Biochemical Pharmacology, Vol. 36, No. 2, pp. 203–210, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

STUDIES ON THE INTERACTION WITH THYMIDYLATE SYNTHASE OF ANALOGUES OF 2′-DEOXYURIDINE-5′-PHOSPHATE AND 5-FLUORO-2′-DEOXYURIDINE-5′-PHOSPHATE WITH MODIFIED PHOSPHATE GROUPS*

WOJCIECH RODE,† TADEUSZ KULIKOWSKI,‡ BARBARA KEDZIERSKA† and DAVID SHUGAR‡§

†Nencki Institute of Experimental Biology, Polish Academy of Sciences, 02-093 Warsaw, Poland; and ‡Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-532 Warsaw, Poland

(*Received* 17 *April* 1986; *accepted* 7 *July* 1986)

Abstract—The role of the phosphate moiety of dUMP, and some analogues, in their interaction with mammalian thymidylate synthase, has been investigated. Substrate and inhibitor activities, and the pH-dependence of these activities, of dUMP and 5-FdUMP, as well as analogues with modified phosphate groups, were compared. The methyl ester of dUMP was neither a substrate nor an inhibitor. By contrast, the methyl ester of 5-FdUMP was a slow-binding inhibitor of the enzyme from L1210, Ehrlich ascites carcinoma and CCRF-CEM cells, with K_i values in the micromolar range. Both 5-FdUrd and the newly synthesized 5′-methylphosphonate of 5-FdUrd were also slow-binding inhibitors of the Ehrlich carcinoma enzyme, but with K_i values in the millimolar range. The interaction of dUMP, 5-FdUMP, and the methyl ester of the latter decreased with increase in pH, whereas that of the 5′-methyl-phosphonate of 5-FdUrd remained unchanged. The results are discussed in relation to the role of the phosphate hydroxyls of dUMP in binding to the enzyme. 5-FdUMP and its analogues exhibited differing interactions with two binding sites on the enzyme molecule, consistent with cooperativity of binding. A convenient procedure is described for the synthesis of 5-fluoro-2′-deoxyuridine-5′-methylphosphonate, applicable also to the preparation of other 5′-methylphosphonate analogues.

Thymidylate synthase (dTMP synthase, EC 2.1.1.45) catalyzes the reductive methylation of dUMP to dTMP, as follows:

$$dUMP + N^{5,10}\text{–}CH_2\text{–}H_4 \text{ folate} \rightarrow dTMP + H_2 \text{ folate}$$

It was initially shown some years ago that the phosphate moiety of the substrate, dUMP, as well as of nucleotide analogue inhibitors, is essential for activity [1]. None the less, despite the multitude of studies on the mechanism of action of this enzyme system, dictated to a considerable extent by the desire to develop effective inhibitors for use in chemotherapy [1–3], only limited attention has been directed to the precise role of the 5′-phosphate group of substrate or inhibitors, or of the phosphate-binding region of the enzyme dUMP-binding site [4–9].

There is now compelling evidence that an arginyl residue is an essential component of the active site of bacterial thymidylate synthase [7, 10], and that binding of the substrate dUMP is bolstered by electrostatic interaction between this arginyl residue and the anionic phosphate moiety. If this were indeed the case, one would anticipate that dissociation of the phosphate secondary hydroxyl ($pK \sim 6.3$) of the substrate or an inhibitor analogue would be accompanied by at least a moderate effect on the reaction in the pH range 5.5–9.5 (where enzyme activity may be readily followed), depending on whether the monoanion or dianion is involved in such binding. Biochemical [7] and physicochemical [11] findings have been interpreted in terms of dUMP binding predominantly as the dianion.

The present investigation is devoted to an examination of the role of the phosphate moiety of dUMP and its analogues in their interaction with mammalian thymidylate synthase, by means of studies on the pH-dependence of activities of substrate and inhibitors, and of both of these with modified phosphate groups.

MATERIALS AND METHODS

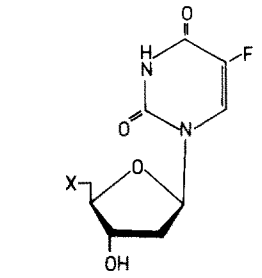
The methyl esters of dUMP and FdUMP were prepared as previously described [12]. (±),L-Tetrahydrofolate was prepared according to Lorenson *et al.* [13], 5-FdUMP was from Sigma (St. Louis, MO), and other reagents as previously described [14].

Analytical TLC made use of Merck (Darmstadt, F.R.G.) cellulose F and silica gel 60 254F plates with the following solvent systems (v/v): (A) chloroform–methanol 6:4; (B) chloroform–methanol 7:3; (C) CCl_4–acetone 7:3; (D) isopropanol–H_2O–conc. NH_4OH (7:2:1); (E) ethanol–0.5 M ammonium acetate (5:2).

