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Thymidylate synthetase purified to homogeneity from human leukemic cells

(affinity chromatography/fluorinated pyrimidines/enzyme–inhibitor complex/neoplastic tissue/amino acid analysis)

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ABSTRACT **Thymidylate synthetase (5,10-methylenetetrahydrofolate:dUMP *C*-methyltransferase, EC 2.1.1.45) from a human leukemic cell line has been purified to homogeneity with one-step affinity column chromatography. The purified enzyme has a specific activity of 3.8 μmol/min per mg of protein, which corresponds to a turnover number of 250. These are the highest values reported for a thymidylate synthetase from neoplastic tissue. A ratio of 1.7 mol of 5-fluoro-2′-deoxyuridylate binds per mol of enzyme in the presence of 5,10-methylenetetrahydrofolate. The ternary complex so formed migrates intact on denaturing gels and can be precipitated with trichloroacetic acid; however, urea dissociates the ternary complex. The human thymidylate synthetase is composed of two subunits of 33,000 daltons each. It contains more residues of cysteine, glycine, and arginine and fewer of histidine than the well-studied thymidylate synthetase from *Lactobacillus casei*.**

Thymidylate synthetase (5,10-methylenetetrahydrofolate: dUMP *C*-methyltransferase, EC 2.1.1.45) has been the object of considerable interest because of its key function in mammalian DNA synthesis as the only *de novo* source of thymidylate. This enzyme is believed to be the major site of action of the cancer chemotherapeutic drug 5-fluorouracil due to inhibition by the metabolite 5-fluoro-2′-deoxyuridylate (FdUMP) (1). FdUMP, 5,10-methylenetetrahydrofolate (5,10-CH_2H_4PteGlu), and thymidylate synthetase form a ternary complex (2), which is joined by covalent bonds in some cases—i.e., is stable to protein denaturants and limited proteolysis (3–6). In other cases, the ternary complex is dissociated partially (7) or completely (8) by denaturants or is entirely disrupted merely upon gel filtration (8). The type of bond FdUMP forms with human thymidylate synthetase has not been determined. Because the nature of this complex in human tumors may be a determinant for the chemotherapeutic effectiveness of 5-fluorouracil, it would be important to isolate and characterize this key enzyme from human tumor tissues and to study its interaction with FdUMP.

Until recently (9), few attempts were made to isolate thymidylate synthetase from human tumors, presumably because of its low levels in human tissues and difficulties in stabilizing the mammalian enzyme (10–12). Most of the mechanistic studies on thymidylate synthetase have been performed with the stable enzyme isolated from a methotrexate-resistant strain of *Lactobacillus casei* (13), which is an especially rich source of the enzyme. Recently, Slavik *et al.* (12) used affinity chromatography with tetrahydromethotrexate (H_4MTX) as the affinity ligand to obtain homogeneous thymidylate synthetase from enzyme-poor sources such as calf thymus; however, much less purification was obtained from tumor materials. We report here a purification to homogeneity of thymidylate synthetase from human neoplastic cells and describe some properties of the pure enzyme, including its interaction with FdUMP.

MATERIALS AND METHODS

Materials. L-1-(+) Tetrahydrofolate and F[^{3}H]dUMP (22 Ci/mmol, 1 Ci = 3.70×10^{10} Bq) were synthesized as described (14, 15). H_4MTX was prepared by catalytic hydrogenation of methothrexate (16) and was used without purification. H_4MTX was lyophilized in ampules, sealed in N_2, and stored in the dark at −25°C.

Aminoethyl polyacrylamide gel beads (Aminoethyl Bio-Gel P-150) were purchased from Bio-Rad, as were the reagents for polyacrylamide gel electrophoresis. Sephadex G-100 was obtained from Pharmacia. Other biochemicals were from Sigma.

Cell Culture. CCRF-CEM human lymphoblastic leukemia cells (17) were thawed from mycoplasma-free stocks at 2½-month intervals and were monitored routinely for absence of mycoplasma. These cells were grown in suspension in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum (GIBCO) and were harvested in late logarithmic growth, washed once with phosphate-buffered saline, and used immediately or stored in liquid N_2.

