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PURIFICATION AND PROPERTIES OF THYMIDYLATE SYNTHASE FROM CALF THYMUS

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SUMMARY

Thymidylate synthase has been purified about 5000-fold from the thymus gland of young calves by $(NH_4)_2SO_4$ fractionation, DEAE, hydroxylapatite, Sephadex G-200 column chromatography and column electrophoresis. The purified enzyme was found to be homogeneous on polyacrylamide gel electrophoresis. The molecular weight, pH values of optimal activity and Michaelis constant were evaluated. A competitive inhibition by dTMP for dUMP was observed and the inhibition constant was determined. The enzyme activity was not stimulated by Mg^{2+}. ATP was found to inhibit the enzymatic activity, while ATP–Mg^{2+} had no effect.

INTRODUCTION

Previous reports on the partial purification and properties of thymidylate synthase of calf thymus have been published by GREENBERG *et al.*[1], JENNY AND GREENBERG[2], and WHITTAKER AND BLAKLEY[3]. The enzyme has been purified more recently from chick embryo by LORENSON *et al.*[4], from Ehrlich ascites cells by FRIEDLAND AND HEIDELBERGER[5], and from *Lactobacillus casei* by CRUSBERG *et al.*[6]. DUNLAP *et al.*[7] have crystallized the enzyme from amethopterin-resistant *Lactobacillus casei*. Enzyme preparations of higher specific activity have been obtained from bacterial than from animal sources[6–11].

This paper reports the results of a further investigation of the purification and properties of the enzyme from the thymus glands of young calves. The enzyme has been purified to a degree that yields a single band by polyacrylamide gel electrophoresis. Some properties of the purified enzyme were investigated.

EXPERIMENTAL PROCEDURE

Materials

Pre-weighed L(±)-tetrahydrofolate (Grade III) and ATP, (disodium salt) were purchased from Sigma Chemical Company. Electrophoretically homogeneous beef

heart lactate dehydrogenase (H_4 isozyme), 2 times crystallized egg white ovalbumin and 2 times crystallized rabbit muscle aldolase were purchased from Worthington Biochemical Corporation. Crystalline bovine serum albumin, 6 times crystallized bovine pancrease, α-chymotrypsinogen-A (Type II) and crystallized porcine pepsin were purchased from Nutritional Biochemical Corporation, Sigma Chemical Company and Armour Laboratories, respectively.

The pH values of buffers used in the present study were adjusted with HCl or KOH. DEAE-cellulose (Cellex -D of Bio-Rad Laboratories) was kept suspended in 0.1 M KOH for 24 h at room temperature and washed with water by decantation until small particles and excess alkali were removed. Hydroxylapatite (Bio-Gel HTP) and Bio-Gel P-2 were obtained from the Bio-Rad Laboratories, Sephadex X-200 was the product of Pharmacia Fine Chemicals. All of the columns were prepared in a cold room (0–5°) according to the directions of the manufacturers. The DEAE-cellulose column (4.5 cm × 37 cm) was used repeatedly for several enzyme purifications without re-packing, but was washed and equilibrated with phosphate after each use. Hydroxylapatite was packed in a 3.0 cm × 21 cm column under a pressure head of about 20 cm water (maintained during chromatography) and equilibrated with 0.01 M Tris buffer (pH 7.6) containing 10 mM 2-mercaptoethanol before use. A freshly prepared column was used for each enzyme purification.

Sephadex G-200 was packed in a column of 1.25 cm × 103 cm under a constant pressure head of less than 15 cm H_2O which was maintained throughout this step. The column was equilibrated with 0.06 M phosphate buffer (pH 7.6) containing 10 mM 2-mercaptoethanol before introduction of the sample. Only 1 cm of column shrinkage was observed with the 103-cm length column after 5 successive runs.

Enzyme activity assays

The spectrophotometer procedure developed by WAHBA AND FRIEDKIN[12] and by BLAKLEY AND MCDOUGALL[13], which is based on the absorbance difference between 5,10-methylenetetrahydrofolate and dihydrofolate at 340 nm ($\Delta\varepsilon = 6400$), was used for assay for thymidylate synthase.