UV absorption spectra were run on a Zeiss (Jena, G.D.R.) Specord instrument. ^1H NMR spectra were recorded on a Bruker 90 with DSS as internal stand-

* This investigation was supported by a grant from the Polish National Cancer Research Program (PR-6/2201).

§ To whom correspondence should be addressed, at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 36 Rakowiecka St., 02-532 Warsaw, Poland.



I X = O-P(=O)(O⊖)-O⊖

II X = O-P(=O)(O⊖)-O-CH3

III X = O-P(=O)(O⊖)-CH3

IV X = CH2-P(=O)(O⊖)-O⊖

Fig. 1. 5-FdUMP (I) and its analogues: 5′-methylphosphate (II), 5′-methylphosphonate (III) and 5′-methylenephosphonate (IV) of 5-FdUrd.

ard. ^{31}P NMR spectra were run on a Jeol FX-90Q at 40 MHz with 85% H_3PO_4 as external standard.

Cell lines. Mouse Ehrlich ascites carcinoma, and leukemia L1210, cells were maintained, harvested and stored as previously described [15]. Human leukemia CCRF-CEM cells were maintained as described by Rode *et al.* [16], and harvested and stored according to Rode *et al.* [17].

Thymidylate synthase. Electrophoretically homogeneous preparations of the enzyme from Ehrlich ascites carcinoma and L1210 cells were prepared as elsewhere described [14, 18]. An affinity chromatographic procedure [18] was employed to purify CCRF-CEM thymidylate synthase with a specific activity of at least 0.7 μmol/min/mg protein.

Enzyme assay. Enzyme activity was assayed as previously described [15], all measurements being done in triplicate. Analogues of dUMP were added to the reaction mixture as neutral aqueous solutions.

Kinetic studies. In order to identify the type of inhibition involved, the effect of a given dUMP analogue on the dependence of reaction rate on dUMP concentration, in the form of a Lineweaver–Burk plot, was analyzed as elsewhere reported [15].

Quantitative analyses of thymidylate synthase inhibition by 5-FdUMP (I) and its analogues (II, III) (Fig. 1), leading to time-dependent inactivation of the enzyme, were performed by following the decrease of enzyme activity over a given time interval during preincubation of the enzyme at 37° in the presence of 1.25 mM (±),L-$N^{5,10}$-methylenetetrahydrofolate, 3.2 μM dUMP (to prevent thermal inactivation), and various concentrations of inhibitor [19]. Activity remaining after preincubation was determined by addition of 25 μM [5-^{3}H]dUMP and measurement of tritium release during 4 min incubation. The slopes of semi-log plots of % remaining activity vs preincubation time, expressing apparent inactivation rate constants (k_{app}), and corresponding inhibitor concentrations ([I]), were then replotted as double reciprocal plots, in accordance with the relationship of Brouillette *et al.* [19]:

$$\frac{1}{k_{app}} = \left[\frac{K_i[S]}{K_m k_2} + \frac{K_i}{k_2}\right]\frac{1}{[I]} + \frac{1}{k_2},$$

where k_2 is the inactivation rate constant. The values of k_2 and K_i were determined from the plot intercept and slope, respectively.

5′-Methylphosphonate of 5-fluoro-2′-deoxyuridine. The procedure normally employed for converting a deoxynucleoside to a 5′-nucleotide analogue is based

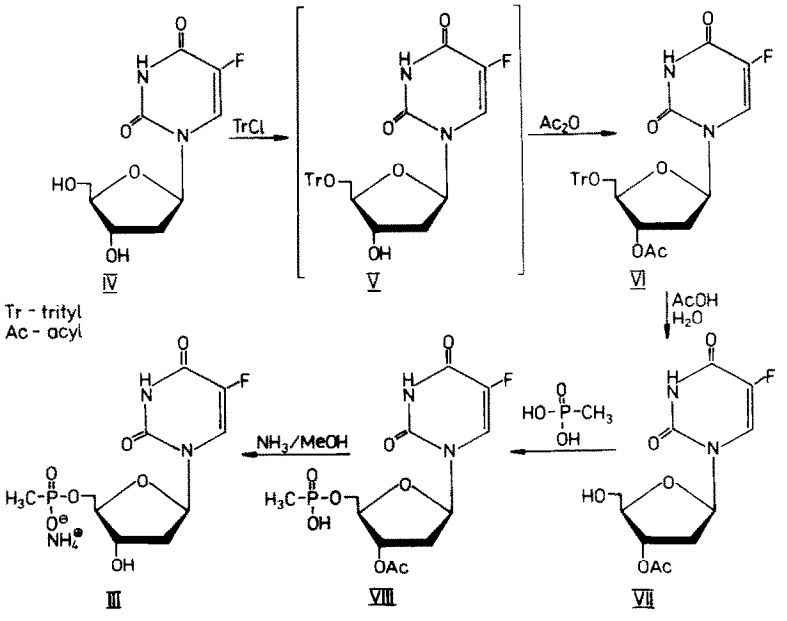


Fig. 2. Synthesis of 5-fluoro-2′-deoxyuridine-5′-methylphosphonate.

