Fluorinated Pyrimidines. XXXIV. Structure–Activity Studies of Methylated 5-Fluoro-2′-deoxyuridine Derivatives[1]

RAYMOND J. KENT, TASNEEM A. KHWAJA, AND CHARLES HEIDELBERGER[2]

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706

Received July 28, 1969

Some methylated derivatives of 5-fluoro-2′-deoxyuridine (FUDR) were tested as substrates or inhibitors in three enzyme systems. None of the methylated derivatives was a substrate for nucleoside phosphorylase. One compound, 3′-O-methyl-5-fluoro-2′-deoxyuridine (3′-OMe-FUDR), inhibited the phosphorylation of thymidine by thymidine kinase, but only when present in a thousandfold excess over the substrate. Studies with methylated FUDR's doubly labeled with ^{3}H in the ring and ^{14}C in the methyl group showed that only 3′-OMe-FUDR is phosphorylated by thymidine kinase and that phosphorylation occurs without prior demethylation. The 5′-monophosphate of 3′-OMe-FUDR is an inhibitor of thymidylate synthetase but has only about 1% the activity of 5-fluoro-2′-deoxyuridine 5′-monophosphate (FUDRP). Structure–activity relationships are discussed.

In the preceding paper, the syntheses of methylated derivatives of FUDR[3] and their effects on the growth of *Escherichia coli*, HeLa, L5178Y, and L5178BF cells were reported.[4] The present paper is concerned with the effect of methylation on the activity of FUDR as a substrate or inhibitor for three enzymes of critical importance in FUDR chemotherapy: (1) nucleoside phosphorylase, which degrades FUDR to FU, thus limiting its cancer chemotherapeutic efficacy;[5-7] (2) thymidine kinase, which phosphorylates FUDR to FUDRP and whose loss causes tumor cells to become resistant to FUDR;[8] and (3) thymidylate synthetase, the target enzyme for the powerful inhibitor FUDRP.[9,10]

Experimental Section

Chemicals.—The methylated derivatives of FUDR were synthesized in this laboratory.[4] All other fluorinated pyrimidines were generously provided by Hoffmann-LaRoche, Inc. ^{3}H-TDR (6.7 Ci/mmole) was purchased from New England Nuclear Corp.

Thymidine Kinase.—The assay has been described.[11]

Nucleoside Phosphorylase.—The optical method of assay was used.[5] Only one standard curve was necessary, since 3′-OMe-FUDR and 5′-OMe-FUDR have the same extinction coefficient as FUDR.[4] 4-OMe-FUDR and 3′,4-di-OMe-FUDR were demethylated at the 4 position by allowing them to stand in alkali for 1 hr at room temperature before optical density readings were made.

Whole Cell Incubations.—The incubation procedure has been described.[11]

Results

Nucleoside Phosphorylase.—Table I shows that none of the methylated derivatives of FUDR was a substrate for nucleoside phosphorylase from Ehrlich ascites cells. This result implies that the 4-hydroxyl of the pyrimidine ring and the 3′- and 5′-hydroxyls of the sugar moiety of these nucleosides must be unsubstituted in order for interaction to occur with this enzyme. Alternatively, methylation might have increased the strength of the glycosidic bond, as was demonstrated for 3′-OMe-FUDR in the previous paper.[4] This, however, could not be the case with 4-OMe-FUDR since it was shown that its glycosidic bond is much weaker than that of FUDR.[4]

TABLE I
NUCLEOSIDE PHOSPHORYLASE ASSAY[a] OF METHYLATED FUDR'S[b]

Nucleoside	% conversion to base/30 min Expt 1	Expt 2
FUDR	66	84
3′-OMe-FUDR	0	0
4-OMe-FUDR	10	0
5′-OMe-FUDR	0	0
3′,4-Di-OMe-FUDR	0	0

[a] Nucleoside (0.4 mM) was incubated at 37° with Ehrlich ascites S3 containing 6 mg of protein, in a total volume of 1.5 ml. At 0 and 30 min 0.5-ml aliquots were removed, the protein was precipitated with PCA and 0.5 N NaOH was added. The optical densities were determined at 260 and 290 mμ, and the per cent conversion of the nucleoside to the base was calculated from a standard curve.

