Fluorinated Pyrimidines

XXVI. Mammalian Thymidylate Synthetase: Its Mechanism of Action and Inhibition by Fluorinated Nucleotides

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(Received February 13, 1965)

SUMMARY

Thymidylate synthetase from Ehrlich ascites carcinoma cells has been assayed by a spectrophotometric method, and several new properties of this enzyme have been discovered. Thymidylate produces a weak noncompetitive product inhibition and mercaptoethanol stimulates the enzyme activity, whereas formaldehyde must be present within a specific concentration range if maximal enzyme activity is to occur. In addition, the results of initial velocity and product-inhibition kinetic studies as well as those of experiments designed to study the kinetics of the inhibition produced by 5-fluoro-2′-deoxyuridine 5′-monophosphate and 5-trifluoromethyl-2′-deoxyuridine 5′-monophosphate made it possible to elucidate the mechanism of action of thymidylate synthetase. The mechanism is ordered and sequential, such that 5,10-methylenetetrahydrofolate interacts with the enzyme before deoxyuridylate, and thymidylate leaves before dihydrofolate. Furthermore, a mechanism without central complexes has been eliminated. 5-Trifluoromethyl-2′-deoxyuridine 5′-monophosphate, unlike 5-fluoro-2′-deoxyuridine 5′-monophosphate, exhibits the ability to combine slowly with the enzyme in an irreversible fashion.

INTRODUCTION

It has been shown in this laboratory that 5-fluorouracil (FU)[2] and 5-fluoro-2′-deoxyuridine (FUdR) inhibit the growth of several transplantable tumors in mice (1) and produce objective responses in humans suffering from solid tumors (2). The antitumor activity of these compounds occurs as a result of their conversion to 5-fluoro-2′-deoxyuridine 5′-monophosphate (FUdRP) (3), which has been shown to be a potent inhibitor of thymidylate synthetase (4–6).

Recently, the syntheses of 5-trifluoromethyluracil (F_3T) and 5-trifluoromethyl-2′-deoxyuridine (F_3TdR) were achieved in this laboratory (7). F_3TdR inhibits the growth of several transplanted tumors in mice (8), is mutagenic to and incorporated into the DNA of bacteriophage T4B (9), renders cultured human cells containing the analog in their DNA more sensitive to ultraviolet (UV) and X-irradiation (10), inhibits the cleavage of FUdR by nucleoside phosphorylase (11), and serves as a powerful antiviral drug against herpes simplex keratitis in the rabbit eye (12).

The earlier observation by Heidelberger

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[2] Abbreviations used in this work: FU, 5-fluorouracil; FUdR, 5-fluoro-2′-deoxyuridine; FUdRP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; F_3T, 5-trifluoromethyluracil; F_3TdR, 5-trifluoromethyl-2′-deoxyuridine; F_3TdRP, 5-trifluoromethyl-2′-deoxyuridine 5′-monophosphate; dUMP, deoxyuridylate; 5,10-methylene-FH_4 or C_1-FH_4, 5,10-methylenetetrahydrofolate; FH_2, dihydrofolate; UMP, uridylate; UdR, deoxyuridine; TdR, thymidine; dTMP, thymidylate; CdR, deoxycytidine; dCMP, deoxycytidylate.

et al. (13), that F_3TdR also inhibits thymidylate synthetase from Ehrlich ascites carcinoma cells, but only in the presence of ATP, suggested that the nucleotide (F_3TdRP) is the actual inhibitor of the enzyme, since the kinases needed for the phosphorylation of nucleosides are known to be present in the crude enzyme preparation (6). It therefore became of interest to determine whether F_3TdRP inhibits the enzyme, and if so, to study and characterize this inhibition in some detail. In addition, the inhibition of thymidylate synthetase by FUdRP has been reinvestigated. This was prompted by reports (14, 15) that the inhibition by FUdRP of this enzyme from several bacterial or bacteriophage sources is noncompetitive against deoxyuridylate (dUMP) following a short preincubation of the enzyme with the inhibitor. However, Hartmann and Heidelberger (6) had found the enzyme from Ehrlich ascites tumor cells to be inhibited competitively whether or not preincubation was employed.

A number of other experiments that have helped to elucidate the mechanism of action of thymidylate synthetase and that describe some additional properties of the enzyme are also reported.

This investigation was considerably facilitated by the use of a modification of the spectrophotometric assay for thymidylate synthetase developed by Wahba and Friedkin (16). This method represents a vast improvement over the previously used isotopic assay (6) since it is much more rapid and convenient and permits the continuous assay of enzymic activity.

MATERIALS AND METHODS

Materials. F_3TdRP was obtained by enzymic phosphorylation of F_3TdR, and was kindly provided by Dr. Mildred Chang of this laboratory. FUdRP was chemically synthesized by Dr. David Remy, formerly of this laboratory. *dl*,L-Tetrahydrofolate was prepared by catalytic hydrogenation of folic acid (Sigma Chemical Company) as previously described (6).

Preparation of enzyme extract. Ehrlich ascites carcinoma cells were harvested 6–7 days after intraperitoneal transplantation into female Swiss mice. The cells were washed 3 times with ice-cold isotonic saline and suspended in 2 volumes of cold 0.05 M phosphate buffer, pH 6.7. The cell suspension was then sonically disrupted for 4 min (20 kc/sec) and centrifuged at 105,000 *g* for 1 hour. The high-speed supernatant fraction was filtered through glass wool and used directly as the source of enzyme. The protein content was determined quantitatively by the biuret method of Gornal *et al.* (17).

Determination of enzyme activity. Thymidylate synthetase was assayed by a slight modification of the spectrophotometric method developed by Wahba and Friedkin (16). This assay is based on the difference spectrum which results when tetrahydrofolate is oxidized to dihydrofolate during the reaction and is characterized by an increase in molar absorbancy of 6400 M^{-1} cm^{-1} at 340 mμ. Since equal amounts of dihydrofolate and thymidylate are produced, the enzyme activity can therefore be expressed in terms of the amount of thymidylate synthesized per unit of time.

Normally the reaction mixture contained the following: *dl*,L-tetrahydrofolate, formaldehyde, mercaptoethanol, dUMP, enzyme extract, FUdRP or F_3TdRP where indicated, and phosphate buffer, pH 6.7, in a total volume of 1.0 ml. For purposes of convenience, *dl*,L-tetrahydrofolate, formaldehyde, mercaptoethanol, and phosphate buffer were combined into a single "cofactor" solution which was then used directly as the source of these components. In such a solution, tetrahydrofolate was found to be stable for several weeks when stored under nitrogen. To prepare this solution, first an appropriate amount of freshly prepared *dl*,L-tetrahydrofolate was dissolved in 3.0 ml of 0.067 M formaldehyde, 1.0 ml of 0.1 M sodium bicarbonate, pH 8.0, and 2.0 ml of 0.375 M mercaptoethanol. Then an aliquot of this mixture was diluted about 125 times in 0.1 M phosphate buffer, pH 7.4, 0.001 M in formaldehyde, to determine the tetrahydrofolate concentration spectrophotometrically at 294 mμ (18). In this way, cofactor solutions of any desired tetrahydrofolate concentration were prepared simply by using the appropriate amount of the undi-

luted mixture together with the required levels of sodium bicarbonate, mercaptoethanol, formaldehyde, and phosphate buffer.