Assay method

The incubation mixture consisted of 100 mM phosphate buffer, 100 mM 2-mercaptoethanol, 14 mM formaldehyde, 0.38 mM L(±)-tetrahydrofolate, 0.65 mM dUMP and 5–500 μl of enzyme solution in a total volume of 1.5 ml. The incubations were performed at pH 6.9, attained by using a stock solution of 1 M phosphate buffer adjusted to pH 6.7 in preparation of the incubation mixture. 25 mg of preweighed L(±)-tetrahydrofolate in an air-evacuated ampule was dissolved in 23.4 ml of a mixture containing 500 mM phosphate buffer, 500 mM 2-mercaptoethanol and 70 mM formaldehyde to prepare a 5 times concentrated solution of these components, and 0.3 ml of the solution was added to each incubation mixture. The L(±)-tetrahydrofolate thus dissolved was stable for at least 12 h at room temperature.

An alternate assay method made use of 50 mM Tris buffer (pH 7.6) instead of the 100 mM phosphate buffer; final pH 6.9.

Thymidylate synthase activity was measured in a 2-ml cuvette of 1.0 cm optical path unless indicated otherwise. The enzymatic reaction at 37° was initiated by the addition of 10 μl of $9.8 \cdot 10^{-2}$ M dUMP, a negligibly small volume as compared with

that of the incubation mixture. Spectrophotometric readings were performed with a Gilford multisample absorbance recorder, thermostated at 37°. Controls were run with all components except dUMP. Spectrophotometric readings were then taken over a period of 10 min from the 10th to 20th min of incubation.

Enzyme units

An enzyme unit is defined as the quantity that synthesized 1 μmole of thymidylate per h. Specific activity is expressed as enzyme units per mg of protein. Protein concentrations were determined by the method of LOWRY *et al.*[14] using bovine serum albumin as the standard. When more than 10 μl of enzyme solution containing 10 mM 2-mercaptoethanol was used, it was diluted to 0.5 ml and dialyzed against water before being added to the 1.5 ml of the protein assay mixture.

Preparative electrophoresis

The final stage of purification was achieved with 2 preparative column electrophoresis runs using Bio-Gel P-2 gel as the carrier*. A 1.0 cm × 23.5 cm Bio-Gel P-2 column, prepared in a vertically held plastic tube, with a polyethylene foam divider at its lower end, was used as the electrophoresis tube. This column was previously equilibrated with 0.03 M Tris (pH 7.6), the running (electrode) buffer for this electrophoresis. To make a run, 0.3 ml of enzyme solution was run onto the top of the gel column and washed down with the same amount of the buffer. The lower end of the plastic tube was dipped in 800 ml of the electrode buffer (anode) and upper end of the gel column was connected to another 800 ml of the electrode buffer (cathode) with the aid of a rubber tube. At the end of a run, the plastic tube was disconnected from the rubber tube and the solution held in the gel column was drawn out in the same manner as in the usual Sephadex column chromatography (*cf.* ref. 16).

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was used to test for purity of the enzyme preparations. It was performed on a 9.3% polyacrylamide gel containing 10 mM 2-mercaptoethanol, as described by DAVIS[15]. The polyacrylamide gel was prepared by photopolymerization of acrylamide in the presence of 10 mM mercaptoethanol using a catalyst system consisting of riboflavin and *N,N,N',N'*-tetramethylethylenediamine[16].

100 μl each of the enzyme from the final step of purification was diluted 2-fold with a buffer solution (pH 8.3) containing 38 mM glycine, 5 mM Tris, 20 mM 2-mercaptoethanol and 40% sucrose, and carefully introduced on the top of the gel column already layered with the glycine–Tris electrode buffer. Electrophoresis was performed at 3 mA per tube for 80 min at 0–5°. After electrophoresis, each gel was kept immersed for 1 h in 10% trichloroacetic acid solution to fix the protein and then for 2 h in 0.025% Coomassie blue in 10% trichloroacetic acid to stain the gels. After staining, the gels were washed with several changes of 10% trichloroacetic acid solution.