Table 1. Thin-layer chromatography

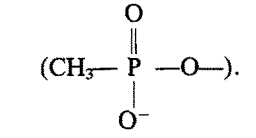
Compound	R_f values in solvent systems				
	$A_{(s)}$	$B_{(s)}$	$C_{(s)}$	$D_{(c)}$	$E_{(c)}$
FdUrd (V)	0.79	0.52	0.04	0.51	0.68
5′-*O*-TrFdUrd	—	0.71	0.20	—	—
3′-*O*-Ac-5′-*O*-TrFdUrd (VI)		0.76	0.50	—	—
3′-*O*-Ac-FdUrd (VII)	0.89	—	—	0.73	0.79
3′-*O*-Ac-FdUMP-CH_3 (VIII)	0.37	—	—	0.41	0.56
FdUMP-CH_3 (III)	0.15	—	—	0.31	0.49

(c) cellulose F, (s) silicagel F_{254}.

on preparation of the 5′-*O*-trityl nucleoside, followed by acetylation of the 3′-OH, detritylation, and then phosphorylation [20]. We have now modified this procedure by preparation of the 3′-*O*-acetyl nucleoside in a "one-pot" synthesis, which not only shortens the method, but leads to a 2-fold increase in yield of the 3′-*O*-acetyl derivative (62% as compared to 33%). Details of this method, which should be of general applicability to the synthesis of 5′-nucleotides and analogues with modified phosphate groups, were as follows:

One-pot synthesis of 3′-O-acetyl-2′-deoxy-5-fluorouridine (VII, Fig. 2). A solution of 2′-deoxy-5-fluorouridine (V, 0.32 g, 1.28 mmol) and triphenylchloromethane (TrCl, 0.42 g, 1.5 mmol) in 3.4 ml anhydrous pyridine was heated under reflux for 2 hr under strictly anhydrous conditions. At this point TLC (with solvents B and C, see Table 1) showed 40% conversion to the 5′-trityl derivative. An additional portion of TrCl (0.42 g) was added and heating continued for 1 hr, leading to more than 90% conversion to the 5′-trityl analogue, and a small quantity of product which migrated with the solvent front on TLC (probably the 3′,5′-di-*O*-trityl derivative). The reaction mixture was cooled to 0°, followed by addition of acetic anhydride (3.5 ml, 37 mmol), and stirring overnight at room temperture, which led to 100% acetylation. The reaction mixture was rapidly added to a vigorously stirred mixture of ice and water (70 ml). The resulting precipitate was collected by filtration, washed with ice-cold water, dissolved in 40 ml hot 40% acetic acid and heated under reflux for 10 min. The mixture was brought to room temperature, poured into a mixture of ice and water (35 ml), and the precipitated triphenylcarbinol removed by filtration. The filtrate was brought to dryness under reduced pressure and dried under vacuum over KOH to give 0.23 g (62% yield) of VII, suitable as such for conversion to a phosphate analogue. Crystallization of VII from methanol gave an analytical sample, m.p. 206°, as compared to 207° reported elsewhere [20].

Synthesis of 5-fluoro-2′-deoxyuridine-5′-methylphosphonate (III). The acetylated nucleoside VII (53 mg, 0.18 mmol) and methylphosphonic acid (140 mg, 1.56 mmol) were dissolved in 3 ml anhydrous pyridine (distilled over CaH_2) and brought to dryness under reduced pressure. This was repeated and the resulting residue dissolved in 6 ml anhydrous pyridine, to which was added 556 mg (2.7 mmol) dicyclohexylcarbodiimide, followed by stirring under anhydrous conditions at room temp. for 5 days. At this point, TLC with solvents A–C demonstrated disappearance of the 3′-*O*-acetyl derivative VI. The reaction mixture was added to 12 ml ice water, stirred for 3 hr at room temp., filtered, and the precipitate washed with 1 ml iced water. The combined filtrates were extracted with 4 × 15 ml petroleum ether, and the aqueous layer brought to dryness under reduced pressure to yield a yellowish oil which was dissolved in 5 ml anhydrous methanol. The solution was saturated with ammonia at 0° and heated in a sealed tube for 16 hr at 50°, filtered, the filtrate concentrated to 0.5 ml and deposited on a Whatman 3MM sheet, which was developed with solvent D. The main band (R_f = 0.68) was eluted with water, the eluate brought to dryness, dissolved in anhydrous methanol and precipitated with anhydrous ethyl ether. The product dried under vacuum over P_2O_5, was 36 mg (51% yield) of the ammonium salt. U.v.: λ_{max} (pH 2–5) 270 nm; λ_{min} (pH 2–5) 235 nm; λ_{max} (pH 13) 269 nm; λ_{min} (pH 13) 249 nm; A_{max} (pH 2–5)/A_{max} (pH 13) 1.26; ^{31}P NMR: (ppm) (D_2O) δ 27.193



