porous glass beads. One of the results in that earlier work was that active immobilized subunits had apparently been obtained. However, it was not shown that the observed activity was physically different (e.g., in stability). The concentration of coupling points was also not regulated to prevent coupling of the enzyme via more than one subunit. Thus it is possible that the reported activity in that case was due to tetrameric LDH bound or adsorbed on the glass surface or in the tubing of the flow assay system. Further characterization of glass beads as a carrier for immobilized subunits would be needed to resolve the present discrepancy. The satisfactory agreement between our results with Sepharose-bound subunits and results of renaturation kinetics studies (Jaenicke, 1974) parallels similar agreement between the two methods in the case of aldolase, although the monomer is active in one case and inactive in the other. Provided that adequate control experiments are performed (as discussed by Chan, 1976), it appears that the study of subunits immobilized on Sepharose can provide information regarding the effects of alterations in quaternary structure on protein function.

References

Anderson, S., and Weber, G. (1966), *Arch. Biochem. Biophys. 116,* 207.

Axén, R., Porath, J., and Ernback, S. (1967), *Nature (London) 214,* 1302.

Chan, W. W.-C. (1970), *Biochem. Biophys. Res. Commun. 41,* 1198.

Chan, W. W.-C. (1973), *Can. J. Biochem. 51,* 1240.

Chan, W. W.-C. (1976), *Methods Enzymol.* (in press).

Chan, W. W.-C., Kaiser, C., Salvo, J. M., and Lawford, G. R. (1974), *J. Mol. Biol. 87,* 847.

Chan, W. W.-C., Mort, J. S., Chong, D. K. K., and Macdonald, P. D. M. (1973a), *J. Biol. Chem. 248,* 2778.

Chan, W. W.-C., Schutt, H., and Brand, K. (1973b), *Eur. J. Biochem. 40,* 533.

Cho, I. C., and Swaisgood, H. E. (1972), *Biochim. Biophys. Acta 258,* 675.

Cho, I. C., and Swaisgood, H. (1973), *Biochemistry 12,* 1572.

Feldman, K., Zeisel, H., and Helmreich, E. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2278.

Green, N. M., and Toms, E. J. (1973), *Biochem. J. 133,* 687.

Griffin, J. H., and Criddle, R. S. (1970), *Biochemistry 9,* 1195.

Jaenicke, R. (1974), *Eur. J. Biochem. 46,* 149.

Jaenicke, R., and Knof, S. (1968), *Eur. J. Biochem. 4,* 157.

Koen, A. L. (1967), *Biochim. Biophys. Acta 237,* 2135.

Kolb, H. J., Renner, R., Hepp, K. D., Weiss, L., and Wieland, O. H. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 248.

March, S. C., Parikh, I., and Cuatrecasas, P. (1974), *Anal. Biochem. 60,* 149.

Markert, C. L. (1963), *Science 140,* 1329.

Mattiasson, B., and Mosbach, K. (1976), *Methods Enzymol.* (in press).

Mort, J. S., Chong, D. K. K., and Chan, W. W.-C. (1973), *Anal. Biochem. 52,* 162.

Woenckhaus, C., Berghauser, J., and Pfleiderer, G. (1969), *Z. Physiol. Chem. 350,* 473.

Purification of Thymidylate Synthetase from Enzyme-Poor Sources by Affinity Chromatography†

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ABSTRACT: The adsorption of thymidylate synthetase from *Escherichia coli* B to aminoalkyl-Sepharose with the increasing length of carbon chain (2–6 carbon atoms) was investigated. A correlation was found between the chain length and adsorption effectiveness, increasing from the two- to the six-carbon chain. A hydrophobic chromatography of the enzyme on aminobutyl-Sepharose gave about 20-fold purification. A new affinity chromatography carrier was synthesized containing tetrahydromethotrexate linked to aminoethyl-Sepharose via its carboxylic groups. The carrier adsorbed the enzyme from the crude preparation only in the presence of deoxyuridine 5′-monophosphate (dUMP) in a concentration of 2×10^{-5} M. The specifically adsorbed thymidylate synthetase was eluted with sacharose-containing buffers in which dUMP was omitted. The purification procedure was applied to a crude thymidylate synthetase preparation from resting *E. coli,* calf thymus, Sarcoma 180, and Gardner lymphosarcoma. The purified enzyme from all mentioned sources showed one protein band on disc electrophoresis corresponding to enzymatic activity. The formation of a reversible noncovalent complex enzyme–tetrahydromethotrexate–dUMP on the affinity column is supposed.

