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ALTERED THYMIDYLATE SYNTHETASE IN 5-FLUORODEOXYURIDINE-RESISTANT EHRLICH ASCITES CARCINOMA CELLS

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Abstract—Thymidylate synthetase from 5-fluorodeoxyuridine-resistant Ehrlich ascites carcinoma cells was purified to a state close to electrophoretical homogeneity (sp. act. = 1.3 μmoles/min/mg protein) and studied in parallel with the homogeneous preparation of the enzyme from the parental Ehrlich ascites carcinoma cells. The enzyme from the resistant cells compared to that from the parental cells showed: (i) a higher turnover number (at least 91 against 31 min^{-1}), (ii) a higher inhibition constant (19 against 1.9 nM) for FdUMP (a tight-binding inhibitor of both enzymes), (iii) a lower activation energy at temps above 36° (1.37 against 2.59 kcal/mole), and (iv) a lower inhibition constant (26 against 108 μM) for dTMP, inhibiting both enzymes competitively vs dUMP.

FdUrd†, a drug used in cancer chemotherapy, acts primarily *via* its phosphorylation product FdUMP, a strong inhibitor of the enzyme thymidylate synthetase [methylenetetrahydrofolate:deoxyuridine-5′-monophosphate *C*-methyltransferase (EC 2.1.1.45)] [1, 2]. Since this enzyme, which converts dUMP to dTMP [3], provides the only known source of dTMP synthesized *de novo*, its inhibition blocks DNA biosynthesis.

One of the major impediments to the effective clinical application of antineoplastic drugs is the development of resistance [4]. Resistance of tumors to FdUrd was found to be accompanied in most cases by impaired phosphorylation of FdUrd in cells [5–10] or elevated thymidylate synthetase activity [11–13]. Another mechanism of resistance to FdUrd, a change in its target enzyme, thymidylate synthetase, was reported by Heidelberger *et al.* [14] for a FdUrd-resistant line of Ehrlich ascites carcinoma cells. Thymidylate synthetase activity studied in crude extracts of those cells revealed an apparent lack of sensitivity to FdUMP. Unfortunately, the resistant cell line had been lost before the enzyme could be purified and studied in detail [15].

We report here distinctly different properties of thymidylate synthetase purified from an FdUrd-resistant Ehrlich ascites carcinoma cell line developed in our laboratory‡ and from the parental line. Both a higher K_i for FdUMP and a higher turnover number of the enzyme from the resistant line indicate a possible resistance mechanism.

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† Abbreviations: FdUrd, 5-fluorodeoxyuridine; FdUMP, 5-fluorodeoxyuridylate; H_2PteGlu, dihydrofolate; 5,10-CH_2-H_4PteGlu, 5,10-methylenetetrahydrofolate.

‡ M. M. Jastreboff and Z. M. Zielińska, paper submitted.

MATERIALS AND METHODS

All reagents used were as described earlier [16].

Maintenance of Ehrlich ascites carcinoma cells. The cells were maintained, harvested during the exponential growth phase (i.e. 5 or 7 days after transplantation in the case of the parental and FdUrd-resistant cells, respectively) and stored as described earlier [16].

Development of FdUrd-resistant line of Ehrlich ascites carcinoma cells‡. The cell-bearing mice were treated with FdUrd (injected intraperitoneally as 0.1–0.25 ml of PBS solution) daily for 5 days, starting 1 day after transplantation. The cells were transplanted to recipients on the seventh day and mice were treated with FdUrd as described earlier. The resistant line was developed as a result of the following schedule of FdUrd treatment: 35 mg/kg/day for four passages, 40 mg/kg/day for two passages, 50 mg/kg/day for two passages, 60 mg/kg/day for two passages and 75 mg/kg/day for five passages.

The resulting cell line showed a longer doubling time (24 hr) than the parental line (17 hr). Treatment with FdUrd (75 mg/kg/day, daily for 5 days, starting on the second day after transplantation) did not influence the time of 50% survival (20 days) of mice bearing the resistant line, whereas it prolonged the time of 50% survival by 70% (from 14 to 24 days) of mice bearing the parental line. Resistance was maintained for about 40 passages without further drug treatment.

Enzyme assays. Thymidylate synthetase activity was determined at pH 7.5 as described previously [16]. The activity of the enzyme is expressed in units defined as the amount required to release 1 μg equivalent of tritium (equivalent to the formation of 1 μmole of dTMP) per minute under the conditions of the assay.

Thymidine kinase activity was determined as described by Münch-Petersen and Tyrsted [17]. The

activity of the enzyme is expressed in units defined as the amount required to phosphorylate 1 μmole of thymidine per minute.

Purification of thymidylate synthetase from the FdUrd-resistant cells. The procedure was as previously used [16] with one additional step: the crude extract was treated at 4° with a tenth of its vol. of 20% (w/v) aqueous streptomycin sulfate, stirred for 15 min, centrifuged at 20,000 *g* for 15 min at 4° and the resulting supernatant fractionated with ammonium sulfate as described earlier [16].

Electrophoretic analysis. Electrophoresis in 7.5% polyacrylamide gels containing 0.1% Triton X-100 [16, 18] was modified due to the recent observation that the tracking dye (bromophenol blue) in such gels is separated into two fractions. The faster fraction, containing a minute amount of the dye and therefore not noticed in earlier experiments [16, 18], was considered as the front marker.