* H. HORINISHI AND D. M. GREENBERG unpublished data.

RESULTS

Enzyme purification and properties

The purification procedure, with some modifications, is similar to the one used by JENNY AND GREENBERG[2] up to the DEAE-cellulose chromatography step. The steps beyond this, starting with chromatography on hydroxylapatite, represent new additions to the purification procedure. These are described below.

All procedures were performed at 0–5°. Buffers were adjusted to pH 7.6 and contained 10 mM 2-mercaptoethanol unless otherwise indicated. The latter exerted a significant protective effect on the enzyme. Measurements of thymidylate synthase activity were made immediately after each purification step.

Hydroxylapatite column chromatography

The eluate from DEAE-cellulose chromatography first dialyzed for first 10 and then 2 additional h against 4-l portions of 0.01 M Tris buffer was poured onto a

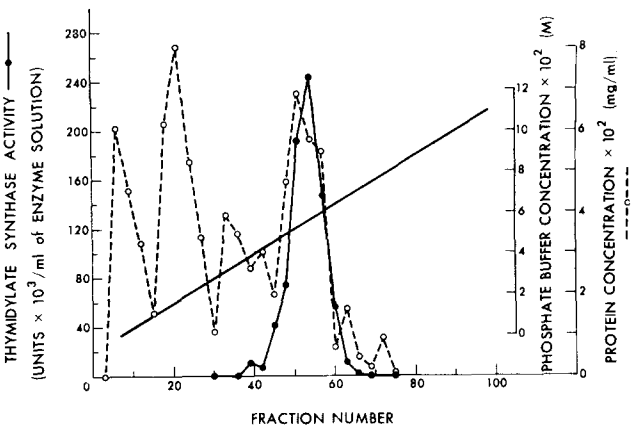


Fig. 1. Elution pattern of thymidylate synthase from hydroxylapatite column.

hydroxylapatite column (3.0 cm × 21 cm) and eluted by a gradient formed by having the mixing chamber contain 1 l of 0.01 M Tris buffer and the reservoir 1 l of 0.11 M phosphate buffer. Fractions of 21.9 ml were collected at a flow rate of 44 ml/h.

Fig. 1 shows the elution pattern of the enzyme from the hydroxylapatite column. The most active 12 fractions (Fractions 48–59 in Fig. 1) were pooled. The pooled eluate (260 ml) was placed in a dialysis tube, and covered with solid sucrose to reduce the volume to about 15 ml. The content of the dialysis tube was then transferred to a vacuum dialysis bag and concentrated to 0.9 ml by vacuum dialysis against 0.06 M phosphate buffer*.

Sephadex G-200 column chromatography

The concentrated enzyme colution (0.9 ml) was passed through a Sephadex

* The vacuum dialysis apparatus consisted of a collodion bag (Schleicher and Schuell, No. 100, 8 ml vol.) mounted on rod which was fitted through a rubber stopper into a glass vessel with a side tube that could be attached to a vacuum pump. The glass container was partially filled with buffer solution.

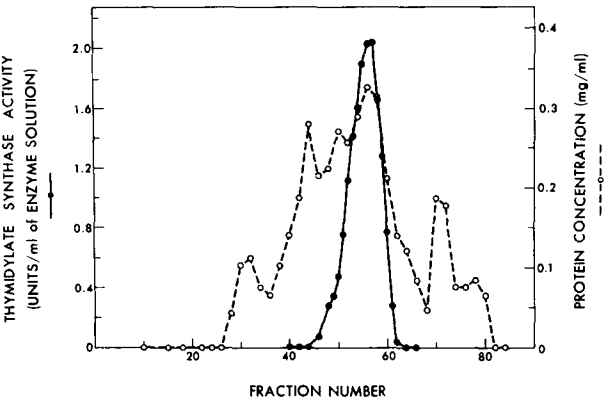


Fig. 2. Gel filtration pattern of thymidylate synthase through Sephadex G-200 column.