Thymidylate synthetase catalyzes the reductive methylation of deoxyuridine 5′-monophosphate (dUMP) to thymidylate with simultaneous conversion of N^5,N^{10}-methylenetetrahydrofolate to dihydrofolate. This enzyme is being intensively studied because of its key function in the DNA synthesis and, therefore, it has been purified from various microbial and animal sources by conventional methods (Greenberg et al., 1961; Mathews and Cohen, 1963; Friedkin et al., 1962;

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Blakley, 1963; Lorenson et al., 1967; Crusberg et al., 1970; Leary and Kisliuk, 1971; Gupta and Meldrum, 1972; Galivan et al., 1974).

Affinity chromatography methods using Sepharose bound with either deoxyuridylate (Danenberg et al., 1972) or 5-fluorodeoxyuridylate (Whiteley et al., 1974)—a strong inhibitor of the enzyme forming a covalent complex with it—were used successfully for purification of thymidylate synthetase from *L. casei* resistant to dichloromethotrexate, which is an exceptionally rich source of the enzyme (Crusberg and Kisliuk, 1969; Dunlap et al., 1971). If applied to the enzyme-poor sources—i.e., resting *E. coli*, calf thymus, or experimental mice tumors—the first method proved to be of very limited effectiveness (15-fold purification) because the enzyme could not have been selectively adsorbed in the presence of excess of other proteins (Rode, 1975). Therefore, another principle has been sought enabling a successful purification of the enzyme by affinity chromatography.

From the tetrahydrofolate analogues, tetrahydromethotrexate appeared to be the most powerful inhibitor ($K_I = 1.2 \times 10^{-7}$ M) (Slavík et al., 1967, 1969). This fact made this compound a promising candidate for an affinity chromatography ligand.

The subject of this paper is the preparation of an affinity carrier containing tetrahydromethotrexate as a ligand and the elaboration of a purification procedure applicable to materials with low enzyme content.

Materials and Methods

Sepharose 4B was obtained from Pharmacia, cyanogen bromide from Schuchardt, ω-alkylenediamines from Lachema, deoxyuridine 5′-monophosphate from Koch-Light. Tetrahydrofolate was prepared from folic acid by catalytic hydrogenation in acetic acid with subsequent precipitation of the product with ether (Slavik et al., 1960). Tetrahydromethotrexate was prepared by a similar procedure from methotrexate Lederle (Slavik et al., 1960). The material contained about 70% of the mixture of two diastereomers of tetrahydromethotrexate, 20% dihydromethotrexate, and a small amount of *p*-methylaminobenzoyl glutamate (as shown by gradient elution on DEAE-cellulose column). (Dimethylaminopropyl)ethylcarbodiimide was synthesised according to Hartman and Drager (1947) and stored in sealed vials in the form of its methiodide (mp 94 °C). Other chemicals were of commercial origin.

E. coli B was cultivated in a 250-l. fermentor on a cornsteep medium, harvested by centrifugation, washed with saline, and kept frozen at −25 °C.

Calf thymus was obtained from a slaughter house and chilled with crushed ice until the enzyme isolation was performed.

Gardner lymphosarcoma were transplanted to C 3H mice subcutaneously and the tumors of 1-g weight were collected after 9 days and kept frozen. Sarcoma 180 was transplanted to H mice subcutaneously and the tumors were collected in a similar manner.

Preparation of the Affinity Carrier

Aminoalkyl–Sepharose. The aminoalkyl derivatives of Sepharose were prepared by the modified method of Porath et al. (1973). Twenty milliliters of Sepharose was suspended in 100 ml of 2 M potassium phosphate buffer, pH 10.5, and chilled with ice under continuous stirring. Nine grams of cyanogen bromide was dissolved in 20 ml of diethyl ether and the solution was mixed with 100 ml of ice-cold water and stirred until the oily drops dissolved. The solution was mixed with the Sepharose suspension and stirred for 15 min. The activated Sepharose was filtered on a Büchner funnel, washed with 1000 ml of ice-cold water, and transferred to a solution of 30 g of ω-alkylenediamine in 100 ml of 1 M sodium bicarbonate (pH 8.5). The reaction proceeded at 20 °C for 18 h. The aminoalkyl–Sepharose was filtered on a Büchner funnel, washed with 2000 ml of water, transferred to a column, and the washing was continued with another 2000 ml of water.

The amount of aminoalkyl groups was estimated by potentiometric titration with 0.02 M hydrochloric acid or checked in a qualitative way by a 0.1% solution of sodium trinitrophenylsulfonate in 5% bicarbonate (orange color of the gel). There was 6–12 μmol of amino alkyl groups bound per 1 ml of activated Sepharose.

The Binding of Tetrahydromethotrexate to Aminoalkyl–Sepharose. A combined glass–calomel electrode set connected with a pH meter was inserted into a rubber stopper and placed in the middle of a three-necked 250-ml round-bottomed flask. Two polyethylene syringes were connected with the flask through the same rubber stopper, one containing 5% $NaHCO_3$, the other 1 N HCl. The flask was connected with a capillary hydrogen inlet on one side and a separatory funnel on the other side. Ten milliliters of aminoalkyl–Sepharose was poured into the flask and washed down with 50 ml of water. Hydrogen was bubbled vigorously through the suspension for 10 min.