All determinations of enzyme activity were made at 37° in a Beckman DK-1A monochromator fitted with thermospacers and a Gilford model 2000 Multisample Absorbance Recorder. The latter is equipped with an absorbancy converter which provides an optical density range up to 3.0. By appropriate scale expansion, the full-scale range in the recorder can be set for a maximum sensitivity of 0.1 optical density unit. As a rule, all reaction components were preincubated in the thermostated chamber of the monochromator for a period of 10–15 min, after which the reaction was begun by the addition of dUMP. In most cases the reaction was found to be linear for a minimum of 8 min. The true initial velocity was determined by correcting the rate of absorbancy change during the first 8 min after dUMP addition for that just prior to dUMP addition. The velocity was generally expressed as millimicromoles of thymidylate synthesized per 5 mg protein per 5 min.

Analyses of kinetic data. All kinetic data were interpreted by applying the theoretical considerations of Cleland (19–21), whose kinetic nomenclature is used throughout this paper. The data derived from initial velocity, product inhibition, or dead-end inhibition studies were analyzed by the double-reciprocal method of Lineweaver and Burk (22). This method expresses the data in accordance with the equation

$$\frac{1}{v} = \frac{K}{V}\left(\frac{1}{\mathrm{A}}\right) + \frac{1}{V} \qquad (1)$$

where v is the initial velocity, V is the maximum velocity at infinite substrate concentration, A is the variable substrate concentration, and K is the Michaelis constant, which is defined as the concentration of A where v is equal to one-half V.

Values for the inhibition constants (k_i, $K_{i,\mathrm{slope}}$, $K_{i,\mathrm{intercept}}$) were obtained either by calculation (23) or by replotting slopes or $1/V$ intercepts versus inhibitor concentration. Furthermore, a digital computor, using a Fortran program described by Cleland (24), was also used to process the data and provide values for the various inhibition and kinetic constants, including standard errors. These values were obtained by fitting experimental points to an overall equation describing the observed type of inhibition or the initial velocity relationships for the appropriate enzyme mechanism.

Dialysis experiments. In general, the following procedure was used for the dialysis experiments to be reported. Enzyme extract and either FUdRP or F_3TdRP, at appropriate concentrations, were mixed and allowed to stand at 4° for 5 min. Then 2.0-ml aliquots were placed in cellulose tubing (size 8, Visking Co.) and dialyzed at 4° with magnetic stirring against 500 ml of 0.1 M phosphate buffer, pH 6.7. This buffer, which was changed several times, is essentially isotonic with the enzyme extract. A control series without added inhibitor was dialyzed simultaneously. At given intervals, samples were removed and the percentage inhibition was determined by comparing the thymidylate synthetase activity of the inhibitor-treated extract to that of the control.

In a number of other experiments, a 2-hr preincubation of the enzyme extract and inhibitor at 4° was carried out prior to dialysis. Furthermore, in some of these experiments, the 2-hr preincubation period was conducted in the presence of *dl*,L-tetrahydrofolate and formaldehyde. As before, the corresponding control without added inhibitor was dialyzed simultaneously.

RESULTS

General Properties and Characteristics of Thymidylate Synthetase from Ehrlich Ascites Carcinoma Cells

The initial objective of this investigation was to test the feasibility of adapting the thymidylate synthetase spectrophotometric assay of Wahba and Friedkin (16) to the Ehrlich ascites carcinoma system. The main obstacle was the fact that the level of this enzyme in mammalian cells is, in general, very low in comparison to the level found in bacteria. However, the required optical sensitivity was achieved by appropriate

scale expansion of the Gilford Multisample Absorbancy Recorder. Table 1 shows the requirements for thymidylate synthetase

TABLE 1

Requirements for thymidylate synthetase activity

The reaction mixture contained 50 mμmoles of dUMP, 6.6 μmoles of formaldehyde, 5 μmoles of $MgSO_4$, 33 μmoles of mercaptoethanol, 200 mμmoles of *dl*,L-tetrahydrofolate, enzyme extract (4.5 mg protein), and 0.10 M phosphate buffer, pH 6.7, in a total volume of 1.0 ml.

System	Thymidylate produced (mμmoles/5 mg protein/5 min)	Relative activity (%)
Complete	6.1	100
Minus *dl*,L-tetrahydrofolate	0	0
Minus dUMP	0.2	3
Minus formaldehyde	2.1	34
Minus $MgSO_4$	5.8	95
Minus dUMP, plus UMP[a]	0.3	5
Boiled enzyme extract	0.2	3
One-half amount of enzyme extract	3.3	54

[a] 50 mμmoles.

activity. Tetrahydrofolate and dUMP are absolutely essential, whereas the absence of formaldehyde does not completely eliminate enzyme activity. The addition of Mg^{++} has little if any effect on enzyme activity, a finding that confirms earlier work from this laboratory (6) and is in agreement with the more recent report by Jenny and Greenberg (25) on the enzyme from calf thymus. In contrast, the enzyme from *Streptococcus faecalis* (26) and *Escherichia coli* (27) has been reported to be activated by Mg^{++}. Table 1 also shows that substitution of uridylic acid (UMP) for dUMP or the use of a boiled extract eliminates all enzyme activity. Furthermore, the enzyme activity is proportional to the amount of enzyme extract used.

The results of experiments dealing with the substrate specificity of the enzyme can be seen in Table 2. None of the nucleosides or nucleotides tested, other than dUMP, is utilized, with the possible exception of

TABLE 2

Substrate specificity of thymidylate synthetase

The experimental conditions were the same as those described in Table 1 except that no $MgSO_4$ was added and 4.0 mg protein was employed. In all cases, 50 mμmoles of the various nucleosides and nucleotides were used.

Substrate	Thymidylate produced (mμmoles/5 mg protein/5 min)	Relative activity
dUMP	4.5	100
UMP	0.2	4
UdR	0	0
TdR	0	0
dTMP	0.2	4
CdR	0	0
dCMP	0.4	8

deoxycytidylate (dCMP). However, the slight activity associated with this compound can be explained on the basis of its deamination to dUMP by enzymes known to be present in the crude enzyme extract.

Thymidylate synthetase activity as a function of pH is illustrated in Fig. 1.

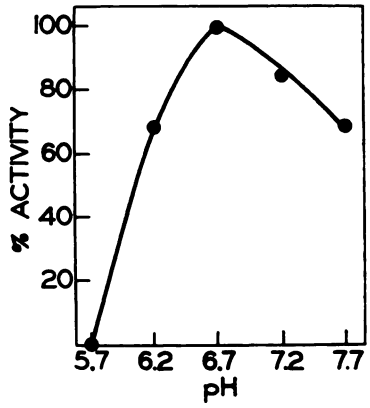


FIG. 1. *Influence of pH on thymidylate synthetase activity in phosphate buffer*

The reaction mixture contained 13.2 μmoles of formaldehyde and enzyme extract (3.0 mg protein); all other components as described in Table 1.

Optimal activity is obtained at pH 6.7 which agrees with the value of 6.5 reported earlier by Hartmann and Heidelberger (6). The enzyme from calf thymus exhibits a pH

optimum of 7.1 (25) whereas the enzyme from bacterial sources usually has an optimum of 7.5–8.0 (26, 27).

The activity of thymidylate synthetase is highly dependent on the concentration of formaldehyde in the reaction mixture (Fig. 2). Enzyme activity is maximal at 3.3×10^{-3} M and diminishes considerably at both the higher and lower formaldehyde concentrations.

