Biochemical Pharmacology, Vol. 31, No. 2, pp. 217–223, 1982.
Printed in Great Britain.

0006–2952/82/020217–07 $03.00/0

PROPERTIES OF THYMIDYLATE SYNTHETASE FROM EHRLICH ASCITES CARCINOMA CELLS

EFFECT OF Mg^{2+} AND $MgATP^{2-}$

MALGORZATA JASTREBOFF, BARBARA KEDZIERSKA and WOJCIECH RODE*
Department of Cellular Biochemistry, Nencki Institute of Experimental Biology, 02-093 Warsaw, Poland

(*Received* 19 *November* 1980; *accepted* 2 *June* 1981)

Abstract—Ehrlich ascites carcinoma thymidylate synthetase was purified to electrophoretic homogeneity by affinity chromatography on 10-formyl-5,8-dideazofolate-ethyl-Sepharose. Electrophoretic analysis of the formation of the enzyme–5–fluorodeoxyuridylate–5,10–methylenetetrahydrofolate complexes showed the presence of two binding sites for 5–fluorodeoxyuridylate on the enzyme molecule. Molecular weight of the native enzyme was found to be 78,500, whereas that of its monomer was 38,500. The apparent Michaelis constants for dUMP and (±)-L-5,10-methylenetetrahydrofolate were 1.3 ± 0.4 and 32.2 ± 0.7 μM respectively. Phosphate acted as a weak inhibitor, competitive toward dUMP. The enzyme reaction exhibited a temperature-dependent change of activation energy, reflected in the binding affinity of dUMP, with a transitional temperature of 35.8°. Both Mg^{2+} and $MgATP^{2-}$ were strong activators of the enzyme, $MgATP^{2-}$ being more effective.

Thymidylate synthetase (methylenetetrahydrofolate:deoxyuridine–5′–monophosphate C-methyltransferase; EC 2.1.1.45) catalyzes the conversion of deoxyuridylate (dUMP) to thymidylate in a concerted reaction involving transfer and reduction of a hydroxymethyl group from 5,10–methylenetetrahydropteroylmono- [1] or oligoglutamate [2, 3]. As the only source of thymine deoxynucleotides synthesized *de novo* in a cell, this enzyme is a target for cancer chemotherapy [4].

Considering the numerous attempts being made to design new thymidylate synthetase-directed antimetabolites active in the chemotherapy of tumors, it is of interest to know whether properties of the enzyme in different tumor tissues of the same specific origin are similar or not. Recently described properties of human thymidylate synthetase isolated from HeLa cells [5] resemble those of the enzyme isolated from human leukemia blast cells [6]. On the other hand, much higher specific activity of homogeneous thymidylate synthetase from human CCRF–CEM leukemic cells [7] than that of the homogeneous enzyme from HeLa cells [5] indicates that human tumor thymidylate synthetases may be different.

Thymidylate synthetases purified to homogeneity from two mouse tumor tissues, Ehrlich ascites carcinoma cells [8] and L1210 leukemic cells [9], appeared to be either monomer (molecular weight of about 70,000) or dimer (subunit molecular weight of 38,500) respectively. Since entirely different methodologies were used to isolate and stabilize the enzyme from each of these materials, we purified Ehrlich ascites carcinoma thymidylate synthetase and studied its properties by the methods applied earlier to the L1210 enzyme [9].

MATERIALS AND METHODS

(±)-L-Tetrahydrofolic acid was prepared by catalytic hydrogenation of folic acid (Merck, Darmstad, Federal Republic of Germany) according to the method of Lorenson *et al.* [10] except that 2-mercaptoethanol was used instead of 2,3-dimercaptopropanol. [5-^{3}H]dUMP (12.7 Ci/mmole), purchased from The Radiochemical Centre (Amersham, U.K.), was purified from tritium not absorbable on charcoal [11]. Other reagents were obtained from the following sources: [6-^{3}H]5-fluorodeoxyuridylate (20 Ci/mmole) from Moravek Biochemicals (City of Industry, CA, U.S.A.); dUMP, 2-mercaptoethanol and ribonuclease from Koch-Light (Colnbrook, U.K.); acrylamide and methylene bisacrylamide from Bio-Rad (Richmond, CA, U.S.A.); *N,N,N′,N′*-tetramethylenediamine from Fluka (Buchs, Switzerland); 5-fluorodeoxyuridylate, bovine serum, albumin, ovalbumin and DEAE-cellulose (coarse mesh) from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); Triton X-100 and Norit A from Serva (Heidelberg, Federal Republic of Germany); Sephadex G-100 and blue dextran 2000 from Pharmacia Fine Chemicals (Uppsala, Sweden); and cytochrome *c* from Reanal (Budapest, Hungary). Homogeneous preparations of actin and tropomyosin were gifts from Dr. E. Nowak-Olszewska from the Nencki Institute of Experimental Biology (Warsaw, Poland). All other reagents used were of the highest quality available from commercial sources.