Affinity Column Preparation. Aminoethyl polyacrylamide (0.5 g) was hydrated with water and reacted with 20 mg of H_4MTX and 150 mg of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide·HCl by the method of Slavik *et al.* (12), except that N_2 gas was used. About 40% of the resultant gel was transferred into a small plastic column (5-ml Pipetman tips partially stuffed with glass wool) in a cold room (4°C). The light-tan beads were washed overnight with 200 ml of 2.5% $NaHCO_3$/0.5 M KCl containing 0.15 M 2-mercaptoethanol. The columns were then washed with 100 ml of 10 mM potassium phosphate buffer (pH 7.4) containing 0.15 M 2-mercaptoethanol, or until the effluent pH was neutral, and then with 15 ml of the same buffer containing 1 mM dUMP (buffer A). Columns were usually used the day after preparation.

Spectrophotometric Assay. A modification of the assay of Wahba and Friedkin (18) was used. 5,10-CH_2H_4PteGlu was prepared by dissolving 3.0 μmol of tetrahydrofolate in 0.5 ml of a deaerated solution of 0.26 M formaldehyde, 80 mM dithiothreitol, and 50 mM Tris·HCl (pH 8.0). The standard reaction mixture contained in a volume of 408 μl: 150 μM 5,10-

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Abbreviations: 5,10-CH_2H_4PteGlu, 5,10-methylenetetrahydrofolate; H_4MTX, tetrahydromethotrexate; $NaDodSO_4$, sodium dodecyl sulfate.

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CH_2H_4PteGlu, 5.0 mM dUMP, 8.0 mM dithiothreitol, 50 mM Tris·HCl (pH 7.4), 204 μg of bovine serum albumin, and 1–5 × 10^{-4} units of enzyme. Enzyme units are defined as μmol of product formed per min at 37°C. The mixture was preincubated at 37°C and enzyme was added to start the reaction. For each assay, a control reaction was run with all components except enzyme, which was replaced by the appropriate buffer. Controls were also run on occasion with enzyme and all other components except dUMP. Reaction rates were linear with time for at least 20 min.

Thymidylate Synthetase–FdUMP–5,10-CH_2H_4PteGlu Ternary Complex Formation and Assay. The ^{3}H-labeled CCRF-CEM thymidylate synthetase ternary complex was formed by incubating in a volume of 240 μl: 200 nM F[^{3}H]-dUMP, 150 μM 5,10-CH_2H_4PteGlu, 120 μg of bovine serum albumin, and enzyme in 50 mM Tris·HCl (pH 7.4). The reaction was allowed to proceed for 2 hr at 37°C. No F[^{3}H]dUMP bound to the enzyme in the absence of 5,10-CH_2H_4PteGlu. The *L. casei* ternary complex was prepared similarly, but without bovine serum albumin. Ternary complex formation was assayed with nitrocellulose filters as described (14, 19), except that solutions to be filtered were made up to 0.2 M KCl in order to obtain quantitative filtration efficiency (20). Radioactivity was determined on a Beckman LS 9000 counter equipped with programs for random coincidence monitoring and data reduction to obtain dpm.

Protein Assay. The method of Bensadoun and Weinstein (21) was modified as follows to increase the sensitivity 4-fold. After two precipitations of protein samples with deoxycholate and trichloroacetic acid, 180 μl of 0.18 M KOH was added to dissolve the precipitate. Protein was then determined by the Hartree method (22) with 60 μl of Hartree reagent A, 7 μl of Hartree reagent B, followed by 133 μl of 1 vol of Folin–Ciocalteu reagent (2N-Sigma) diluted with 5 vol of water. The precipitate that formed was removed by centrifugation. Absorbance of the samples was determined at 750 nm. As little as 0.5 μg of protein could be measured readily, and the assay was linear from 0.5 to 5 μg of protein.