Forty milligrams (0.09 mmol) of tetrahydromethotrexate was dissolved in 5 ml of 5% sodium bicarbonate and the solution was poured into the flask through the separatory funnel immediately. pH was brought to 6.0 by addition of 1 N HCl from the syringe. Then a solution of 250 mg of (dimethylaminopropyl)ethylcarbodiimide methiodide in 4 ml of water was added dropwise over a 5-min period and the pH was adjusted to 5.5–6 again. The reaction was allowed to proceed for 1 h in a hydrogen stream while pH was maintained in the range of 5.5–6.0 by the addition of bicarbonate or HCl from the syringes. The suspension occasionally changed its color to light brown. Then 5 ml of 1 M mercaptoethanol was added to terminate the reaction and to protect the product against oxidation. The carrier was transferred to a column 1.5 cm in diameter and washed with 100 ml of 2.5% sodium bicarbonate in 0.5 M NaCl containing 0.1 M mercaptoethanol and then equilibrated with the starting buffer for chromatography containing mercaptoethanol. The column was stable for 2 days if kept in darkness at 5 °C.

Test for Binding of Tetrahydromethotrexate. The amount of bound tetrahydromethotrexate was titrated with 0.01 M iodine in a sample freed from mercaptoethanol by washing with oxygen-free water and was found to be 5–7 μmol/ml of the gel. (Not corrected for dihydromethotrexate content.) A simple qualitative test was performed in the following way. A small aliquot of the carrier (about 1 ml) was transferred to a small column (0.3 × 1 cm) and washed with 25 ml of oxygen-free water. The gel was transferred to a test tube and 0.2 ml of 0.5 M phosphate, pH 7.5, was added, followed by the solution of 20 mg of nitrotetrazolium bromide in 2 ml of 20% aqueous dimethylformamide. The color of the gel turned to violet in the course of 1 min and later to black during 30 min.

The Crude Enzyme Preparations from E. coli, Thymus, and Tumor. Preparation was performed as described previously (Slavik et al., 1967, 1969) using streptomycin sulfate precipitation of the homogenate followed by ammonium sulfate fractionation. The active fractions (30–70% saturation) were kept frozen at −20 °C.

Hydrophobic Chromatography. Twenty milliliters of am-

TABLE I: Effect of Length of the Aminoalkyl Spacer on the Nonspecific Sorption of Thymidylate Synthetase to Aminoalkyl-Sepharose.[a]

	Spacer Group				
	None	Aminohexyl	Aminobutyl	1-Amino-2-hydroxypropyl	Aminoethyl
Activity in the supernatant (0.01 M phosphate buffer)	360.70	0	18.98	264.51	194.91
Activity in the eluate (0.2 M phosphate buffer)	11.39	37.96	216.42	30.38	72.14

[a] The gels containing various spacers (1 ml of bed volume containing 10–12 μmol of amine) were added in the form of their 50% aqueous suspension to 2-ml samples of the *E. coli* enzyme dialyzed against 0.01 M phosphate containing 0.025 M mercaptoethanol. The suspension was filled up with the same buffer to a total volume of 5 ml and allowed to stand for 30 min. The gel was separated by centrifugation and the supernatant was assayed for enzyme activity (nmol of dihydrofolate/h). The gel was washed with 5 ml of 0.01 M phosphate buffer containing 0.025 M mercaptoethanol and separated by centrifugation. The washing buffer was removed and the gel was suspended in 5 ml of 0.2 M phosphate buffer containing 0.025 M mercaptoethanol. After 30-min standing, the gel was centrifuged and the eluate was assayed for thymidylate synthetase activity. All operations were carried out at 0–5 °C.

monium sulfate fraction from *E. coli* equilibrated with 0.01 M phosphate, pH 7.5, by dialysis was adsorbed on a 1.5 × 10 cm column of aminobutyl-Sepharose. The column was washed subsequently with 0.01, 0.1, and 0.2 M phosphate, pH 7.5, containing 0.025 M mercaptoethanol. Ten-milliliter fractions were assayed for enzyme activity and protein content.

Affinity Chromatography. (a) Preparation from *E. coli.* Twenty milliliters of the crude enzyme preparation (= 1.6 g of protein as determined by absorption at 280 nm) was dialyzed overnight against 2000 ml of 0.01 M phosphate buffer, pH 7.5, containing 0.025 M mercaptoethanol. The unsoluble material was removed by centrifugation. To the clear supernatant 1/20 of its volume of 4×10^{-4} M dUMP was added (final concentration 2×10^{-5} M). The resulting solution was passed through a 1.5 × 7 cm column of the affinity carrier containing tetrahydromethotrexate linked via its carboxylic groups to aminoethyl-Sepharose.