RESULTS

Interaction of enzyme with 2′-deoxyuridine-5′-methyl-phosphate

The methyl ester of dUMP appeared to be completely devoid of substrate or inhibitor activity with both mouse tumour thymidylate synthases employed in the present study, as previously reported for the bacterial enzyme [4]. The analogue, at concentrations of up to 2 mM, did not perceptibly affect the Ehrlich carcinoma enzyme activity, measured as release of tritium from [5-^{3}H]dUMP, even with the latter at a concentration as low as 3 μM (not shown). This result suggests that, if 2′-deoxyuridine-5′-methylphosphate possesses any substrate or inhibitor activity towards the enzyme, its K_m or K_i values must be higher than 10 mM.

Inhibition of enzyme by the 5′-methylphosphate (II) and 5′-methylphosphonate of 5-FdUrd (III)

Inhibition of the Ehrlich carcinoma enzyme by each of the analogues was examined by varying the dUMP concentration with different concentrations of inhibitor, added simultaneously to the reaction

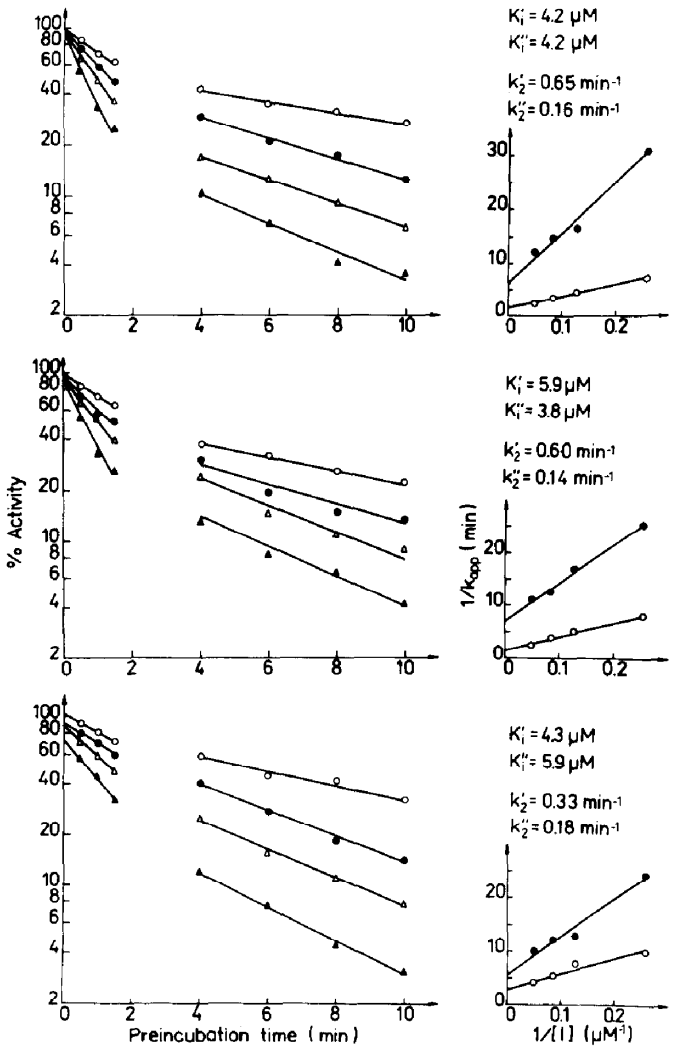


Fig. 3. Inhibition of Ehrlich carcinoma (upper panels), L1210 (middle panels) and CCRF-CEM (bottom panels) thymidylate synthases by the methyl ester of 5-FdUMP: *Left panels:* Semi-log plots of % remaining enzyme activity vs time of preincubation, with inhibitor concentrations of: ○, 3.9 μM; ●, 7.8 μM; △, 11.7 μM; ▲, 19.5 μM. *Right panels:* Double reciprocal plots of apparent inactivation rate constants of enzyme vs inhibitor concentration (open circles are for inactivation rates with 0–1.5 min preincubation, and filled-in circles with 4–10 min preincubation). Note: In a duplicate series of experiments, the results were: Ehrlich carcinoma enzyme, $K_i' = 3.0\ \mu M$, $k_2' = 0.34\ min^{-1}$, $K_i'' = 6.1\ \mu M$, $k_2'' = 0.15\ min^{-1}$; L1210 enzyme: $K_i' = 7.7\ \mu M$, $k_2' = 0.50\ min^{-1}$, $K_i'' = 5.4\ \mu M$, $k_2'' = 0.15\ min^{-1}$; CCRF-CEM enzyme, $K_i' = 5.2\ \mu M$, $k_2' = 0.20\ min^{-1}$, $K_i'' = 6.0\ \mu M$, $k_2'' = 0.07\ min^{-1}$.