The mol. wt of denatured thymidylate synthetase was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate [19]. Lysozyme (M_r = 14,300), β-lactoglobulin (subunit M_r = 18,400), trypsinogen (M_r = 24,000), pepsin (M_r = 34,700), ovalbumin (M_r = 45,000) and bovine serum albumin (M_r = 66,000), components of "Dalton Mark VI" (Sigma Chemical Co. St. Louis, MO), were used as mol. wt standards.

Kinetic studies. The Lineweaver–Burk plots were constructed using values of the overall velocity instead of the initial velocity and those of the arithmetical mean substrate concn over the course of the assay instead of the initial substrate concn [20]. All plots were fitted using the linear regression program of the "Sharp" EL-5100 scientific calculator.

Statistically evaluated results. These are presented as means ± S.E., followed by the number of experiments (N) in parentheses.

All other methods used were as described earlier [16].

RESULTS

Thymidylate synthetase and thymidine kinase levels

Crude extracts from exponentially growing parental and FdUrd-resistant Ehrlich ascites carcinoma cells showed thymidylate synthetase sp. acts of 274.0 ± 19.3 μunits/mg protein (N = 10) and 1586 ± 57.7 μunits/mg protein (N = 8), respectively. Thymidine kinase sp. act. measured in the same extracts were 289.0 ± 5.3 μunits/mg protein (N = 6) and 3.3 ± 0.48 μunits/mg protein (N = 6), respectively.

Thymidylate synthetase purification

The enzyme from the resistant line could be isolated by the same affinity chromatography method applied earlier to the enzyme from the Ehrlich ascites carcinoma cells [16]. However, the purification procedure had to be modified by introducing the streptomycin sulfate precipitation before the ammonium sulfate fractionation (see Materials and Methods) in order to keep the yield of the latter step high. This modification applied to the resistant cells resulted in over 150% of the crude extract thymidylate synthetase total activity being found in the 30–70%

Table 1. Purification of thymidylate synthetase from FdUrd-resistant Ehrlich ascites carcinoma cells

Purification stage	Vol. (ml)	Total activity (10^{-3} units)	Total protein (mg)	Sp. act. (10^{-3} units/mg protein)	Purification	Yield (%)
Crude extract*	75	342.6	332.6	1.0	1.0	100
Streptomycin sulfate fraction	73	548.1	313.2	1.8	1.7	160
30–70% $(NH_4)_2SO_4$ fraction	72	568.6	220.4	2.6	2.5	166
Pooled fractions after first affinity chromatography	16	500.1	0.58	866.2	841	146
Pooled fractions after second affinity chromatography	10	479.6	0.36	1332.1	1293	140

For details see Materials and Methods.
* Corresponding to 17 g of the cells.

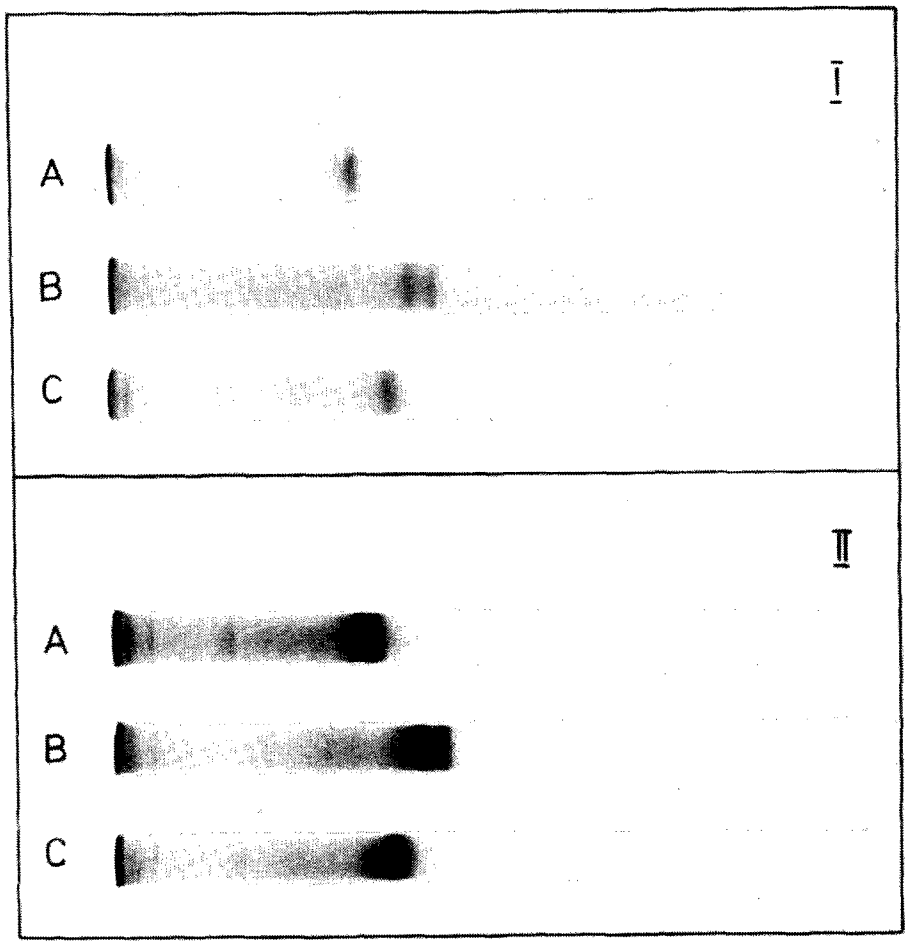

Fig. 1. Polyacrylamide gel electrophoresis of thymidylate synthetase preparations from Ehrlich ascites carcinoma parental (I) and FdUrd-resistant (II) cells. Samples contained 2 μg (I) or 5 μg (II) of protein. (A) The enzyme preparations were not treated before electrophoresis; (B) the enzyme preparations treated before electrophoresis for 15 min at 37° with 50 μM FdUMP and 500 μM (±)-L-5,10-CH_2-H_4PteGlu; (C) 20 μM dUMP present in both the enzyme preparations and the electrode buffer.

ammonium sulfate fraction (Table 1), whereas without the streptomycin sulfate precipitation the yield of the ammonium sulfate fractionation dropped to about 25% (not shown). The modified purification procedure applied to the parental cells resulted in a yield similar to that obtained earlier [16] (not shown).