The majority of contaminant proteins was eluted with about 200 ml of 0.01 M phosphate containing 0.025 M mercaptoethanol and 2×10^{-5} M dUMP, followed by 0.2 M phosphate buffer, pH 7.5, containing 0.5 M NaCl with the same concentration of mercaptoethanol and dUMP. The elution of ballast proteins was finished when the eluate did not show any absorbance at 280 nm and any turbidity with equal volume of 20% sulfosalicylic acid. The enzyme was eluted with 0.2 M phosphate containing 0.025 M mercaptoethanol and 20% sacharose. The eluate (about 10 ml) was concentrated to the final volume of 1.5 ml by ultrafiltration in Amicon-UF cell on Diaflo PM 10 membranes. About 0.16 mg of enzyme protein in total was obtained from 20 ml of the crude enzyme preparation.

(b) Preparation from Calf Thymus and Animal Tumors. Thirty-five milliliters of frozen ammonium sulfate fraction (= 10.29 g of protein) was diluted with 105 ml of 0.01 M phosphate buffer, pH 7.2, containing 0.025 M mercaptoethanol and 14 ml of 4×10^{-4} M dUMP was added. The insoluble material was removed by centrifugation at 25 000*g* and the opalescent pink supernatant was passed through a 30 × 25 mm column of the tetrahydromethotrexate–aminoethyl-Sepharose by the speed 0.3–0.6 ml/min. The column was washed with 500 ml of 0.01 M phosphate, pH 7.2, containing 0.025 M mercaptoethanol and 2×10^{-5} M dUMP. The column material was suspended into 200 ml of 0.5 M phosphate, pH 8.2, containing 0.5 M NaCl, 0.025 M mercaptoethanol, and 4×10^{-5} M dUMP, and stirred for 10 min. Then the affinity carrier was brought back to the column and washed with 1000 ml of the same buffer. The enzyme was eluted by 0.5 M phosphate, pH 7.2, containing 0.5 M NaCl, 20% sacharose or glycerol, and 6.81×10^{-5} M methylenetetrahydrofolate. The active fraction eluted usually in 50 ml of buffer and was concentrated by ultrafiltration to a final volume 2–3 ml. Disc electrophoresis was carried out in 7.5% acrylamide gel using Tris-HCl–Tris-glycine, pH 9.0, buffer systems (Gordon et al., 1967).

Protein concentration was determined either by absorption at 280 nm or according to the method of Lowry et al. (1951).

The activity of thymidylate synthetase was estimated by the modified (Slavik et al., 1967) method of Wahba and Friedkin (1962) based on the oxidation of N^5,N^{10}-methylenetetrahydrofolate to dihydrofolate. Nanomoles of dihydrofolate formed per hour at 20 °C was defined as the enzyme unit.

Results

The affinity carriers were prepared by the reaction of tetrahydromethotrexate with aminoalkyl-Sepharose promoted by water-soluble carbodiimide at pH 5–6 in water in the absence of oxygen. The reaction had to proceed in absence of any reducing agent (mercaptoethanol, ascorbate) because they strongly interferred with the formation of amide bonds.

Initial experiments dealing with the purification of thymidylate synthetase on tetrahydromethotrexate bound with Sepharose were performed using buffers without addition of any substrate or inhibitor. Different carriers were employed containing tetrahydromethotrexate linked to the Sepharose matrix via aminohexyl, aminobutyl, and 1-amino-(2-hydroxy)propyl or aminoethyl spacers. The carriers with the aminohexyl or aminobutyl spacers adsorbed the enzyme from 0.01 M phosphate buffer strongly, whereas the absorption by the carriers containing the aminoethyl or 1-amino-2-hydroxypropyl spacers was poor.

The comparison of the carriers containing tetrahydromethotrexate with those containing the free spacer groups only showed that tetrahydromethotrexate did not participate in the adsorption of the enzyme under the conditions described. Therefore, the observed adsorption was considered to be of nonspecific ("hydrophobic") nature and attempts were made to purify the enzyme by a method based on this principle (Table I).

In column arrangement, the enzyme from *E. coli* could be adsorbed from 0.01 M phosphate to aminobutyl-Sepharose and eluted by 0.2 M phosphate (purified about 20 times) (Table II). A similar column of aminoethyl-Sepharose did not adsorb thymidylate synthetase from *E. coli* from 0.01 and 0.05 M phosphate buffers. Therefore, aminoethyl-Sepharose has been chosen as the suitable carrier for affinity chromatography experiments.

Because the enzyme was not adsorbed to tetrahydrometh-

TABLE II: Purification of *E. coli* Thymidylate Synthetase by Means of Hydrophobic Chromatography on Aminobutyl-Sepharose.