G-200 column (1.25 cm × 103 cm) using 0.06 M phosphate as the running buffer. Fractions of 1.36 ml were collected at a flow rate of 2.2 ml/h, and the enzyme was detected by the alternate assay. The gel filtration pattern is shown in Fig. 2. An activity peak was found approximately at the middle portion of the distribution of the eluted proteins. The enzyme solution in tubes 51–60 was pooled (13.2 ml) and concentrated to approx. 0.3 ml by vacuum dialysis against 0.03 M Tris buffer.

Preparative electrophoresis

The concentrated enzyme solution (approx. 0.3 ml) was subjected to preparative column electrophoresis using Bio-Gel P-2 as a supporting material, as described above. The electrophoresis was carried out for 15.5 h in 0.03 M Tris buffer (pH 7.6) at a current of 3–4 mA. The solution being held in the supporting column was drawn out at a flow rate of 1 ml/h and collected into fractions of 7 drops (0.185 ml) with an ISCO fraction collector equiped with a drop counting device. The enzyme activity was found in Fractions 6–18 with the largest amount of activity in Fraction 11. The highest

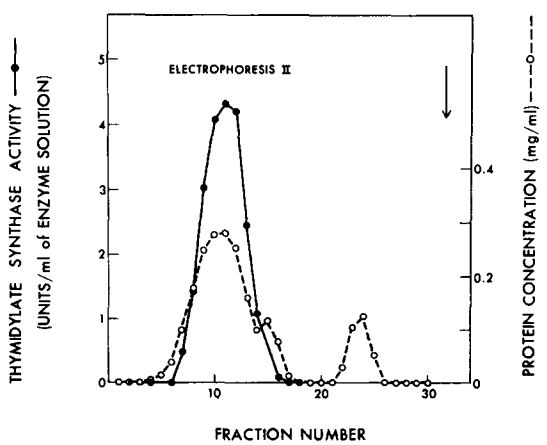


Fig. 3. Preparative electrophoresis pattern of thymidylate synthase and Bio-Gel P-2.

specific activity was found in Fraction 12. Fractions 7–14 (1.4 ml) were pooled and concentrated to approx. 0.3 ml by vacuum dialysis against 0.03 M Tris buffer. The distribution pattern of protein and enzyme in the first electrophoresis run was similar to that in the second run and a figure is considered redundant and is not included.

An additional electrophoresis was done in the same manner as above with the concentrated enzyme solution (approx. 0.3 ml) from the above electrophoresis step. Fig. 3 shows the result of the second preparation electrophoresis. In the absence of an applied electric field in this step, the enzyme was found to remain at the position indicated by an arrow in Fig. 3, which shows that the enzyme migrated toward the anode during preparative electrophoresis. The highest specific activities were observed in Fractions 10–13, and these fractions were pooled for use in the following experiments on enzyme kinetics.

Summary of purification

A summary of a typical purification of thymidylate synthase from young calf thymus is reported in Table I.

TABLE I

SUMMARY OF PURIFICATION

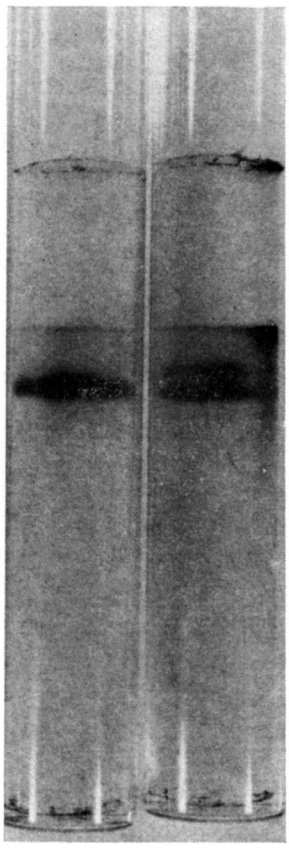
	Vol. (ml)	*Total protein (mg)*	*Specific activity (units/mg)*	*Total enzyme units*	*Recovery (%)*	*Purification*
Crude extract	7040	88 600	0.0044	399	100	1.0
$(NH_4)_2SO_4$ fraction						
Before freezing	447	9840	0.022	215	54.0	5.0
After thawing*	447	9840	0.015	416	36.6	3.4
After dialysis*	515	9940	0.0094	92.5	23.2	2.1
DEAE chromatography						
Pooled fractions	540	185	0.368	68.1	17.1	83.6
After dialysis*	535	185	0.324	62.6	15.7	76.8
Hydroxylapatite chromatography						
Pooled fractions	260	14.9	2.91	43.4	10.9	661
Sephadex G-200 chromatography						
Pooled fractions	13.2	3.7	6.47	23.8	6.0	1470
Electrophoresis I						
Pooled fractions	1.40	0.80	12.9	10.3	2.58	2940
Electrophoresis II						
Pooled fractions	0.64	0.15	21.0	3.2	0.80	4780