The final preparation of thymidylate synthetase from the FdUrd-resistant cells (Table 1) was close to electrophoretical homogeneity (Fig. 1). Its sp. act. [1.3 units/mg protein (Table 1)] was over 3 times higher than that (0.4 units/mg protein) of the electrophoretically homogeneous preparation of the enzyme from the parental line [16].

Electrophoretic properties

Native thymidylate synthetase from the resistant line incubated with FdUMP and 5,10-CH_2-H_4PteGlu formed two types of electrophoretically separable complexes (Fig. 1) allowing us to identify the thymidylate synthetase band on the gel. When [6-^{3}H]FdUMP was used both bands corresponding to the complexes were found to contain the label (not shown). Electrophoresis of the enzyme preparation with dUMP present in an electrode buffer showed a new band which probably represents a thymidylate synthetase–dUMP complex, instead of the band representing the enzyme alone (Fig. 1). Electrophoretical properties of native thymidylate synthetase alone or bound in the complexes were identical when the enzyme from either the parental or the resistant cells was studied (Fig. 1).

Mol. wt

The mol. wts of the denatured enzymes from the parental and resistant cell lines, determined by sodium dodecyl sulfate electrophoresis, were found to be 35,500 ± 1100 (N = 6) and 34,200 ± 1200 (N = 7), respectively. Since gel filtration of the native enzymes in Sephadex G-100 showed their mol. wts to be 70,500 ± 3800 (N = 4) and 58,300 ± 3900 (N = 3), respectively, both seem then to be dimers.

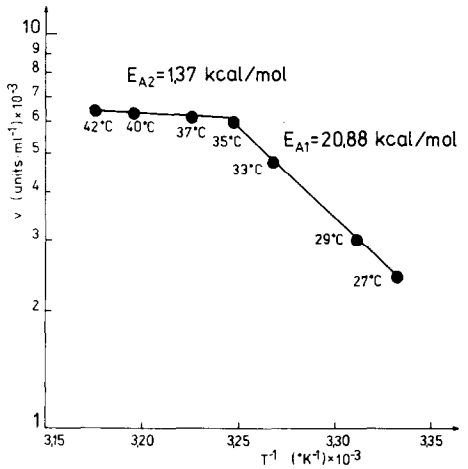

Fig. 2. Dependence of the reaction of thymidylate synthetase from FdUrd-resistant Ehrlich ascites carcinoma cells on temp (Arrhenius plot).

Turnover number

Based on sp. acts of the purified preparations of the enzymes and their mol. wts, turnover numbers have been calculated (turnover number = sp. act. expressed in units/mg protein × mol. wt/1000) as 31 and at least 91 min^{-1} (preparation was not completely homogeneous) for thymidylate synthetase from the parental and resistant cells, respectively. For the reasons given in Discussion doubled values of the subunit mol. wts were used in calculations as mol. wts of the native enzymes.

Temp dependence of reaction velocity

The dependence of the reaction catalyzed by thymidylate synthetase from the resistant cells on temp, expressed as an Arrhenius plot, exhibited a biphasic curve with a transitional temp at 35.5° (Fig. 2). This result is similar to that obtained when the enzyme from the parental cells was studied [16] but for one detail: a distinctly higher activation energy above the transitional temp was found for the latter (2.59 kcal/mole) than for the former (1.37 kcal/mole) enzyme.

Interaction with substrates and product inhibition

The kinetics of the interaction of the enzymes with the substrates was studied either in 100 mM phosphate or in 100 mM Tris–HCl buffer (pH 7.5 in both cases) by keeping dUMP at certain fixed concns and varying the concn of 5,10-CH_2–H_4PteGlu. For both enzymes studied, independently of the buffer used, linear plots obtained when $1/v$ was plotted against 1/[dUMP] or 1/[5,10-CH_2–H_4PteGlu] intersected to the left of the ordinate [Fig. 3 (results in Tris not shown)], suggesting that the reaction mechanism is sequential [21–23]. The Michaelis constants determined in the presence of phosphate were found to be 2.0 and 2.5 μM for dUMP and 33.6 and 25.3 μM for (±)-L-5,10-CH_2–H_4PteGlu for the enzymes from the parental and resistant cells, respectively. Those determined in the presence of Tris were 1.5 and 1.6 μM for dUMP and 33.6 and 39.3 μM for (±)-L-5,10-CH_2–H_4PteGlu for the enzymes from the parental and resistant cells, respectively.

