive inhibitors of dehalogenation of BrdUMP. These data, as well as similarities in the catalytic mechanisms of the reactions discussed below, support the view that the same site of the enzyme is involved in the binding of nucleotide substrates and in catalysis of both dehalogenation and dTMP formation.

During the enzymic debromination of BrdUMP in the presence of cysteine, a minor side product is formed which we identified to be

CysdUMP. This is directly analogous to the cysteine-promoted dehalogenation of BrdUrd in which CysdUrd is formed as a side product (Wataya et al., 1973.) Enzymic debromination of BrdUMP in the presence of other thiols also produces side products with chromatographic properties characteristic of 5-substituted dUMP's, and it is reasonable to conclude that these are also corresponding 5-alkylthio-2'-deoxyuridylates. For reasons unclear, no such side products are formed during the dehalogenation of IdUMP; this too is in accord with chemical studies in which the cysteine-promoted dehalogenation of IdUrd is not accompanied by significant formation of CysdUrd (H. Hayatsu, personal communication.)

Numerous chemical studies on the thiol-mediated dehalogenation of 1-substituted-5-bromo(iodo)uracils have resulted in the proposal of the mechanisms depicted in Figure 8 (for a review see Sander, 1978.) The initial and perhaps rate-determining step almost certainly involves attack of thiolate anion at the 6-position of the heterocycle (1a); subsequent protonation of C-5 yields the 5-bromo-6-cysteinyl-5,6-dihydrouracil 2a. Two general pathways have been proposed to account for subsequent steps leading to the dehalogenated product. The first, E2 Hal, involves abstraction of bromonium (Br^+) ion from 2a to provide intermediate 3a and a sulfenyl halide. The latter would rapidly react with thiol to yield the halide ion and disulfide, and β-elimination of 3a would yield products. The second mechanism (S_N2) involves nucleophilic displacement of Br^- from 2a by thiolate to give intermediate 5a. Further reaction with

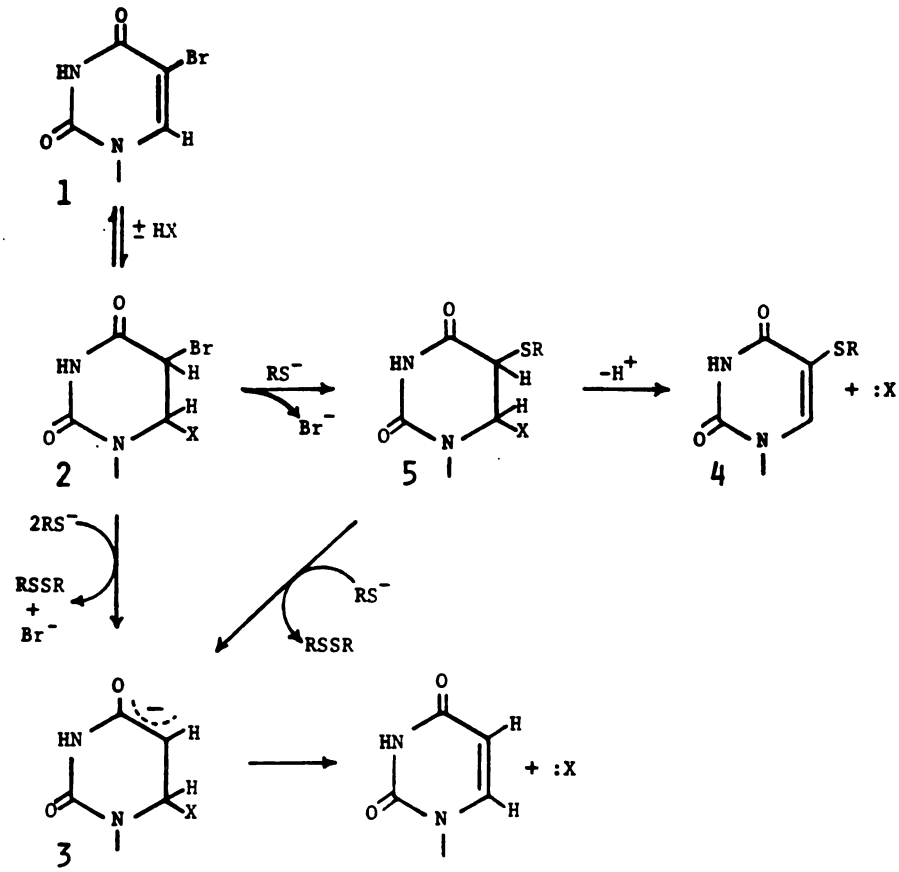

FIGURE 8: Proposed mechanism for the debromination of BrdUMP by (a) thiols and (b) thymidylate synthetase.

RS^- would yield the oxidized thiol (RS_2) and intermediate $\underline{\underline{3a}}$, which is common with the E2 Hal mechanism and would yield the dehalogenated pyrimidine upon β-elimination. As previously mentioned, the cysteine-induced dehalogenation of BrdUrd is accompanied by formation of CysdUrd, the mechanism of which has been proposed to involve conversion of $\underline{\underline{5a}}$ to $\underline{\underline{4a}}$. While the mechanism proposed for the thiol-mediated dehalogenation is in accord with kinetic data, it has largely rested upon analogy with the extensively studied bisulfite-mediated dehalogenation where direct evidence of 5,6-dihydropyrimidine intermediates has been firmly established. In the case of the thiol-mediated reaction, the proposed intermediates are unstable and their existence has not heretofore been directly demonstrated.

The observation of a deuterium isotope effect of greater than 10%, equivalent to a tritium effect of 15% (Swain et al., 1958), is generally accepted to provide conclusive evidence for carbon rehybridization at or before the rate-limiting step in a reaction. Thus, the magnitude of the inverse secondary isotope effect reported here for the cysteine-promoted, non-enzymatic dehalogenation of 6-tritiated BrdUrd (k_T/k_H = 1.174) demonstrates the occurence of sp^2 to sp^3 rehybridization at the 6-carbon of the pyrimidine heterocycle at or before the rate-determining step of the reaction, and confirms the existence of 5,6-dihydropyrimidine intermediates early in this reaction pathway. Likewise, the substantial secondary isotope effect in the thymidylate synthetase-catalyzed conversion of BrdUMP to dUMP (k_T/k_H = 1.212-1.258) convincingly demonstrates that

an early catalytic event in the enzymic reaction also involves nucleophilic attack at the 6-position of the pyrimidine heterocycle and consequent formation of a 5,6-dihydropyrimidine intermediate. We therfore conclude that the thymidylate synthetase-catalyzed dehalogenation reaction proceeds as described in Figure 8. Since the 5-alkylthio-dUMP 4b is formed during debromination of BrdUMP, it would appear that intermediate 5b is formed and that the S_N2 mechanism plays a significant role. As is the case with the non-enzymatic reaction, the relative contribution of the E2 Hal mechanism (2b → 3b) is not easily determined.

In summary, studies of model chemical counterparts and interactions with mechanism-based inhibitors have resulted in general acceptance that the mechanism of thymidylate synthetase involves nucleophilic attack at the 6-position of dUMP to form 5,6-dihydropyrimidine intermediates in which normally inert moities of the pyrimidine heterocycle are activated. However, until this time no direct evidence has been reported which demonstrates that reactions catalyzed by this enzyme proceed in this manner. In this and the accompanying paper (Pogolotti et al., 1979) we demonstrate that thymidylate synthetase catalyzes the exchange of the 5-H of dUMP for protons of water and the dehalogenation of BrdUMP and IdUMP. From chemical precedent and analysis of α-secondary isotope effects, conclusive evidence has been obtained that these enzyme-catalyzed reactions proceed via nucleophilic attack at the 6-position of the heterocycle to form transient 5,6-dihydropyrimidine intermediates.

These results are in complete accord with and verify important aspects of the mechanism originally proposed for this enzyme (Santi and Brewer, 1968; Pogolotti and Santi, 1974; 1977.)

CHAPTER 3: INVESTIGATION OF THE MECHANISM OF URACIL-DNA GLYCOSYLASE BY THE USE OF α-SECONDARY HYDROGEN ISOTOPE EFFECTS

Uracil-DNA glycosylase catalyzes the release of free uracil from uracil-containing DNA by hydrolysis of N-glycosidic bonds in deoxyuridine residues (Lindahl, 1974; Lindahl *et al.*, 1977). The enzyme is the first in a series, involving exonuclease II, DNA polymerase, and DNA ligase, which acts to replace anomalous uracil residues with the appropriate thymine base. Uracil-DNA glycosylase is apparently widespread in living organisms, having been found in *E. coli* (Lindahl, 1974), *B. subtilis* (Freidberg *et al.*, 1975), human placenta (Sekiguchi *et al.*, 1976), and calf thymus (Lindahl *et al.*, 1977).

As mentioned in the introductory chapter, we feel that a number of enzymic reactions involving conversions of pyrimidine nucleosides and nucleotides, including N-glycosidic cleavage, might proceed *via* 5,6-dihydropyrimidine intermediates. The chemical mechanism of non-enzymic glycosidic cleavage of pyrimidine nucleosides is poorly understood at the present time, but it is not unlikely that it proceeds through acid-catalyzed hydration of the 5,6-C=C portion of the base. Hydration occurs much more rapidly than glycosidic bond cleavage (Santi *et al.*, 1970), and 5,6-dihydropyrimidine nucleosides are well known to be much more sus-

ceptible to cleavage of the glycosidic bond than unsaturated nucleosides (Levene and LaForge, 1912; Kochetkov and Budovskii, 1972; Capon, 1969). As an example, the glycosidic bond of uridine is stable toward 1 M HCl at 60° for at least 1 mo and has $k_{obs} < 2 \times 10^{-8}$ sec^{-1} while under identical conditions the glycosidic bond of 5,6-dihydrouridine hydrolyzes with $k_{obs} = 3 \times 10^{-5}$ sec^{-1}, which represents a minimum of 1500-fold enhancement in the rate of hydrolysis (Santi and Gravitz, unpublished results).

We investigated the possibility that uracil-DNA glycosylase might act via such a mechanistic pathway using [6-^{3}H] substrate in an attempt to detect an inverse α-secondary hydrogen isotope effect in the reaction. The observation of a kinetic isotope effect under these conditions would be indicative of carbon rehybridization at the substituted center at or before the rate-determining step in the reaction (Kirsch, 1977).

A 100-fold purified preparation of uracil-DNA glycosylase (specific activity ca. 0.35 U/mg protein) and bacteriophage PBS 2 DNA labelled with [6-^{3}H, 2-^{14}C]uracil (^{14}C specific activity 1000 dpm/μmol, $^{3}H/^{14}C = 3.34$) were kindly provided by Dr. H. Warner. The reaction was performed by incubating a mixture (1.1 ml) containing 67 mM HEPES (pH 7.8), 1.33 mM ethylenediamine tetraacetate, 1.33 mM dithiothreitol, 33 mg/ml bovine serum albumin, 0.07 μCi/ml (^{14}C) heat-denatured PBS 2 DNA, and 8 U uracil-DNA glycosylase at 37°. 150 μl aliquots were removed and quenched by heating for 2 min at 100°. 450 μl of water was added to each

sample and the mixture was applied to a 3.3 x 30 mm column of Dowex 1 ($HCOO^-$). Uracil was eluted with two 0.45 ml washes of water and the combined eluate (1.5 ml) was collected directly into scintillation vials. After 30 min of reaction, 145 U of enzyme was added to the remaining mixture and incubation was continued another 30 min; an aliquot from this was taken to represent 100% reaction. Samples were mixed with 10 ml toluene-based scintillation cocktail (0.4% Omnifluor in toluene:Triton X-100, 3:1) and counted 7 x 20 min each. ^3H and ^{14}C dpm's were calculated by the external standards method, and the isotope effect was calculated by the method of Melander (1960).

As shown in Table 4, the $^3H/^{14}C$ ratios of uracil product formed during the course of the reaction are not significantly different from that expected for a reaction with $k_T/k_H = 1$. At maximal extent of the reaction, 69.4% of the ^{14}C dpm's eluted from Dowex 1 in the wash. This is consistent with the known intracellular equilibration of uracil and cytosine nucleotide pools during labelling and the fact that PBS 2 DNA contains 36% uracil and 14% cytosine residues, the latter being unreactive towards uracil-DNA glycosylase.

The absence of an apparent α-secondary hydrogen isotope effect, as observed here, does not disprove the occurence of carbon rehybridization in a reaction. Such a result would also occur if (a) the rehybridization occured after the rate-determining step in the reaction, or (b) rehybridization occurs at a transition

TABLE 4: α-Secondary Hydrogen Isotope Effect in the Uracil-DNA Glycosylase Reaction[a]

Time (min)	^{14}C dpm	$^{3}H/^{14}C$	% Reaction	k_T/k_H
0	10	---	<0.1	---
2.5	479 ± 2	3.315 ± 0.028	3.0	1.002
5.0	938 ± 2	3.365 ± 0.010	5.8	1.018
10.0	2004 ± 2	3.287 ± 0.005	12.5	0.994
15.0	3165 ± 13	3.259 ± 0.011	19.7	0.984
30.0	7767 ± 28	3.438 ± 0.007	48.4	1.057
Final[b]	16054 ± 41	3.307 ± 0.006	(100)	
Initial[c]	22713 ± 50	3.335 ± 0.002		

[a]Reaction conditions and isotope effect calculations are described in the text. All statistical estimates are presented as mean ± S.E. [b]After incubation with excess enzyme. [c]A 150 µl aliquot of the reaction mixture was counted directly.

state which is rate-determining in the reaction and resembles closely the structure of starting material (Kirsch, 1977). Thus the results described here may not be interpreted either in support or denial of the proposed 5,6-dihydropyrimidine intermediates in this reaction.