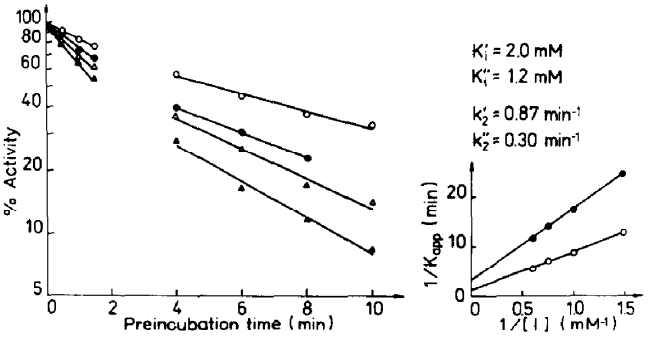


Fig. 4. Inhibition of Ehrlich carcinoma thymidylate synthase by 5-FdUrd-5′-methylphosphonate. Description of plots as in Fig. 3. Inhibitor concentrations (left panel, from upper plot to lower), 0.68 mM, 1.01 mM, 1.35 mM, 1.69 mM. Results of a duplicate experiment were: $K_i' = 2.4\ mM$, $k_2' = 0.87\ min^{-1}$, $K_i'' = 1.8\ mM$, $k_2'' = 0.30\ min^{-1}$.

mixture. Both analogues exhibited competitive inhibition with respect to substrate, manifested by intersection at the ordinate of Lineweaver–Burk plots. Apparent inhibition constants were 1.2 μM and 0.4 mM for the methylphosphate and methylphosphonate analogues, respectively (not shown).

Both analogues also exhibited time-dependent inhibition of the enzyme, consistent with the behaviour of each as a slow-binding inhibitor [21]. Furthermore, the time-dependent inactivation of the enzyme with each inhibitor was biphasic (Figs. 3 and 4), suggesting different interactions of inhibitor with two binding sites on the thymidylate synthase molecule. Consequently, inhibition constants and inactivation rate constants were calculated with the use of apparent inactivation rate constants during the initial (0.0–1.5 min) and later (4–10 min) periods of preincubation with a given inhibitor at various concentrations. The corresponding inhibition constants and inactivation rate constants were then K_i' and k_2' and K_i'' and k_2'', respectively.

Inhibition constants for inhibition of Ehrlich carcinoma, L1210 and CCRF-CEM thymidylate synthases by 5-fluoro-2′-deoxyuridine-5′-methylphosphate (Fig. 1-II) were all in the micromolar range and were not significantly dependent on preincubation time (Fig. 3). By contrast, the inactivation rate constants did distinctly decrease with preincubation time (Fig. 3). Inhibition of the Ehrlich carcinoma cell enzyme by 5-fluoro-2′-deoxyuridine-5′-methylphosphonate (III) (Fig. 1) was similar in all respects, with the exception that inhibition constants were in the millimolar range (Fig. 4).

Inhibition by 5-FdUMP

The dependences of inactivation of the Ehrlich carcinoma and CCRF-CEM enzymes on preincubation time were also biphasic, but the two preparations differed quantitatively in inhibition patterns. Whereas, with the mouse enzyme, the inhibition constant increased after 4 min preincubation by an order of magnitude, with the inactivation rate