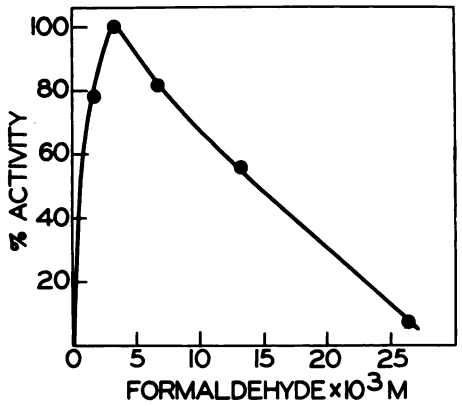


FIG. 2. *Effect of formaldehyde concentration on thymidylate synthetase activity*

The conditions were the same as for Table 1 except that 1.7, 3.3, 6.6, 13.2, or 26.4 μmoles of formaldehyde were used.

In the earlier publication from this laboratory (6) it was reported that mercaptoethanol does not stabilize thymidylate synthetase. Jenny and Greenberg (25), however, have found that the activity of the enzyme from calf thymus is greatly increased by sulfhydryl compounds such as mercaptoethanol, cysteine, and glutathione. An enhancing as well as a stabilizing effect by sulfhydryl compounds on the enzyme from several bacteria has also been reported (15, 26). Blakley (14) has concluded that sulfhydryl compounds not only act to protect tetrahydrofolate from oxidation, but also function to activate the enzyme from *S. faecalis*. In view of this difference, it was decided to reinvestigate the effect of mercaptoethanol on thymidylate synthetase from Ehrlich ascites carcinoma cells. It is clear from Fig. 3 that mercaptoethanol does affect enzyme activity. Maximal activity

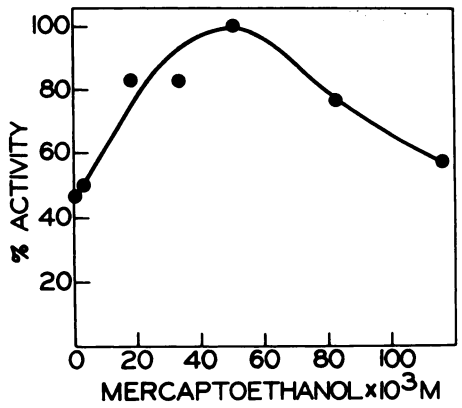


FIG. 3. *Thymidylate synthetase activity as a function of mercaptoethanol concentration*

The reaction mixture consisted of the following: 200 mμmoles of dUMP; 3.3 μmoles of formaldehyde; 200 mμmoles of *dl*,L-tetrahydrofolate; ascorbic acid at a final concentration of 5×10^{-3} M; enzyme extract (3.4 mg protein); 3.3, 16.5, 33, 49.5, 82.5, or 116 μmoles of mercaptoethanol; and 0.1 M phosphate buffer, pH 6.7; all in a total volume of 1.0 ml.

was obtained at a mercaptoethanol concentration of 50×10^{-3} M. In these experiments ascorbic acid (5×10^{-3} M) was included in every reaction mixture to prevent the oxidation of tetrahydrofolate at the low mercaptoethanol levels.[3] Thus, it appears that

TABLE 3

Inhibition of thymidylate synthetase by thymidylate

All components in the reaction mixture as for Table 1 except that 200 mμmoles of dUMP were employed.

Thymidylate added (μmoles)	Thymidylate produced (mμmoles/5 mg protein/5 min)	Inhibition (%)
0	7.3	0
0.02	7.3	0
0.05	7.4	0
0.10	7.1	3
0.25	6.2	15
0.50	5.6	24
1.00	4.7	36

[3] The protective effect of ascorbic acid was shown by its ability to prevent the oxidation of tetrahydrofolate solutions left in the air overnight.

the effect of mercaptoethanol is on the enzyme and not on the coenzyme or tetrahydrofolate.

Product inhibition of thymidylate synthetase had not previously been detected in this laboratory, although such inhibition is known for the enzyme obtained from *S. faecalis* (14). Weak product inhibition has now been observed (Table 3) but is significant only at thymidylate concentrations in excess of the dUMP level. The data in Table 4 demonstrate that the slight inhibition is produced specifically by thymidylate and thus represents true product inhibition.

TABLE 4

Effect of various nucleosides and nucleotides on thymidylate synthetase activity

Experimental conditions as described for Table 1 except that 200 mμmoles of dUMP and 500 mμmoles of each of the nucleosides and nucleotides were used.

Addition	Thymidylate produced (mμmoles/5 mg protein/5 min)	Relative activity (%)
None	6.8	100
UdR	7.0	103
UMP	6.4	94
CdR	6.4	94
dCMP	6.8	100
TdR	6.8	100
dTMP	5.4	80

Inhibition of Thymidylate Synthetase by FUdRP and F_3TdRP

In agreement with the earlier results of Hartmann and Heidelberger (6), at pH 6.7 FUdRP inhibits competitively, with respect to dUMP, when preincubated with the enzyme for 10 min as well as when no preincubation is employed (Fig. 4). Although the kinetics of inhibition is not altered by preincubation, $K_{i,slope}$ decreases by a factor of 10 (3.1×10^{-8} to 3.1×10^{-9} M) with preincubation.[4] Figure 5 shows that when no preincubation is used, F_3TdRP also inhibits competitively versus dUMP at pH 6.7. However, in contrast to the above results with FUdRP, the inhibition becomes noncompetitive after a 10-min preincubation. Nevertheless, preincubation is again

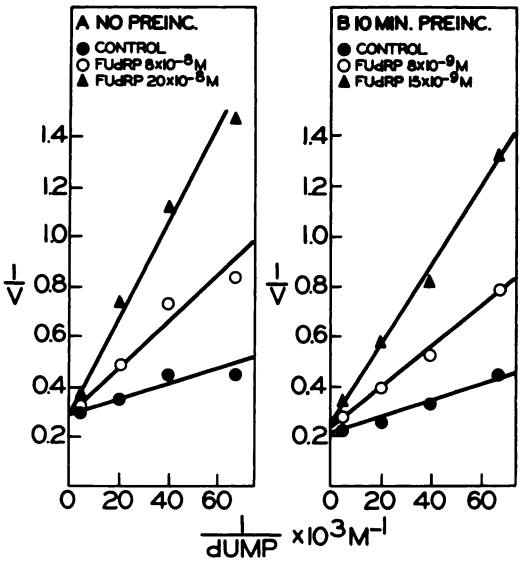


FIG. 4. *Double-reciprocal plots for thymidylate synthetase and its inhibition by FUdRP*

The reaction mixtures contained varying amounts of dUMP (15, 25, 50, and 200 mμmoles) while the *dl*-tetrahydrofolate level was held constant at 200 mμmoles. In addition, 3.3 μmoles of formaldehyde, 33 μmoles of mercaptoethanol, enzyme extract (4.6 mg protein), and 0.1 M phosphate buffer, pH 6.7, were added to a total volume of 1.0 ml. (A) dUMP and FUdRP were premixed and added together after a 10-min preincubation of all other components. (B) dUMP was added after a 10-min preincubation of all other components, including FUdRP. Enzyme activity is expressed as mμmoles of thymidylate produced per 5 mg protein per 5 min. The apparent inhibition constant, $K_{i,slope}$ is 3.1 (±0.6) × 10^{-8} M and 3.1 (±0.6) × 10^{-9} M for (A) and (B), respectively.