Thymidine Kinase.—FUDR is phosphorylated by TDR kinase to the active drug.[6,10] Unfortunately from the clinical standpoint, FUDR is also readily cleaved to FU by nucleoside phosphorylase.[5-7] Since none of the methylated FUDR's was a substrate for nucleoside phosphorylase, it became of interest from the standpoint of drug design to know whether any of these derivatives would be phosphorylated by TDR kinase and whether any nucleotide thus formed would have activity as an inhibitor of thymidylate synthetase.

Figures 1A and 1B show the result of an experiment in which the methylated FUDR's were tested as competitive inhibitors of TDR kinase. At 100 times the level of the substrate, none of the derivatives exerted any effect on the rate of phosphorylation of TDR. At 1000 times the level of ^{3}H-TDR, only 3′-OMe-FUDR

(1) This work was supported in part by grants CA-07175 and CRTY-5002 from the National Cancer Institute, National Institutes of Health.

(2) American Cancer Society Professor of Oncology.

(3) Abbreviations used in this work are: FU, 5-fluorouracil; FUDR, 5-fluoro-2′-deoxyuridine; FUDRP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; 3′-OMe-FUDR, 3′-O-methyl-5-fluoro-2′-deoxyuridine; 5′-OMe-FUDR, 5′-O-methyl-5-fluoro-2′-deoxyuridine; 4-OMe-FUDR, 1-(2′-deoxy-β-D-ribofuranosyl)-4-methoxy-5-fluoro-2(1H)-pyrimidone; 3′,4-di-OMe-FUDR, 1-(2′-deoxy-3′-O-methyl-β-D-ribofuranosyl)-4-methoxy-5-fluoro-2-(1H)-pyrimidone; 3-NMe-FUDR, 3-N-methyl-5-fluoro-2′-deoxyuridine; 3-N,3′-O-di-Me-FUDR, 3-N,3′-O-dimethyl-5-fluoro-2′-deoxyuridine; F$_3$TDR, 5-(trifluoromethyl-2′-deoxyuridine; TDR, thymidine; TMP, thymidine 5′-monophosphate; DUMP, deoxyuridine 5′-monophosphate.

(4) T. A. Khawaja and C. Heidelberger, *J. Med. Chem.*, **13**, 64 (1970).

(5) G. D. Birnie, H. Kroeger, and C. Heidelberger, *Biochemistry*, **2**, 566 (1963).

(6) C. Heidelberger, G. D. Birnie, J. Baolar, and D. Wentland, *Biochim. Biophys. Acta*, **76**, 315 (1963).

(7) C. Heidelberger and J. Baolar, *ibid.*, **91**, 639 (1964).

(8) P. A. Morse, Jr., and V. R. Potter, *Cancer Res.*, **25**, 499 (1965).

(9) C. Heidelberger, *Progr. Nucleic Acid Res. Mol. Biol.*, **4**, 1 (1965).

(10) P. Reyes and C. Heidelberger, *Mol. Pharmacol.*, **1**, 14 (1965).

(11) R. J. Kent and C. Heidelberger, *Biochem. Pharmacol.*, in press.