* See the text for details.

The specific activity of calf thymus homogenates ranged from $2 \cdot 10^{-3}$–$6 \cdot 10^{-3}$ μmole/mg per h. When the mean value of the specific activities of the 4 homogenates, which were combined after the $(NH_4)_2SO_4$ fractionation step, was less than $4 \cdot 10^{-3}$ μmole/mg per h, an additional Sephadex G-100 column chromatography step was inserted between the hydroxylapatite and Sephadex G-200 column chromatography step. The additional Sephadex step was carried out in the same manner as the Sephadex G-200 step, and a 2.5-fold purification was normally attained with about 75% recovery of the activity.

Stability of enzyme

The enzyme was comparatively unstable before the DEAE-cellulose step as previously reported from this laboratory by JENNY AND GREENBERG[2], but became relatively stable after this step (Table I). The enzyme in the pooled fractions from the final electrophoresis was unstable at 0–5°, losing 30–50% of its activity in 3 days. The enzyme was more stable in 20% sucrose solution but still lost 25% of its activity in 6 days.



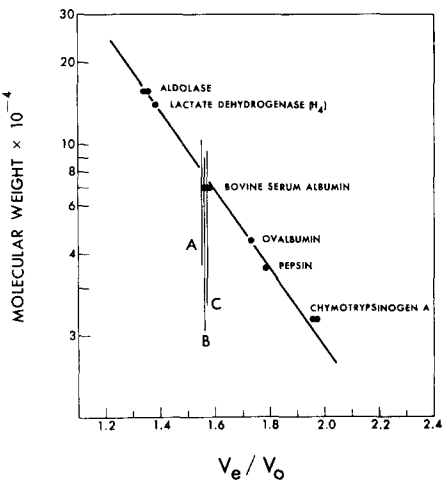


Fig. 4. Polyacrylamide gel electrophoresis examination of preparative electrophoresis II fractions. Tube on left, pooled fractions from fraction 10–13; Tube on right, Fraction 14 (see text for explanation).

Fig. 5. Molecular weight determination with Sephadex G-200 column. V_0 = void volume; V_e = elution volume.

Polyacrylamide gel electrophoresis of the pooled fractions of the final purification step (Fractions 10–13, Fig. 3) showed only one band (left) in Fig. 4. The tube on the right shows the result obtained with Fraction 14 of the same step. This yielded two colored bands, the darker band being located at exactly the same position as the band in tube on the left.

Molecular weight determination

During the course of purification the molecular weight of thymidylate synthase was determined on the preparative Sephadex G-200 column by the method of WHITTAKER[17] using the enzymatic activity to locate the elution position of the enzyme. The column was calibrated with the pure proteins as shown in Fig. 5 and the void volume was determined to be 48.0 ml with blue dextran.

The elution positions of the enzyme were examined on 3 separate batches from the hydroxylapatite column chromatography step. The 3 samples applied had specific activities of 1.30, 2.68 and 2.49 μmoles/mg per h, respectively, by the alternate assay. The elution positions are shown by vertical lines A, B and C in Fig. 5. Estimated molecular weights are 80 000, 77 5000 and 75 000, respectively, with an average molecular weight of 77 500. Molecular weights have been reported of 58 000 for chick embryo thymidylate synthase[4], 67 000 for the Ehrlich ascites tumor[5] and 70 000–72 000 for the *L. casei*[7] enzyme.