Purification Stage	Total Act. (nmol/h)	Sp. Act. (nmol h^{-1} mg^{-1})	Purification	Yield (%)
Crude enzyme preparation (30–70% $(NH_4)_2SO_4$ fraction)	21 390	51.3	1	100
0.1 M buffer eluate	28 266	936.0	18	132[a]

[a] Apparent increase of the yield over the initial value could be caused by the separation of the enzyme from its natural inhibitor.

TABLE III: Effect of Two Different Concentrations of Deoxyuridylate on the Specific Sorption and Elution of Thymidylate Synthetase on Tetrahydromethotrexate-Aminoethyl-Sepharose.[a]

	Sample No.			
	1	2	3	4
Concn of dUMP (M)	0	4×10^{-6}	2×10^{-5}	2×10^{-5}
Act. in the supernatant (0.01 M phosphate)	567.00	221.48	31.64	32.90
Act. in the eluate (0.2 M phosphate)	11.39	36.70	20.25	212.10[b]

[a] Ten milliliters of the crude enzyme preparation from *E. coli* was dialyzed overnight against 2000 ml of 0.01 M phosphate buffer, pH 7.5, containing 0.025 M mercaptoethanol and cleared by centrifugation. The enzyme solution was distributed into four centrifugation tubes in 2-ml portions and diluted with 0.01 M phosphate buffer to 5 ml. To each tube, 2 ml of 50% suspension of tetrahydromethotrexate-aminoethyl-Sepharose equal to 1-ml bed volume containing 5.7 μmol of tetrahydromethotrexate was added. A 4×10^{-4} M dUMP solution was added to the requested concentration in the suspension. The samples were stirred and allowed to stand for 30 min. The gel was separated by centrifugation and the supernatant was assayed for enzyme activity. The sedimented samples of the gel were washed with 0.01 M phosphate buffer containing 0.025 M mercaptoethanol and the same concentration of dUMP as in the incubation mixture. The gel was eluted either with 5 ml of 0.2 M phosphate buffer containing 0.025 M mercaptoethanol and dUMP in the same concentration as in the incubation mixture for 30 min (samples 2 and 3) or with the same buffers but without dUMP (sample 4). The carrier was separated by centrifugation and the supernatant was assayed for the enzyme activity. All operations were carried out at 0–4 °C. [b] dUMP in the elution buffer omitted.

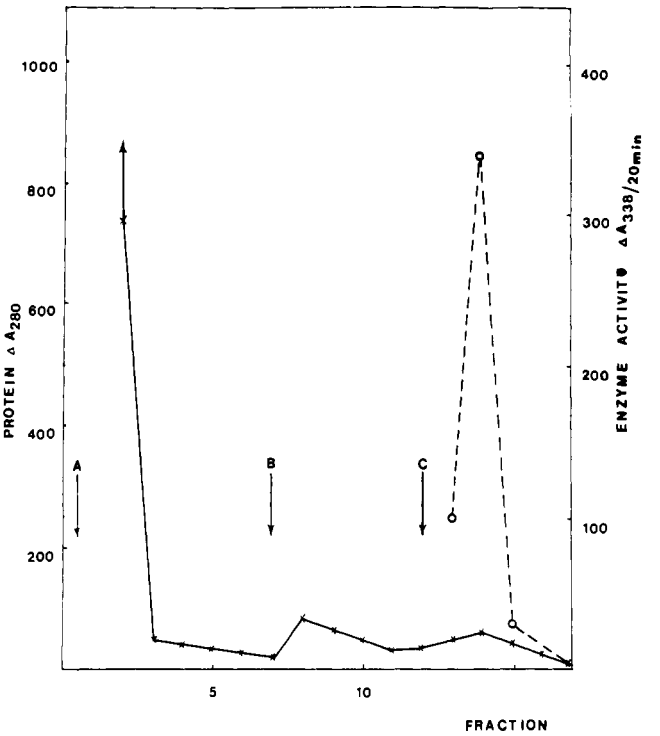

FIGURE 1: Affinity chromatography of *E. coli* thymidylate synthetase on tetrahydromethotrexate-aminoethyl-Sepharose in the presence of dUMP. A_{280} (—), $A_{338}/20$ min (- - -). (A) Phosphate buffer, 0.01 M, containing 2×10^{-5} M dUMP; (B) 0.2 M phosphate buffer containing 2×10^{-5} M dUMP; (C) 0.2 M phosphate buffer containing 20% sacharose. All buffers contained 0.025 M mercaptoethanol. Arrows indicate buffer change.

otrexate aminoethyl-Sepharose from dilute buffers, the effect of the addition of deoxyuridine 5′-monophosphate on its selective sorption was investigated.