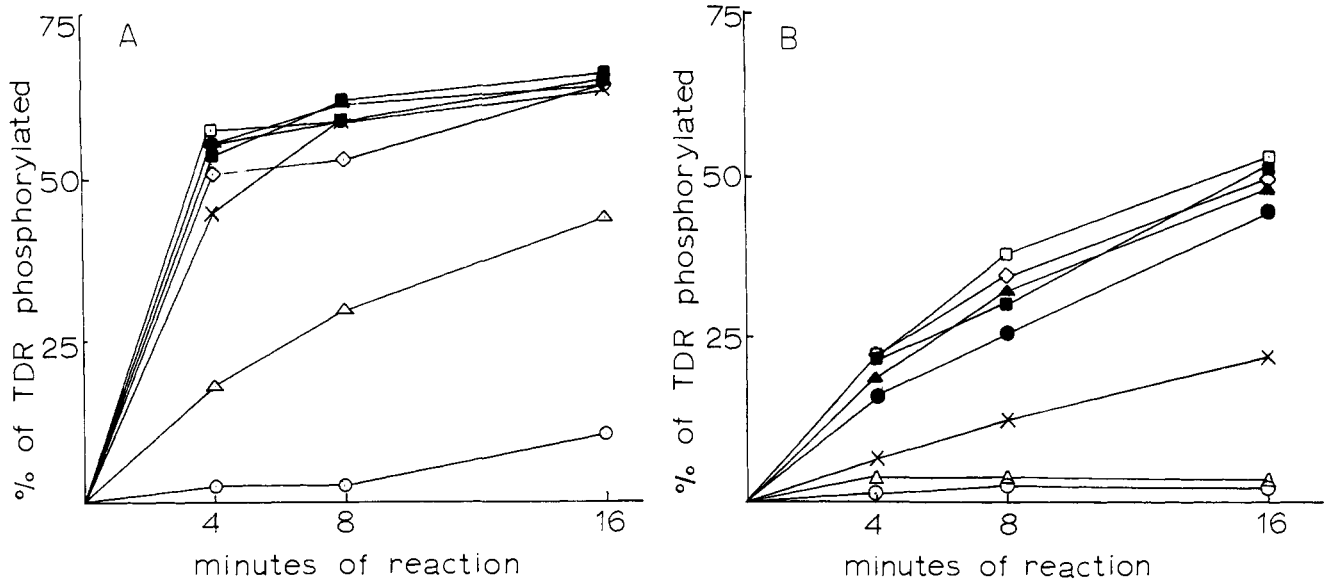

Figure 1.—Effect of methylated FUDR's on the phosphorylation of ^{3}H-TDR by thymidine kinase. A. ^{3}H-TDR at 2×10^{-7} M was incubated with a 100,000g supernatant fraction from Ehrlich ascites cells containing 0.5 mg of protein in the thymidine kinase assay procedure described by Kent and Heidelberger.[11] The following nucleosides were added to the incubation mixture at a concentration 100 times that of ^{3}H-TDR: □ no addition; ○, TDR; △, FUDR; × 3'-OMe-FUDR; ●, 4-OMe-FUDR; ▲, 3-NMe-FUDR; ■, 3,4-di-OMe-FUDR; ◇, 5'-OMe-FUDR. B. Same as A except that the methylated derivatives were present at 1000 times the concentration of ^{3}H-TDR.