ENZYME KINETICS

Measurements of thymidylate synthase activity were calculated from the reaction rate in a time period of 10 min from 5 min before to 5 min after the stated incubation time.

Dependency of activity on pH

Fig. 6 shows the pH–activity curves of the enzyme determined by 2 different procedures. The pH value of each incubation mixture was determined, and did not change during the incubation at all pH values employed here.

In one procedure the complete reaction media was incubated without preincu-

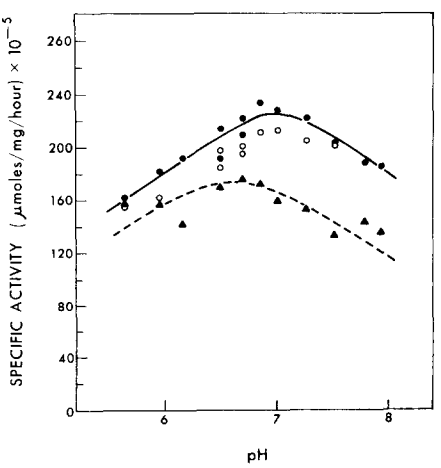


Fig. 6. pH dependency of thymidylate synthase activity. Enzyme activities shown by closed triangles were taken at an incubation time of 10 min after 14-min preincubation of enzyme at 37° with all of the other components of incubation mixture except dUMP. Activities shown by circles were determined without preincubation of enzyme; values of closed and open circles were taken at incubation times of 10 and 24 min, respectively.

TABLE II

EFFECT OF CERTAIN COMPOUNDS ON THYMIDYLATE SYNTHASE ACTIVITY

The incubation mixture consisted of 100 mM phosphate buffer*, 100 mM 2-mercaptoethanol, 15 mM formaldehyde, 0.38 mM L(±)-tetrahydrofolate, 0.65 mM dUMP and thymidylate synthase. The protein concentration of the enzyme preparation used was 0.23 mg/ml and 10 µl of the preparation was used for each assay of 1.5 ml in total volume of the incubation mixture.

	Concn. (mM)	*Thymidylate produced (µmoles/ml of enzyme solution per h)*	*Activity relative (%)*
None	—	3.39	100
KCl	10	3.30	97
$(NH_4)_2SO_4$	100	3.08	91
	10	3.67	108
$ZnCl_2$**	5	3.30	97
$MgCl_2$	25	3.33	98
	5	3.35	99
ATP	1	2.15	65
	1	1.99	59
	0.1	3.49	103
ATP–Mg^{2+}	1	3.37	99
	0.1	3.47	102
EDTA	5	3.49	103
	0.5	3.35	99

* See the text on the pH value.
** Measurement at 25 mM $ZnCl_2$ was precluded by formation of a precipitate.

bation and the enzyme activities at incubation times of 10 and 24 min were measured. The filled circles indicate the activities at the 10 min and a relatively flat pH–activity curve shown by the solid line was obtained. Maximum activity in this curve is at pH 6.9. The activities at the 24 min are represented by the open circles. These occupy positions relatively close to those shown by the closed circles.

In the alternate procedure, the enzyme was first preincubated with all components of the mixture except dUMP for 14 min. The latter was then added to the incubation mixture and the activities were measured at an incubation time of 20 min. The preincubation resulted in a shift of the above pH–activity curve to the one represented by the broken line and closed triangles and a maximum enzyme activity was found at pH 6.6.

Effect of certain additives on enzyme activity

Table II shows the effect of several additives on the thymidylate synthase activity. LORENSON *et al.*[4] added 0.12 M $(NH_4)_2SO_4$ to their incubation mixture. No effect of $(NH_4)_2SO_4$ on the activity of the highly purified enzyme were observed at the concentrations used here (0.10 and 0.01 M). The addition of KCl_2 and $ZnCl_2$ also had no effect on the activity. The addition of Mg^{2+} did not bring about any change of the enzyme activity at concentrations of 25 and 5 mM, which confirmed the previous observation from this laboratory[2] with a less purified enzyme preparation of calf thymus. EDTA had no effect on the activity even at a relatively high concentration of 5 mM.