CHAPTER 4: EFFECTS OF FLUOROURACIL AND METHOTREXATE ON NUCLEOSIDE TRIPHOSPHATE POOLS IN L1210 CELLS

5-Fluorouracil (FUra) and methotrexate (MTX) have been used both separately and in combination to treat a variety of neoplasms. Studies on the effects of these drugs in experimental systems have yielded conflicting reports both as to their major metabolic consequences, as well as to whether these antimetabolites are additive, synergistic, or antagonistic in their effects on cell growth (Bertino, 1979). Because of their clinical utility and the controversy over their major sites of action, further effort is warranted towards understanding their precise effects on cellular metabolism.

In vivo FUra undergoes rapid conversion to both ribo- and deoxyribonucleotides (for a review see Heidelberger, 1975). The cytotoxic effects of FUra are thought to be primarily due to inhibition of thymidylate synthetase by the active metabolite 5-fluorodeoxyuridylate (FdUMP). This compound has been shown to form a covalent complex with thymidylate synthetase in the presence of 5,10-methylenetetrahydrofolate, the folate cofactor utilized for dTMP synthesis (Santi and McHenry, 1972; Langenbach et al., 1972). The immediate result of this inhibition of thymidylate synthetase is depletion of intracellular dTTP pools and cessation of DNA synthesis. Another effect, which may represent an important side

reaction of FUra is its incorporation, at the level of FUTP, into cellular RNA. Reported effects of this incorporation include disruption of normal RNA maturation (Wilkinson and Pitot, 1973; Wilkinson et al., 1975) and formation of tRNAs with low transcriptional activity (Ramberg et al., 1978).

MTX is thought to act by disrupting the availability of tetrahydrofolate cofactors by inhibition of dihydrofolate reductase (for a review see Blakley, 1969). Reduced folates are required for a number of cellular reactions, the most sensitive to antifolate treatment being de novo purine and dTMP synthesis. Whether purine or dTMP depletion is the more serious metabolic effect depends on the cell line studied. Thymidine, but not purines, protects L60T cells (Borsa and Whitmore, 1969); purines, but not thymidine, protect S180 cells (Hakala et al., 1961); while both purines and thymidine are required to fully protect L1210 cells (Tattersall et al., 1974).

Studies have appeared in which the effects of FUra and MTX (Tattersall and Harrap, 1973; Fridland, 1974; Adams et al., 1971) on intracellular nucleoside triphosphate pools are described. Nevertheless, there is a lack of information on the effects of these drugs together or in combination with exogenously supplied purine of pyrimidine sources. This chapter presents experiments designed to more fully characterize the alterations in rNTP and dNTP pools upon treatment with these antimetabolites and to describe in detail the complex in vivo regulation of these important nucleotide compounds. For this purpose a new assay for deoxyribonucleoside triphosphates, involving

treatment of cell extracts with periodate and methylamine to destroy ribonucleotide compounds followed by HPLC analysis, was employed.

In subsequent discussion reference will be made to the allosteric regulation by nucleoside triphosphates of a crucial enzyme in deoxyribonucleotide synthesis, ribonucleotide reductase. The complex regulatory scheme of this enzyme, determined *in vitro* for the purified enzyme from *E. coli*, is depicted in Figure 9 (Brown and Reichard, 1969). Briefly, ATP activation is required for pyrimidine, but not purine, nucleoside diphosphate reduction. dATP is a general inhibitor of the enzyme. dTTP inhibits pyrimidine nucleotide reduction but stimulates GDP reduction, and dGTP in turn stimulates reduction of ADP. Results from mammalian systems indicate so far that the basic regulatory characteristics of the enzyme are the same (see Reichard, 1978).

Experimental Procedures.

Cells and Media. Suspension cultures of L1210 murine leukemia cells (obtained from the National Institute of Health) were grown in Dulbecco's Modified Eagles medium supplemented with 10% heat-inactivated horse serum. Doubling times were *ca*. 11 hr. Cell concentrations were determined using a Coulter ZB1 particle counter.

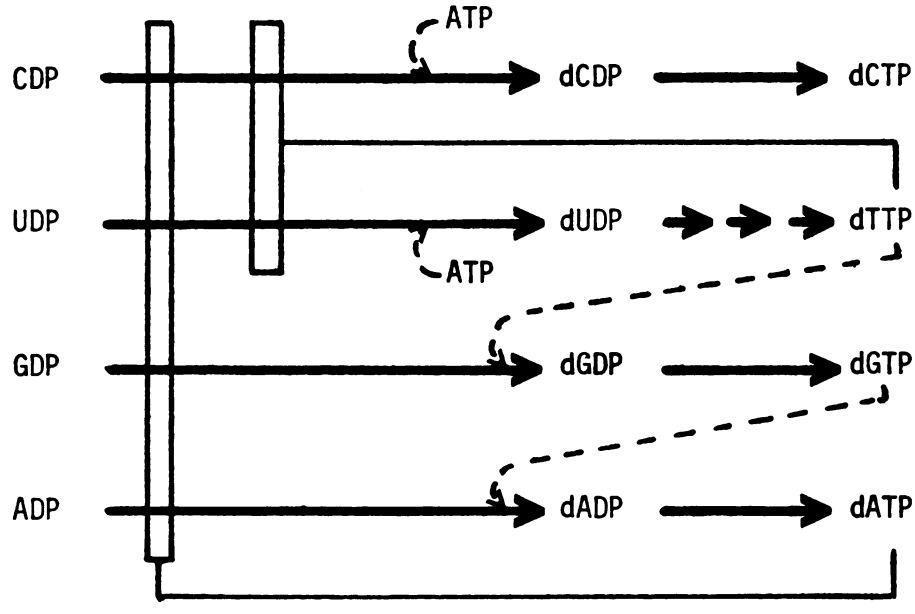

FIGURE 9: Model for the in vivo regulation of ribonucleotide reductase activity. Bars indicate inhibitions, broken arrows indicate activations.

Cell Harvesting and Determination of Nucleoside Triphosphate Pools. Cells were grown to a concentration of 5-7 x 10^5/ml, when treatment with antimetabolites or nucleosides was initiated. At specified times (1, 3, and 7.5 hr) cell concentrations were determined and 50 ml portions of the suspension were harvested by centrifugation (4 min, 1000 g, ambient temperature). Unless otherwise specified, cells were washed in 1 ml ice-cold phosphate-buffered saline solution (PBS) containing 0.1% glucose and repelleted (10 sec, 13000 g, 4°). Pellets were resuspended in 0.1 ml 1 M $HClO_4$, and after 20 min on ice the extract was cleared by centrifugation. Extracts were then neutralized (to pH 6.5-7.5) by addition of 4 M KOH containing 0.4 M K_2HPO_4. After precipitation of $KClO_4$ the extracts were again cleared and stored at -20° until analysis. Ribo- and deoxyribonucleotide pools were determined by the method of Garrett and Santi (1979, Appendix 2).

Results and Discussion.

Effect of Harvesting Procedure. To determine the optimal method for extracting nucleotides with minimal hydrolyses of labile triphosphates, six 50 ml portions of a cell suspension were harvested and extracted with the modifications of the standard procedure described in Table 5. It is seen that the standard procedure (adopted for all subsequent experiments) gives the highest yield of ATP and GTP and

TABLE 5: Effect of Extraction Procedure on Measured Pool Sizes and Phosphorylation Ratios in L1210 Cells[a]

Conditions	Pool sizes (pmol/10^6 cells)		ATP/ADP	GTP/GDP
	ATP	GTP		
1. 0.5 M $HClO_4$, no PBS wash	2190	612	4.6	4.3
2. 0.5 M $HClO_4$, no glucose in PBS	2050	537	4.4	3.5
3. 0.5 M $HClO_4$	2200	599	8.8	7.4
4. As in 3, PBS warmed to 37°	2240	641	8.6	6.8
5. Standard procedure	2455	710	10.2	9.6
6. Same as 3, last extract treated at each step	2360	643	8.4	7.9

[a]A 300 ml culture of L1210 cells was divided into 6 equal portions which were harvested as described in the text. The six pellets were extracted using the indicated modifications of the standard procedure. At each step the extracts were treated sequentially, beginning with 1 and ending with 6.

the highest ratios of ATP to ADP and GTP to GDP. (Results for CTP and UTP were similar, but HPLC separation of pyrimidine diphosphates was inadequate for calculating phosphorylation ratios of these compounds.) Washing with PBS alone was inferior to no washing at all, but inclusion of 0.1% glucose in the PBS wash was better than either. The temperature of the PBS wash had little effect. Extraction with 1 M $HClO_4$ gave higher nucleotide yields and phosphorylation ratios than did 0.5 M $HClO_4$, apparently due to more complete cell lysis and more rapid inactivation of cellular nucleotidases. In addition, little effect was seen between the third and seventh extractions, both of which were prepared identically but the seventh having a ca. 5 min lag at each step in the procedure; thus, four extracts (the usual number in subsequent experiments) could safely be prepared in parallel. Since storage of extracts at 30° resulted in a loss of ATP of only ca. 4%/hr (data not shown), subsequent handling of extracts had minimal effect on nucleoside triphosphate concentrations.

Nucleoside Triphosphate Pools in Exponentially Growing L1210 Cells. The rNTP pools determined from 19 samples in 5 control cultures over the course of this work were, in pmol/10^6 cells: CTP, 362 ± 52 (± S.D.) UTP, 792 ± 105; ATP, 2350 ± 289; and GTP, 533 ± 53. Table 6 shows the sizes of the dNTP pools similarly determined in control cultures, and for comparison reported pool sizes for this and other lymphoma and leukemic cell types are also given.

Comparison of the dNTP pools between cell types is complicated by

TABLE 6: Comparison of Deoxyribonucleoside Triphosphate Pools in Various Lymphoma and Leukemic Cell Types

Cell type	Pool size (pmol/10^6 cells)			
	dCTP	dTTP	dATP	dGTP
1. L1210 Murine leukemia[a]	36.2 ± 6.4	24.1 ± 3.4	16.3 ± 3.0	5.9 ± 0.9
2. L1210[b]	56	86	36	36
3. L1210[c]	1.8	8.5	0.8	0.6
4. 5178Y Murine lymphoma[d]	1.7 ± 0.1	74 ± 20	6.5 ± 1.5	2.5 ± 0.5
5. CCRF-CEM Human lymphocytic[e]	13	39	13	16
6. S-49 Murine lymphoma[f]	67.9	74.2	65.2	24.0

[a]Determined here. Results are expressed as mean ± S.D. (n = 17).

[b]Lowe and Grindley (1976).

[c]Tattersall et al. (1974). Calculated from results expressed as per µg of DNA, assuming 10 µg DNA/10^6 cells.

[d]Tattersall and Harrap (1973). Mean ± extreme values in 6 experiments.

[e]Fridland (1974).

[f]Garrett and Santi (1979) (Appendix 2).

the fact that even in the same cells widely different values are reported by different authors. Virtually identical extraction and assay techniques were described for the data on L1210 cells in lines 2 and 3, underscoring the importance of carefully controlled methodology in dNTP pool measurements.

Changes in dNTP pools upon treatment with fluorouracil. Figures 10 and 11 show the nucleoside triphosphate pool changes upon treatment of cells with 1 and 10 μM FUra, respectively. Little effect on rNTP pools is apparent, though a slight general rise in both purine and pyrimidine triphoshpates is accentuated at the higher drug concentration. In the case of dNTP's, however, characteristic and dramatic changes are observed. The primary event is presumably a lowering of dTTP pools upon inhibition of thymidylate synthetase, dTTP pools stabilize at ca. 65% of normal at 1 μM FU, and steadily decrease to 25% after 7.5 hr at the higher drug concentration. dTTP represses CDP reduction and activates GDP reduction by ribonucleotide reductase; this regulatory mechanism explains the observed expansion of dCTP and contraction of dGTP pools. The cause of the rise in dATP pools, most apparent at 10 μM FUra after 7.5 hr, is less easily explainable. dGTP is proposed to activate ADP reduction, and the fall in dGTP would be expected to lead to lowered dATP levels. Inhibition of ADP reduction by dTTP, or activation by dCTP, either of which could explain these results, have not been observed in vitro. One possible explanation for the rise in dATP pools is that in the absence of dTTP and dGTP incorporation of dATP into DNA ceases while a near normal rate of dATP synthesis

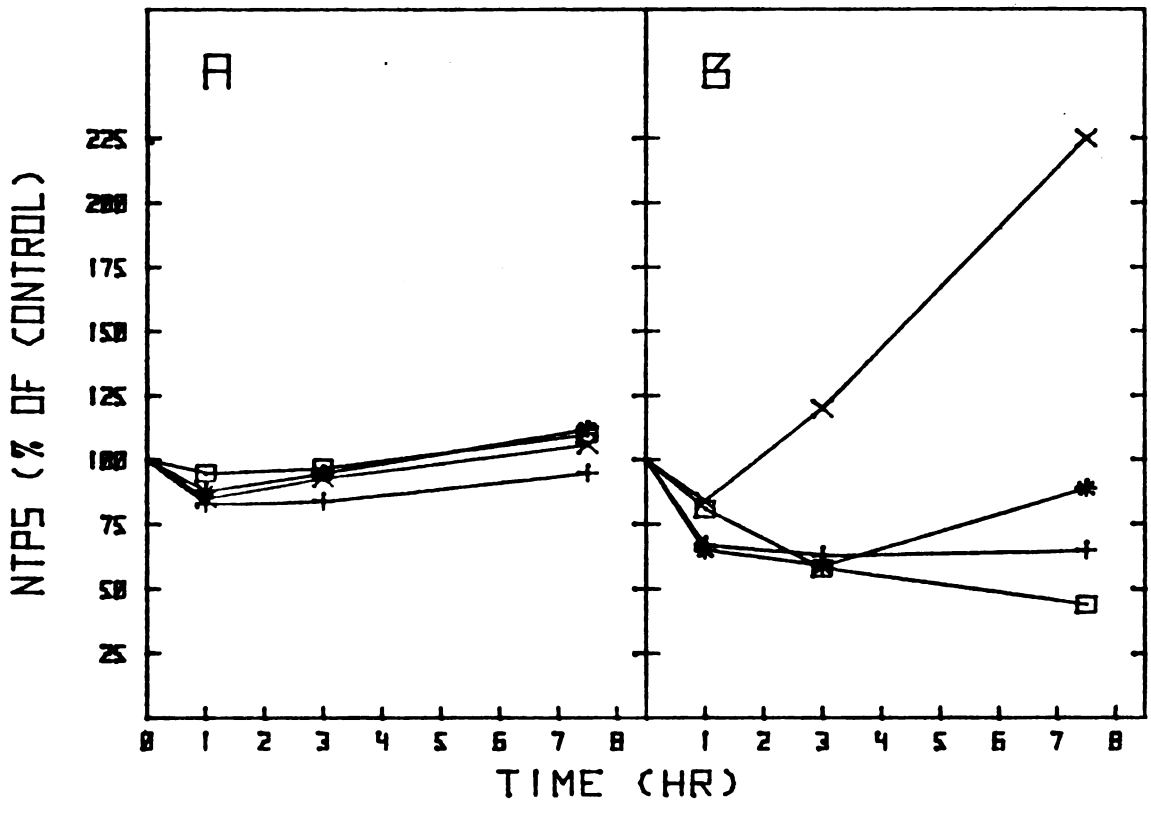

FIGURE 10: Nucleoside triphosphate pool changes in L1210 cells treated with 1 μM FUra.
Panel A: ×—×, CTP; +—+, UTP; ✱—✱, ATP; □—□, GTP.
Panel B: ×—×, dCTP; +—+, dTTP; ✱—✱, dATP; □—□, dGTP.