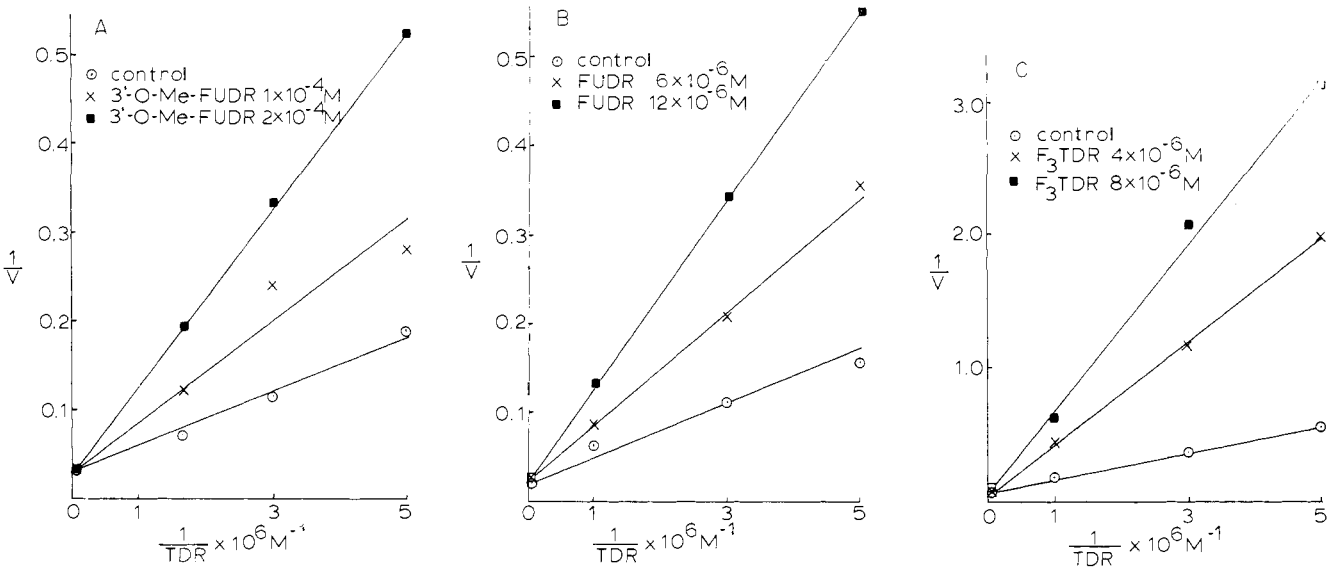

Figure 2.—Lineweaver-Burk plots of the inhibition of TDR kinase by 3'-OMe-FUDR, FUDR, and F_3TDR. The thymidine kinase was done according to the method of Kent and Heidelberger.[11] The concentrations of the inhibitors are given on each graph. The enzyme source was a 100,000g supernatant fraction (S3) of Ehrlich ascites cells. Incubation was carried out for various times up to 20 min, depending upon the ^{3}H-TDR concentration. In all cases the velocity of the reaction was linear throughout the incubation. Enzyme activity is expressed in mμmoles of TDR phosphorylated/hr per mg of protein. The K_m's and K_i's were calculated from the slopes of the lines.[12] A: 3'-OMe-FUDR, $K_m = 0.9 \times 10^{-6}$ M, $K_i = 8.2 \times 10^{-5}$ M; B: FUDR, $K_m = 1.4 \times 10^{-6}$ M, $K_i = 4.4 \times 10^{-6}$ M; C: F_3TDR, $K_m = 1.2 \times 10^{-6}$ M, $K_i = 1.4 \times 10^{-6}$ M.

was capable of inhibiting the rate of phosphorylation of the substrate.

Thus, it appears that in order for a nucleoside analog to combine with TDR kinase, the 4-, 5'-, and 3'-OH and the 3-N must be free. The data suggest that some interaction with TDR kinase can occur when the 3'-OH is blocked by methylation.

Figures 2A–C are Lineweaver–Burk[12] plots of the inhibition of TDR kinase by 3'-OMe-FUDR, by the parent compound FUDR, and, as a further comparison, by F_3TDR, another of the fluorinated pyrimidines studied extensively in this laboratory.[9] The inhibition of TDR kinase by all three compounds is competitive with TDR. The K_i/K_m ratios for 3'-OMe-FUDR, FUDR, and F_3TDR, respectively, are 90, 3.1 and 1.2. The latter value confirms that of Bresnick and Williams who obtained a value of K_m for TDR of 3.3×10^{-6} M, a K_i for F_3TDR of 3.7×10^{-6} M,[13] or a K_i/K_m ratio of 1.1 with TDR kinase from regenerating rat liver. The strength of binding of 3'-OMe-FUDR to TDR kinase is, therefore, much less than that of either FUDR or F_3TDR.

(12) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

(13) E. Bresnick and S. S. Williams, *Biochem. Pharmacol.*, **16**, 503 (1967).

TABLE II
PHOSPHORYLATION OF DOUBLE-LABELED METHYLATED FUDR'S[a]

	Incubation, min								
	0			45			90		
Marker	^{3}H, dpm	^{14}C, dpm	^{3}H/^{14}C	^{3}H, dpm	^{14}C, dpm	^{3}H/^{14}C	^{3}H, dpm	^{14}C, dpm	^{3}H/^{14}C
FUDR (^{14}C only)	0	12,800	0	0	1,730	0	20	960	0.02
FUDRP				0	10,500	0	160	9800	0.02
3'-OMe-FUDR	1050	2,920	0.36	920	2,950	0.31	490	1140	0.43
FUDR				120	80	1.5	60	20	3.0
3'-OMe-FUDRP				160	220	0.73	210	420	0.50
FUDRP				40	0		25	0	
3-NMe-FUDR	1880	1,090	1.73				2120	1140	1.85
FUDR							110	10	11
3-NMe-FUDRP							0	0	
FUDRP							50	0	
5'-OMe-FUDR	0	2,000	0				0	1980	0
FUDR							70	0	0
5'-OMe-FUDRP							0	0	
FUDRP							30	0	
4'-OMe-FUDR	3990	2,130	1.87				3270	1670	1.96
FUDR							100	10	10
4-OMe-FUDRP							0	0	
FUDRP							60	0	
3-N,3'-O-diMeFUDR	5050	4,860	1.04				6150	5890	1.04
FUDR							30	0	
3-N,3'-O-diMeFUDRP							0	0	
FUDRP							30	0	
3',4-diOMe-FUDR	1560	1,130	1.38				1710	1140	1.50
FUDR							20	0	
3',4-diOMe-FUDRP							0	0	
FUDRP							20	0	
3'-OMe-FUDRP	300	200	1.50				440	280	1.57
FUDR							30	0	
FUDRP							30	0	