Maintenance of Ehrlich ascites carcinoma cells. The cells were maintained in albino Swiss mice. Transplantations were done once each 5–8 days by

* Author to whom all correspondence should be addressed: Wojciech Rode, Ph.D., Department of Cellular Biochemistry, Nencki Institute of Experimental Biology, 3 Pasteur St., 02-093 Warsaw, Poland.

intraperitoneal inoculation of 5×10^5–10^6 cells in 0.5 ml of ascites fluid diluted once with phosphate buffered saline.

The cells were harvested 5–8 days after inoculation by centrifugating the ascites fluid at 300 *g* for 10 min, washing the cells twice with phosphate buffered saline, and storing them as a pellet at −20° until use.

Affinity adsorbent. The same adsorbent, 10-formyl-5,8-dideazafolate-ethyl-Sepharose, used earlier to purify L1210 thymidylate synthetase [9], was used to purify the enzyme from Ehrlich ascites carcinoma cells.

Enzyme assay. Thymidylate synthetase activity was estimated by a modification of the isotopic method of Roberts [12]. Unless otherwise indicated the standard reaction mixture in a total volume of 40 μl contained: 2.0 nmoles [5-^{3}H]dUMP (about 3×10^7 cpm/μmole), 20 nmoles (±)-L-tetrahydrofolate, 0.2 μmole formaldehyde, 4 μmoles 2-mercaptoethanol, 2 μmoles NaF, 2 μmoles phosphate buffer pH 7.5, 0.4 μmole ascorbic buffer, pH 7.5, and the enzyme ($\leq 3.3 \times 10^{-6}$ units). In controls, the enzyme was substituted with buffer. The reaction was started by addition of the enzyme and was terminated after incubation (≤ 1 hr) at 37° by addition of 200 μl of a charcoal suspension (Norit A, 100 mg/ml) in a 2% solution of trichloroacetic acid. The mixture was centrifuged at 14,000 *g* for 0.5 min. A 100 μl sample of the supernatant fraction was added to 5 ml of scintillant [2.8 g of 2,5-diphenyloxazol and 0.07 g of 1,4-di-2-(5-phenyloxazoyl)-benzene dissolved in 700 ml of toluene and mixed with 300 ml of ethanol] and counted in a Packard 2003 liquid scintillation counter. All assays were performed in duplicate. Activity of the enzyme is expressed in units defined as the amount required to release 1 μg equivalent of tritium (equivalent to formation of 1 μmole of TMP) per min under conditions of the assay.

Purification procedure. The cell pellets were thawed with 3 vol. of 0.01 M phosphate buffer, pH 7.5, containing 0.1 M KCl and 0.01 M 2-mercaptoethanol. The resulting mixture was sonicated (MSE ultrasonic power unit, 5 × 20 sec at 1.5A), and centrifuged at 20,000 *g* for 30 min at 4°. The supernatant, fraction further referred to as the crude extract, was saved. All subsequent steps were carried out at 4°.

To the stirred extract solid ammonium sulfate was added slowly to bring it to 30% saturation. The suspension was stirred for an additional 15 min and centrifuged at 20,000 *g* for 20 min, and the pellet was discarded. The supernatant fraction was brought to 70% saturation with ammonium sulfate, stirred, and centrifuged as described above. The pellet was dissolved in a small amount of 0.01 M phosphate buffer, pH 7.5, containing 0.01 M 2-mercaptoethanol and 0.1% Triton X-100 and was dialyzed for 2 hr at 4° against the same buffer. The resulting preparation is referred to as the 30–70% ammonium sulfate fraction.

To the 30–70% ammonium sulfate fraction (corresponding to 0.25 unit of enzyme activity), 20 μM (final concentration) dUMP was added and the solution was passed slowly (about 0.5 ml/min) through the affinity column (2.5 × 6 cm) previously saturated with 0.01 M phosphate buffer, pH 7.5, containing

Table 1. Purification of thymidylate synthetase from Ehrlich ascites carcinoma cells*

Purification stage	Volume (ml)	Total activity (10^{-3} units)	Total protein (mg)	Specific activity (10^{-3} units/mg protein)	Purification	Yield (%)
Crude extract†	254	247	1004	0.247	1.0	100
30–70% $(NH_4)_2SO_4$ fraction	251	266	724	0.380	1.5	101
Pooled fractions after first affinity chromatography	42	184	0.63	292.0	1184	74
Pooled fractions after second affinity chromatography	26	175	0.41	427.2 (1366.7‡)	1732	71

* For details see Materials and Methods.
† Corresponding to 100 g of the cells.
‡ Assayed in the presence of 40 mM $MgCl_2$.