Product inhibition was examined in 100 mM Tris–HCl buffer by varying the concn of either substrate at different concns of product. The results are summarized in Table 2. With both enzymes inhibition by dTMP was competitive with respect to dUMP (Fig. 4) and noncompetitive with respect to 5,10-CH_2–H_4PteGlu, whereas inhibition by H_2PteGlu was noncompetitive with respect to either substrate (Table 2). These data are in agreement with both an ordered mechanism where dUMP binds before 5,10-CH_2–H_4PteGlu and H_2PteGlu is released before dTMP and an Iso Theorell–Chance mechanism where 5,10-CH_2–H_4PteGlu binds to the free enzyme

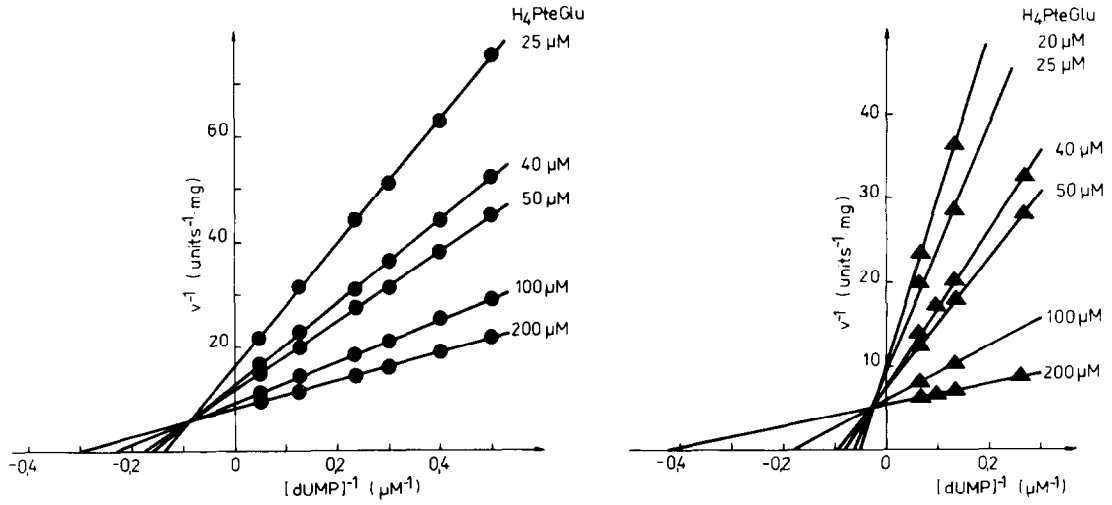

Fig. 3. Kinetics of substrate interaction with thymidylate synthetase from Ehrlich ascites carcinoma parental (●) and FdUrd-resistant (▲) cells (Lineweaver–Burk plots). Incubations were for 2, 4 and 6 min in duplicates. dUMP concn was varied from 2 to 20 μM.

Table 2. Product inhibition of thymidylate synthetase from Ehrlich ascites carcinoma parental and FdUrd-resistant cell lines*

		Source of the enzyme			
		Parental line		Resistant line	
Product	Variable substrate	K_i (μM)	Inhibition type	K_i (μM)	Inhibition type
dTMP	dUMP	108	Competitive	26	Competitive
dTMP	5,10-CH_2-H_4PteGlu	82	Noncompetitive	78	Noncompetitive
H_2PteGlu	dUMP	392	Noncompetitive	250	Noncompetitive
H_2PteGlu	5,10-CH_2-H_4PteGlu	208	Noncompetitive	223	Noncompetitive

* [5-^{3}H]dUMP concn was varied from 0.5 to 8 μM. (±)-L-5,10-CH_2-H_4PteGlu concn was varied from 20 to 100 μM. H_2PteGlu concns were constant at 0, 50 and 100 μM (the parental cell enzyme) or 0, 25 and 50 μM (the resistant cell enzyme) with dUMP as the variable substrate (0.5 mM 5,10-CH_2-H_4PteGlu), but at 0, 25, 50 and 100 μM (the parental cell enzyme) or 0, 50 and 100 μM (the resistant cell enzyme) with 5,10-CH_2-H_4PteGlu as the variable substrate (50 μM [5-^{3}H]dUMP). dTMP concns were constant at 0, 25, 50 and 100 μM (the parental cell enzyme) or 0, 25, 50 and 82 μM (the resistant cell enzyme) with dUMP as the variable substrate (0.5 mM 5,10-CH_2-H_4PteGlu), but at 0, 25, 50 and 82 μM (the parental cell enzyme) or 0, 25, 50 and 100 μM (the resistant cell enzyme) with 5,10-CH_2—H_4PteGlu as the variable substrate (50 μM [5-^{3}H]dUMP). Incubations were for 2, 4 and 6 min in duplicates.