[a] $^{14}CH_3$ and 6-^{3}H methylated derivatives of FUDR[4] were combined to give doubly labeled compounds. These derivatives, at a final concentration of 2×10^{-5} *M*, were tested in a TDR kinase assay system.[11] At 0, 45, and 90 min, 0.3-ml aliquots were removed from the incubation tubes, boiled for 2 min to stop the reaction, centrifuged to remove coagulated protein, and chromatographed on paper in a system of 1-butanol–AcMe–formic acid–5.5% aqueous NH_4O_2CH (35:25:15:25, v:v:v:v). In this system all the methylated FUDR's migrated with a mobility relative to FUDR of 1.2 while 3'-OMe-FUDRP and FUDRP migrated with relative mobilities of 0.87 and 0.73, respectively. On each chromatogram, uv-visible markers of FUDR and FUDRP were spotted. After development the paper strips were scanned in a Packard strip scanner to locate the radioactive peaks. Unchanged nucleoside, nucleotide, and the FUDR and FUDRP markers were cut from the strips, eluted with water, and portions of the eluate counted in a Packard scintillation counter equipped with an automatic activity analyzer for simultaneous computation of ^{3}H and ^{14}C activities. The table gives the ^{3}H and ^{14}C disintegrations per minute (dpm) and the ratio of ^{3}H to ^{14}C for every radioactive peak and uv-visible marker on each chromatogram.

TABLE III
PHOSPHORYLATION OF 3'-OMe-FUDR BY A CELL-FREE EXTRACT FROM EHRLICH ASCITES CELLS[a]

	Incubation, min								
	0			45			90		
	^{3}H, dpm	^{14}C, dpm	^{3}H/^{14}C	^{3}H, dpm	^{14}C, dpm	^{3}H/^{14}C	^{3}H, dpm	^{14}C, dpm	^{3}H/^{14}C
3'-OMe-FUDR	930	2860	0.32	1620	4880	0.33	2080	6200	0.33
FUDR				20	50	0.4	30	160	0.18
3'-OMe-FUDRP				630	2000	0.32	270	780	0.34
FUDRP				0	0		0	0	

[a] Same conditions as for Table II except that after elution from the chromatograms, the eluates were evaporated to a small volume and rechromatographed on isobutyric acid–NH_4OH–H_2O (66:4:30, v:v:v). The eluates from this second chromatogram were counted in a scintillation counter as in Table II.

Phosphorylation Studies of ^{3}H- and ^{14}C-Labeled Methylated FUDR's.—The synthesis of the ^{3}H-ring- and ^{14}C-methyl-labeled derivatives of FUDR described in the previous paper[4] permitted us to test these compounds directly as substrates for thymidine kinase and to determine whether the derivatives were demethylated prior to phosphorylation.

The previous results led us to expect that if any of the methylated FUDR's could be phosphorylated, the most likely would be 3'-OMe-FUDR, which inhibited TDR kinase at high concentrations and hence was bound to some extent to the enzyme. The results in Table II show that 3'-OMe-FUDR was, in fact, the only methylated derivative of FUDR to be phosphorylated.

The $^3H/^{14}C$ ratio of the nucleotide produced by phosphorylation indicated a slight loss of ^{14}C, and hence there was a possibility of some demethylation. An additional experiment was performed in which ^3H- and ^{14}C-labeled 3′-OMe-FUDR was incubated in the TDR kinase system in the same way as previously, but an additional chromatographic purification was included in the work-up. The results in Table III show that the ratio of $^3H/^{14}C$ in 3′-OMe-FUDRP was identical with that of unchanged 3′-OMe-FUDR on the same chromatogram and to 3′-OMe-FUDR on the zero-time chromatogram, indicating that no demethylation had taken place.