Table III shows the enhancing effect of the addition of dUMP on the sorption of thymidylate synthetase by tetrahydromethotrexate-aminoethyl-Sepharose as estimated by the batchwise procedure. It has been shown simultaneously that the elimination of dUMP from the buffer liberates the enzyme from the carrier. As a consequence of above mentioned observations, a new affinity chromatography method was elaborated in which *E. coli* thymidylate synthetase was adsorbed from the crude enzyme preparation on the tetrahydromethotrexate-aminoethyl-Sepharose carrier in the presence of dUMP (2×10^{-5} M). Under given conditions, quantitative adsorption occurred. Subsequent stepwise elution with 0.01 and 0.2 M phosphate buffers containing 0.025 M mercaptoethanol, 0.5 M NaCl and 2×10^{-5} M dUMP led to the elution of practically all ballast proteins. The enzyme was liberated from the carrier by elution with 0.2 M phosphate containing 0.025 M mercaptoethanol and 20% sacharose in a single peak (Figure 1). The disc electrophoresis of the concentrated eluate showed one peak of protein corresponding to the peak of the activity (Figure 2a). Therefore, it can be concluded that the presence of dUMP in the crude enzyme preparation and in the elution buffers promotes the specific adsorption of thymidylate synthetase to the tetrahydromethotrexate-aminoethyl-Sepharose carrier enabling effective elution of ballast proteins. The specifically adsorbed enzyme can be eluted with a phosphate buffer of the same concentration in which dUMP is omitted.

Besides *E. coli*, the described procedure was applied to the crude enzyme preparations from calf thymus, Gardner lymphosarcoma, and Sarcoma 180. The latter materials contained a considerable amount of lipoproteins and chylomicrones and were turbid, but the turbidity did not affect the result of the affinity chromatography. Thymidylate synthetase from all animal sources was adsorbed from 0.01 M phosphate, pH 7.2, containing mercaptoethanol and 4×10^{-5} M dUMP. Previous desalting of crude ammonium sulfate fractions was not necessary. The contaminating proteins were eluted by extensive washing with 0.5 M phosphate, pH 8.2, containing 0.5 M NaCl, 0.025 M mercaptoethanol, and 4×10^{-5} M dUMP. The enzyme was liberated by 0.5 M phosphate with 20% sacharose

TABLE IV: Comparison of the Purification of Thymidylate Synthetase from Different Sources by Affinity Chromatography on Tetrahydromethotrexate–Aminoethyl-Sepharose.

Fraction[a]	Volume (ml)	Total Protein (mg)	Total Act. (units)	Sp. Act. (units/mg of protein)	Purification	Recovery (%)
(a) Streptomycin supernatant	1000	21 680	749 000	34.50	1.00	100.00
30–70% ammonium sulfate fraction	90	6 800	236 311	43.58	1.26	39.50
Affinity fraction	8.10	0.75	509 742	677 848	19 647	68.05
(b) Streptomycin supernatant	460	36 800	89 305	2.42	1.00	100.00
30–70% ammonium sulfate fraction	80	23 520	66 208	2.81	1.16	74.10
Affinity fraction	1	0.28	29 438	102 352	42 368	32.96
(c) Streptomycin supernatant	420	10 091	53 760	5.32	1.00	100.00
30–70% ammonium sulfate fraction	60	3 406	72 960	21.32	4.02	26.60
Affinity fraction	3	7.74	27 249	3 519	661	28.10
(d) Streptomycin supernatant	260	19 660	67 000	3.40	1.00	100.00
30–70% ammonium sulfate fraction	41	2 589	17 835	6.90	2.02	135.70
Affinity fraction	4	7.95	18 875	2 135	625	50.68

[a] (a) *E. coli;* (b) calf thymus; (c) Sarcoma 180; (d) Gardner lymphosarcoma.

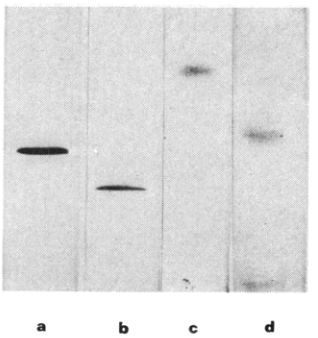

FIGURE 2: Disc electrophoresis of thymidylate synthetase from different sources purified by affinity chromatography on tetrahydromethotrexate-aminoethyl-Sepharose. (A) *E. coli;* (B) thymus; (C) Sarcoma 180; (D) Gardner lymphosarcoma.

and it was eluted in a larger volume (about 50 ml). The addition of methylenetetrahydrofolate in a concentration of 6×10^{-5} M to the elution buffer lowered the elution volume to about half. The active fraction from calf thymus and Sarcoma 180 gave a single peak on disc electrophoresis (Figure 2b,c). The enzyme from Gardner lymphosarcoma showed beside of the main protein zone two minor faster moving impurities (Figure 2d). Table IV shows the recovery and degree of purification of enzymes isolated from various sources. The overall yield varied from 30 to 60% with respect to the streptomycin supernatant. Thymidylate synthetase was purified about 19 000 times from *E. coli* and more than 42 000 times from thymus (as estimated by increase of specific activity) but only about 600 times from tumor materials.