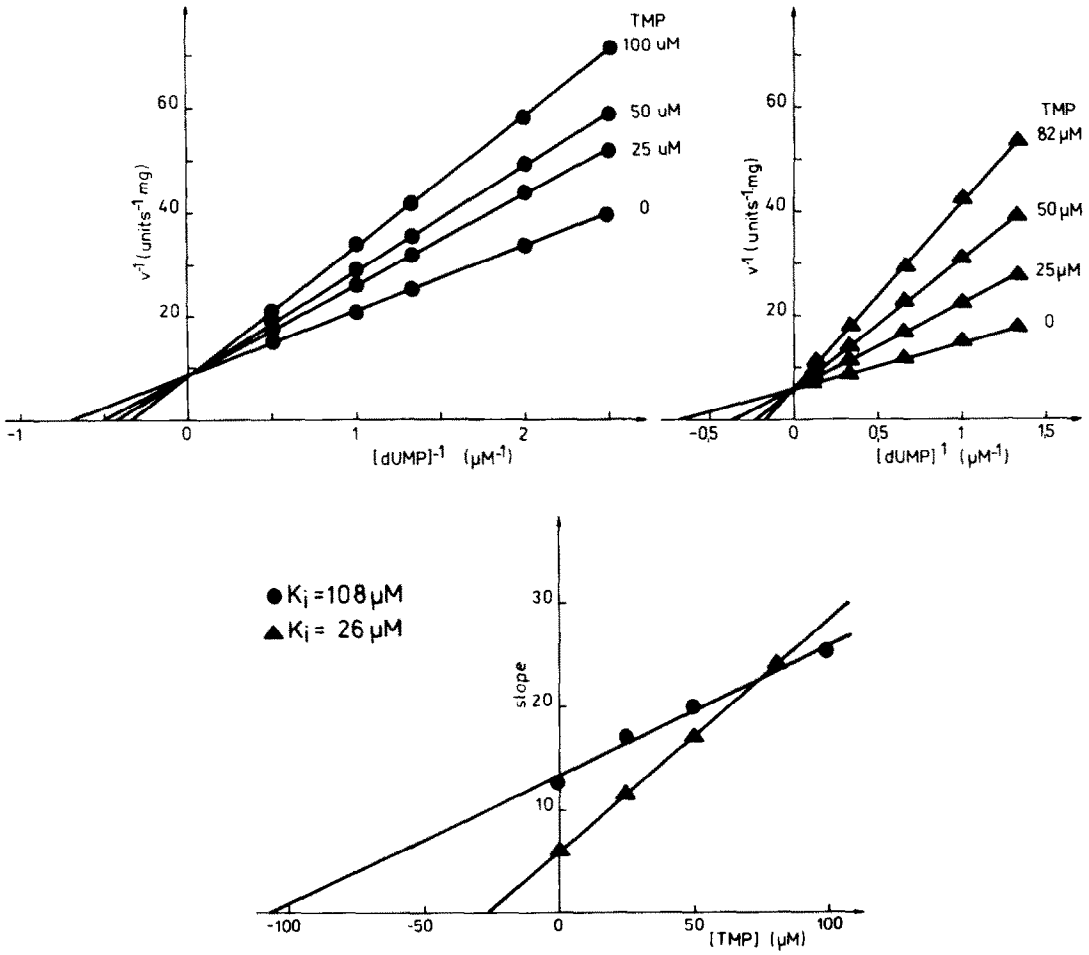

Fig. 4. Inhibition of thymidylate synthetase from Ehrlich ascites carcinoma parental (●) and FdUrd-resistant (▲) cells by dTMP vs dUMP (Lineweaver–Burk plots). For details see Table 2.

and H_2PteGlu is released after dTMP has been formed [21–23].

Interaction with FdUMP

Inhibition by FdUMP was examined at a few different fixed concns with varied dUMP concn. When the reaction was started by addition of thymidylate synthetase, FdUMP inhibited both enzymes competitively towards dUMP (not shown). The apparent K_i determined for the parental cell enzyme was 11 nM, whereas that for the FdUrd-resistant cell enzyme was 182 nM. On the other hand, inhibition of the parental cell enzyme became noncompetitive (apparent K_i = 6 nM) when [^{3}H]dUMP was added after a 10-min preincubation of all other components, including FdUMP, 5,10-CH_2–H_4PteGlu and the enzyme (not shown; the resistant cell enzyme was not examined). Such a dependence of the pattern of inhibition of an enzymic reaction on the order of addition of the components may indicate that the inhibitor acts as a tight-binding one [24, 25].

In order to determine whether FdUMP interacts as a tight binder with the enzymes studied, the method of Ackermann and Potter [26] has been employed. The reaction velocity in samples containing various amounts of thymidylate synthetase and different concns of FdUMP was measured after 2, 4 and 6 min and was linear with time. Ackermann–Potter plots (Fig. 5) demonstrate that FdUMP acts as a tight-binding inhibitor towards both thymidylate synthetases, as manifested in each plot by a series of lines describing inhibition by FdUMP which are parallel to the uninhibited control and which intersect the enzyme axis [24–26]. The data from the Ackermann–Potter plots were plotted also as v_o/v_i (ratio of uninhibited to inhibited reaction velocities) vs FdUMP concn and from this plot I_{50} values (FdUMP concns at which $v_o/v_i = 2$) were estimated (not shown). The plot of I_{50} values against the enzyme concn intersects the I_{50}-axis at K_i [25]. Such plots (Fig. 5) allowed us to determine the FdUMP inhibition constants for thymidylate synthetase from the parental and resistant cell lines to be 1.9 and 19 nM, respectively.