[4] Equations (2), (3), and (4) represent linear competitive, linear uncompetitive, and linear noncompetitive inhibition, respectively,

$$\frac{1}{v} = \frac{K}{V}\left(1 + \frac{I}{K_{i,slope}}\right)\frac{1}{A} + \frac{1}{V} \quad (2)$$

$$\frac{1}{v} = \frac{K}{V}\left(\frac{1}{A}\right) + \frac{1}{V}\left(1 + \frac{I}{K_{i,intercept}}\right) \quad (3)$$

$$\frac{1}{v} = \frac{K}{V}\left(1 + \frac{I}{K_{i,slope}}\right)\frac{1}{A} + \frac{1}{V}\left(1 + \frac{I}{K_{i,intercept}}\right) \quad (4)$$

where $K_{i,slope}$ and $K_{i,intercept}$ are inhibition constants, and I is the inhibitor concentration [Cleland (20)].

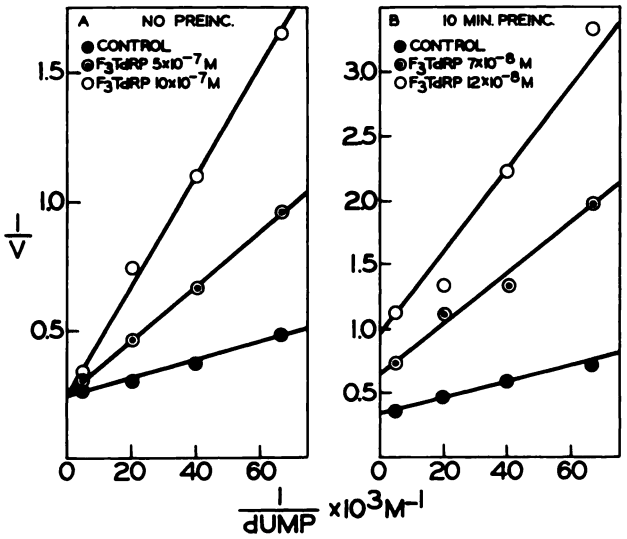


FIG. 5. *Double-reciprocal plots for thymidylate synthetase and its inhibition by F_3TdRP*

All conditions as for Fig. 4 except that 5.2 mg protein was used. The apparent inhibition constants are: (A) $K_{i,slope} = 2.3\ (\pm 0.5) \times 10^{-7}$ M and (B) $K_{i,slope} = 3.8\ (\pm 0.7) \times 10^{-8}$ M; $K_{i,intercept} = 7.1\ (\pm 0.8) \times 10^{-8}$ M.

found to decrease $K_{i,slope}$ (2.3×10^{-7} to 3.8×10^{-8} M). At the more physiologic pH of 7.4, the inhibition by FUdRP with respect to dUMP is also competitive with ($K_{i,slope} = 4.9 \times 10^{-9}$ M) and without ($K_{i,slope} = 2.7 \times 10^{-8}$ M) a preincubation period (Table 5). At this same pH, F_3TdRP inhibits competitively without preincubation ($K_{i,slope} = 1.7 \times 10^{-7}$ M) and noncompetitively when preincubated for 10 min ($K_{i,slope} = 6.2 \times 10^{-8}$ M; $K_{i,intercept} = 6.6 \times 10^{-8}$ M).

Figure 6 illustrates that, at pH 6.7, both FUdRP and F_3TdRP inhibit uncompetitively with respect to 5,10-methylenetetrahydrofolate (5,10-methylene FH_4), which serves as coenzyme in the thymidylate synthetase reaction. In these experiments a 10-min preincubation of inhibitor and enzyme was used prior to addition of dUMP. The $K_{i,intercept}$ for FUdRP is 9.9×10^{-9} M whereas that for F_3TdRP is 3.1×10^{-8} M.

Since the inhibition produced by FUdRP is competitive against dUMP and uncom-

TABLE 5

Inhibition of thymidylate synthetase by FUdRP and F_3TdRP at pH 7.4

The experimental conditions were the same as for Fig. 4 except that 4.0–4.6 mg protein was used and the experiments were conducted at pH 7.4. In the experiments with preincubation, the final FUdRP concentrations were 6 and 10×10^{-9} M while those for F_3TdRP were 4 and 6×10^{-8} M. In the experiments without preincubation, the concentrations of FUdRP were 8 and 15×10^{-8} M and those for F_3TdRP were 2 and 4×10^{-7} M.

Experimental condition	Inhibitor	Inhibition	Inhibition constant
No preincubation	FUdRP	Competitive with dUMP	$K_{i,slope} = 2.7\ (\pm 0.5) \times 10^{-8}$ M
No preincubation	F_3TdRP	Competitive with dUMP	$K_{i,slope} = 1.7\ (\pm 0.2) \times 10^{-7}$ M
10-min preincubation	FUdRP	Competitive with dUMP	$K_{i,slope} = 4.9\ (\pm 2.4) \times 10^{-9}$ M
10-min preincubation	F_3TdRP	Noncompetitive with dUMP	$K_{i,slope} = 6.2\ (\pm 3.7) \times 10^{-8}$ M $K_{i,intercept} = 6.6\ (\pm 1.3) \times 10^{-8}$ M

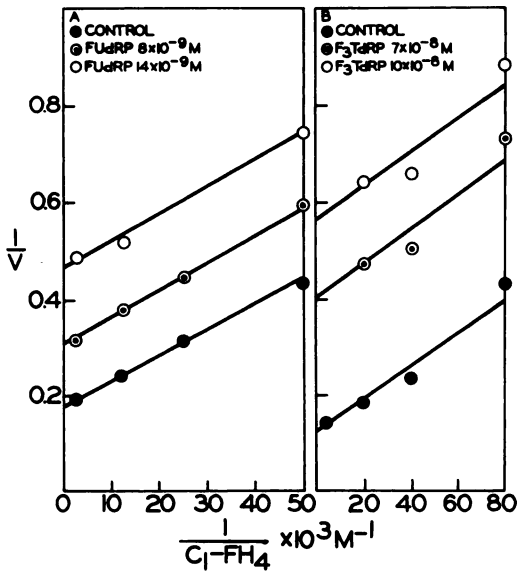


FIG. 6. *Double-reciprocal plots for thymidylate synthetase and its inhibition by FUdRP and F_3TdRP*

The reaction mixtures in (A) contained 20, 40, 80, and 400 mμmoles of *dl*,L-tetrahydrofolate, while those in (B) contained 12.5, 25, 50, and 250 mμmoles. The amount of added dUMP in all cases was held constant at 200 mμmoles. 5,10-Methylene-FH_4(C_1-FH_4) concentrations were assumed to equal the concentration of added *dl*, L-tetrahydrofolate (see text). The other components included: 3.3 μmoles of formaldehyde; 50 μmoles of mercaptoethanol; enzyme extract (4.2 mg protein), and 0.1 M phosphate buffer, pH 6.7, in a total volume of 1.0 ml. In all cases, dUMP was added after a 10-min preincubation of all other reactants, including FUdRP or F_3TdRP. The apparent inhibition constant, $K_{i,\,intercept}$ is 9.9 (± 0.6) $\times 10^{-9}$ M and 3.1 (± 0.3) $\times 10^{-8}$ M for FUdRP and F_3TdRP, respectively.

petitive versus the coenzyme, it follows that the coenzyme interacts with the enzyme before dUMP, since it is known that dead-end inhibitors do not affect the slope of the double-reciprocal plot (uncompetitive inhibition) when the variable substrate interacts with the enzyme before the inhibitor (21). This same reaction sequence is also suggested by the results with F_3TdRP although these are complicated by the fact that the inhibition versus dUMP becomes noncompetitive following a 10-min preincubation (Fig. 5).