An inhibition of about 40% of the enzyme activity by ATP was observed at a concentration in excess of the level of the dUMP concentration (see Table II), while

no inhibition occurred at a concentration of ATP less than that of dUMP. The inhibition by ATP could not be observed at all in the presence of Mg^{2+} at the same concentration as that of ATP despite that Mg^{2+} itself did not stimulate the activity as mentioned above.

Michaelis constant and inhibition constant

The enzyme was warmed up for 7 min at 37° with all of the components of the incubation mixture except dUMP prior to the enzymatic reaction, and the reaction was initiated by addition of dUMP. The reaction was found to be linear for at least 8 min from the 4th to 12th min of incubation at various concentrations of dUMP, and the rate of reaction was measured in the above time period.

An apparent Michaelis constant of $9.0 \cdot 10^{-6}$ M was obtained from a Lineweaver–Burk plot of the data in the absence of dTMP as shown in Fig. 7. JENNY AND

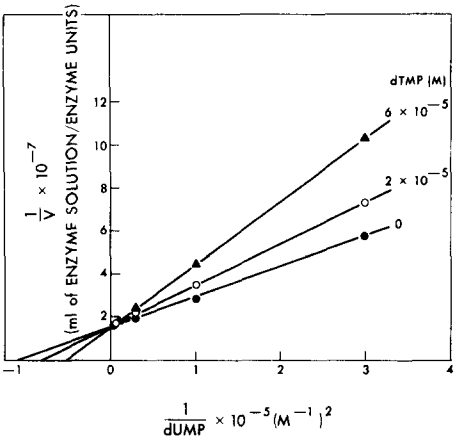


Fig. 7. Lineweaver–Burk plot. The specific activity of the enzyme used was 19.7 μmoles/mg per h by Assay I.

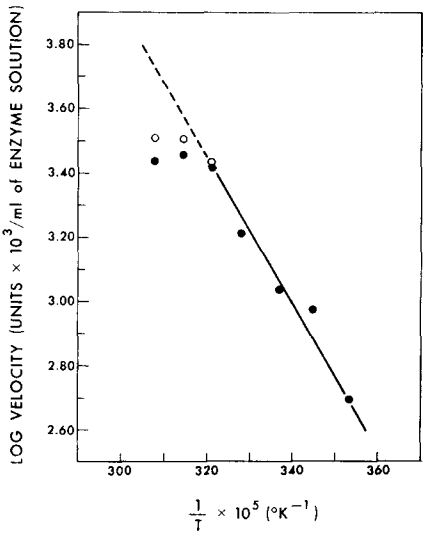


Fig. 8. Arrhenius plot. The specific activity of the enzyme used was 15.7 μmoles/mg per h by Assay I.

GREENBERG[2] previously reported a K_m value for dUMP of $2 \cdot 10^{-5}$ M with the less purified thymus enzyme. A competitive inhibition of thymidylate synthase activity by dTMP for dUMP was observed and the mean value of the inhibition constant, K_i for dTMP was $5.9 \cdot 10^{-5}$ M.

Temperature dependency of enzyme activity

Fig. 8 shows an Arrhenius plot of the data measured at various temperatures. The values shown by the closed circles were taken in a time period of 20 min from the 7th to 27th min of the incubation, while open circles represent measurement of 10 min from the 7th to 27th min.

The enzyme was found to be relatively unstable above 38° in the reaction mixture. The energy of activation was calculated from the slope of an Arrhenius plot as 10.4 kcal/mole. LORENSON *et al.*[4] calculated the activation energy of the chicken embryo enzyme to be 12 kcal/mole.

DISCUSSION

The highly purified enzyme preparation was found to give only one band on polyacrylamide gel electrophoresis. This does not necessarily imply that the band consists of a single protein, unless the specific activity of the pure thymus thymidylate synthase is much lower than that of the corresponding bacterial enzyme.

The highest specific activity reached in this investigation was about 21 μmoles/mg per h. This could not be increased by the further addition of a hydroxylapatite or a Sephadex G-200 column chromatography step.