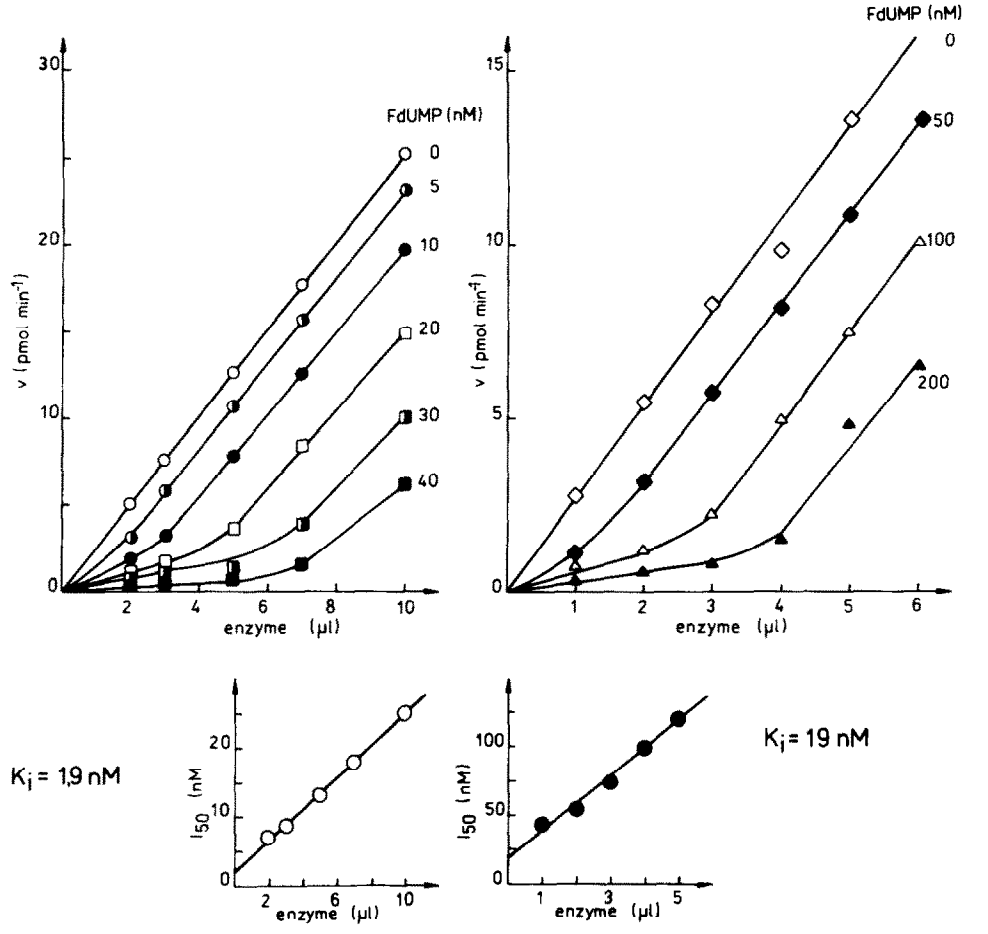

Fig. 5. Inhibition of thymidylate synthetase from Ehrlich ascites carcinoma parental (left plots) and FdUrd-resistant (right plots) cells by FdUMP vs enzyme concn [Ackermann–Potter (upper) plots and I_{50} vs enzyme concn (lower) plots]. Reaction mixtures contained 0.1 M Tris–HCl, pH 7.5, instead of phosphate buffer. The reaction was started by addition of the enzyme. Incubations were 2, 4 and 6 min in duplicates. I_{50} values were estimated from the data presented in the Ackermann–Potter plots (see text for details).

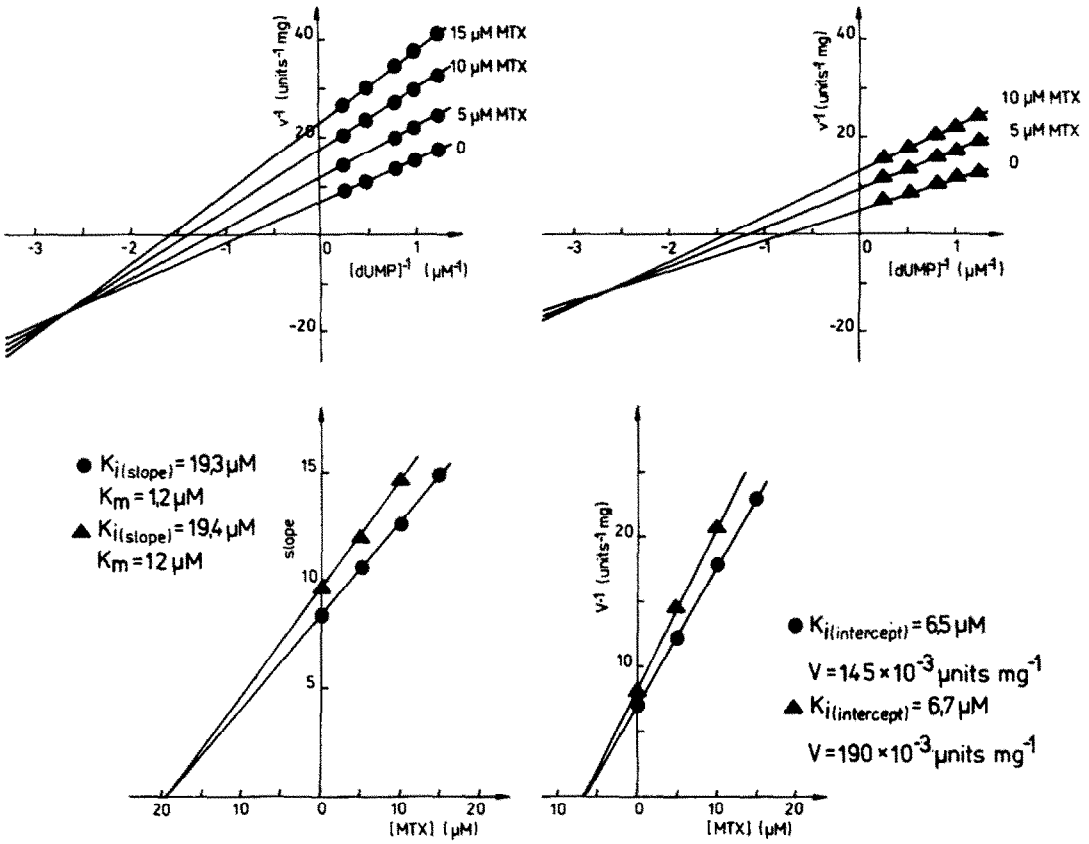

Fig. 6. Inhibition of thymidylate synthetase from Ehrlich ascites carcinoma parental (●) and FdUrd-resistant (▲) cells by amethopterin vs dUMP [Lineweaver–Burk (both upper), slope vs amethopterin concn (left lower) and intercept vs amethopterin concn (right lower) plots]. Reaction mixtures contained 0.1 M Tris–HCl, pH 7.5, instead of phosphate buffer. Incubations were for 2 and 4 min in duplicates. dUMP concn was varied from 0.8 to 4 μM.