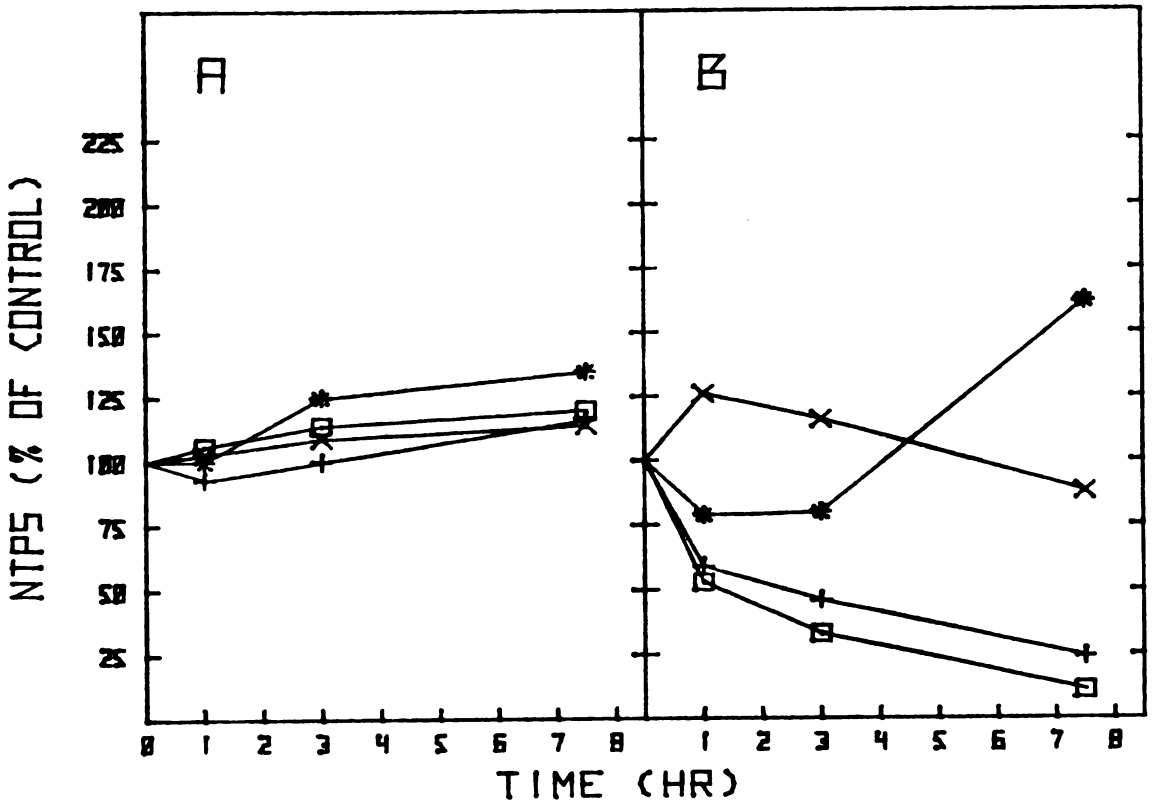

FIGURE 11: Nucleoside triphosphate pool changes in L1210 cells treated with 10 μM FUra.
Panel A: ×—×, CTP; +—+, UTP; ✱—✱, ATP; □—□, GTP.
Panel B: ×—×, dCTP; +—+, dTTP; ✱—✱, dATP; □—□, dGTP.

continues, the net result being an increase in dATP pools.

This general pattern of rNTP and dNTP pool changes shown in Figures 10 and 11, i.e., decreased dTTP and increased dCTP and dATP, is consistent with the patterns observed during thymine starvation of bacteria (Neuhard and Thomassen, 1971), and during FUra treatment of L5178Y mouse lymphoma cells (Tattersall and Harrap, 1973). In neither case, however, was the striking decrease in dGTP pools observed.

Effects of MTX on Nucleoside Triphosphate Pools. Figures 12 and 13 show the effects of 1 and 10 μM MTX on rNTP and dNTP pools of L1210 cells. Dihydrofolate reductase activity is blocked by MTX, and dTMP synthesis rapidly depletes intracellular pools of reduced folates which are required for purine, as well as for continued dTMP, synthesis. This explains the observed severe depletion of intracellular purine ribonucleoside triphosphates. The concomitant rise in pyrimidine ribonucleotides is perhaps best explained as a result of increased de novo pyrimidine synthesis upon release of purine nucleotide inhibition of dihydroorotase (Bresnick and Blatchford, 1964), together with decreased consumption of pyrimidine nucleotides in nucleic acid synthesis. With regard to deoxyribonucleotides, the most dramatic finding is that all the dNTPs are lowered. This might result since purine substrates are in short supply (implying that their concentrations are near or below their K_m values for ribonucleotide reductase), and since ATP is a requisite activator of pyrimidine nucleotide reduction. A transient rise in

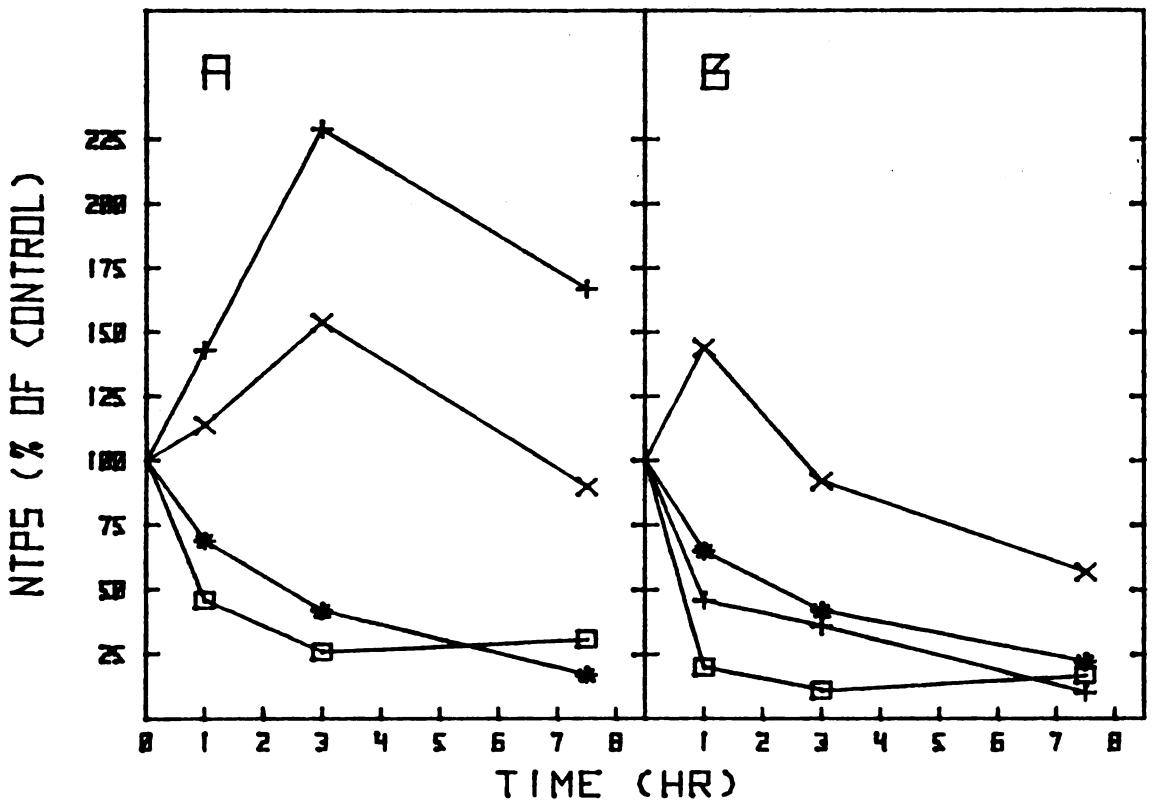

FIGURE 12: Nucleoside triphosphate pool changes in L1210 cells treated with 1 μM MTX.
Panel A: ×—×, CTP; +—+, UTP; ✱—✱, ATP; □—□, GTP.
Panel B: ×—×, dCTP; +—+, dTTP; ✱—✱, dATP; □—□, dGTP.

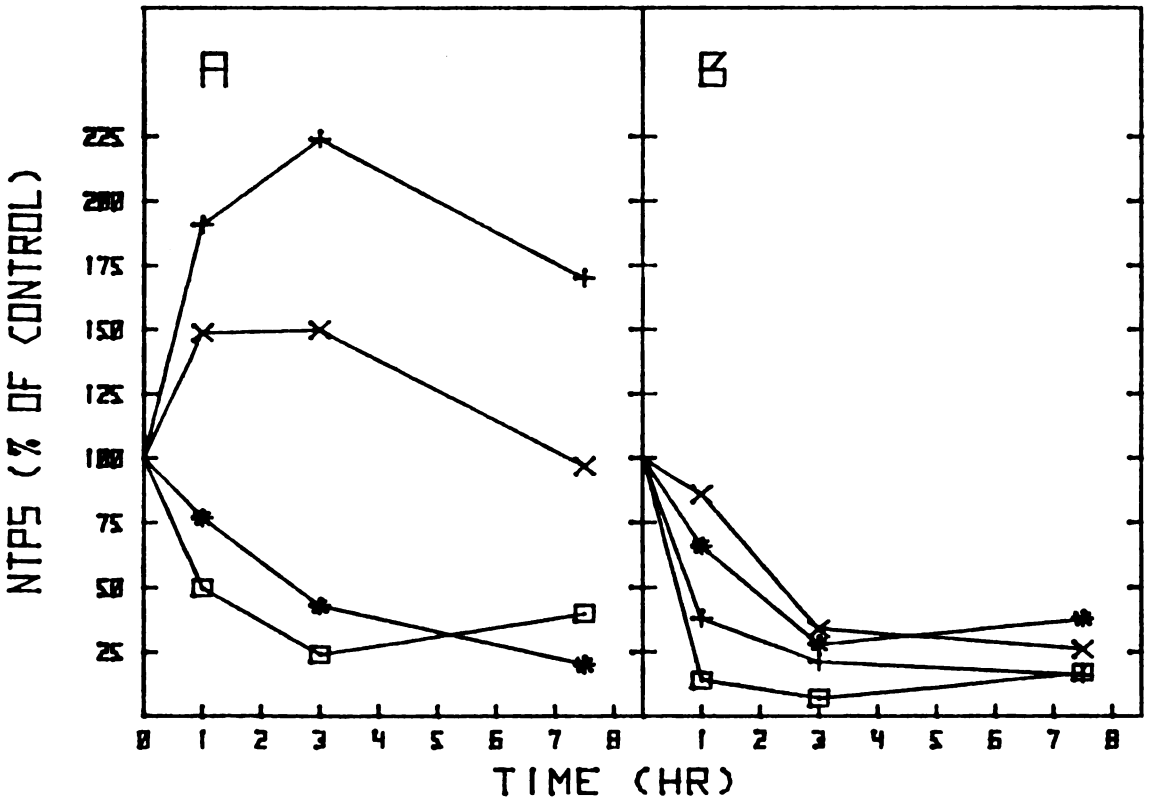

FIGURE 13: Nucleoside triphosphate pool changes in L1210 cells treated with 10 μM MTX.
Panel A: ×—×, CTP; +—+, UTP; ✱—✱, ATP; □—□, GTP.
Panel B: ×—×, dCTP; +—+, dTTP; ✱—✱, dATP; □—□, dGTP.

dCTP, resulting from loss of dTTP inhibition of CDP reduction, is soon overcome.

These results with MTX treatment differ from those of Tattersall and Harrap (1973) upon treatment of L5178Y cells with 1 μM MTX. These workers found unchanged dATP concentrations and a persistant 300% increase in dCTP pools. It would appear that with respect to dNTP pool changes L1210 cells are simply more sensitive to MTX than are L5178Y cells. This is not unexpected since they are also more sensitive to growth inhibition by MTX (the EC_{50} is 9×10^{-9} M, compared to 8×10^{-8} for L5178Y cells) (Tattersall et al., 1974).