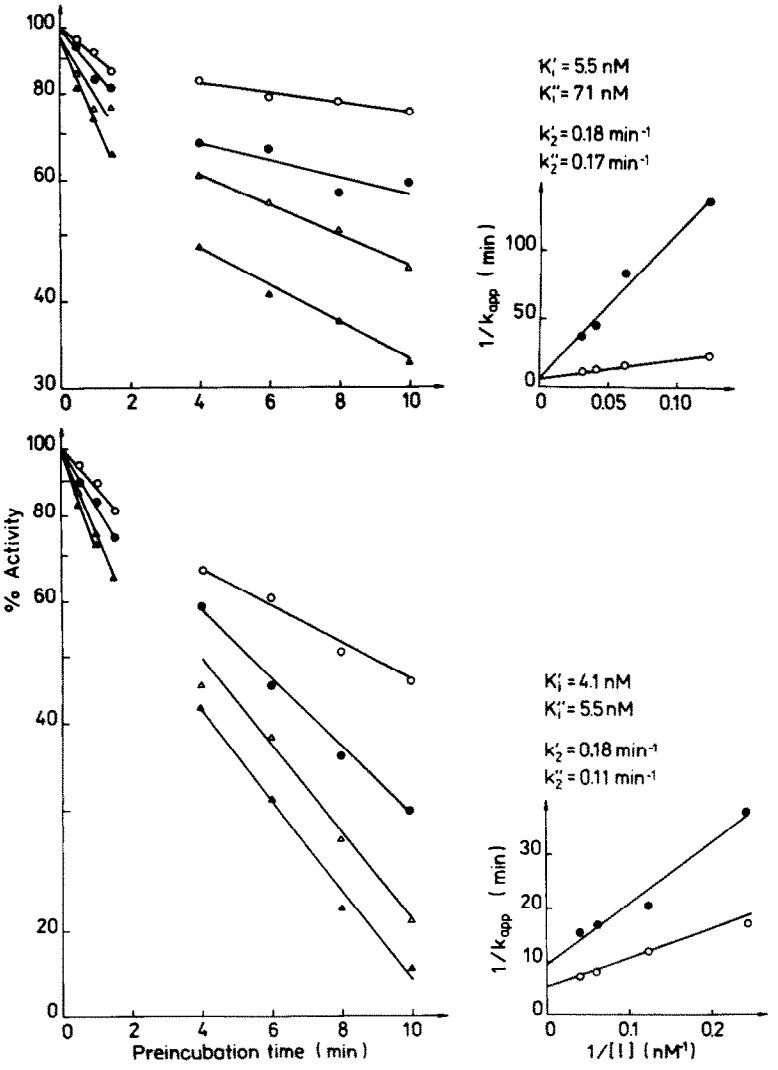


Fig. 5. Inhibition of Ehrlich carcinoma (upper panels) and CCRF–CEM (lower panels) thymidylate synthases by 5-FdUMP. Description of plots as in Fig. 3. Inhibitor concentrations were (from upper plot to lower in left panels) 8.1, 16.3, 24.4 and 32.5 nM with the Ehrlich ascites enzyme, and 4.1, 8.1, 16.3 and 24.4 nM with the CCRF–CEM enzyme.

constant unchanged, for the human enzyme only the inactivation rate constant decreased with preincubation time (Fig. 5).

Inhibition of Ehrlich carcinoma enzyme by 5-FdUrd

The nucleoside itself was found to exhibit feeble, but time-dependent, inhibition of the enzyme. In the presence of 16 mM FdUrd in the preincubation mixture (see Materials and Methods), thymidylate synthase activity remaining after 2, 5 and 10 min preincubation was 72%, 45% and 28%, respectively (not shown). These results permitted of a rough estimation of the K_i for the nucleoside, approximately 30 mM.

pH-dependence of interaction of dUMP and its analogues with thymidylate synthase

In order to gain some appreciation of the role of the phosphate group at the thymidylate synthase dUMP binding site(s), an examination was made of the effect of the pH of the incubation medium on (i) the dependence of reaction rate on dUMP concentration, (ii) the inactivation of the enzyme by 5-FdUMP, and its methylphosphate and methylphosphonate analogues, as a function of time of preincubation. From Table 2 and Fig. 6, it will be seen that the affinity of dUMP, and the inhibitory activity of 5-FdUMP and its 5′-methylphosphate, towards Ehrlich carcinoma thymidylate synthase decreased with an increase in pH and was appreciably lower at pH 7.5 than at pH 6.0. On the other hand, and in striking contrast, inhibition of the enzyme by the 5′-methylphosphonate analogue of 5-FdUMP was unaltered when the pH was increased from 6.0 to 7.5 (Fig. 6).

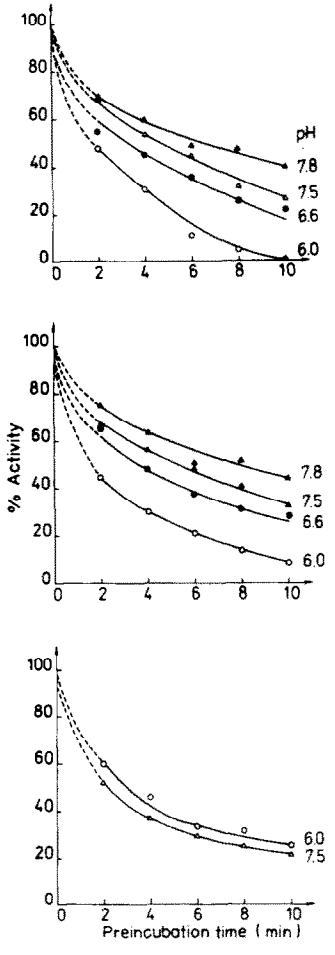


Fig. 6. pH-dependence of inhibition of Ehrlich carcinoma thymidylate synthase by 5-FdUMP (33 nM, upper plot), and its 5′-methylphosphate analogue (8 μM, middle plot), and its 5′-methylphosphonate analogue (3.6 mM, bottom plot). Experiments were as described in Materials and Methods, except that preincubation was in the presence of 13 μM dUMP, and 50 μM [5-^{3}H]dUMP was added to assay remaining activity.