0.01 M 2-mercaptoethanol, 0.1% Triton X-100 and 20 μM dUMP (Buffer A). The column was washed with 0.2 M phosphate buffer, pH 7.5, containing 0.5 M KCl, 0.01 M 2-mercaptoethanol, 0.1% Triton X-100, and 20 μM dUMP (Buffer B, 1000 ml), equilibrated again with Buffer A (500 ml), and thymidylate synthetase eluted with Buffer A without dUMP (Buffer C, 1000 ml). Before the elution step a small DEAE-cellulose column (0.9 × 4 cm) equilibrated with Buffer C was connected to the affinity column in a series. The enzyme adsorbed on DEAE-cellulose was eluted with 0.2 M phosphate buffer, pH 7.5, containing 0.01 M 2-mercaptoethanol, 0.1% Triton X-100 and 20% sucrose. To the pooled active fractions dUMP was added (final concentration 20 μM) and the affinity chromatography, followed by concentration of thymidylate synthetase on DEAE-cellulose, was repeated as described above.

Electrophoretic analysis. The samples of thymidylate synthetase preparations were tested for homogeneity by polyacrylamide gel electrophoresis as described earlier [9]. Identification of thymidylate synthetase on gels was accomplished either through testing its ability to form a ternary complex with 5-fluorodeoxyuridylate and 5,10-methylenetetrahydrofolate (Fig. 1) or by assaying its activity in gel slices (both as described earlier [9]).

Molecular weight of denatured thymidylate synthetase was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as described by Weber and Osborn [13]. Bovine serum albumin (M_r = 67,000), ovalbumin (M_r = 45,000), actin (M_r = 42,000), tropomyosin (M_r = 36,000), ribonuclease (M_r = 13,700) and cytochrome *c* (M_r = 12,400) were used as molecular weight standards.

Analytical gel filtration. Molecular weight of native thymidylate synthetase was determined by gel filtration on a Sephadex G-100 column (2.6 × 64 cm). The column was developed at room temperature with 0.05 M Tris–HCl buffer, pH 7.5, containing, 0.1 M KCl and 0.01 M 2-mercaptoethanol. Fractions 3.3 ml were collected at a flow rate of 1 ml/min. The ratio of the elution volume of reference proteins