An apparent increase of the total enzyme amount could be observed during some purification steps from *E. coli,* probably due to the removal of natural inhibitors. The purified enzyme rapidly lost its activity but it could be maintained in its active form in the presence of sacharose or glycerol for several days at 0 °C.

The tetrahydromethotrexate–aminoethyl–Sepharose column could be used only for one purification. Repeated use did not give reproducible results. However, once purified enzyme could be rechromatographed on another freshly prepared column without substantial loss of activity.

If commercial CNBr-Sepharose or a Sepharose preparations stored for a longer period of time were used as the starting material, nonspecific sorption of ballast proteins disturbed the purification, which gave a preparation containing only 50–60% of enzyme and several contaminants.

Discussion

Two main problems should be solved if a one-step purification procedure for thymidylate synthetase by affinity chromatography had to be elaborated: first, to overcome the low affinity of the enzyme to the substrates or inhibitors bound to macromolecular matrix and second, to eliminate the nonspecific "hydrophobic" sorption (O'Carra et al., 1974) of thymidylate synthetase to the spacer groups together with other "sticky" proteins.

Tetrahydromethotrexate bound via its carboxylic groups to aminoalkyl-Sepharose did not exert itself appropriate binding of the enzyme from *E. coli* like dUMP or fluorodeoxyuridylate.

Our previous experiments have shown that the principle of ternary complex formation can be exploited for the purification of enzymes by affinity chromatography especially if the affinity of the individual substrates towards the enzymes was low (high apparent K_M).

Experimental evidence has shown that a binary complex enzyme–substrate has higher affinity to a second substrate bound to the affinity carrier than the enzyme itself. Formiminotransferase was adsorbed to tetrahydrofolate bound to aminohexyl-Sepharose matrix in the form of its complex with formimino glutamate (Slavík et al., 1974). Tetrahydrofolate formylase and methylenetetrahydrofolate dehydrogenase (an enzyme complex) could be adsorbed to tetrahydrofolate–aminohexyl-Sepharose in the form of its complex with Mg^{2+} and ATP and eluted with formate buffers (Pohl, 1975).

The formation of an analogous complex of thymidylate synthetase with tetrahydromethotrexate and deoxyuridylate was expected because thymidylate synthetase has been found to form ternary complexes of the enzyme–substrate–inhibitor type. Two covalent complexes of thymidylate synthetase with 5-fluorodeoxyuridylate and methylenetetrahydrofolate were described (Aull et al., 1974; Danenberg et al., 1974). Besides, evidence has been obtained that thymidylate synthetase forms dissociable noncovalent complexes with fluorodeoxyuridylate and some tetrahydrofolate analogues, i.e., N^{10}-methyltetrahydrofolate (Danenberg et al., 1974). Therefore, greater affinity of a binary complex of thymidylate synthetase with dUMP, existence of which has been shown by circular dichroism (Leary et al., 1975), towards tetrahydro-

methotrexate, was assumed. The presence of dUMP in the concentration of 2×10^{-5} M in the crude enzyme preparation appeared to be high enough to bring the enzyme into its binary complex and to enhance its affinity towards bound tetrahydromethotrexate, leading to the complete specific sorption of the enzyme. The fact that the enzyme can be liberated from the carrier by the elimination of dUMP from the elution buffer supports the reversible nature of the complex.

The comparison of the carriers with the different lengths of the spacers has shown that strong nonspecific sorption of thymidylate synthetase together with other proteins occurs if the aminoalkyl residue contains 4–6 carbon atoms. The sorption increases with the length of the carbon chain.

This hydrophobic sorption—perhaps of limited use for purification experiments—concerned thymidylate synthetase from all sources investigated and can be explained by the supposed existence of a hydrophobic area in the molecule of the enzyme both of bacterial and of animal origin. Such a possibility would be interesting from the point of view of the hypothesis that thymidylate synthetase could participate in the multienzyme complex of DNA synthesis (Baril et al., 1974).

On the other hand, the hydrophobic character of the enzyme led to the necessity of choosing a hydrophilic spacer for binding of tetrahydromethotrexate to Sepharose. Experimental evidence has been obtained that the aminoethyl chain does not effect any steric hindrance in the accessibility of the affinant for the enzyme-dUMP complex. All crude enzyme preparations used as starting materials contained considerable amounts of other hydrophobic proteins that were adsorbed nonspecifically. If the aminoethyl spacer was used, the undesirable proteins could be eluted by appropriate buffers. But if the 1-amino-2-hydroxypropyl chain was used for binding of tetrahydromethotrexate to the matrix some contaminating proteins were eluted together with the enzyme, although their amount was quite small. This fact could be detectable only if the enzyme purity was checked by disc electrophoresis. Therefore, the aminoethyl spacer was chosen as the optimal one.