Initial Velocity Studies

The mechanism of enzyme-catalyzed reactions involving two or more substrates or products can be classified into two general types, according to Alberty (28). In the first type, all substrates must combine with the enzyme before any product is released. In the second type, one or more products are released before all substrates have been added to the enzyme. Cleland (19) has designated the former type as "sequential" and the latter as "ping pong."

The results presented in the previous section indicate that methylene-FH_4 combines with thymidylate synthetase before dUMP. It therefore became of interest to determine which of the above two mechanisms applies in the present case, since the possibility was considered that coenzyme and dUMP might combine with the enzyme in a "sequential" manner or that the coenzyme first might react with the enzyme to produce a "methylated" enzyme plus dihydrofolate, a possibility considered by Blakley (14). The "methylated" enzyme might then react with dUMP to yield thymidylate via a "ping pong" mechanism.

Initial velocity experiments where one substrate is varied at several fixed levels of another, while keeping any others constant, can differentiate between "ping pong" and sequential mechanisms (29). When these results are expressed as a double-reciprocal plot, a sequential mechanism yields a family of lines which intersect to the left of the vertical axis. On the other hand, a "ping pong" mechanism yields parallel lines. Such an initial velocity study is shown in Fig. 7. The results clearly support a sequential mechanism. Furthermore, the designation "ordered" can be applied since the order of combination of coenzyme and dUMP is restricted, not random. The rate equation for this mechanism and to which the data were fitted is:

$$1/v = \frac{1}{V}\left(\frac{K_{ia}K_b}{AB} + \frac{K_a}{A} + \frac{K_b}{B} + 1\right) \quad (5)$$

where A and B are substrate concentrations, K_a and K_b are the Michaelis constants for A and B, respectively, K_{ia} is the dissocia-

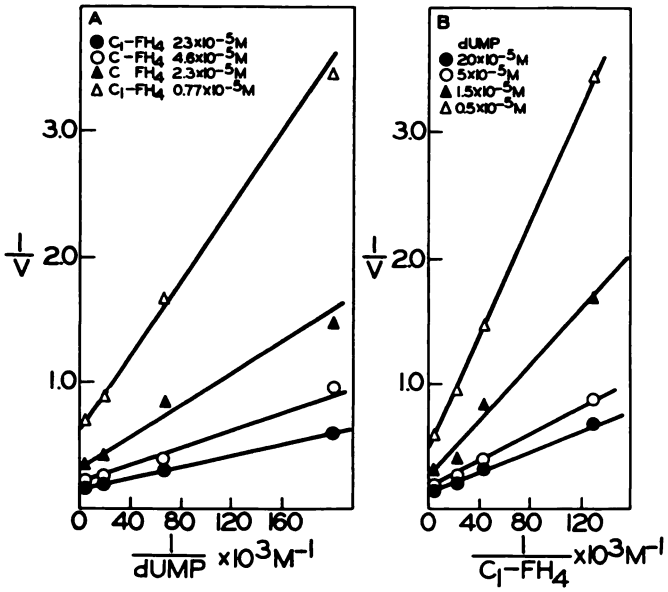


FIG. 7. *Initial velocity plots for thymidylate synthetase with dUMP or 5,10-methylene-FH$_4$ as the variable substrate*

The two plots were derived from a single experiment in which the initial velocity was determined with four different levels of dUMP at each of four different 5,10-methylene-FH$_4$ concentrations. In all cases, all components were preincubated for 10 min prior to the addition of the variable substrate. The amounts of dUMP employed were 5, 15, 50 and 200 mμmoles while those for 5,10-methylene-FH$_4$ were 7.7, 23, 46, and 230 mμmoles. As in Fig. 6, the coenzyme concentration was assumed to equal that of the added *dl*,L-tetrahydrofolate. All other components were the same as for Fig. 6 except that 4.6 mg protein was added. The apparent Michaelis constant for both dUMP and 5,10-methylene-FH$_4$ is 1.4 ($\pm$0.2) $\times$ 10^{-5} M.

tion constant for the enzyme–A complex, v is the initial velocity, and V is the maximum velocity (29).

The apparent Michaelis constants for dUMP and 5,10-methylene-FH$_4$ were both found to be 1.4 $\times$ 10^{-5} M. The value for 5,10-methylene-FH$_4$ was corrected for the fact that the enzymically active diastereoisomer of tetrahydrofolate possesses the *l*,L-configuration, whereas catalytic hydrogenation of folate produces equal amounts of the *d*,L- and *l*,L-diastereoisomers (30). This has been demonstrated in a more direct manner recently by Blakley (14), who reported that the Michaelis constant for thymidylate synthetase obtained with the *dl*,L-mixture of 5,10-methylene-FH$_4$ is twice the value found with the *l*,L-diastereoisomer. At any rate, the Michaelis constant for 5,10-methylene-FH$_4$ reported here must of necessity represent a maximum value, since the assumption was made that the nonenzymic reaction of formaldehyde with tetrahydrofolate to yield 5,10-methylene-FH$_4$ is essentially complete. However, it is possible that under the present experimental conditions, the reaction does not go to completion.

Product Inhibition Studies

In order to obtain additional evidence in support of the proposed ordered and sequential mechanism for thymidylate synthetase, as opposed to a random one where substrates combine with the enzyme in a random fashion, several product inhibition experiments were conducted. The rate equation for a random bi bi mechanism (19) predicts that inhibition by either thymidylate or dihydrofolate will be noncompetitive and nonlinear, i.e., $1/V$ intercepts will be hyperbolic functions of inhibitor while the slopes will often be a more complicated

function of inhibitor. On the other hand, bireactant ordered mechanisms, which do not have alternate reaction sequences, always yield linear product inhibition (20).

Figures 8 and 9 illustrate that the inhibition by thymidylate is linear and noncom-

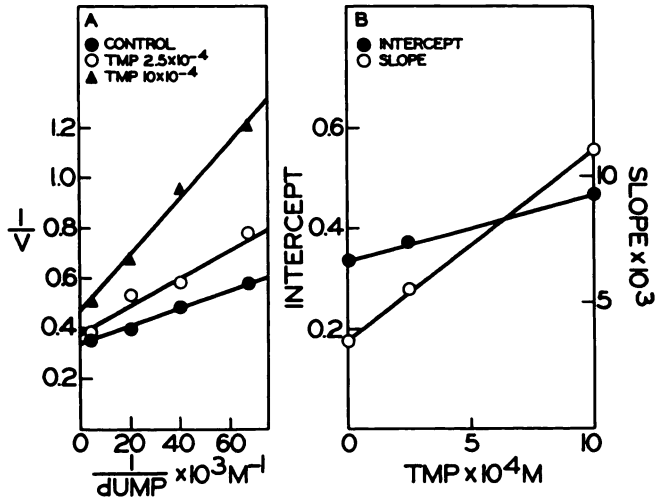


FIG. 8. *Product inhibition of thymidylate synthetase with dUMP as the variable substrate*

The reaction mixture consisted of 200 mμmoles of *dl*,L-tetrahydrofolate; 15, 25, 50, and 200 mμmoles of dUMP; with formaldehyde, mercaptoethanol, enzyme extract, and buffer as described for Fig. 6. All components were preincubated for 10 min prior to the addition of dUMP. (A) Double-reciprocal plot. (B) Replot of slopes and $1/V$ intercepts versus added thymidylate concentration. The apparent inhibition constants for thymidylate are: $K_{I,\,slope} = 4.1$ (± 1.2) $\times 10^{-4}$ M; $K_{I,\,intercept} = 3.3$ (± 1.2) $\times 10^{-3}$ M.