Interaction with amethopterin

Amethopterin inhibited both thymidylate synthetases in an identical way. Inhibition was linear-non-competitive with respect to dUMP (Fig. 6). Lineweaver–Burk plots intersected below the $1/S$ axis. The slope and intercept replots showed the K_i slope to be 19.3 and 19.4 whereas the K_i intercept was 6.5 and 6.7 μM for the enzymes from the parental and resistant cell lines, respectively (Fig. 6).

Effect of amethopterin on FdUMP inhibition

As Fig. 7 demonstrates, in the case of both thymidylate synthetases studied, amethopterin (20 μM) reduced the time-dependent inactivation of the enzymes by FdUMP (10 and 200 nM with the parental and resistant cell enzymes, respectively).

DISCUSSION

FdUrd-resistant Ehrlich ascites carcinoma cells compared with the sensitive line seemed to possess at least two possible elements of a resistance mechanism: increased thymidylate *de novo* synthesis and decreased phosphorylation of FdUrd, manifested by higher thymidylate synthetase and lower thymidine kinase sp. acts, respectively. A low thymidine kinase activity was found in several cell lines resistant to FdUrd [5–10]. However, although it is obvious to assume that in consequence of such a phenomenon cells are resistant to FdUrd, further investigation is necessary to evaluate an extent to which a given activity of thymidine kinase present in a cell may influence the sensitivity of this cell to FdUrd. It should be considered that: (i) the relatively low activity of this enzyme may suffice to supply enough FdUMP to inhibit thymidylate synthetase, and (ii) FdUrd might probably be phosphorylated by nucleoside phosphotransferases [27, 28] if those were present in examined cells. On the other hand, any changes in thymidylate synthetase in cells should directly influence sensitivity to FdUrd, since this enzyme is a target for the drug. Heidelberger *et al* [14] described 5-fluorouracil- and FdUrd-resistant Ehrlich ascites cells with thymidylate synthetase apparently insensitive to FdUMP but no difference in the enzyme sp. acts between the resistant and sensitive cells has been mentioned. Therefore a detailed comparison of thymidylate synthetase isolated from the FdUrd-resistant cells developed in our laboratory with the enzyme from the parental Ehrlich ascites carcinoma cells was of interest.

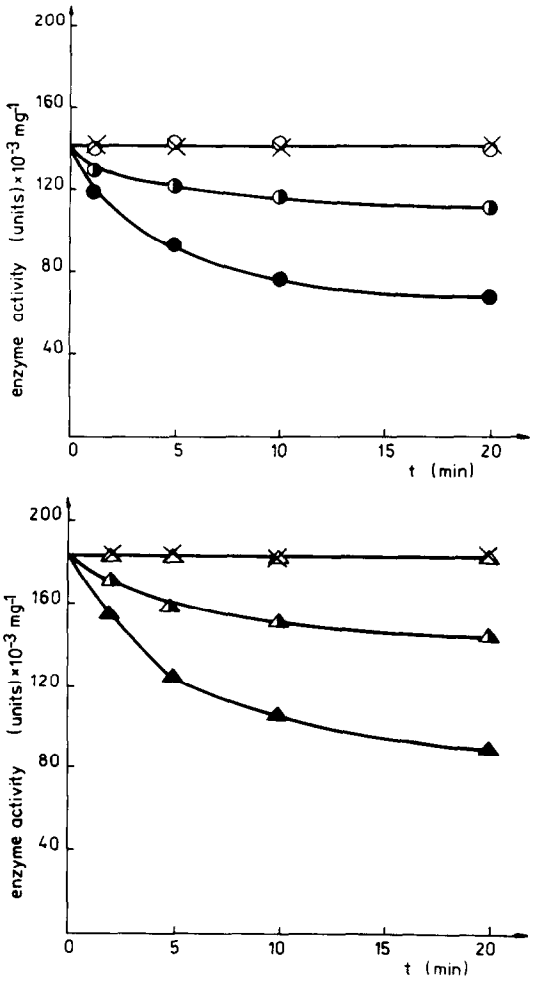

Fig. 7. Effect of amethopterin on inhibition of thymidylate synthetase from Ehrlich ascites carcinoma parental (upper plot) and FdUrd-resistant (lower plot) cells by FdUMP. Reaction mixtures contained 0.1 M Tris–HCl, pH 7.5, instead of phosphate buffer. 20 μM amethopterin (○, △), 20 μM amethopterin and 10 nM FdUMP (◑), 20 μM amethopterin and 200 nM FdUMP (▲), 10 nM FdUMP (●), 200 nM FdUMP (▲) or no inhibitors (×) present.

Results presented in Table 1 show that thymidylate synthetase activity in the extracts from the resistant but not from the sensitive cells is probably partially masked by some material removable by streptomycin sulfate. It seems therefore that the molecular concn of the enzyme present in the exponentially growing resistant cells is almost 2 times higher than that indicated by its sp. act. determined in the crude extract and by its turnover number (see Results). If so, then both the turnover number and the molecular concn of thymidylate synthetase in the resistant cells are higher than in the sensitive cells.