Higher specific activities have been reported for bacterial preparations of the enzyme. FRIEDKIN *et al.*[10] reached a specific activity of about 67 for the *Escherichia coli* enzyme measured at 30°, and BLAKLEY AND MCDOUGAL[13] a specific activity of 54 for the enzyme from *Streptococus faecalis* at 37°. DUNLAP *et al.*[7] reported that the crystalline enzyme isolated from amethopterin resistant *L. casei* had a specific activity of 150 at 30°. It is to be noted that measurements of the bacterial enzymes were made in the presence of Mg^{2+}, which enhances the activity of bacterial thymidylate synthase[7,10,11,20], but not of the enzyme from mammalian sources[2,18,19]. BLAKLEY AND MCDOUGALL[11] found that the activity of their enzyme was decreased by about 70% in the absence of Mg^{2+}. The different effect of Mg^{2+} may explain in part the difference between the specific activity reached in the present study and that obtained for the bacterial preparations.

The enzyme is inhibited by an excess of ATP over dUMP and this is counteracted by Mg^{2+} (Table II). This may explain the observation of FRIEDKIN AND KORNBERG[20] who assayed their enzyme preparation in the presence of 200 times more ATP than dUMP. Practically no activity was detected in the absence of Mg^{2+}.

The optimum pH value of 6.9 is nearly to same as that reported by JENNY AND GREENBERG[2] (pH 7.1). HARTMAN AND HEIDELBERGER[18] and REYES AND HEIDELBERGER[19], who performed preincubation of the enzyme in 0.1 M phosphate buffer at 37°, found the maximal activity was at pH 6.5 and 6.7 for the enzyme from Ehrlich ascites carcinoma cells. These values agree with the optimum pH value of pH 6.6 obtained with preincubation in the present study. LORENSON *et al.*[4] reported an optimum pH value of pH 7.5 for the chick embryo thymidylate synthase. They, however, used 2 different buffer systems to obtain a single pH dependency curve and they exchanged one buffer system with another at the optimum pH value of pH 7.5, without mention of the particular buffer used at this pH. Thymidylate synthase activity is strongly buffer-dependent according to WAHBA AND FRIEDKIN[8] and JENNY AND GREENBERG[2]. The effect of pH on the *L. casei* enzyme was found to be complex and was dependent on the nature of the buffer and the presence or absence of Mg^{2+} (ref. 7). In phosphate buffer the pH optimum occurs in the range of 6.5–6.8. The enzyme of *E. coli* seems to have a little higher optimum pH value of 7.5–8.0 (ref. 8).

A Michaelis constant of $9.0 \cdot 10^{-6}$ M was obtained for dUMP in the present study. Other reported values of K_m are $2 \cdot 10^{-5}$ M for the thymus gland enzyme[2]

$7.5 \cdot 10^{-6}$ M for chick embryo enzyme[4], $3.7 \cdot 10^{-5}$ M and $1.4 \cdot 10^{-5}$ M for the enzyme of Ehrlich ascites carcinoma cells[18,19]. Values of K_m for dUMP for the bacterial enzyme are $4 \cdot 10^{-6}$ M for *E. coli*[8], $1.8 \cdot 10^{-5}$ M for T2 phage-infected *E. coli*[21], $3.0 \cdot 10^{-5}$ M for T6 phage-infected *E. coli*[21], $5.7 \cdot 10^{-6}$ M for *S. faecalis*[22], and $5.1 \cdot 10^{-6}$ M (ref. 7 for the *L. casei* enzyme in the presence of Mg^{2+} (10 mM).

dTMP was observed to be a competitive inhibitor of dUMP for enzyme activity, giving an inhibition constant of $5.9 \cdot 10^{-5}$ M. LORENSEN *et al.*[4] and BLAKLEY[21] also reported the competitive inhibition of the activities of chick embryo and *S. faecalis* enzyme by dTMP for dUMP, with K_i values of $1.4 \cdot 10^{-4}$ and $2.4 \cdot 10^{-5}$ M, respectively. REYES AND HEIDELBERGER[19] interpreted the inhibition of the activity of Ehrlich ascites carcinoma cell enzyme by dTMP for dUMP as being non-competitive.

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