MTX and Hypoxanthine or Thymidine Treatment. Since depletion of intracellular tetrahydrofolate cofactors most seriously affects purine and dTMP synthesis, addition of a source of preformed purines to MTX treated cells would be expected to yield a pattern of nucleotide pool changes similar to that seen upon direct inhibition of thymidylate synthetase by FUra. To test this supposition, we treated cells with 10 μM MTX together with 50 μM Hypoxanthine (Hyp). As seen in Figure 14, this treatment completely reversed the purine depletion of MTX alone (cf. Figure 13) and resulted in a moderate increase over control cells in purine rNTPs and a small, transient decrease in pyrimidine rNTPs. dTTP pools contract as expected, and dGTP pools also are low, again indicating that dTTP is a necessary as well as potent activator of GDP reduction. dATP pools expand greatly after initially normal levels, much as is seen upon FUra treatment (Figure 11). dCTP pools, however, remain essentially

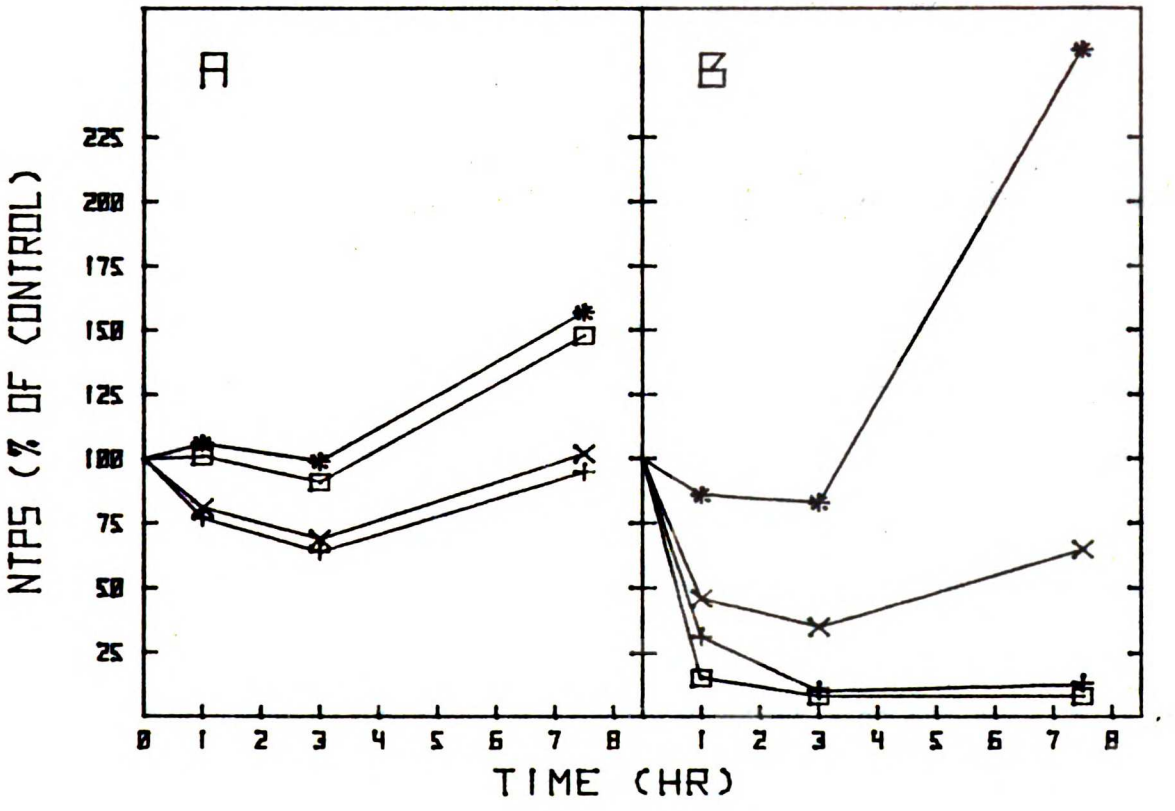

FIGURE 14: Nucleoside triphosphate pool changes in L1210 cells treated with 10 μM MTX and 50 μM Hyp.
Panel A: ×—×, CTP; +—+, UTP; ✱—✱, ATP; □—□, GTP.
Panel B: ×—×, dCTP; +—+, dTTP; ✱—✱, dATP; □—□, dGTP.

unchanged from those seen with MTX alone. This result is troublesome since in this case the activator ATP is above normal in concentration, while dATP levels initially are only slightly altered from normal cells and dTTP, the only other known inhibitor of CDP reduction, is greatly lowered in concentration. Especially in comparison with FUra treatment alone, in which all the other nucleoside triphosphate pools are affected similarly and yet dCTP levels rise, this behavior suggests either an effect of MTX *per se* on dCTP synthesis or the existence of a mechanism for its regulation by some other intracellular compound whose altered concentration is not reflected by detectible changes in the nucleoside triphoshpate pools.

The effects of simultaneous treatment of cells with MTX and dThd are shown in Figure 15. Here, rNTP pools are virtually the same as with MTX alone. Depletion of dTTP pools is reversed as expected, although they decline at later times presumably as the pool is depleted by continuing DNA synthesis; this would be consistent with the fact that dThd partially reverses MTX inhibition of cell growth in L1210 cells (Tattersall *et al.*, 1974). The extreme dependence of dGTP formation on dTTP activation discussed above is confirmed by the fact that dGTP pools fall to a lesser degree compared to the situation with MTX alone (*cf.* Figure 13), and this fall is transient in nature. In addition, dATP pools are unchanged compared to the situation without dThd, and again, at least over the time course of this experiment, no stimulation of ADP reduction by dGTP as observed *in vitro* was noted. Lastly, depletion of dCTP pools is explainable

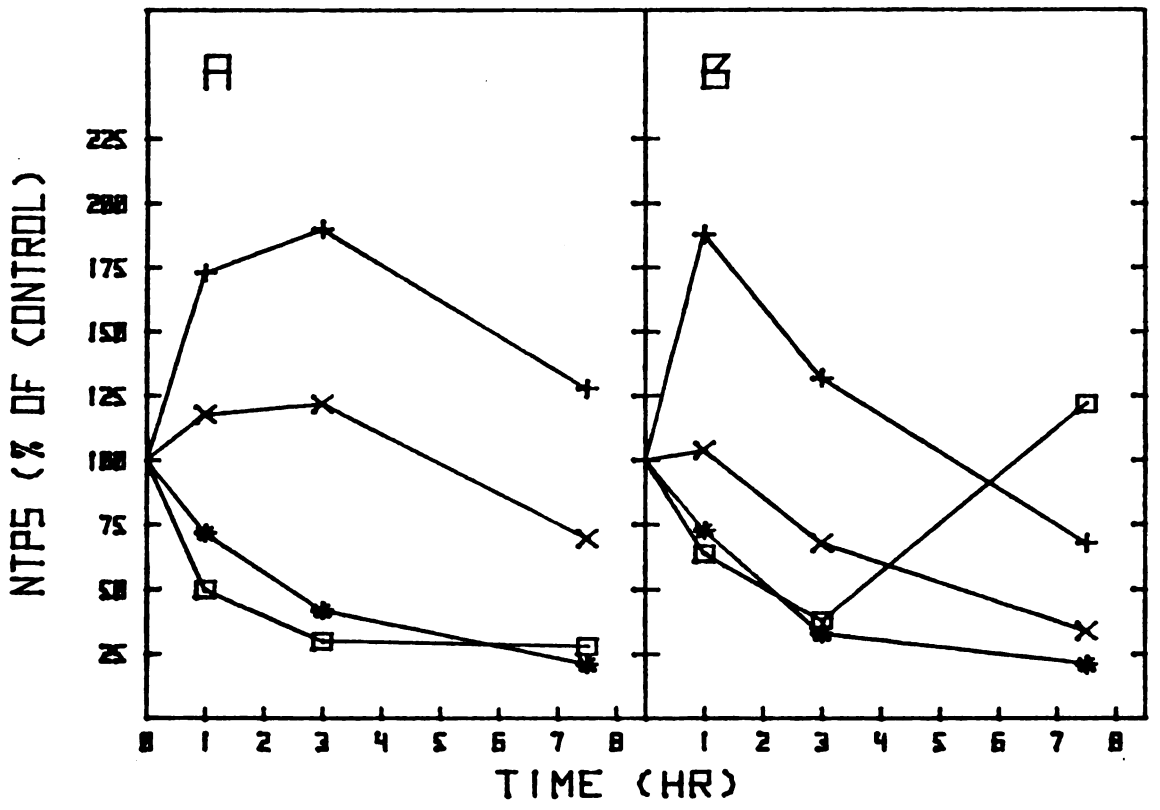

FIGURE 15: Nucleoside triphosphate pool changes in L1210 cells treated with 10 μM MTX and 20 μM dThd.
Panel A: ×—×, CTP; +—+, UTP; ✱—✱, ATP; □—□, GTP.
Panel B: ×—×, dCTP; +—+, dTTP; ✱—✱, dATP; □—□, dGTP.

as being due to the known dTTP inhibition of CDP reduction.

MTX and FUra in Combination. Since thymidylate synthetase catalyzes the only intracellular reaction in which reduced folates are consumed (i.e., oxidized), it has been suggested that direct inhibition of the enzyme by FUra or fluorodeoxyuridine (FdUrd) during MTX treatment might retard the loss of reduced folates and ameliorate the purine-depleting effects of MTX. This idea is supported for L5178Y cells by the work of Tattersall et al. (1973) who found that while dThd alone was uanble to rescue these cells from MTX inhibition, the addition of FUra together with dThd allowed significant cell growth. We tested this hypothesis in L1210 cells by measuring nucleoside triphosphate pools upon simultaneous treatment of cells with 10 μM FUra and 1 and 10 μM MTX (Figures 16 and 17, respectively). In both cases the nucleotide patterns are nearly identical to those of MTX alone at the same concentration (cf. Figures 12 and 13). A moderate rise in GTP pools as compared to MTX treatment alone is not matched by similar increases in ATP, dATP, or dGTP, and from these data it would appear that the purine-sparing effect of FUra, if any, is small under these conditions.

dThd is reported to inhibit formation of dUMP, the nucleotide substrate for dTMP synthesis (Jackson, 1978). Since decreased dUMP levels would be expected to slow de novo dTMP synthesis and potentiate any purine-sparing effect of FUra during MTX treatment, we examined the nucleotide pool changes upon treatment of cells with

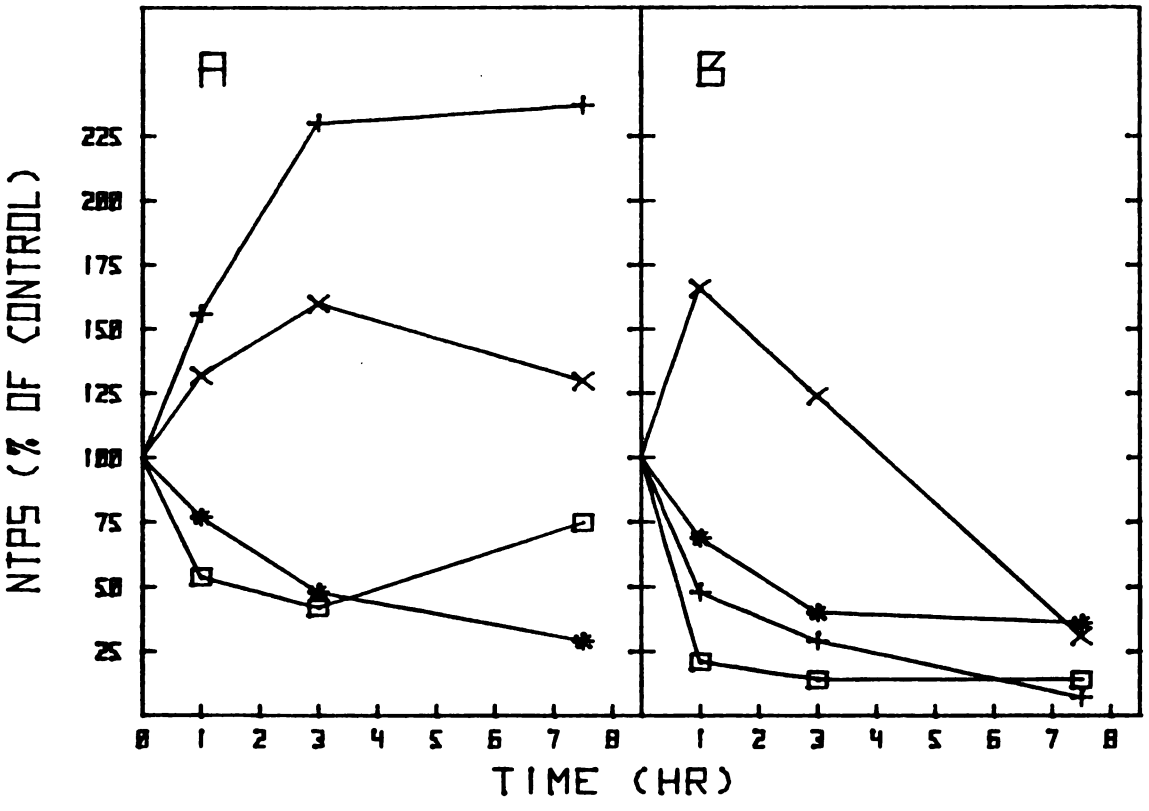

FIGURE 16: Nucleoside triphosphate pool changes in L1210 cells treated with 1 μM MTX and 10 μM FUra.
Panel A: ×—×, CTP; +—+, UTP; ✱—✱, ATP; □—□, GTP.
Panel B: ×—×, dCTP; +—+, dTTP; ✱—✱, dATP; □—□, dGTP.