Table 2. pH-dependence of apparent K_m for dUMP with Ehrlich carcinoma thymidylate synthase

pH	6.0	7.5	9.5
K_m (μM)*	1.2	3.4	6.7

* Mean values of two independent series of experiments, with differences from the means of less than 20%.

DISCUSSION

Although replacement of the phosphate moiety of FdUMP by a methylphosphate group leads to an appreciable decrease in affinity for thymidylate synthase, with K_i increasing by a factor of 10^3 (Figs 3 and 5, cf. refs. 22 and 23), it does not abolish the ability of this analogue to exhibit slow binding to the enzyme. This is all the more interesting in that the methyl ester of dUMP exhibited no substrate or inhibitor properties (Results and cf. ref. 4).

Relevant to the foregoing is the observation that esterification of the phosphate moiety of 5-FdUMP with *p*-azidophenol (a group much bulkier, and with appreciably different electronic properties, than a methyl group) led to a decrease in affinity for bacterial thymidylate synthase (to $K_i = 1$ μM) [5], similar to that observed here with the methylphosphate of 5-FdUMP (Fig. 3).

On the other hand, replacement of the phosphate moiety of 5-FdUMP by methylphosphonate led to an even more striking decrease in affinity for thymidylate synthase, by a factor of about 10^6. None the less this analogue still exhibited slow binding to the enzyme (Figs 4 and 5).

By contrast, the methylenephosphonate (or 6′-phosphonate) analogue of 5-FdUMP (see Fig. 1-IV) was found to exhibit an affinity for bacterial and phage T2 thymidylate synthases 10^3–10^5-fold higher [6] than that of our methylphosphonate analogue for the mammalian enzyme (Fig. 4). The 6′-phosphonate analogue is clearly isosteric with respect to 5-FdUMP. Furthermore, in contrast to our methylphosphonate analogue, it possesses a secondary hydroxyl with $pK_2 \sim 8$ [24]. However, in the presence of saturating concentrations of Mg^{2+}, the pK_2 of the secondary hydroxyl is reduced to about 6.3 [24], hence similar to that for the parent 5-FdUMP. The experiments of Montgomery *et al.* [6] were con-

ducted in the presence of a 5-fold excess of Mg^{2+} at pH 7.4, so that their 6′-phosphonate analogue was in the dianionic form. It would have been of obvious interest to examine the behaviour of this analogue in the absence of Mg^{2+}, so that at pH 7.4 it was predominantly (>80%) in the monoanionic form. It would be equally pertinent to examine the substrate properties of the 6′-phosphonate analogue of dUMP both in presence and absence of Mg^{2+}, and as a function of pH. The 6′-phosphonate of 5-FdUrd, like our 5′-methylphosphonate, exhibited time-dependent inhibition against all three enzymes. It was, however, only moderately cytotoxic towards H.Ep.-2 cells, pointing to the need for an examination of its behaviour towards the purified mammalian enzymes, the more so in that its inhibitory properties towards two bacterial and one phage enzyme appeared to vary by up to three orders of magnitude.

The much feebler inhibitory properties of the 5′-methylphosphonate of 5-FdUrd as compared to the 5′-methylphosphate (Fig 3 and 4) underlines the importance of the secondary hydroxyl. Moreover, comparison of the inhibitory properties of 5-FdUMP and its methylphosphonate congener with those of 5-FdUrd shows that, while the replacement of one hydroxyl group increases K_i by a factor of about 10^6, removal of the phosphate moiety leads to a further increase in K_i of only 15-fold. It thus appears that the two hydroxyls of the phosphate moiety of 5-FdUMP are not of equal importance in binding to the enzyme.

Inspection of the effects of pH on the properties of the substrate and inhibitors, while furnishing interesting new data, does not provide an unequivocal interpretation of the role of the phosphate group. The almost 6-fold increase in K_m of dUMP with increase in pH, from 1.2 μM at pH 6 to 6.7 μM at pH 9.5 (Table 2), may be attributed to dissociation of the phosphate secondary hydroxyl, so that the monoanion binds more strongly than the dianion. But two other interpretations are equally plausible, viz. involvement of dissociable histidine imidazole residues ($pK \sim 5.6$–7.0; cf. ref. 25), elsewhere proposed as essential for enzyme activity [26], and/or dissociation of the uracil N(3)–H of dUMP ($pK \sim 9.5$).

The time-dependent inhibition of the enzyme by 5-FdUMP (Fig. 6), which decreases with increase in pH from 6 to 7.8, is also consistent with involvement of the phosphate monoanion. But the analogous behaviour of the methyl ester of dUMP (Fig. 6), in which the phosphate secondary hydroxyl is blocked, invalidates such an interpretation, and is rather consistent with the methoxy oxygen acting as a weak acceptor in hydrogen bonding to some amino acid side chain of the enzyme. The decrease in inhibitory properties with increase in pH from 6 to 7.8 may then be accounted for on the assumption that the hydrogen donor is a histidine imidazole N–H. This would also explain why the methyl phosphonate shows no difference in time-dependent inhibition between pH 6 and 7.5.