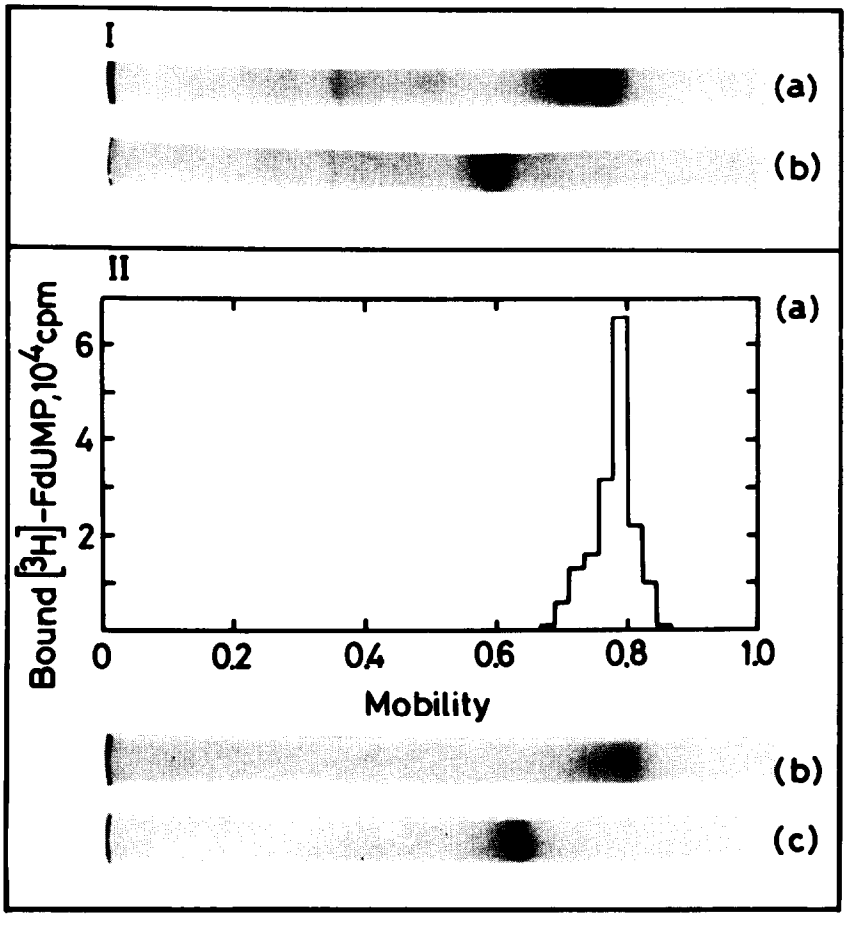

Fig. 1. Polyacrylamide gel electrophoresis of Ehrlich ascites carcinoma thymidylate synthetase preparation after first (I) and second (II) affinity chromatography (see Table 1). (I) The enzyme preparation was treated before electrophoresis for 15 min at 37° with 30 μM 5-fluorodeoxyuridylate and 100 μM 5,10-methylenetetrahydrofolate (a) or was untreated (b). (II) Each sample applied to a gel contained 5 μg of protein. The samples were treated for 15 min at 37° with 30 μM [^{3}H]-5-fluorodeoxyuridylate and 100 μM 5,10-methylenetetrahydrofolate. Gels were either sliced and assayed for bound label (a) or were stained for protein (b); in (c), the sample was not treated before electrophoresis.

(bovine serum albumin, ovalbumin, ribonuclease and cytochrome *c*) to the void volume of the column, plotted against the logarithm of molecular weight of the reference proteins, formed a standard curve [14].

A sample of purified thymidylate synthetase (5–10 μg protein) in 0.2 M Tris–HCl buffer, pH 7.5, containing 0.01 M 2-mercaptoethanol was incubated for 15 min at 37° with 1 μM [6-^{3}H]-5-fluorodeoxyuridylate, 100 μM (±)-L-tetrahydrofolate, and 1 mM formaldehyde. Gel filtration of the enzyme was performed in the presence of 100 μM (±)-L-tetrahydrofolate and 1 mM formaldehyde in the buffer. In order to identify thymidylate synthetase, collected fractions were monitored for radioactivity.

Protein determination. The procedure of Sedmak and Grossberg [15] was used with bovine serum albumin as a standard.

RESULTS

Results of the purification of Ehrlich ascites carcinoma thymidylate synthetase are presented in Table 1. A single affinity chromatography step did not result in a homogeneous preparation of the enzyme [Fig. 1 (I)]. This preparation, however, could be purified further by simply repeating the same affinity chromatography step.

Native Ehrlich ascites carcinoma thymidylate synthetase incubated with 5-fluorodeoxyuridylate (FdUMP) and 5,10-methylenetetrahydrofolate formed two types of electrophoretically separable complexes (Fig. 1), both containing FdUMP [Fig. 1 (IIa)]. Formation of the complexes depended on the concentration of FdUMP in the incubation mixture, provided 5,10-methylenetetrahydrofolate was present in an excess (not shown).

Electrophoresis of the thymidylate synthetase preparation with dUMP present in electrode buffer showed a new form of the enzyme ($R_F = 0.7$); (Fig. 2), presumably thymidylate synthetase-dUMP complex.

A complex of Ehrlich ascites carcinoma thymidylate synthetase with 5-fluorodeoxyuridylate and 5,10-methylenetetrahydrofolate, denatured with trichloroacetic acid, was stable to 6 M urea and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Fig. 3). The molecular weight of the denaturated enzyme was found to be 38,500 (Fig. 3).

Gel filtration of thymidylate synthetase in Sephadex G-100 under conditions preserving native structure of the enzyme showed its molecular weight to be 78,500. However, when the gel filtration of a sample of the enzyme containing 0.1% Triton X-100 was performed, its molecular weight was 88,000 (not shown).

Cofactor requirements of the thymidylate synthetase reaction were studied by omitting appropriate components of the reaction mixture. The rate of the tritium release was negligible (about 3 per cent of control) in the absence of (±)-L-tetrahydrofolate and approached 22 per cent of control in the absence of formaldehyde (not shown).

Dithiothreitol and 2-mercaptoethanol were both potent activators of the enzyme, with the optimal concentration of the former being lower than of the latter (Fig. 4).