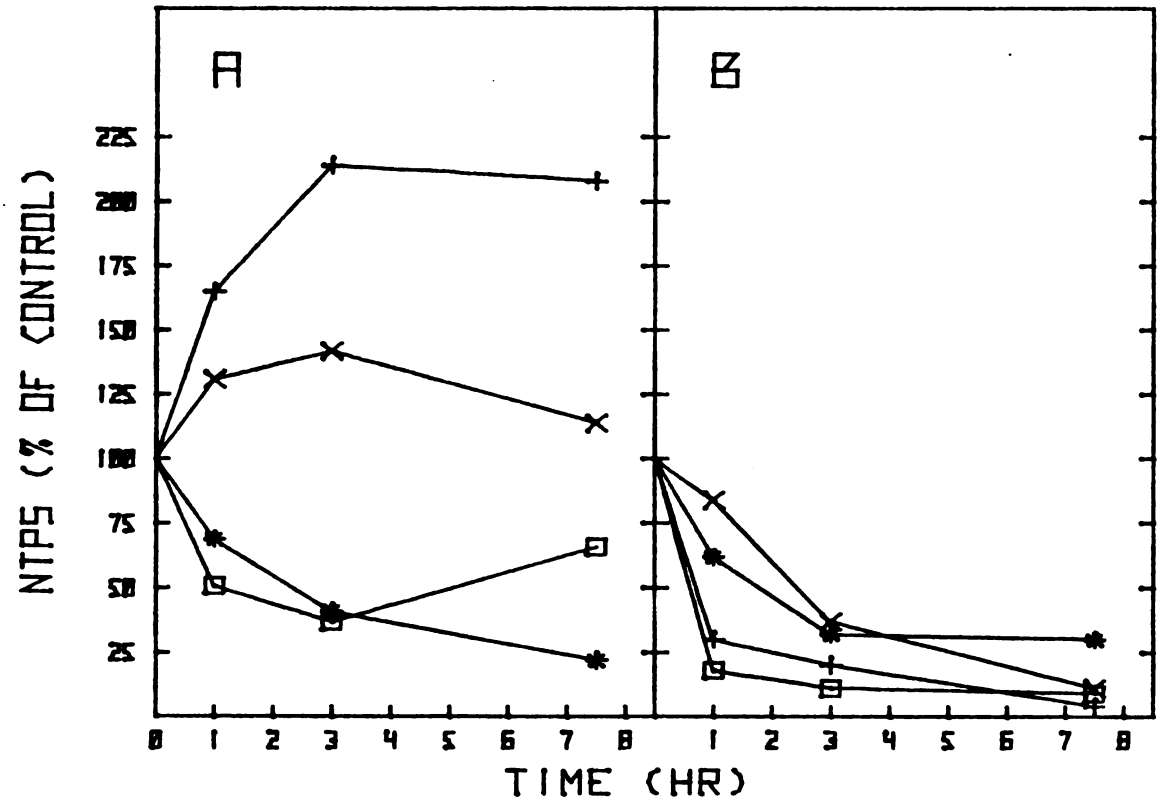

FIGURE 17: Nucleoside triphosphate pool changes in L1210 cells treated with 10 μM MTX and 10 μM FUra.
Panel A: ×—×, CTP; +—+, UTP; ✱—✱, ATP; □—□, GTP.
Panel B: ×—×, dCTP; +—+, dTTP; ✱—✱, dATP; □—□, dGTP.

10 μM MTX, 10 μM FUra, and 10 μM dThd. As seen in Figure 18, the effects of added dThd are essentially the same as those found in the absence of FUra (cf. Figure 15). Neither ATP nor GTP levels are further increased by addition of dThd, and it must be concluded that in L1210 cells the purine sparing-effect of FUra during MTX treatment is small.

Conclusions. Several important points concerning the interrelationship of the various dNTP pools have become apparent from this study:

(1) dGTP levels are intimately tied to dTTP levels in L1210 cells. Since dGTP pools are the smallest of the dNTP pools, it appears possible, even likely, that the primary interference of dTTP depletion on DNA synthesis at least in this cell line is not direct, but rather via depletion of already limiting dGTP pools, and that the most serious metabolic consequence of thymidylate synthetase inhibition is in fact dGTP pool depletion. Furthermore, inhibition of GDP reduction by dATP proposed for the E. coli enzyme is not apparent in this in vivo situation. Whether this is due to differences in the mammalian enzyme, or to apparent differences between the in vitro and in vivo situation is not known.

(2) The regulation of CDP reduction is more complex than previously realized. In vitro, ATP stimulates CDP reduction while only dTTP and dATP inhibit it. We find here, however, that other influences being equal, lowered dTTP levels do not necessarily result in elevated dCTP levels as predicted. Under conditions of near normal CTP, ATP and dATP, but lowered dTTP and dGTP

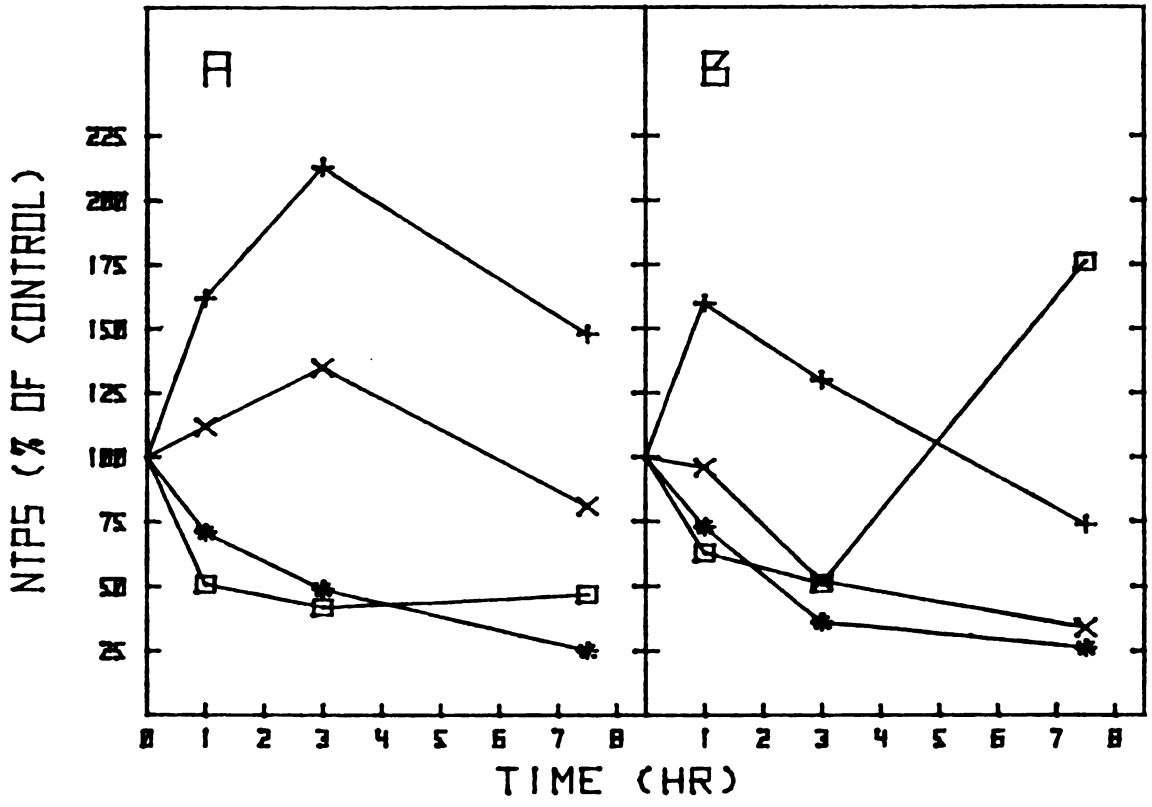

FIGURE 18: Nucleoside triphosphate pool changes in L1210 cells treated with 10 μM MTX, 10 μM FUra, and 20 μM dThd.
Panel A: ✕—✕, CTP; +—+, UTP; ✱—✱, ATP; □—□, GTP.
Panel B: ✕—✕, dCTP; +—+, dTTP; ✱—✱, dATP; □—□, dGTP.

(MTX + Hyp, Figure 14), dCTP was actually lower than control. Further experiments will be required to determine whether this is due to an as yet unknown regulatory aspect of ribonucleotide reductase or another enzyme of deoxycytidine metabolism, or if it is due to peculiar effects of MTX itself.

(3) High-dose MTX, an increasingly common chemotherapeutic treatment, appears to have a qualitatively different effect on deoxyribonucleotide pools than lower dosages in that it induces collapse of all dNTP pools, rather than just dTTP and dGTP. Whether this leads to a different or more efficient mode of cell killing is worthy of further investigation.

(4) Little evidence was found to support the hypothesis that inhibition of de novo dTMP synthesis spares intracellular pools of reduced folates during MTX treatment. The effect in L1210 cells, if any, is small, and further investigation will be required for its accurate quantitation.

In conclusion, this study establishes the utility of HPLC analysis of dNTPs in periodate and amine treated cell extracts. Because of its simplicity and the rapidity with which numerous assays may be performed as compared to previous methods, it is ideally suited for detailed analysis of the complex regulatory interactions of nucleoside triphosphate metabolism.

CHAPTER 5: SENSITIVITY TO THYMINE STARVATION OF *E. COLI* STRAINS DEFICIENT IN DEOXYURIDINE TRIPHOSPHATE PYROPHOSPHORYLASE AND URACIL-DNA GLYCOSYLASE

Although E. coli is capable of synthesizing 2'-deoxyuridine-5'-triphosphate (dUTP) (Okazaki and Kornberg, 1964; Bertani et al., 1963), and DNA polymerase I is known to accept dUTP as a substrate in DNA synthesis (Bessman et al., 1958), E. coli does not contain uracil in its DNA. At least two metabolic processes exist to account for this fact. First, E. coli has an active dUTP pyrophosphate (Bertani et al., 1963), thus removing it as a substrate for DNA synthesis. Second, it has been found that the bacteria contain an N-glycosidase activity (uracil-DNA-glycosylase) which releases free uracil from deoxyuridine-containing DNA (Lindahl, 1974; Lindahl, et al., 1977); this activity, in conjunction with repair mechanisms which recognize apyrimidinic sites (Cerutti, 1974), presumably functions to remove any deoxyuridine which escapes the action of dUTPase.

In the absence of thymine, bacterial strains which require this compound for growth lose viability in a characteristic manner. This "thymineless death" is accompanied by DNA chain breakage, mutagenesis, non-conservative DNA replication, prophage induction, and gross alterations in deoxyribonucleoside pools (Cohen, 1971). Though less well studied, a similar pattern is found when cells

are treated with 5-fluorodeoxyuridine (Cohen et al., 1958), an agent known to result in severe depletion of intracellular dTTP pools (Taylor, 1963, Tattersall and Harrap, 1973). Numerous proposals have been set forth to explain the mechanism by which the reduction of this DNA precursor pool results in irreversible and presumably lethal inhibition of DNA synthesis, but the underlying mechanism has not been established.

One possible cause for this aberrant DNA metabolism is that during thymineless states the incorporation of uracil into DNA increases dramatically due to an increased intracellular ratio of dUTP to dTTP, and that base excision repair of these misincorporated residues is responsible for the DNA damage observed. Thymine starvation is accompanied by a substantial decrease in dTTP pools, and this alone could account for an increased dUTP to dTTP ratio. There is reason to suspect, however, that thymine starvation is accompanied by a large increase in dUTP pools as well. dCTP pools increase greatly under these conditions (Neuhard and Thomasson, 1971), and in enteric bacteria dCTP is converted to dUTP via dCTP deaminase enzyme 1 in Figure 19 (Beck et al., 1975). dUTP is converted to dUMP via dUTPase, but in the absence of thymidylate synthetase activity (which is the case in thymine requiring strains or upon treatment with FdUrd) this represents entry into a deoxyuridine nucleotide loop defined by dUTPase and nucleoside mono- and diphosphate kinases. The net increase in dUTP will of course depend on the relative activities of the enzymes involved, but in this regard dUMP levels are reported

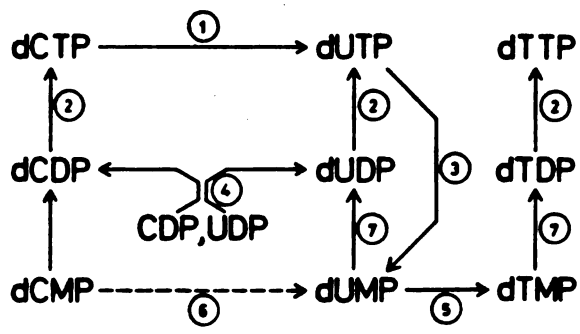

FIGURE 19: Pyrimidine deoxyribonucleotide metabolism in enteric bacteria. Dashed line indicates reaction nonexistant in enteric bacteria, but generally present in other bacteria and in mammalian cells. The enzymes are identified by numbers as follows: 1, dCTP deaminase; 2, nucleoside diphosphate kinase; 3, dUTPase; 4, ribonucleotide reductase; 5, thymidylate synthetase; 6, dCMP deaminase; 7, thymidylate kinase.

to expand by more than 20-fold in thymine requiring cells (Munch-Peterson, 1970).

We investigated this possible role of dUTP by examining the sensitivity to FdUrd-induced thymine starvation of a set of congeneic strains of E. coli deficient in the enzymes dUTPase and uracil-DNA glucosylase. The stains used, kindly provided by Dr. H. Warner, were BD1153 (dut$^+$ ung-1), BD1154 (dut$^+$ ung$^+$), BD1156 (dut-1 ung$^+$), and BD1157 (dut-1 ung-1) (Duncan et al., 1978; Tye and Lehman, 1977). Cells were grown at 37° in Minimal A medium supplemented with 0.5% casamino acids, 0.2% glucose, and 1 µg/ml thiamine; doubling times were, for BD1153, 33 min; for BD1154, 38 min; for BD1156, 50 min; and for BD1157, 81 min. Cultures (10 ml) were grown to a concentration of 2 to 4 x 10^7 cells/ml, at which time 80 µM FdUrd was added to initiate cell killing. Cell viability was determined by spreading appropriate dilutions of the cultures onto tryptone agar plates and counting colony-forming loci.