In retrospect, bearing in mind possible steric effects of the methyl groups in the methylphosphate and methylphophonate congeners of 5-FdUMP, more suitable model compounds might be the corresponding fluorophosphates, which have already been used to determine whether the mono- or dianionic phosphate is involved in binding to other enzymes [27]. We are presently synthesizing the 5′-fluorophosphates of dUrd, dThd and 5-FdUrd for such an investigation.

In was previously shown that the kinetics of thymidylate synthase inhibition by dUMP congeners may be affected by interaction between two dUMP binding sites [15]. In keeping with this are the present findings on inhibition of the enzyme by 5-FdUMP and its analogues. The biphasic dependence of the rate of enzyme inactivation on time of interaction with the inhibitors (Figs. 3–5) is consistent with involvement of cooperative binding. The apparent cooperativity is negative and, in all but a single instance, results in slower inactivation of the second dUMP binding site, without affecting K_i. Only inhibition of the Ehrlich ascites carcinoma enzyme by 5-FdUMP exhibited a different pattern, with a change in the values of K_i between the two sites by an order of magnitude, the value of k_2 being unaffected (Fig. 5).

REFERENCES

1. D. V. Santi, *J. Med. Chem.* **23**, 103 (1980).
2. C. A. Lewis and R. B. Dunlap, in *Topics in Molecular Pharmacology* (Eds. A. S. V. Burgen and G. C. K. Roberts), p. 169. Elsevier, Amsterdam (1981).
3. P. V. Danenberg and A. Lockshin, *Mol. Cell. Biochem.* **43**, 49 (1982).
4. A. Holy and J. Votruba, *Collection Czechoslov. Chem. Commun.* **39**, 1646 (1974).
5. L. G. Stephanson and J. M. Whiteley, *J. Med. Chem.* **22**, 953 (1979).
6. J. A. Montgomery and H. J. Thomas, *J. Med. Chem.* **22**, 109 (1979).
7. M. Belfort, G. F. Maley and F. Maley, *Archs Biochem. Biophys.* **204**, 340 (1980).
8. N. V. Beaudette, N. Langerman and R. L. Kisliuk, *Archs Biochem. Biophys.* **200**, 410 (1980).
9. A. Srinivasan, V. Amarnath, A. D. Broom, F. C. Zou and Y.-C. Cheng, *J. Med. Chem.* **27**, 1710 (1984).
10. K. L. Cipollo, C. A. Lewis, Jr., P. D. Ellis and R. B. Dunlap, *J. biol. Chem.* **257**, 4398 (1982).
11. M. J. Beckage, M. Blumenstein and R. L. Kisliuk, *Biochim. biophys. Acta* **571**, 157 (1979).
12. W. Rode, T. Kulikowski, B. Kedzierska and D. Shugar, *Drugs Exp. Clin. Res.* **XII**, 545 (1986).
13. M. Y. Lorenson, G. F. Maley and F. Maley, *J. biol. Chem.* **242**, 3332 (1967).
14. M. M. Jastreboff, B. Kedzierska and W. Rode, *Biochem. Pharmac.* **31**, 217 (1982).
15. W. Rode, T. Kulikowski, B. Kedzierska, M. Jastreboff and D. Shugar, *Biochem. Pharmac.* **33**, 2699 (1984).
16. W. Rode, M. M. Jastreboff and J. R. Bertino, *Biochem. biophys. Res. Commun.* **128**, 345 (1985).
17. W. Rode, B. J. Dolnick and J. R. Bertino, *Biochem. Pharmac.* **29**, 723 (1980).
18. W. Rode, K. J. Scanlon, J. Hynes and J. R. Bertino, *J. biol. Chem.* **254**, 11538 (1979).
19. C. B. Brouillette, C. T.-C. Chang and M. P. Mertes, *J. Med. Chem.* **22**, 1541 (1979).
20. H. J. Thomas and J. A. Mongomery, *J. Med. Pharm. Chem.* **5**, 24 (1962).
21. J. F. Morrison, *Trends Biochem. Sci.* **7**, 102 (1982).
22. A. Lockshin and P. V. Danenberg, *Biochem. Pharmac.* **30**, 247 (1981).
23. M. M. Jastreboff, B. Kedzierska and W. Rode, *Biochem. Pharmac.* **32**, 2259 (1983).

24. H. J. Vogel and W. A. Bridger, *Biochemistry* **21**, 394 (1982).
25. E. A. Dawes, in *Comprehensive Biochemistry*, Vol. 12 (Eds. M. Florkin and E. H. Stotz), p. 89 (1964).
26. H. H. Daron and J. L. Aull, *Biochim. biophys. Acta* **658**, 132 (1981).
27. S. G. Withers and N. B. Madsen, *Biochem. biophys. Res. Commun.* **97**, 513 (1980).