All preparations of tetrahydromethotrexate were mixtures of two diastereomers and contained dihydromethotrexate. The presence of variable amounts of dihydromethotrexate did not affect the results because dihydromethotrexate has lower affinity (one order of magnitude higher K_I) towards thymidylate synthetase from all sources reported here.

Concerning the affinity of the carrier for enzymes of bacterial and animal origin, the animal enzymes seem to be bound more firmly, since their K_I for tetrahydromethotrexate is about ten times lower than that for *E. coli* enzyme. Therefore, buffers of higher ionic strength and substrate addition were necessary to liberate the animal enzymes from the affinity carrier.

The relatively lower increase of the specific activity of tumor enzymes in comparison with those from thymus and *E. coli* in the course of their purification cannot be explained satisfactorily at present and it will need further investigation. Despite some disadvantages represented mainly by the chemical instability of the affinity carrier, the described method can be applied to different sources of thymidylate synthetase rather poor in the enzyme content and it represents a tool for comparative studies of thymidylate synthetase of different origin.

References

Aull, J. L., Lyon, J. A., and Dunlap, B. R. (1974), *Arch. Biochem. Biophys. 165,* 805.

Baril, E., Baril, B., Elford, H., and Luftig, R. B. (1974), *Mech. Regul. DNA Replication,* 275.

Blakley, R. L. (1963), *J. Biol. Chem. 238,* 2113.

Crusberg, T. C., and Kisliuk, R. L. (1969), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 28,* 473.

Crusberg, T. C., Leary, R., and Kisliuk, R. L. (1970), *J. Biol. Chem. 245,* 5292.

Danenberg, P. V., Langenbach, R. J., and Heidelberger, C. (1972), *Biochem. Biophys. Res. Commun. 49,* 1029.

Danenberg, P. V., Langenbach, R. J., and Heidelberger, C. (1974), *Biochemistry 13,* 962.

Dunlap, R. B., Harding, N. C. L., and Huennekens, F. M. (1971), *Biochemistry 10,* 88.

Fridland, A., Langenbach, J., and Heidelberger, C. (1971), *J. Biol. Chem. 246,* 7110.

Friedkin, M., Crawford, E. J., Donovan, E. J., and Pastore, E. J. (1962), *J. Biol. Chem. 237,* 3811.

Galivan, J., Maley, G. F., and Maley, F. (1974), *Biochemistry 13,* 2282.

Gordon, A. H. (1967), in Laboratory Techniques in Biochemistry and Molecular Biology, Work, T. S., and Work, E., Ed., Amsterdam, London, Academic Press.

Greenberg, D. M., Nath, R., and Humphreys, G. K. (1961), *J. Biol. Chem. 236,* 2271.

Gupta, V. S., and Meldrum, J. B. (1972), *Can. J. Biochem. 50,* 352.

Hartman, W. W., and Drager, E. E. (1947), *Org. Synth. 2,* 150.

Leary, R. P., Beaudette, N., and Kisliuk, R. L. (1975), *J. Biol. Chem. 250,* 4864.

Leary, R. P., and Kisliuk, R. L. (1971), *Prep. Biochem. 1,* 47.

Lorenson, M. Y., Maley, G. F., and Maley, F. (1967), *J. Biol. Chem. 242,* 3332.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 242,* 3332.

Mathews, C. K., and Cohen, S. S. (1963), *J. Biol. Chem. 238,* 367.

O'Carra, P., Barry, S., and Griffin, I. (1974), *FEBS Lett. 43,* 169.

Pohl, J. (1975), The Use of the Complexes Enzyme—Substrates for Purification of Tetrahydrofolate Formylase, Dissertation Thesis, Charles University, Prague, Czechoslovakia.

Porath, J., Apsberg, K., Drevin, H., and Axén, R. (1973), *J. Chromatogr. 86,* 53.

Rode, W. (1975), unpublished results.

Slavík, K., Slavíková, V., and Kolman, Z. (1960), *Collect. Czech. Chem. Commun. 25,* 1929.

Slavík, K., Slavíková, V., Motyčka, K., and Hermanová, E. (1969), *Fed. Eur. Biochem. Soc., Meet., 5th 16,* 65.

Slavík, K., and Zakrzewski, S. F. (1967), *Mol. Pharmacol. 3,* 370.

Slavík, K., Žižkovský, V., Slavíková, V., and Fořt, P. (1974), *Biochem. Biophys. Res. Commun. 59,* 1173.

Wahba, A. J., and Friedkin, M. (1962), *J. Biol. Chem. 273,* 3794.

Whiteley, J. M., Jerkunica, I., and Deits, T. (1974a), *Biochemistry 13,* 2044.

Whiteley, J. M., Jerkunica, I., and Deits, T. (1974b), in Immobilized Biochemicals and Affinity Chromatography, Dunlap, B., Ed., New York, N.Y., Plenum Press, p. 135.