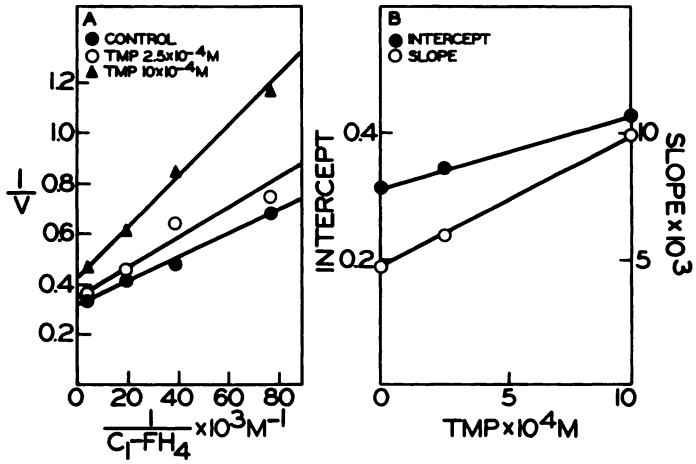


FIG. 9. *Product inhibition of thymidylate synthetase with 5,10-methylene-FH$_4$ as the variable substrate*

The reaction mixture contained 200 mμmoles of dUMP, and 13, 26, 52, and 260 mμmoles of 5,10-methylene-FH$_4$. Formaldehyde, mercaptoethanol, enzyme extract, and phosphate buffer were the same as for Fig. 6. All components were preincubated for 10 min prior to dUMP addition. The 5,10-methylene-FH$_4$ concentration was again assumed to equal the *dl*,L-tetrahydrofolate level. (A) Double-reciprocal plot. (B) Replot of slopes and $1/V$ intercepts versus added thymidylate concentration. The apparent inhibition constants for thymidylate are: $K_{I,\,slope} = 7.9$ (± 2.2) $\times 10^{-4}$ M; $K_{I,\,intercept} = 3.2$ (± 0.8) $\times 10^{-3}$ M.

petitive against both dUMP and coenzyme, as seen from the replots of intercepts and slopes versus thymidylate concentration. Thus, these data are not compatible with a random bi bi mechanism and therefore provide further evidence in support of the ordered and sequential mechanism whereby coenzyme combines with the enzyme before dUMP. A rapid equilibrium random bi bi mechanism has also been ruled out, since it predicts that the inhibition by thymidylate would be linear and competitive (19).

No attempt has been made to distinguish between the various ordered mechanisms possible, although the Theorell-Chance mechanism (no central complex) (31) has been eliminated since it would predict competitive inhibition by thymidylate versus dUMP (19).

Dialysis Experiments

The proposed ordered and sequential mechanism further predicts that both FUdRP and F_3TdRP will not normally combine with the enzyme in the absence of coenzyme, since these inhibitors can combine only with the enzyme–coenzyme complex. Thus, it should be possible to dialyze away both inhibitors, from an enzyme–inhibitor mixture, in the absence of coenzyme. In the presence of coenzyme, however, it is expected that the loss of inhibitors might be retarded because of their tight combination with the enzyme, as judged from their very small K_I values. It also follows that the loss of inhibitor will be reflected by a reversal of inhibition during dialysis.

Figure 10 illustrates this type of experiment, in which either the enzyme or the enzyme–coenzyme mixture was simultaneously dialyzed in the presence and absence of inhibitor. A complete reversal of the inhibition by FUdRP arose during dialysis and, as predicted, was significantly retarded by the presence of coenzyme. In contrast, F_3TdRP produces inhibition that is not completely reversed by dialysis, even in the absence of coenzyme. The level of this residual inhibition is increased by preincu-

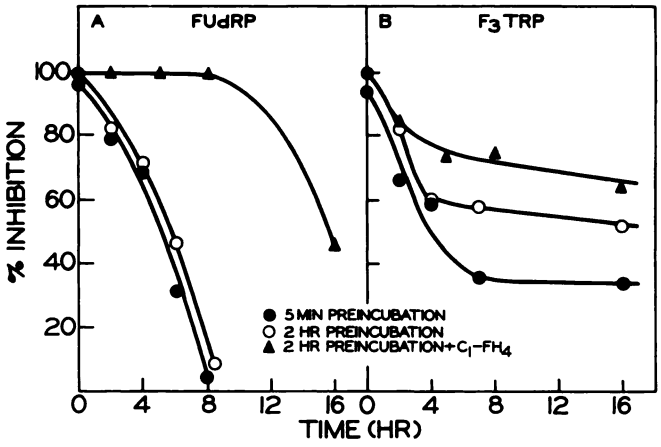


FIG. 10. *The effect of dialysis on the inhibition of thymidylate synthetase by FUdRP and F_3TdRP*

(A) Enzyme extract (12 mg protein per milliliter) was mixed with FUdRP to give a final inhibitor concentration of 4.5×10^{-8} M. After 5 min or 2 hr at 4°, 2.0-ml aliquots were dialyzed, with magnetic stirring, against 500 ml of 0.1 M phosphate buffer, pH 6.7, which was changed after 4 and 8 hr. A control extract without added FUdRP was dialyzed simultaneously. At appropriate time intervals, samples were removed, and the thymidylate synthetase activity was measured. In experiments with a 2-hr preincubation in the presence of coenzyme (C_1-FH_4), the following components, in addition to FUdRP, were added to the enzyme extract in the indicated concentrations: *dl*,L-tetrahydrofolate, 1.9×10^{-4} M; formaldehyde, 1.7×10^{-3} M; and mercaptoethanol, 6×10^{-3} M. (B) The experimental conditions were identical to those in (A) except that F_3TdRP at a final concentration of 4.5×10^{-7} M was used in place of FUdRP.

bating the enzyme with F_3TdRP for 2 hr prior to dialysis; no such effect is observed with FUdRP. Again, the presence of the coenzyme slows the rate of reversal of the inhibition by F_3TdRP. Thus, the retarding effect of the coenzyme on the rate of reversal of inhibition by both inhibitors offers additional support for the ordered and sequential mechanism.

The reason for the incomplete reversal of the inhibition by F_3TdRP is not entirely clear. The observation that the level of residual inhibition is dependent on the length of the preincubation period prior to dialysis suggests that F_3TdRP either slowly combines somehow with thymidylate synthetase in an irreversible manner, or is able slowly to inactivate the enzyme.

Similar dialysis experiments were repeated using a 2-hr preincubation at 4° without coenzyme and with a concentration of F_3TdRP 10 times that previously employed. Experiments with FUdRP at a concentration 10 times the previous one were also conducted. The results (Fig. 11)

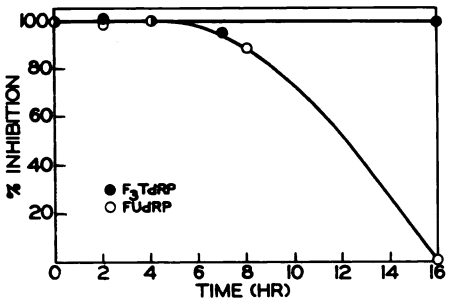


FIG. 11. *The effect of dialysis on the inhibition of thymidylate synthetase by FUdRP and F_3TdRP*

The experimental conditions were identical to those described in Fig. 10 for experiments with a 2-hr preincubation in the absence of coenzyme, except that FUdRP and F_3TdRP were present in concentrations of 4.5×10^{-7} M and 4.5×10^{-6} M, respectively. In addition, the dialysis buffer was changed after 2, 4, and 8 hr.

clearly show that the inhibition by F_3TdRP is now completely unaffected by dialysis, indicating that the interaction with the enzyme which produces the residual inhibition is time dependent (Fig. 10) as well as dependent on the concentration of F_3TdRP. On the other hand, the inhibition by FUdRP is again completely reversed, although at a slower rate, apparently because of the increased amount of inhibitor that must be dialyzed away from the enzyme.