After 90 min of incubation there was radioactivity associated with the FUDR and FUDRP markers on the chromatograms of all the methylated FUDR's. This radioactivity, mostly ^3H, amounted to only a few per cent of the total label in all cases. It is not known whether this represents demethylation, or counting and computation error.

Phosphorylation of 3′-OMe-FUDR by Ehrlich Ascites Cells.—The previous experiment showed that 3′-OMe-FUDR is phosphorylated without prior demethylation. To determine whether demethylation would occur in intact cells, doubly labeled 3′-OMe-FUDR was incubated with Ehrlich ascites cells and the ratio of $^3H/^{14}C$ was determined in the various metabolites that were formed. The results in Table IV indicate that demethylation occurs only to a very small extent in the intact cell. There is some radioactivity associated with the FUDR marker but the $^3H/^{14}C$ ratio is nearly the same as that of 3′-OMe-FUDR on the same paper strip, suggesting that the 3′-OMe-FUDR had tailed slightly on the chromatogram. The FUDRP marker contained a slight amount of ^3H and no ^{14}C, which would indicate that some demethylation had occurred, but this ^3H activity represents 0.5% of the total ^3H dpm. Thus, if demethylation occurs it is only to a very slight extent.

TABLE IV
PHOSPHORYLATION OF ^3H-, ^{14}C-3′-OMe-FUDR IN INTACT EHRLICH ASCITES CELLS[a]

	^3H, dpm	^{14}C, dpm	$^3H/^{14}C$
0 min			
3′-OMe-FUDR	11,500	940	12.2
90 min			
3′-OMe-FUDR	17,600	1470	12.0
FUDR	220	20	11.0
3′-OMe-FUDRP	3,750	260	14.4
FUDRP	120	0	

[a] 3′-OMe-FUDR (10^{-4} *M*) containing ^{14}C and ^3H labels was incubated with Ehrlich ascites cells by the method of Kent and Heidelberger.[11] At 0 min an aliquot of the medium was removed for chromatography. At 90 min, a 75% EtOH-soluble fraction of the cells was prepared and chromatographed as described in Table III. Uv-visible markers of 3′-OMe-FUDR, FUDR, 3′-OMe-FUDRP, and FUDRP were spotted on the chromatogram. After development of the chromatogram, the markers were cut out, eluted with water, and counted as described in Table II.

Chemically prepared 3′-OMe-FUDRP[4] inhibited thymidylate synthetase from Ehrlich ascites cells, but to only 1% of inhibition produced FUDRP (A. Fridland and C. Heidelberger, unpublished data.)

Discussion

We have studied the effect of methylation on the activity of FUDR as a substrate or inhibitor for three enzymes of importance in cancer chemotherapy with this drug. The results indicate that methylation of any of the OH groups of FUDR destroys its activity as a substrate for nucleoside phosphorylase. Methylation of any of the hydroxyls or of the 3-N of FUDR causes a loss of substrate activity for thymidine kinase, although 3′-OMe-FUDR is a weak substrate for this enzyme.

Although the 5′-monophosphate of 3′-OMe-FUDR is an inhibitor of thymidylate synthetase, it is doubtful that 3′-OMe-FUDR could be of value in chemotherapy, since its nucleotide is only 1% as effective as is FUDRP against this enzyme. In addition, 3′-OMe-FUDR is phosphorylated at a slow rate, and 3′-OMe-FUDRP is readily dephosphorylated by phosphomonoesterases.[4] Since 3′-OMe-FUDR is phosphorylated by thymidine kinase, it would be as ineffective as FUDR in resistant cells, which had lost this enzyme.

The results suggest that very slight demethylation of the FUDR derivatives occurred during incubations with whole cells or cell-free extracts. This demethylation could explain the observation reported in the previous paper that some of the methylated FUDR'S were inhibitors of the growth of HeLa and L5178Y cells in culture, but only at a concentration 100-fold higher than that of FUDR required for the same effect.[4]

The following conclusions with respect to structure–activity relationships and enzyme affinities can be drawn from the present study: (1) all the OH groups of FUDR must be unblocked in order for binding to nucleoside phosphorylase to occur; and (2) a free OH at 3′ is not necessary in order for FUDR to bind to thymidine kinase or for FUDRP to interact with thymidylate synthetase, although methylation decreases the affinity of the analog toward both enzymes.