As seen in Figure 20, the dut-1 strain was strikingly sensitive to FdUrd treatment as compared to the control ($t_{½}$ for logrithmic phase of killing of 18 min and 58 min respectively). In addition, this increased sensitivity was completely reversed by co-introduction of the ung-1 mutation ($t_{½}$ = 76 min). Conversely, the strain containing the ung-1 mutation alone was clearly more resistant to FdUrd than the ung$^+$ control ($t_{½}$ = 96 min).

Transient uracil incorporation into DNA is known to be elevated in dut mutants under normal conditions (Tye and Lehman, 1977). As

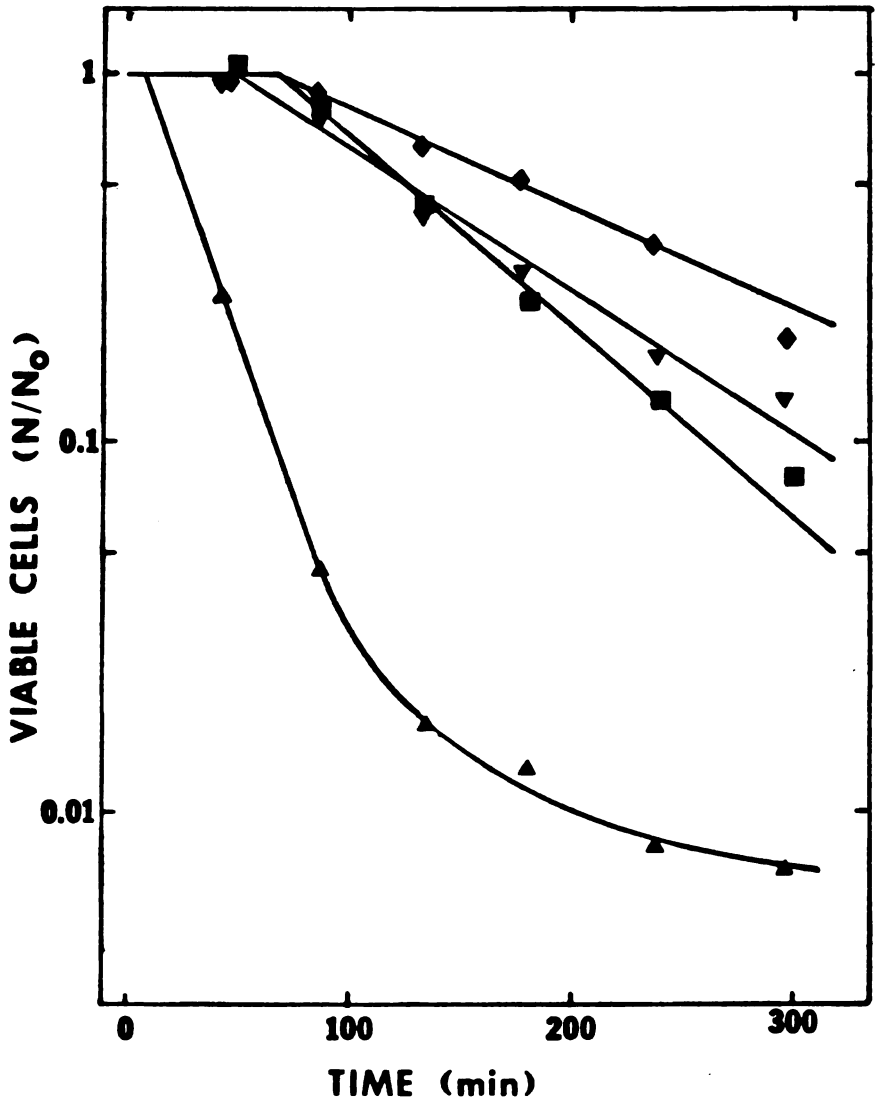

FIGURE 20: Survival of E. coli cells treated with 80 μM FdUrd. Strains used were BD1153, dut$^+$ung-1$^+$, (◆); BD1154, dut$^+$ung$^+$, (■); BD1156, dut-lung$^+$, (▲); BD1157, dut-lung-1, (▼).

demonstrated here, this same strain is extremely sensitive to FdUrd-induced thymine starvation, and the ameliorating effect of co-introduction of the ung-1 mutation argues that this increased sensitivity is specifically due to uracil incorporation into DNA. Furthermore, the protective effect of the ung mutation alone as compared to wild-type indicates that uracil excision repair is detrimental to normal cells during thymine depletion. Thus, these results are in complete agreement with the proposed role for the uracil excision repair system inthe DNA damage observed during thymineless death.

The latter result, i.e. the protective effect of mutation of the uracil-DNA glycosylase gene, was independently reported by Makino and Munakata (1978) for ung mutants of B. subtilis. These workers also demonstrated both increased incorporation of uracil and decreased DNA degradation for this strain during thymine starvation. It is important to note that dUTP, the putative precursor to DNA-uracil, has not been detected in cellular extracts.

CHAPTER 6: PRODUCTS OF ALKALINE HYDROLYSIS OF p-DEOXYRIBO-p-dT

In principle DNA synthesis on the leading strand of the replication fork can be synthesized continuously, i.e., without repeated de novo priming events. Nevertheless, evidence has accumulated from several experimental systems that DNA synthesis on both the leading and lagging strands proceeds discontinuously (Kurosawa and Okazaki, 1975; Sternglanz et al., 1976). It has been suggested (Alberts and Sternglanz, 1977) that one source of these leading strand fragments is transient uracil incorporation and repair via the DNA-uracil excision-repair pathway (Lindahl, 1974).

The initial intermediate in uracil excision-repair is an apyrimidinic DNA segment, consisting of DNA with an intact sugar-phosphate backbone but lacking the excised base (Lindahl, 1974). We undertook to examine the products resulting from alkaline hydrolysis of such apyrimidinic sites to determine (a) the identity of the hydrolysis products and (b) whether such sites could give rise to the 5'-OH termini found after alkaline hydrolysis of Okazaki fragments (Hirose et al., 1973). Alkaline hydrolysis of Okazaki fragments stoichiometrically yields DNA fragments with 5'-OH ends (Hirose et al., 1973); this has been supposed to result from intramolecular catalysis of phosphodiester hydrolysis by the 2'-OH of adjacent ribonucleotide

residues. 5'-Phosphorylated termini are not found to any great extent in such experiments (≤ 6%), and the formation of this product from apyrimidinic sites would indicate that excision repair intermediates do not contribute substantially to Okazaki fragment hydrolysis products. On the other hand, formation of 5'-OH termini would be consistent with the possibility of excision-repair involvement.

We examined the products of the reaction using the model compound p-deoxyribo-p-dT. Two possible reaction pathways for alkaline hydrolysis of this nucleotide are illustrated in Figure 21. Pathway (a) involves a β-elimination reaction yielding the dTMP (1)and the α,β-unsaturated aldehyde phosphate 2 (Brown and Todd, 1955). Pathway (b) proceeds via a 3',4' cyclization to give dThd (3) and the 3',4'-cyclic phosphate 4 (Tamm et al., 1953).

p-Deoxyribo-p-dT was prepared in the following manner: To 9 mg (230 A_{260} units) of d(pGpT) dissolved in 2.0 ml of 1 M sodium acetate (pH 5.0), was added 150 µl of $(CH_3)_2SO_4$. The mixture was incubated at ambient temperature (24°) for 2 hr to methylate the purine heterocycle (Pochon and Michelson, 1967), followed by 20 min at 75° to hydrolyze the methylated purine base (Lawley and Brookes, 1963). The crude product was desalted and separated from 7-methylguanine by chromatography on Sephadex G-10 using water as the eluting solvent, and the product was concentrated by lyophilization. The product had a uv abosrbance maximum in water identical to that of dTMP. Its elution from Lichrosorb C_{18}, using aqueous 5 mM tetrabutylammonium hydrogensulfate:acetonitrile (9:1) as buffer, was clearly different from dTMP

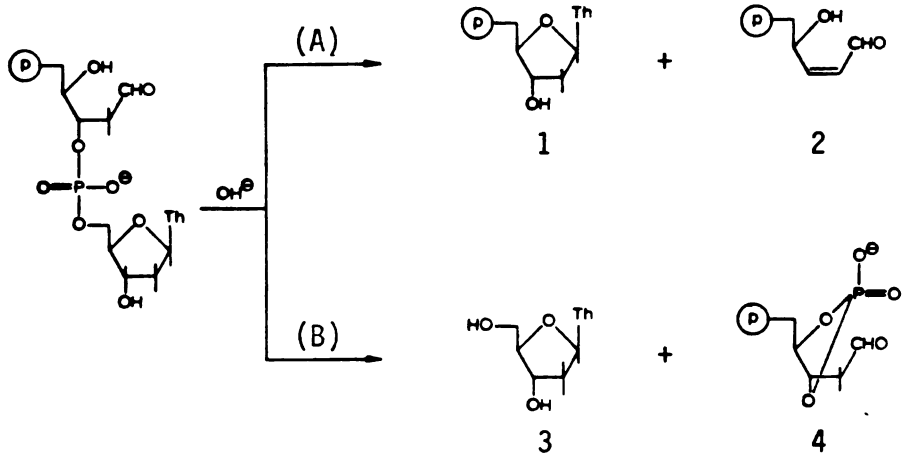

FIGURE 21: Possible reaction pathways for alkaline hydrolysis of p-deoxyribo-p-dT.

and indicative of a dinucleotide compound. Barely detectible amounts of starting material were observed, indicating virtual completion of the reaction. 88 A_{260} units were recovered; 91% of the expected value.

Alkaline hydrolysis of p-deoxyribo-p-dT was performed by incubating 3 A_{260} units of the compound in 0.11 ml of 0.3 M NAOH for 14 hr at 37°. Glacial acetic acid was added to give a pH of 3, and the mixture was analyzed on Lichrosorb C_{18} as described above. Less than 1% of the uv-absorbing product eluted as dThd, the remainder being dTMP. Thus, alkaline hydrolysis of this nucleotide clearly results in formation of a 5'-phosphoryl and not a 5'-OH terminus. By analogy, the results from this nucleotide model indicate that the 5'-OH termini of alkali-treated Okazaki fragments do not arise via hydrolytic cleavage at uracil-DNA glycosylase generated apyrimidinic sites.

We note that these results are in disagreement with those of Seidel (1967) who found 27% dThd and 4% dTMP after alkaline hydrolysis of a similar apyrimidinic dinucleotide. In this case, however, the apyrimidinic compound was formed by oxidizing the 3-N of cytosine in d(pCpT) followed by acid hydrolysis of the N-glycosidic bond. The presumed product of this sequence of reactions is p-deoxyribo-p-dT, but the compound was not isolated nor was product analysis performed.

APPENDICES

APPENDIX 1: SEPARATION OF 5-SUBSTITUTED 2'-DEOXYURIDINES AND DEOXYURIDYLATES BY HIGH-PRESSURE ANION EXCHANGE LIQUID CHROMATOGRAPHY

Investigations in this laboratory have been concerned with enzymatic modifications at the 5-position of 2'-deoxyuridine and 2'-deoxyuridylate. Simple methods which permit separation and quantitative recovery of reactants and products were required for monitoring such conversions. For example, separation of dUMP from dTMP, the reactant and product of the thymidylate synthetase reaction, is usually performed using paper chromatography (Wahba and Friedkin, 1962), but with relatively poor resolution. An earlier observation by Cohn and Bollum (1961) indicated that the slight difference in pK_a values of the 3-NH of the heterocycles ($\Delta pK_a = 0.5$) would result in their separation by anion-exchange chromatography at the proper pH. Using Aminex A-27, this separation was indeed achieved (Weill, 1974; Santi et al., 1976) in a rapid and efficient manner.

Based on this observation, the ability to separate other similar compounds on a small Aminex A-27 column at moderately high operating pressures with single buffer systems was investigated in detail, and we achieved highly effective separations of virtually all the 5-substituted 2'-deoxyuridines and 2'-deoxyuridylates available to us. This method is now used routinely in this laboratory for monitoring the thymidylate synthetase-catalyzed conversion of dUMP to dTMP, and of BrdUMP or IdUMP to dUMP, as well as for purification of radioac-

tively labelled nucleosides and nucleotides used as substrates in these reactions.

MATERIALS AND METHODS

Separations were performed using a Microbore column, 3 mm x 15 cm (Altex, Berkeley, CA), packed with Aminex A-27 (Bio-rad, Richmond, CA). Pressure was maintained at ca. 500 psi using a Milton Roy pump and a Matheson pressure guage. Samples were injected via a slider-type injection valve (Laboratory Data Control, Riviera Beach, FA), and column effluent was monitored at 254 nm.

5-CldUMP and 5-CF_3dUMP were gifts of Dr. Yusuke Wataya, this laboratory. 5-FdUrd was a gift of the National Cancer Institute. All other nucleoside and nucleotides were purchased from commercial sources. Other chemicals were of reagent grade quality.

Buffer A was prepared as follows: to 0.11 mol NH_4HCO_3 in 800 ml water was added 80 ml n-propanol and NH_4OH to give a pH of 9.4. Water was then added to give a final volume of 1 liter. Specific conductance was 8.0 mmho/cm. Buffer B was prepared in the the same manner as buffer A, except that 0.3 mol of the salt was used, and the pH was adjusted to 9.0 (19 mmho/cm). Buffer C was 0.75 M ammonium acetate, pH 4.4, containing 8% n-propanol (15 mmho/cm).

The column was equilibrated with elution buffer prior to use, and aqueous solutions of samples, containing ca. 0.5 μmol of each substance to be separated, were applied. Flow rates were ca. 23

ml/hr for buffers A and B, and 13 ml/hr for buffer C.

RESULTS AND DISCUSSION

Table 7 gives the retention volumes for dUrd, dUMP, and various 5-substituted derivatives of each, using the chromatographic systems described. Although the absolute volumes shown varied slightly between runs, perhaps due to aging or settling of the column resin, relative retention volumes were highly reproducible.