It must be pointed out that the apparent irreversible inhibition of thymidylate synthetase by F_3TdRP, which is detected only after several hours of preincubation, does not apply to the earlier kinetic studies where only a 10-min preincubation period was used. Evidence to support this contention is presented in the last section below.

In Vivo Inhibition Experiments

The inhibitory effects on thymidylate synthetase of single chemotherapeutic doses of FUdR and F_3TdR injected intraperitoneally into intact mice, bearing the Ehrlich ascites carcinoma, was measured at various times in the high-speed supernatant fraction, as shown in Fig. 12. The inhibition

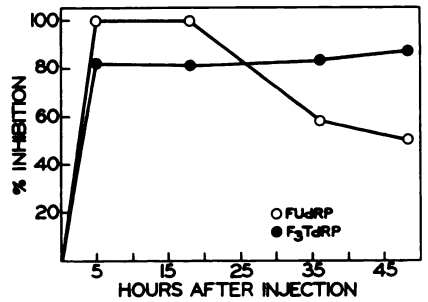


FIG. 12. *The effect of in vivo chemotherapeutic doses of FUdR or F_3TdR on thymidylate synthetase activity measured in vitro*

Groups of 10–15 female Swiss mice bearing the Ehrlich ascites carcinoma were injected once intraperitoneally with chemotherapeutic doses of either FUdR (50 mg/kg) or F_3TdR (200 mg/kg). A similar group injected with physiological saline (0.9%) served as the control. After given intervals of time, high-speed supernatant enzyme extracts were prepared from each group as already described, and the percentage inhibition was determined by comparing the thymidylate synthetase activity of the control with that of extracts obtained from drug-treated mice.

produced by FUdR, which is converted *in vivo* to FUdRP, begins to decrease about 20 hr after the injection. On the other hand,

the inhibition produced by F_3TdR, which is similarly converted to F_3TdRP, remains constant throughout the entire experimental period of 48 hr. The decrease in inhibition after an injection of FUdR probably results from its metabolic degradation, according to Mukherjee and Heidelberger (32), and from the fact that the nucleotide combines reversibly with thymidylate synthetase, whereas under these conditions F_3TdR could combine irreversibly with the enzyme and would not be expected to be degraded under such circumstances. Thus, the slowly occurring irreversible inhibition produced by F_3TdRP *in vitro* (Figs. 10 and 11) is paralleled by a similar inhibition *in vivo*.

Further Inhibition Studies

The treatment of enzyme inhibition by Michaelis-Menten kinetics (33) is based on the assumption that enzyme (E) and inhibitor (I) interact in a completely reversible manner. Moreover, the determination of inhibition constants by the method of Lineweaver and Burk (22) further assumes that the amount of inhibitor combined with enzyme is negligible in comparison to the total inhibitor concentration. However, the very small K_i values reported for F_3TdRP in the present investigation, as well as the results of the dialysis and *in vivo* experiments, suggest that after some time F_3TdRP combines with the enzyme to produce "stoichiometric" inhibition, as designated by Werkheiser (34).

The method of Ackermann and Potter (35) has been employed to determine whether the inhibition of thymidylate synthetase by F_3TdRP, as well as by FUdRP, is stoichiometric (irreversible) after a 10-min preincubation of the enzyme with inhibitor. This method involves a study of the effect of increasing enzyme concentration on the inhibition produced by a constant concentration of inhibitor. A stoichiometric inhibitor, by this method, yields a series of lines which are parallel to the uninhibited control and which intersect the enzyme axis. By contrast, reversible inhibition, where the amount of inhibitor combined with the enzyme is negligible, yields a family of lines of decreasing slope which pass through the origin.

Figure 13 shows that, under these experimental conditions involving a 10-min preincubation, the inhibition by FUdRP or F_3TdRP cannot be considered to be stoichiometric. Further support for this conclusion is obtained by applying the criteria of Werkheiser (34), who has pointed out that

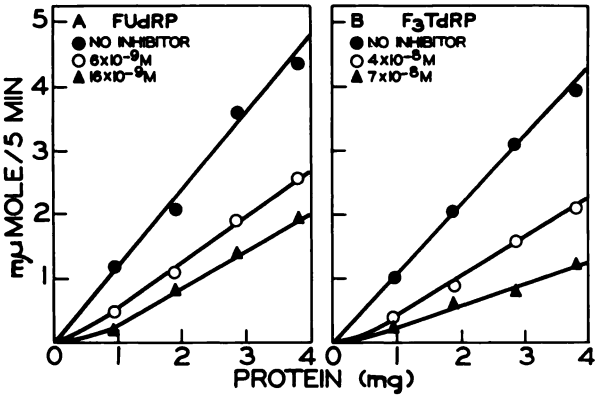


FIG. 13. *Ackermann-Potter plot for the inhibition of thymidylate synthetase*

(A) The reaction mixture contained 50 mμmoles of dUMP; 200 mμmoles of *dl*,L-tetrahydrofolate; 3.3 μmoles of formaldehyde; 50 μmoles of mercaptoethanol; enzyme extract (0.95, 1.90, 2.85, and 3.80 mg protein) and 0.1 M phosphate buffer, pH 6.7, in a total volume of 1.0 ml. All components, including FUdRP at 6 and 16 × 10^{-9} M, were preincubated for 10 min before dUMP addition. (B) The reaction mixture was the same as in (A) except that F_3TdRP (4 and 7 × 10^{-8} M) was used in place of FUdRP. In all cases, enzyme activity was compared to a control without added inhibitor.

stoichiometric inhibition produces a double-reciprocal plot similar to noncompetitive inhibition, but with $1/V$ intercepts whose reciprocals are linear with inhibitor concentration. In this respect the finding that FUdRP and F_3TdRP inhibit competitively (Figs. 4 and 5A) and uncompetitively (Fig. 6) against dUMP and coenzyme, respectively, also argues against stoichiometric inhibition. As shown in Fig. 5B, inhibition by F_3TdRP versus dUMP becomes noncompetitive after a 10-min preincubation of enzyme and inhibitor. Nevertheless, the inhibition in this instance yields $1/V$ intercepts that are themselves linear with inhibitor concentration, thereby arguing against stoichiometric inhibition.

Straus and Goldstein (36) and Goldstein (37) have divided enzyme inhibition into three zones (A, B, C) depending on the relationship between the inhibition constant (K_i) and the enzyme concentration (E). They have designated the ratio E/K_i as the specific concentration of enzyme (E'). When K_i is very small (stoichiometric inhibition, zone C), E' is large and the inhibition is directly proportional to the concentration of inhibitor. Thus, a plot of enzyme activity versus inhibitor concentration at a constant level of enzyme, would produce a straight line which intersects the inhibitor axis. The inhibition produced by either F_3TdRP or FUdRP, after a 10-min preincubation of enzyme with inhibitor, was studied by this type of experiment (Fig. 14). These experiments do not yield straight lines, but instead give curves, as expected for reversible inhibition where the inhibitor is essentially free in solution.