The apparent Michaelis constant of the enzyme reaction for dUMP, but not that for (±)-L-5,10-meth-

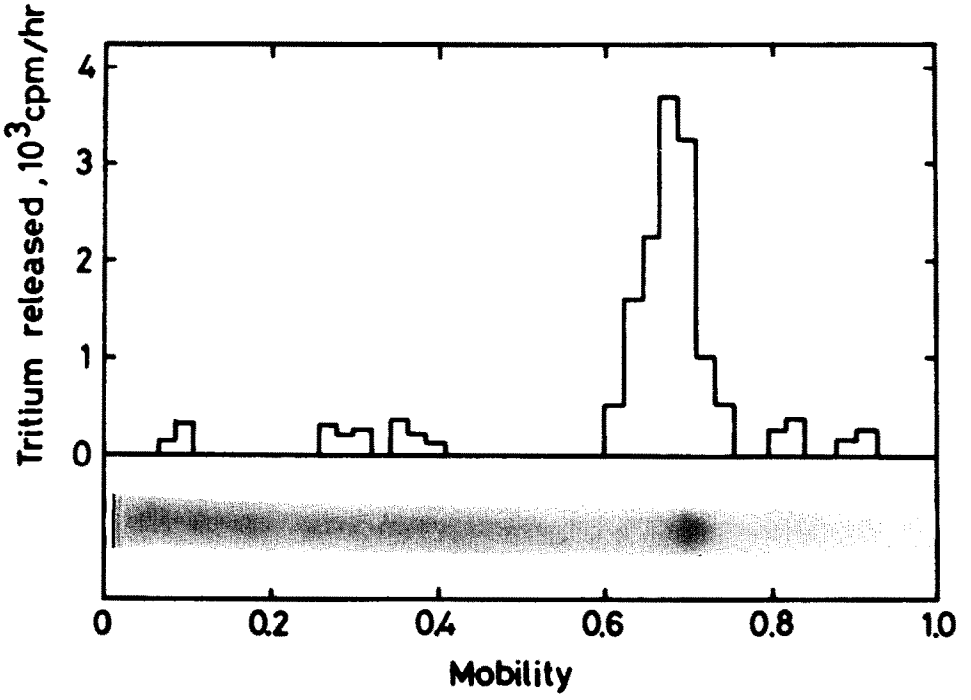

Fig. 2. Polyacrylamide gel electrophoresis of Ehrlich ascites carcinoma thymidylate synthetase with dUMP (20 μM) present in electrode buffer. Gels were either stained (lower panel) or sliced and assayed for the enzyme activity (upper panel).

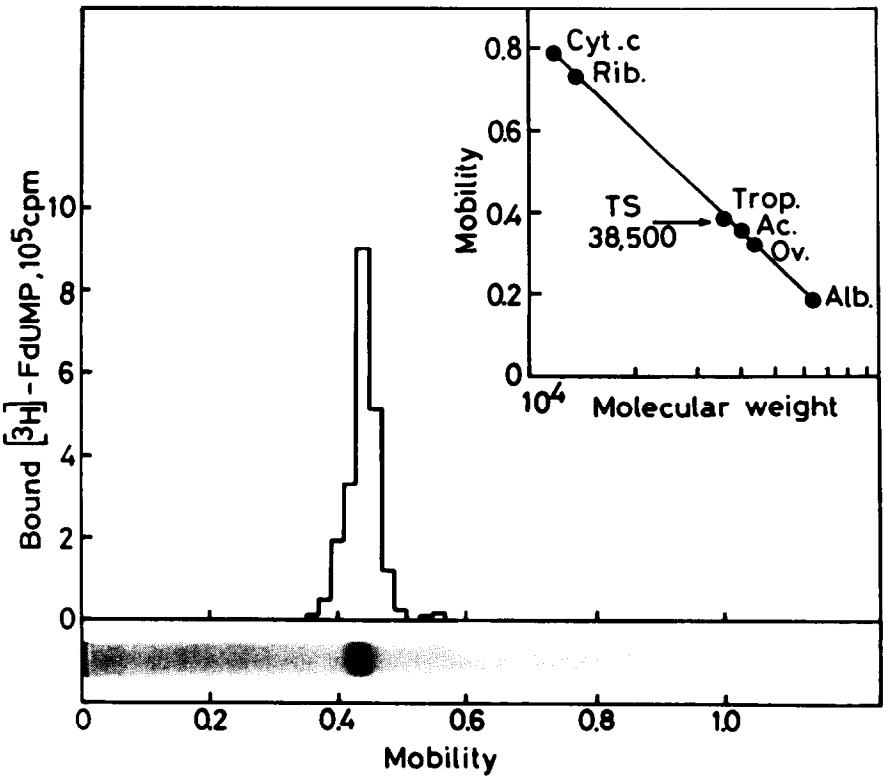

Fig. 3. SDS polyacrylamide gel electrophoresis and molecular weight determination of denatured Ehrlich ascites carcinoma thymidylate synthetase. Before electrophoresis, samples were treated with [^{3}H]-5-fluorodeoxyuridylate and 5,10-methylenetetrahydrofolate and were prepared as described earlier [9]. Upper gel was sliced and assayed for radioactivity. Abbreviations: Cyt. c, cytochrome *c*; Rib., ribonuclease; Trop., tropomyosin; Ac., actin; Ov., ovalbumin; Alb., bovine serum albumin; and TS, thymidylate synthetase.

ylenetetrahydrofolate, was dependent on the buffer present in the reaction mixture. Phosphate buffer (0.1 M) increased the K_m value for dUMP from 1.3 ± 0.4 μM (assayed in 0.1 M Tris–HCl buffer) to 2.4 ± 0.2 μM. No change of the apparent V_{max} value was observed (Table 2).