With buffer A, dUrd and dUMP can be separated from dTMP and all of the 5-substituted nucleosides except CF_3dUrd. Other nucleotides, however, in which the 3-NH is relatively more acidic, were not recovered from the column even with extended washings. For separating the various nucleotides buffer B was optimal, and was also satisfactory for separating dUMP and the nucleosides. In this case resolution of dUMP and dTMP is lost; also most 5-substituted nucleosides cannot be separated from their corresponding nucleotides. The latter separations are conveniently accomplished using buffer C.

For 0.5 μmol of a substance injected in 100-150 μl of solution, peak width depended primarily on the retention volume of the substance; a substance eluting at 2 ml shows one-half peak width (W) of ca. 0.6 ml, while at 50 ml retention volume W ranged from 3.5 to 4.0 ml. With buffer A, up to 2.0 μmol each of dUMP and dTMP, applied in 250 μl of solution, could be completely resolved. Recovery of dUMP, BrdUMP, and dTMP was determined to be virtually

TABLE 7 : Separation of 5-Substituted 2'-Deoxyuridines and Deoxyuridylates on Aminex A-27[a]

Compound	Retention volume (ml)[b]		
	Buffer A	Buffer B	Buffer C
dUrd	5	2.0	1.4
FdUrd	16	7.0	1.5
CldUrd	34	14	2.3
BrdUrd	53	20	2.5
CF_3dUrd	68		3.0
IdUrd	77	28	3.6
dTMP	48	3.8	5.4
dUMP	65	4.3	5.6
FdUMP		12	7.1
CldUMP		20	11
BrdUMP		27	12
CF_3dUMP			14
IdUMP		36	16

[a]Buffer compositions and column description are given in Materials and Methods. [b]Volume of eluate from sample injection to peak concentration.

quantitative by the use of tritium labelled compounds.

For some purposes, modifications of the described buffers have been found useful; for a discussion of the subject see Singhal and Cohn (1972). Resolution of peaks is improved upon lowering the ionic strength of the elution buffer, but the retention time increases. Conversely, separation may be speeded by increasing the ionic strength, but at the expense of resolution. The omission of n-propanol retards the passage of the compounds tested with no increase in resolution. The effect of elevated temperatures was not investigated. Recently we have adapted this method for use at higher operating pressures (4000 psi) with greatly increased speed of separation and no loss in resolution.

APPENDIX 2: A RAPID AND SENSITIVE HPLC ASSAY FOR DEOXYRIBONUCLEOSIDE TRIPHOSPHATES IN CELL EXTRACTS

Investigations of deoxyribonucleoside triphosphate (dNTP) metabolism require the ability to detect and quantitate these compounds at the low levels found in most biological materials. Although high pressure liquid chromatography (HPLC) has become a valuable tool for separation and quantitation of ribonucleotides in cell extracts (Brenton et al., 1977; Gudas et al., 1978), this technique has not been directly applicable to estimation of dNTP pools since these compounds are in general only incompletely separated from the more abundant ribose-containing compounds present. A number of laboratories have studied the degradation of ribonucleotides to their respective bases by the combined action of periodate and amines, primarily as a method for the sequence analysis of RNA (Neu and Heppel, 1964; Rammler, 1971; Keith and Gilham, 1974). Here we describe a simple procedure, utilizing a modification of these techniques, for the selective destruction of ribonucleotides in cell extracts; after such treatment dNTPs may be easily and directly quantitated by HPLC analysis.

Methods

Periodate oxidation procedure. To 1.0 ml of a neutralized (pH 6.5-7.5) cell extract or nucleotide solution was added 40 µl of 0.5 M $NaIO_4$, followed within several minutes by 50 µl of a 4 M solution of methylamine which had been (slowly) brought to pH 7.5 with H_3PO_4. After mixing, reactions were incubated at 37° for 30 min. 10 µl of 1 M rhamnose was then added to destroy remaining IO_4^-, and the samples were immediately put on ice. For smaller reactions all volumes were scaled proportionately.

HPLC separation of nucleotides. For all HPLC a Hewlett-Packard model 1084 liquid chromatograph was used. Aliquots of untreated or periodate-treated samples were injected onto a 4.6 x 250 mm Partisil-10 SAX column and eluted with 0.4 M ammonium phosphate, pH 3.25 - spectrophotometric grade acetonitrile (10:1) at a flow rate of 2 ml/min and a column temperature of 30°. Column eluate was monitored at 254 nm, and compounds were identified by their retention time.[1]

HPLC separation of bases. Separation of bases in periodate-amine reaction mixtures was performed on an Aminex A-5 column

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[1]Because of the high resolution obtained and the extreme reproducibility of the separations, peak identification was unambiguous. A large number of cell extracts have also been analyzed using a uv column monitor (Altex) capable of simultaneous monitoring at 254 and 280 nm. The spectral ratios of dNTP peaks at these wavelengths consistently matched those of dNTP standards, confirming the accuracy of peak identification and the absence of significant contamination by other co-eluting cellular components.

(3 x 150 mm). In order to speed analysis time, two buffer systems were used: (a) For uracil and guanine, 0.3 M NH_4HCOO (pH 4.75) containing 7% n-propanol and (b) for adenine and cytosine, 0.3 M NH_4HCOO (pH 6.2) containing 1.5% n-propanol. Compounds were eluted at a flow rate of 1 ml/min and a column temperature of 70°. These separation conditions are essentially those described by Demushkin and Plyashekevich (1978) with modifications to optimize separating the particular compound of interest. In all cases the bases were separated from nucleosides, nucleotides and IO_4^-.

Quantitation of peaks. Peak areas of nucleotides or bases at levels greater than ca. 500 pmol could be accurately quantitated using the integrating function of the Hewlett-Packard 1084 liquid chromatograph. For lower levels of nucleotides, as found in periodate-treated cell extracts, automatic integration was found to be unreliable, and quantitation was performed by manually measuring the height of the peak (Hartwick and Brown, 1975); this method was found to be accurate for as little as 30 pmol of nucleotide. In either case, concentration of the compound was determined by comparison of peak area or height to that of an accurately prepared standard solution of approximately the same concentration. Standard nucleotide triphosphate solutions were analyzed to correct for contamination by mono- and diphosphates using the HPLC procedure of Hartwick and Brown (1975).

Preparation of cell extracts. S-49 mouse lymphoma cells were grown to a density of 4 x 10^5/ml in Dulbecco's modified Eagles medium

(Dulbecco and Vogt, 1954) supplemented with 10% heat-treated horse serum. Fifty to 300 ml of cells were harvested by centrifugation, washed twice with 0.5 ml ice-cold phosphate-buffered isotonic saline solution (PBS), and resuspended in PBS at a concentration of 0.5 - 3.0 x 10^8 cells/ml. [^{3}H]dUrd was added as a concentration marker (final concentration ca. 50,000 cpm/10 µl), and aliquots of the suspension were removed for determination of radioactivity and of cell concentration (using a Coulter ZBl counter). Recovery of cells was 94%. Cellular integrity was confirmed by the absence of extracellular ATP (1.5% of the total ATP in the suspension) and by the absence of increased trypan blue uptake as compared to cells before harvesting. Upon dilution of S-49 cells into large volumes of PBS, however, substantial lysis was observed. 60% $HClO_4$ was then added to the suspension to give a final concentration of 0.5 M. After 30 min on ice the precipitate was removed by centrifugation and the solution neutralized sith 4 M KOH containing 0.4 M KH_2PO_4. The neutralized extract was kept on ice at least 5 min to allow precipitation of $KClO_4$ and the solution was again cleared by centrifugation. An aliquot of the extract was then removed and counted for calculation of dilution factors.

Results

Destruction of rNTPs with periodate and methylamine. When a solution containing 1 mM each of the four common rNTPs was treated with periodate and methylamine as described in Methods and injected onto Partisil-10 SAX there was a complete (>99.9%) loss of uv absorbance in the triphosphate region of the chromatogram. With periodate treatment alone only a relatively small change in chromatographic mobility was observed, but in the presence of methylamine the products of the reaction eluted near the void volume of the column. By analysis of the reaction mixture on Aminex A-5 it was determined that under these conditions the products formed are the corresponding purine and pyrimidine bases. The time course of base formation under these conditions is shown in Figure 22, and it is clear that under these conditions the reaction is essentially complete after 20 min.

Stability of dNTPs during periodate-amine treatment. Separate solutions (2.0 ml each) containing 10 mg of dATP, dCTP, dGTP, dUTP or dTTP were prepared. 1.0 ml aliquots of each were removed and treated with periodate and methylamine. Three or more aliquots (50 µl) from each solution were injected onto Partisil-10 SAX and peak areas before and after periodate-amine treatment were compared. After correcting for dilution factors, recoveries of the dNTPs were as follows: dATP, 101%; dCTP, 98%; dGTP, 100%; dUTP, 101% dTTP, 100%; standard errors for these determinations were ca. 1%. Thus the

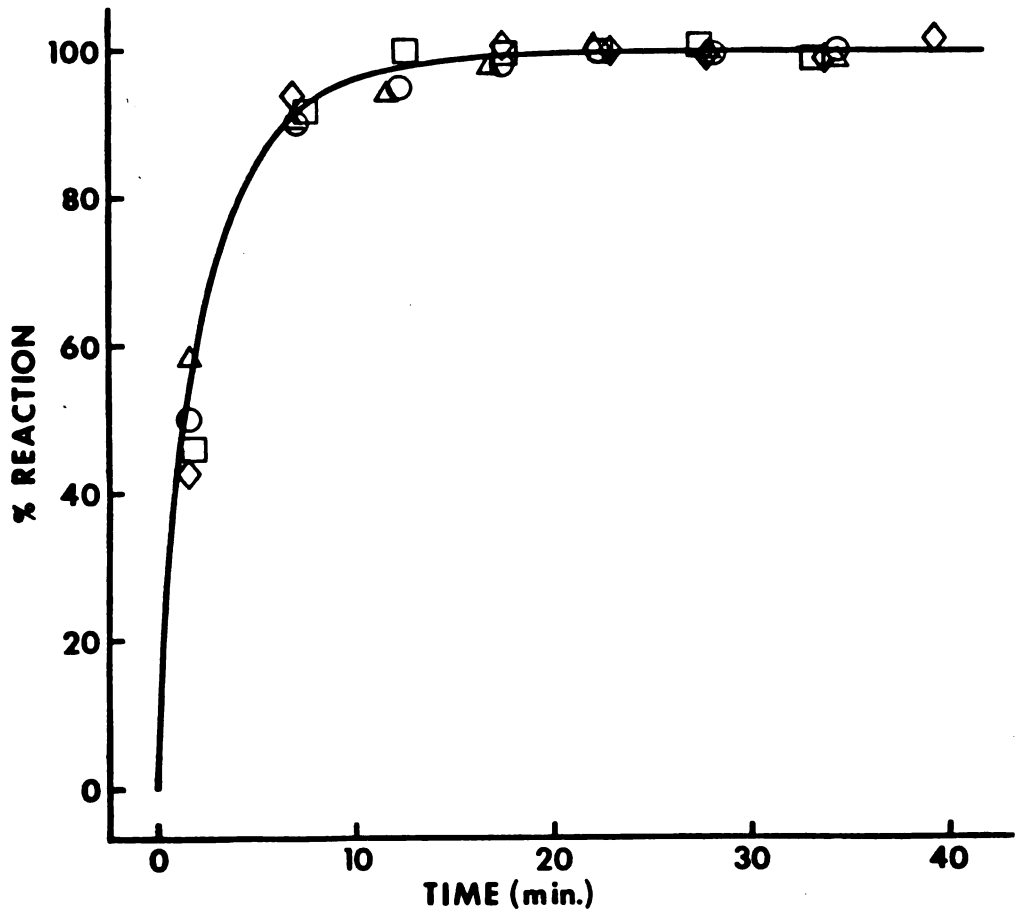

FIGURE 22: Rate of base formation from rNTPs. Separate solutions (1.0 ml) containing 1 mM ATP, CTP, or UTP, or 33 μM GTP were treated with periodate and methylamine as described in Methods. 25 μl aliquots were removed and the formation of the corresponding base determined from the area of the appropriate peak after separation on Aminex A-5. Data points represent the area of the base formed from △, CTP; ◇, UTP; □, ATP; and ○, GTP, normalized to the mean of the two final points.

dNTPs are quantitatively recovered after periodate-amine treatment.

Periodate-amine treatment of a mixture of rNTPs and dNTPs. As shown in Figure 23, deoxyribonucleotides are easily quantitated after destruction of ribonucleotides in an artificial mixture initially containing a ten-fold excess of the latter. After correcting for dilution, recoveries of the dNTPs were as follows: dCTP, 95%; dTTP, 99%; dATP, 100%; and dGTP, 88%. Despite the fact that dGTP by itself is stable to periodate-amine treatment, recovery of dGTP in such experiments was consistently 11-14% lower than expected. Addition of dGTP to periodate-oxidized rNTPs resulted in no such loss, and it would appear that dGTP reacts with some intermediate formed during periodate oxidation of ribonucleotides.