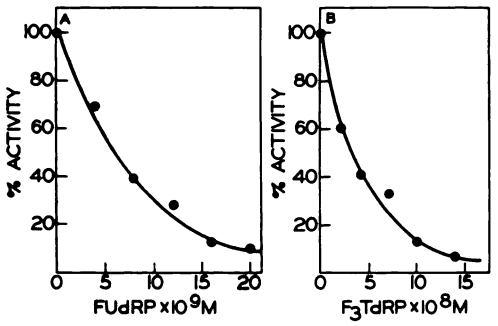


FIG. 14. *The influence of FUdRP or F_3TdRP concentration on thymidylate synthetase activity*

The reaction conditions were the same as for Fig. 13 except that 200 mμmoles of dUMP was used while the amount of extract protein was held constant at 4.2 mg. All components were preincubated for 10 min prior to the addition of dUMP. Enzyme activity was compared to a control without added inhibitor. (A) FUdRP was present in final concentrations of 4, 8, 12, 16, and 20×10^{-9} M. (B) F_3TdRP was added at concentrations of 2, 4, 7, 10, and 14×10^{-8} M.

The results presented in this section show that the inhibition of thymidylate synthetase by F_3TdRP or F_3TdRP, as measured after a 10-min preincubation of enzyme with inhibitor, is not stoichiometric but is reversible. Hence, the treatment by conventional Michaelis-Menten methods of the inhibition produced in the kinetic studies (Figs. 4–6), which also involved a similar 10-min preincubation, is valid. However, after a longer preincubation F_3TdRP combines with the enzyme in an irreversible fashion, as shown by the dialysis experiments.

DISCUSSION

A slight modification to achieve greater sensitivity of the spectrophotometric assay for thymidylate synthetase developed by Wahba and Friedkin (16) was used throughout this investigation. This assay, which represents a vast improvement over the previously used isotopic assay of Hartmann and Heidelberger (6), because of its rapidity and convenience and because it allows the continuous measurement of enzyme activity, made it possible to discover several new properties of thymidylate synthetase assayed in extracts of Ehrlich ascites carcinoma cells. These include the weak product inhibition by thymidylate and the stimulatory effect of mercaptoethanol on enzyme activity, neither of which could previously be detected (6). Thymidylate was found to be a weak noncompetitive inhibitor against both dUMP and 5,10-methylene-FH_4. The evidence indicates that the stimulatory effect of mercaptoethanol is primarily on the enzyme and does not reflect a protective effect on the easily oxidized tetrahydrofolate. It has also been

shown that the concentration of formaldehyde in the reaction mixture markedly affects the enzyme activity, which is considerably reduced at high levels of formaldehyde.

Blakley (14) has interpreted the results of his investigation of bacterial thymidylate synthetase as favoring a sequential mechanism without a central complex, although a "ping pong" mechanism as well as one involving central complexes was also considered possible. In that instance, a "ping pong" mechanism was defined as one where the coenzyme initially methylates the enzyme with a concomitant production of dihydrofolate. It was proposed that the methylated enzyme might then react with dUMP to produce thymidylate. However, we have conducted initial velocity experiments which demonstrated that the mechanism is sequential, not "ping pong." Furthermore, product-inhibition kinetic studies showed that the sequence of substrate and coenzyme addition to the enzyme is ordered, not random, and also eliminated a mechanism without a central complex. The kinetics of the inhibition by FUdRP or F_3TdRP have made it possible further to define the reaction mechanism of thymidylate synthetase from Ehrlich ascites carcinoma cells as one where 5,10-methylene-FH_4 interacts with the enzyme before dUMP, and dTMP leaves before dihydrofolate, as indicated schematically in Fig. 15. It should be

The powerful inhibition of thymidylate synthetase by FUdRP or F_3TdRP has been examined in considerable detail. These two fluorinated pyrimidine nucleotides produce inhibitions that show several interesting differences. Thus, FUdRP acts as a competitive inhibitor with respect to dUMP, whether or not it is preincubated with the enzyme for 10 min prior to initiating the reaction by the addition of dUMP. This agrees with earlier findings from this laboratory (6) and is different from the results of Blakley (14) and Mathews and Cohen (15), who reported that the enzyme from bacterial and bacteriophage sources, respectively, is inhibited noncompetitively after a similar preincubation. The reason for this kinetic difference is not apparent, but the difference may be due to the fact that the above workers used partially purified enzymes whereas a crude extract has been used in the present investigation. Alternatively, intrinsic differences in the properties of the mammalian and of the other enzymes may account for this difference in kinetics. F_3TdRP, in contrast to FUdRP, produces inhibition with respect to dUMP which changes from competitive to noncompetitive when a 10-min preincubation period is employed. Both compounds, however, are uncompetitive against the coenzyme.

One additional difference in the inhibition produced by FUdRP and F_3TdRP is that the latter compound exhibits the ability to

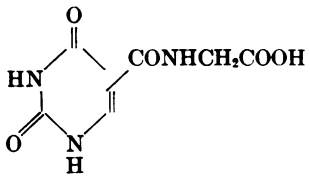
FIG. 15. *Schematic representation of the time sequences involved in the mechanism of thymidylate synthetase, assayed in extracts of Ehrlich ascites carcinoma cells*

E = enzyme.

emphasized that the scheme in Fig. 15 depicts only a time sequence and makes *no* inferences about the spatial arrangements at the active site of the enzyme. The elucidation of this reaction sequence has added considerably to our present understanding of the mechanism of action of thymidylate synthetase.

interact slowly, in the presence or absence of coenzyme, with thymidylate synthetase in an irreversible manner. This is detected only when the inhibition is allowed to progress for a number of hours, as in the dialysis and *in vivo* experiments reported here. A number of other experiments showed, however, that the inhibition by

F_3TdRP or FUdRP is reversible when only a 10-min preincubation is used. The exact nature or cause of this slow and irreversible interaction is not known, but it may be related to the increased acidity of the proton on N-3 of F_3T (pK_a $F_3T = 7.35$; pK_a FU = 8.15; pK_a uracil = 9.45; pK_a thymine = 9.82) [Gottschling and Heidelberger (9)], if it is assumed that this portion of the pyrimidine molecule acts as a binding site in the combination of dUMP and of the fluorinated pyrimidines with thymidylate synthetase. The data suggest that a higher degree of ionization of the N-3 proton may favor the formation of a more tightly bound complex with the enzyme, so that on this basis F_3TdRP might be very tightly bound while FUdRP would be less tightly held. By the same argument, the lower degree of ionization of dUMP and thymidylate, suggested by the less acidic pK_a values for the corresponding base, would not be conducive to the formation of a tightly held complex. In this regard, the very weak product inhibition by thymidylate reported in the present investigation, might be taken as evidence to support this hypothesis. It is tempting to speculate that F_3TdRP, after its attachment to the active site of the enzyme, would alkylate it to produce the observed irreversible inhibition. This is not farfetched, since Heidelberger *et al.* (7) prepared from F_3T, compounds in which an amino acid was N-acylated, cf:



Whether this slowly occurring irreversible inhibition of thymidylate synthetase might increase the chemotherapeutic efficacy of F_3TdR, in comparison to FUdR, as an inhibitor of the growth of human tumors is not known at the present time but will be investigated.

ACKNOWLEDGMENT

The authors would like to thank Professor W. W. Cleland for several valuable and helpful discussions during the course of this investigation. This work was supported in part by grants CRTY-5002 and CA-07175 from the National Cancer Institute, United States Public Health Service, Bethesda, Maryland.

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