The dependence of the thymidylate synthetase reaction on temperature expressed as an Arrhenius plot (Fig. 5A) exhibited a biphasic curve with a transitional temperature at 35.8°. Different activation energies below and above this temperature were found to reflect different changes of standard enthalpy for the dissociation of the enzyme–dUMP–5,10-methylenetetrahydrofolate complex (Fig. 5B).

It was found that Ehrlich ascites carcinoma thymidylate synthetase was activated strongly up to over 300 per cent of its control activity by magnesium

Table 2. Kinetic properties of the reaction catalyzed by thymidylate synthetase from Ehrlich ascites carcinoma cells: effect of phosphate buffer*

Buffer present in the reaction mixture	Variable substrate			
	dUMP		5,10-CH_2-H_4PteGlu	
	K_m (μM)	V_{max} (10^{-3} units/mg protein)	K_m (μM)	V_{max} (10^{-3} units/mg protein)
0.1 M Phosphate (pH 7.5)	2.4 ± 0.2	138.3 ± 3.3	25.2 ± 3.2	131.7 ± 5.0
0.1 M Tris–HCl (pH 7.5)	1.3 ± 0.4	136.7 ± 1.7	32.2 ± 0.7	138.3 ± 1.7

* The concentrations of dUMP as the variable substrate were 0.37, 0.74, 0.92, 1.15, 1.42, 1.80, 2.70 and 6.73 μM [183 μM (±)-L-5,10-methylenetetrahydrofolate (5,10-CH_2-H_4PteGlu)] whereas those of 5,10-CH_2-H_4PteGlu as the variable substrate were 9.13, 10.95, 14.60, 18.25, 27.37, 45.60 and 54.75 μM [6.75 μM dUMP]. Tritium release was measured after 2, 4 and 6 min of incubation. All assays were done in triplicate.

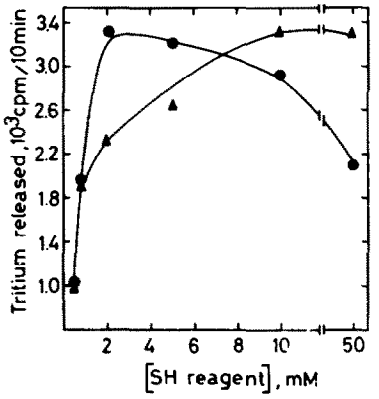

Fig. 4. Effect of SH-reagents on Ehrlich ascites carcinoma thymidylate synthetase activity. Key: (●—●) dithiothreitol; and (▲—▲) 2-mercaptocthanol.

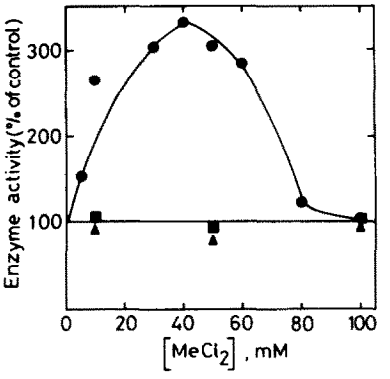

Fig. 6. Effect of divalent cations on Ehrlich ascites carcinoma thymidylate synthetase activity. Key: (●—●) $MgCl_2$; (■—■) $CaCl_2$; and (▲—▲) $MnCl_2$.

ions. Optimal activation was observed at 40–60 mM Mg^{2+}. At concentrations higher than 80 mM, Mg^{2+} lost its activating properties (Fig. 6). The K_m for dUMP was not dependent on the presence of 50 mM Mg^{2+} (not shown). Neither Ca^{2+} nor Mn^{2+} had any influence on the enzyme activity (Fig. 6).