Sodium Dodecyl Sulfate ($NaDodSO_4$)/Polyacrylamide Gel Electrophoresis. In order to concentrate protein samples for visual observation of stained bands, we precipitated protein with deoxycholate and trichloroacetic acid (21). Excess acid and most of the deoxycholate were removed with two ether washes followed by lyophilization for at least 2 hr. The protein was resuspended in sample buffer (23) containing 1.5% $NaDodSO_4$, denatured by heating for 2 min at 100°C, and then applied to $NaDodSO_4$ stacking gels (23). Proteins used as molecular weight standards migrated identically with or without pretreatment with deoxycholate and trichloroacetic acid, as did the bands of an overloaded, partially purified preparation of thymidylate synthetase from *L. casei*.

To locate the radioactivity in gels containing the ^{3}H-labeled thymidylate synthetase ternary complex, we sliced the gels into uniform 1-mm sections with an Aliquogel Fractionator (Gilson Medical Electronics, Inc., Middleton, WI). A solution of 0.5 M ammonium bicarbonate containing 0.1% $NaDodSO_4$ (1 ml) was added to each section in a scintillation vial, and the vials were capped tightly and incubated for 18 hr at 37°C. RIA-Solve II (Research Products Intl. Corp., Elk Grove Village, IL) was added to each vial for scintillation counting.

Amino Acid Analysis. Analyses were performed at AAA Laboratory (Mercer Island, WA) on two 20-μg samples of the pure enzyme. One sample was hydrolyzed for 24 hr by the method of Hirs (24) and the other for 24 hr according to Simpson *et al.* (25).

RESULTS

Purification of thymidylate synthetase

CCRF-CEM cells (25 g) were suspended in approximately 80 ml of ice-cold 50 mM Tris·HCl, pH 7.4/0.1 M 2-mercaptoethanol, and were sonicated (Biosonik IV) five times for 12–15 sec with a medium probe. All preliminary operations were carried out at 0–4°C.

The cell extract was separated from debris by centrifugation at 25,000 × *g* for 25 min. The precipitate was discarded, and 30% (wt/vol) streptomycin sulfate was added dropwise to the cell extract to bring the final concentration to 1%. After additional stirring for 10 min, the precipitate was removed by centrifugation at 18,000 × *g* for 15 min. This supernatant was brought to 30% saturation by slow addition of solid ammonium sulfate with stirring, and this mixture was centrifuged at 25,000 × *g* for 25 min. Additional ammonium sulfate was added to this supernatant to bring the concentration to 62% saturation, and the material was again centrifuged at 25,000 × *g* for 25 min.

The precipitate obtained was resuspended in 15 ml of 10 mM potassium phosphate, pH 7.4/0.15 M 2-mercaptoethanol. dUMP was added to a concentration of 1 mM, and the mixture was added directly to the H_4MTX-aminoethyl polyacrylamide gel column in a cold room (4°C). The column was washed with 100 ml of buffer A (10 mM phosphate, pH 7.4/0.15 M 2-mercaptoethanol/1 mM dUMP) followed by a linear gradient of 100 ml of buffer A and 100 ml of buffer B (0.2 M phosphate, pH 7.4/0.5 M KCl/0.15 M 2-mercaptoethanol/2 mM dUMP). Then, 150 ml of buffer C (0.2 M phosphate, pH 7.4/1.0 M KCl/0.15 M 2-mercaptoethanol/3 mM dUMP) was passed through the affinity column. The column was finally washed with 35 ml of buffer D (50 mM Tris·HCl, pH 7.4/1.0 M KCl/20 mM dithiothreitol/2 mM dUMP/10% glycerol). The affinity column was allowed to warm up to room temperature (20°C) and was washed with an additional 15 ml of buffer D. Thymidylate synthetase was eluted in about 35 ml of buffer E (50 mM Tris·HCl, pH 7.4/1.0 M KCl/20 mM dithiothreitol/10% glycerol) and placed in ice. The column was eluted at a rate of 0.2–0.6 ml per min, and 5-ml factions were collected.

A 5200-fold purification of thymidylate synthetase was obtained with 34% recovery of the enzyme from the cell-free extract (Table 1). In a typical purification, 65.6 μg of enzyme was obtained from 25.5 g of CCRF-CEM cells. The specific activity of the purified enzyme is 3.8 μmol of product per min per mg of protein.