The experiment shown in Figure 23 was repeated using several variations of the standard periodate-amine treatment described in Methods. With one-half the concentration of $NaIO_4$ or methylamine, destruction of ribonucleotides was not reproducibly complete in 30 min; while using twice the amount of either of these reagents gave no apparent advantage. At pH 5.9 the amine-promoted elimination of phosphate was incomplete in 30 min. At pH 10 the reaction proceeded satisfactorily, but overall recovery of dNTPs was somewhat lower (by ca. 5%), presumably due to increased hydrolysis of the phosphate esters. When cyclohexylamine-HCl (pH 10) was used in place of methylamine, all rNTP peaks were removed in less than one minute, but the resulting reaction mixture was unsuitable for direct separation by HPLC since severe trailing from the

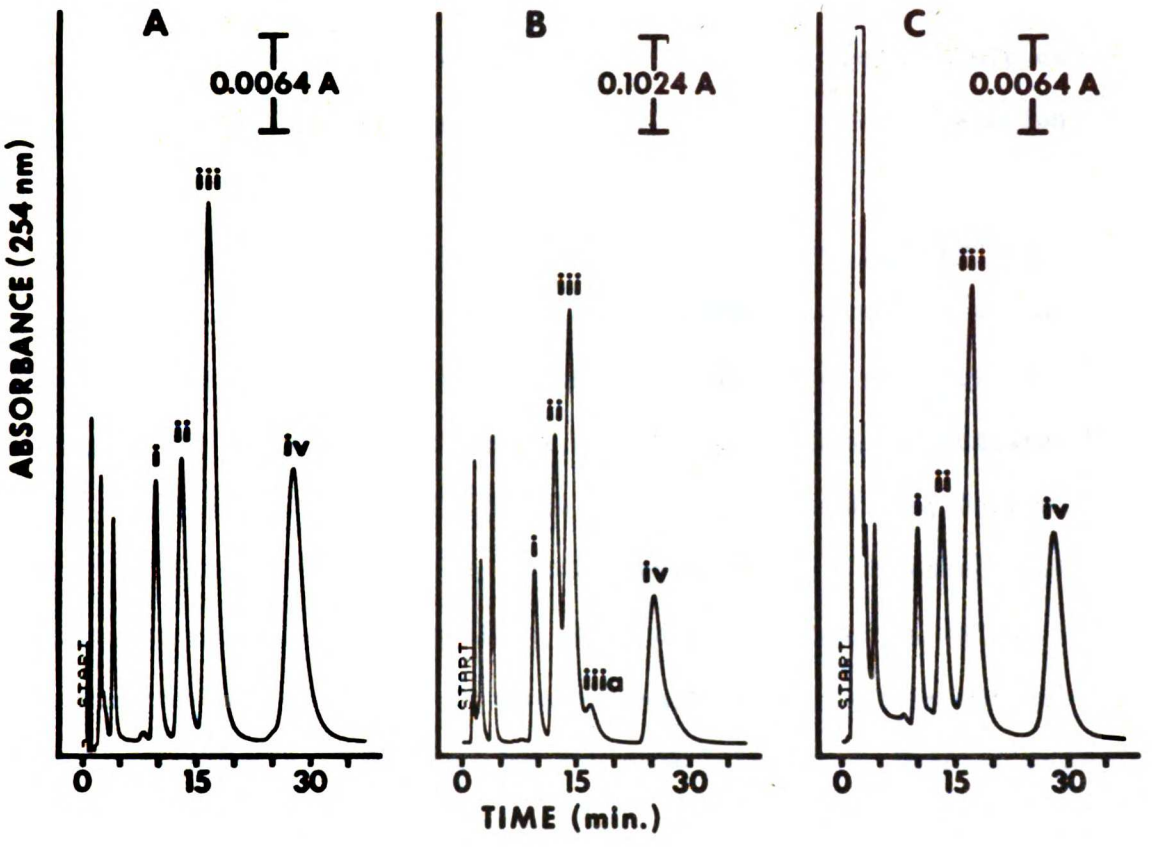

FIGURE 23: Periodate treatment of an artificial mixture containing dNTPs and a ten-fold excess of rNTPs. A solution containing 0.1 mM each of dCTP, dTTP, dATP, and dGTP was prepared and an aliquot removed for chromatographic separation (A). To the remainder was added a solution containing 10.4 mM each of CTP, UTP, ATP, and GTP to give a final concentration of 1.0 mM of the rNTPs (B), followed by periodate-amine treatment (C). Separations were performed as described in Methods; in each case 25 µl aliquots were injected. Peak identification is as follows: Panels A and C, i, dCTP; ii, dTTP; iii, dATP; and iv, dGTP. Panel B, i, CTP; ii, UTP; iii, ATP; iiia, dATP; and iv, GTP. Note differences in scale.

void-volume peak occurred (even after neutralization with HCl) and precluded resolution of dCTP. The anomalous loss of dGTP encountered with methylamine was not observed under these reaction conditions; recoveries of dNTPs were ca 95%.

Periodate oxidation of S-49 cell extracts and recovery of dNTPs. Chromatograms of nucleoside triphosphates in an S-49 cell extract before and after periodate-amine treatment are shown in Figure 24. Complete destruction of ribonucleotides is indicated by the absence of ATP and GTP, which would be resolved just before dATP and dGTP, respectively, and by the absence of oxidized nucleotide intermediates, which elute after the corresponding dNTPs. The concentrations of ribo- and deoxyribonucleotides in this extract was determined by comparison of the peak heights to those of standard nucleotide mixtures and the calculated pool sizes are given in Table 8.[2] The high reproducibility of the assay is also indicated by the data in Table 8; standard deviations for four separate determinations on aliquots of the same extract ranged from 1.2% (dCTP) to 5.4% (dGTP).

To determine the actual recoveries of the four dNTPs in treated cell extracts the following experiment was performed. To 90 µl of

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[2]The dNTP levels reported here are 3- to 5-fold higher than those determined for the same cell line using a DNA polymerase assay [Ullman et al. (1975) Cell 14, 365-375]. We feel these differences likely reflect different techniques in cell counting or extract preparation, particularly since the relative values obtained agree well. Relative to dTTP, the concentrations determined here are dCTP, 0.92; dATP, 0.88; dGTP, 0.32. Ullman et al. (op cit.) reported 0.84, 0.77, and 0.52 respectively.

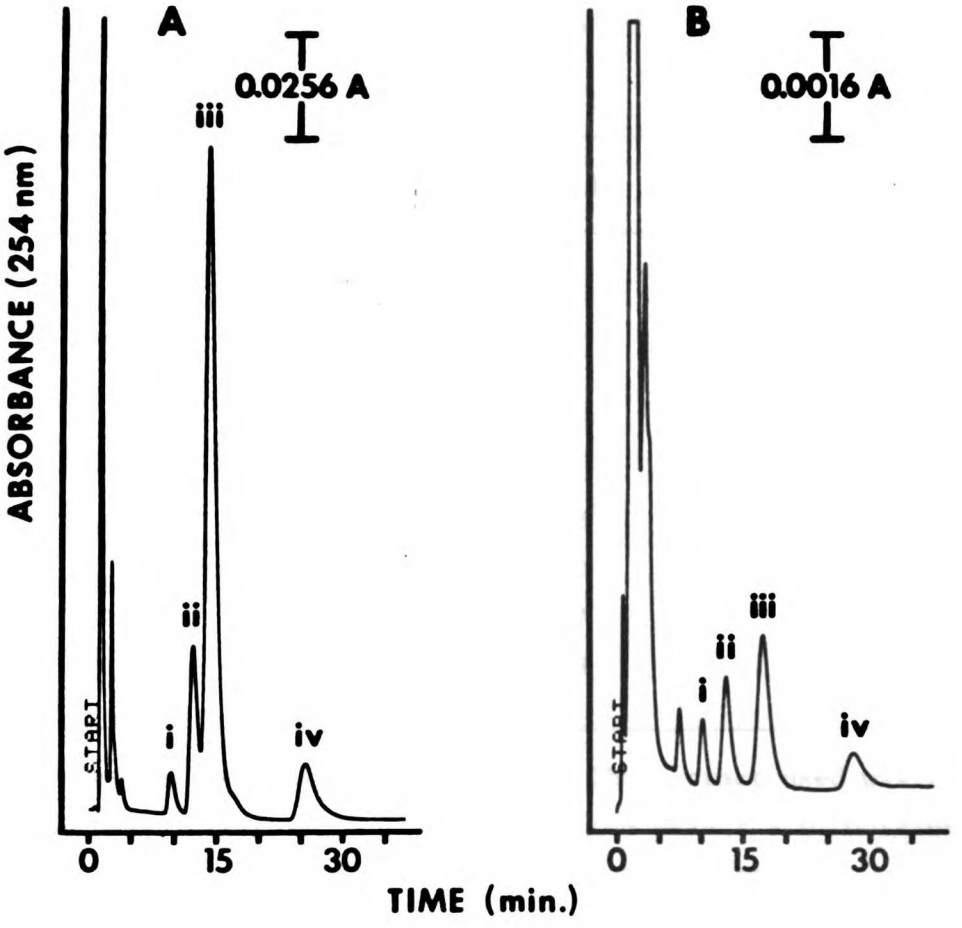

FIGURE 24: S-49 cell extract before and after periodate treatment. A) 25 µl of an untreated extract prepared as described in Methods, equivalent to 5.1 x 10^6 cells. Peak identification: i, CTP; ii, UTP; iii, ATP; and iv, GTP. B) The same extract after treatment with periodate and methylamine; 25 µl, equivalent to 4.5 x 10^6 cells, was injected. i, dCTP; ii, dTTP; iii, dATP; and iv, dGTP. Note differences in scale. ADP is represented by the peak eluting at 2.8 min in Panel A, and the ATP to ADP ratio of the extract was greater than 9.

TABLE 8: Ribonucleotides and Deoxyribonucleotides in S-49 Cell Extract.[a]

	pmol/10^6 cells
CTP	465
UTP	998
ATP	2810
GTP	484
dCTP	67.9 ± 0.8[b]
dTTP	74.2 ± 2.0
dATP	65.2 ± 1.7
dGTP	24.0 ± 1.3

[a]Determined as described in the text. The extract used was the same as that in Figure 24. [b]Values are the mean ± S.D. obtained from four separately treated aliquots of the extract.

an extract (equivalent to 8.9 x 10^6 cells) was added 10 µl of a solution containing 10 mM each of dCTP, dTTP, dATP and dGTP. The mixture was treated with periodate and methylamine and analyzed for dNTP concentrations. Another aliquot of the same extract was treated similarly except that the internal standards were added after periodate-amine treatment. After correcting for the initial dNTP content of the extract (ca. 10% of the total) recoveries of the added dNTP standards were: dCTP, 96%; dTTP, 97%; dATP, 100%; and dGTP, 93%. Thus the recovery of these compounds in cell extracts is near quantitative and agrees well with the values obtained upon periodate-amine treatment of artificial nucleotide solutions containing the rNTPs.

Storage of periodate-amine treated samples. After periodate-amine treatment, samples could be stored for 24 hours at 4° with minimal (<5%) change in dNTP concentrations. Upon storage overnight at -20°, however, a distinct loss of dGTP (21%) was observed, and a new peak appeared, eluting just after dATP. This problem may be avoided by rapid freezing of treated samples in a dry ice-acetone bath and storage at -80°; periodate-amine treated extracts stored in this manner for three weeks showed no detectable loss of dNTPs.

Interference of Tris(hydroxymethyl)aminomethane (Tris) in the assay. As mentioned above, dGTP by itself is stable to periodate-amine treatment. When a 0.1 mM solution of dGTP, containing 2.5 mM Tris-HCl (pH 7.5) was similarly treated, however, recovery of dGTP

was 3.5% and a new peak eluting at the position of dATP was observed. The combined areas of the two peaks equalled that expected for dGTP itself. No effect on the recoveries of other dNTPs was observed.

Discussion

The assay for dNTPs described here exploits the known reactivity of the cis-2',3'-hydroxyls of ribonucleotides toward periodate oxidation. The resulting dialdehyde products, which themselves have only moderately altered chromatographic properties, undergo subsequent reaction with methylamine, which results in elimination of phosphate and cleavage of the N-glycosidic bond (Neu and Heppel, 1964; Rammler, 1971; Keith and Gilham, 1974). In this manner the interfering ribonucleotides are converted to their respective bases and full resolution of the dNTPs by HPLC is made possible. Destruction of rNTPs is complete (>99.9%), and the treated extracts may be injected directly onto Partisil-10 SAX for analysis. dNTPs are easily quantitated by comparison of peak heights to those of a standard mixture, and recoveries of dNTPs are reproducible and near quantitative. In our hands the lower limit for accurate quantitation of the dNTPs by HPLC is ca. 30 pmol, the dGTP content of 10^6 S-49 cells. This limit will undoubtedly be lowered with future

improvements in HPLC instrumentation and technology.[3]

When small amounts of Tris were present during the periodate-amine oxidation recoveries of dGTP were considerably reduced, and the presence of Tris in reaction mixtures must be avoided. Although the exact reason for this loss of dGTP is unknown, caution is also warranted in the use of similar oxidizable reagents. Other compounds present in cell extracts prepared as described here do not interfere with the assay, since good agreement was obtained between recoveries of dNTPs in standard nucleotide solutions and in cell extracts containing internal standards.

Current methods for measuring dNTP levels in cell extracts are difficult and time-consuming. The most widely-used involves quantitating the amount of DNA synthesized by an in vitro polymerizing system with the dNTP of interest as the limiting factor in the reaction (Solter and Handschumacher, 1969; Skoog, 1970; Lindberg and Skoog, 1970). This technique, while extremely sensitive, requires extensive calibration, numerous manipulations of small volumes, and separate reaction mixtures for each dNTP. Another assay utilizes thin-layer chromatography to separate dNTPs remaining in periodate-treated (but not amine-treated) extracts (Yegian, 1974; Reynolds and Finch, 1977). With this method complex

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[3]Initially we investigated the use of elevated temperatures (65°) for the separation of dNTPs. Resolution and sensitivity were greatly improved, but the short lifetime of the columns prohibited the use of high-temperature separations on a routine basis. Using the chromatographic conditions described in Methods, Partisil-10 SAX columns have a useful life of at least several hundred assays.

chromatographic procedures are required and the relatively low sensitivity obtained necessitates prelabeling of cellular nucleotides with ^{32}P.

The assay described here offers significant advantages over these methods in speed and simplicity. It is rapid, and both the rNTP and dNTP content of an extract can be determined in approximately one hour. The four dNTPs are quantitated simultaneously, allowing direct and unambiguous comparison of their concentrations. In addition, the necessity of working with ^{32}P is eliminated. Preliminary results indicate that the technique is directly applicable to measurements of deoxyribonucleoside mono- and diphosphate pools also.

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