The activity of thymidylate synthetase in the presence of 40 mM Mg^{2+} was increased further by 50 μM ATP (Table 3) whereas the same nucleotide at concentrations below 20 μM and above 500 μM was

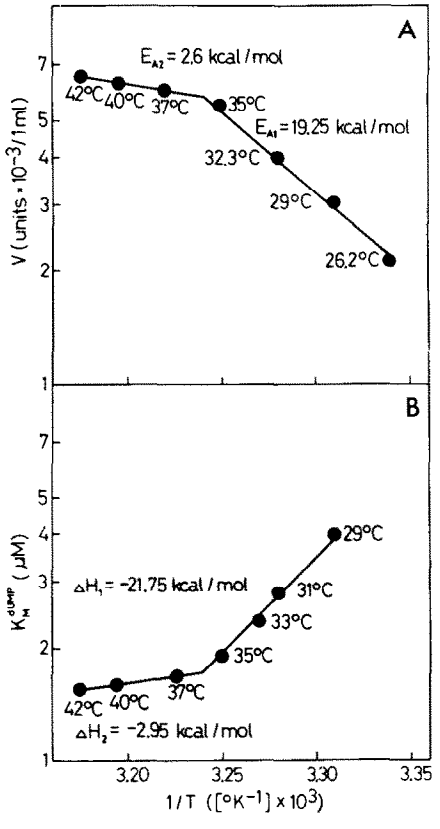

Fig. 5. Dependence of the thymidylate synthetase reaction on temperature. Arrhenius plot (A) and log K_m^{dUMP} vs $1/T$ plot (B).

Table 3. Effect of adenine nucleotides on thymidylate synthetase activity*

Additions	Relative activity (%)
None	100
$MgCl_2$ (40 mM)	368
ATP (50 μM)	96
$MgCl_2$ (40 mM) + ATP (50 μM)	641
ADP (50 μM)	98
$MgCl_2$ (40 mM) + ADP (50 μM)	73
AMP (50 μM)	95
$MgCl_2$ (40 mM) + AMP (50 μM)	114

* All assays were done as described in Materials and Methods.

inhibitory (not shown). No effect of ATP could be observed in the absence of Mg^{2+}. Both ADP and AMP in the presence but not in the absence, of Mg^{2+} inhibited the enzyme (Table 3).

DISCUSSION

Thymidylate synthetase from Ehrlich ascites carcinoma cells, purified as described here, resembled that from mouse leukemia L1210 cells [9]. Both enzymes interacted in an identical way with 10-formyl-5,8-dideazafolate-ethyl-Sepharose column so that their affinity chromatography on this material could be performed using the same conditions (for a comparison see conditions of affinity chromatography of human thymidylate synthetase on the same column [5]. Electrophoretic properties, molecular weights, and subunit compositions were also similar. In both cases thymidylate synthetase formed two types of complexes in the presence of 5-fluorodeoxyuridylate and 5,10-methylenetetrahydrofolate, showing the presence of two binding sites for 5-fluorodeoxyuridylate on the enzyme dimeric molecule.

On the other hand, some properties of the enzyme presented here, i.e. subunit composition (a dimer of 78,500 mol. wt) and the apparent Michaelis constants [2.4 ± 0.2 μM for dUMP and 25 ± 0.2 μM for

(±)-L-5,10-methylenetetrahydrofolate, both in 0.1 M phosphate buffer, pH 7.5, or 1.3 ± 0.4 μM for dUMP in 0.1 M Tris–HCl buffer, pH 7.5; Table 2] differ distinctly from the properties of Ehrlich ascites carcinoma thymidylate synthetase described earlier (a monomer of about 70,000 mol. wt, K_m for dUMP 6.3 μM in 0.1 M phosphate buffer, pH 6.7 [7] or 4.0 μM in 0.02 M Tris–HCl buffer, pH 7.4 [16], and for (±)-L-5,10-methylenetetrahydrofolate 43 μM in 0.1 M phosphate buffer, pH 6.7 [8]). We feel that application of Triton X-100 as a stabilizing agent for thymidylate synthetase might have been responsible for this situation.

Triton X-100 was found to stabilize L1210 thymidylate synthetase [9], and we present evidence for its interaction with Ehrlich ascites carcinoma enzyme in this paper. Such an interaction, resulting in formation of a complex of the enzyme with the detergent, has to take place since addition of the latter to a sample of homogeneous thymidylate synthetase decreased its elution volume on analytical gel filtration (see Results).

The results of electrophoretic analysis of Ehrlich ascites carcinoma thymidylate synthetase in the presence of dUMP in the electrode buffer (Fig. 2) revealed that the enzyme may form a binary complex with dUMP. Formation of such a complex by thymidylate synthetase from amethopterin-resistant *Lactobacillus casei* was shown by circular dichroic [17] and equilibrium dialysis [18] studies.

The Ehrlich ascites carcinoma enzyme catalyzed tetrahydrofolate-dependent release of tritium from [5-^{3}H]dUMP (see Results). This activity has been described previously for the enzyme from both *Escherichia coli* [19] and human leukemia cells [3].

Phosphate has been shown to decrease the affinity of amethopterin-resistant *L. casei* thymidylate synthetase for dUMP [18] and in the present experiments it also acted as a weak competitive inhibitor of the Ehrlich ascites carcinoma enzyme (Table 2).