Purified thymidylate synthetase from the resistant cells has a sp. act. (1.3 units/mg protein) greater than the sp. acts of most homogeneous preparations of mammalian thymidylate synthetase so far reported [16, 18, 29–32]. Its turnover number (at least 91 min^{-1}) is 3 times higher than that of the Ehrlich ascites cell enzyme (31 min^{-1}) and over 7 times higher than that of the human leukemia blast cell enzyme (12 min^{-1} assuming two FdUMP-binding sites per molecule [33]), but almost 3 times lower than that of the homogeneous human leukemic CCRF-CEM cell enzyme (250 min^{-1} [34]).

Native thymidylate synthetases from the parental and FdUrd-resistant Ehrlich ascites carcinoma cells showed markedly different Sephadex G-100 gel filtration behavior reflected in different mol. wts estimated in those experiments (see Results). This result is rather unexpected in view of almost identical subunit mol. wts determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, and probably indicates a distinct difference in the Stokes' radii [35] rather than in the mol. wts of the native dimers. Since in our gel filtration experiments elution of the enzyme-[^{3}H]FdUMP–5,10-CH_2–H_4PteGlu complex was monitored (for details of the method see Ref. 16), then the different Stokes' radii may not necessarily characterize molecules of the free enzymes.

Thymidylate synthetase from the FdUrd-resistant Ehrlich ascites carcinoma cells underwent a temp-dependent conformational change at 35.5° manifested by a biphasic Arrhenius plot (Fig. 2). The reaction catalyzed by thymidylate synthetases from Ehrlich ascites parental cells [16], human leukemic cells [33] and HeLa cells [32] showed a similar dependence on temp but the resistant cell enzyme exhibited the lowest activation energy above the transitional temp.

The kinetics of interactions of thymidylate synthetase from both sensitive and resistant Ehrlich ascites cells with the substrates and products were very much alike (Fig. 3 and Table 2) but for a striking difference found between the K_i for dTMP when the dUMP concn was varied (Fig. 4). Both enzymes act by a sequential mechanism. The results of product inhibition studies do not allow us to distinguish whether it is an ordered mechanism with dUMP adding to thymidylate synthetase before 5,10-CH_2–H_4PteGlu and dihydrofolate leaving the central complex before dTMP or an Iso Theorell-Chance mechanism with 5,10-CH_2–H_4PteGlu adding first and H_2PteGlu released after dTMP formation. However, the ordered mechanism is favored by the observation that both enzymes examined seem to bind dUMP in the absence of 5,10-CH_2–H_4PteGlu, forming electrophoretically distinguishable binary complexes (Fig. 1C) and by the fact that both enzymes could be adsorbed only in the presence of dUMP on the column containing an immobilized 5,10-CH_2–H_4PteGlu analogue, 10-formyl-5,8-dideazafolate. Such a mechanism is consistent with that proposed for thymidylate synthetase from human tumor cells [33] and *Lactobacillus casei* [36], as well as with the results of product inhibition kinetic studies on the enzyme from the chicken embryo [37] and of studies of FdUMP binding by the human CCRF-CEM cell enzyme [38], but not with the mechanism suggested by Reyes and Heidelberger [39] for Ehrlich ascites thymidylate synthetase (sequentially ordered but with 5,10-CH_2–H_4PteGlu added before dUMP and dTMP released before dihydrofolate).

FdUMP interacts with thymidylate synthetase

from both sensitive and resistant Ehrlich ascites carcinoma cells as a tightly-binding inhibitor, as indicated by the dependence of the inhibition on enzyme concn (Fig. 5) and time (Fig. 7). A similar type of inhibition by FdUMP was reported for the enzyme from *Streptococcus faecalis* [40], the chicken embryo [37], human leukemic blast cells [33] and human CCRF-CEM cells [38]. On the other hand, inhibition of thymidylate synthetase activity in crude extracts of Ehrlich ascites carcinoma by FdUMP has been reported to be not of a tight-binding type [15, 39]. FdUMP behaved as a classical inhibitor competitive with dUMP and only the apparent K_i changed from 31 nM when the reaction was started without preincubation to 3.1 nM when the enzyme was preincubated for 10 min with FdUMP and 5,10-CH_2–H_4PteGlu [39]. Obviously these results as well as the results describing the kinetics of Ehrlich ascites carcinoma thymidylate synthetase inhibition by dTMP (noncompetitive; K_i slope = 0.41 mM [39]), presented by the same laboratory, are virtually different from ours (Fig. 4 and Table 2) concerning the same enzyme. It is not clear whether this is due to the various states of purity of the thymidylate synthetase preparations used or to the fact that Reyes and Heidelberger [39] studied the enzyme reaction at pH 6.7 whereas we did it at pH 7.5.

The kinetics of amethopterin inhibition of thymidylate synthetase from Ehrlich ascites cells, both parental and FdUrd-resistant (Fig. 6), and the reduction of tight-binding FdUMP inhibition of the enzyme by amethopterin (Fig. 7) were similar to those reported for the enzyme from human leukemia blast cells [33].

The results presented here demonstrate that thymidylate synthetase from the FdUrd-resistant Ehrlich ascites carcinoma cells has distinctly different properties from the enzyme from the parental cells. A 3 times higher turnover number and a 10 times lower affinity of the resistant cell enzyme towards FdUMP in connection with a 2 times higher concn of this enzyme in the cells seem to be elements of the resistance mechanism which may also involve thymidine kinase deficiency.

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