Ehrlich ascites carcinoma thymidylate synthetase underwent a temperature-dependent conformational change at 35.8° manifested by biphasic dependencies of both log V and log K_m on $1/T$ (Fig. 5). A similar result was described for the enzyme from both human leukemia blast cells [6] and HeLa cells [5].

Sensitivity to activation by Mg^{2+} is known to be a common property of bacterial thymidylate synthetase [20–24]. As far as the enzyme of mammalian origin is concerned, only thymidylate synthetase from human leukemic leukocytes [25] and one of two forms of the enzyme found in pig thymus [26] were reported to respond to such an activation. Besides, Mg^{2+} was found to counteract an inhibition of calf thymus thymidylate synthetase by ATP [27]. We present here results indicating that Ehrlich ascites carcinoma thymidylate synthetase was activated by high concentrations of Mg^{2+} (Fig. 6) as well as by $MgATP^{2-}$ complex (Table 3). Narrow ranges of effective concentrations of either Mg^{2+} or $MgATP^{2-}$ may indicate allosteric effects. This phenomenon, as well as inhibition of the Mg^{2+}-activated enzyme by both ADP and AMP, raise the question of whether Ehrlich ascites carcinoma thymidylate synthetase is under the control of energy charge in the cells. This problem is currently under investigation.

REFERENCES

1. G. K. Humphreys and D. M. Greenberg, *Archs Biochem. Biophys.* **78**, 275 (1958).
2. R. L. Kisliuk, Y. Gaumont and C. M. Baugh, *J. biol. Chem.* **249**, 4100 (1974).
3. B. J. Dolnick and Y.-C. Cheng, *J. biol. Chem.* **253**, 3563 (1978).
4. P. V. Danenberg, *Biochim. biophys. Acta* **473**, 73 (1977).
5. W. Rode, B. J. Dolnick and J. R. Bertino, *Biochem. Pharmac.* **29**, 723 (1980).
6. B. J. Dolnick and Y.-C. Cheng, *J. biol. Chem.* **252**, 7697 (1977).
7. A. Lockshin, R. G. Moran and P. V. Danenberg, *Proc. natn. Acad. Sci. U.S.A.* **76**, 750 (1979).
8. A Fridland, R. J. Langenbach and C. Heidelberger, *J. biol. Chem.* **246**, 7110 (1971).
9. W. Rode, K. J. Scanlon, J. Hynes and J. R. Bertino, *J. biol. Chem.* **254**, 11538 (1979).
10. M. Y. Lorenson, G. F. Maley and F. Maley, *J. biol. Chem.* **242**, 3332 (1967).
11. W. Rode and H. Szymanowska, *Insect Biochem.* **6**, 333 (1976).
12. D. Roberts, *Biochemistry* **5**, 3546 (1966).
13. K. Weber and M. Osborn, *J. biol. Chem.* **244**, 4406 (1969).
14. J. R. Whitaker, *Analyt. Chem.* **35**, 1950 (1963).
15. J. J. Sedmak and S. E. Grossberg, *Analyt. Biochem.* **79**, 544 (1977).
16. A. Kampf, R. L. Barfknecht, P. J. Shaffer, S. Osaki and M. P. Mertes, *J. med. Chem.* **19**, 903 (1976).
17. R. P. Leary, N. Beaudette and R. L. Kisliuk, *J. biol. Chem.* **250**, 4864 (1975).
18. J. H. Galivan, G. F. Maley and F. Maley, *Biochemistry* **15**, 356 (1976).
19. M. J. S. Lomax and G. R. Greenberg, *J. biol. Chem.* **242**, 1302 (1967).
20. A. J. Wahba and M. Friedkin, *J. biol. Chem.* **237**, 3794 (1962).
21. B. M. McDougall and R. L. Blakley, *J. biol. Chem.* **236**, 832 (1961).
22. T. C. Crusberg, R. Leary and R. L. Kisliuk, *J. biol. Chem.* **245**, 5292 (1970).
23. R. B. Dunlap, N. G. L. Harding and F. M. Huennekens, *Biochemistry* **10**, 88 (1971).
24. R. W. McCuen and F. M. Sirotnak, *Biochim. biophys. Acta* **384**, 369 (1975).
25. R. Silber, B. W. Gabrio and F. M. Huennekens, *J. clin. Invest.* **42**, 1913 (1963).
26. V. S. Gupta and J. B. Meldrum, *Can. J. Biochem.* **50**, 352 (1972).
27. H. Hornishi and D. M. Greenberg, *Biochim. biophys. Acta* **258**, 741 (1972).