Table 1. Purification of CCRF-CEM thymidylate synthetase

Step	Total units	Total protein, mg	Specific activity, units/mg protein	Purification	Yield, %
Cell-free extract	—	1010	—	1*	100*
Streptomycin sulfate	—	666	—	1.48*	98*
$(NH_4)_2SO_4$ fraction	0.588	468	0.00126	1.72*	80*
H_4MTX column	0.249	0.0656	3.8	5200†	34†

* Determined by F[^{3}H]dUMP binding.

† The affinity column gave a 3000-fold purification of the ammonium sulfate fraction as determined by enzyme activity and F[^{3}H]dUMP binding.

Properties of purified enzyme

Enzyme Stability. The enzyme was stabilized by addition of high concentrations of dithiothreitol (20–120 mM) plus bovine serum albumin (1 mg/ml). With 20 mM dithiothreitol and bovine serum albumin, the enzyme retained activity for 3 months when stored at 4° or −25°C. 2-Mercaptoethanol was much less effective than dithiothreitol for maintaining activity. Without thiols, CCRF-CEM thymidylate synthetase lost all activity in 2 days.

Effect of Dithiothreitol, pH, and Cations on Enzyme Activity. Enzyme activity was stimulated at least 2-fold by 8–20 mM dithiothreitol, compared with 2 mM dithiothreitol. (With less than 2 mM dithiothreitol, the nonenzymatic rate of absorbance change was too great for accurate enzyme assay.) Above 20 mM dithiothreitol, the thiol reagent inhibited the enzyme. The enzyme demonstrated optimal activity between pH 7.0 and 8.1. CCRF-CEM thymidylate synthetase was not activated by the chloride salts of Ca, Mg, Ba, Li, or K.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. As shown in Fig. 1, 8.3 μg of purified enzyme gave a single band when subjected to $NaDodSO_4$ gel electrophoresis after concentration with deoxycholate and trichloroacetic acid. An impurity of less than 5% could have been detected, indicating that the preparation is minimally 95% pure. When the concentration step with deoxycholate and trichloroacetic acid was omitted, a single faint band was located at the same position. (Attempts to concentrate the pure enzyme by ultrafiltration resulted in apparent loss of protein.) The band was identified as thymidylate synthetase by location of protein-bound F[^{3}H]dUMP when the CCRF-CEM ternary complex was subjected to electrophoresis on a parallel gel (Fig. 1). As with *L. casei* thymidylate synthetase (26), there was no significant difference in mobility between the CCRF-CEM enzyme and its ternary complex.

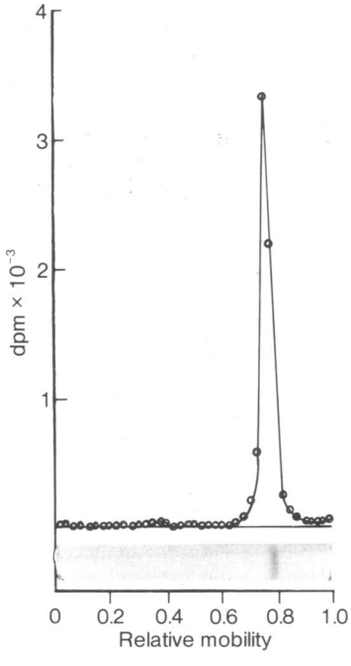

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of purified CCRF-CEM thymidylate synthetase and location of its F[^{3}H]dUMP ternary complex. Two gels were run together. In the first gel (*Lower*), 8.3 μg of purified thymidylate synthetase was concentrated, denatured by heating for 2 min at 100°C in 1.5% $NaDodSO_4$, and subjected to electrophoresis. The gel was fixed in 10% trichloroacetic acid and stained with Coomassie brilliant blue R. In the second gel (*Upper*), 0.08 μg of the F[^{3}H]dUMP–5,10-CH_2H_4PteGlu–thymidylate synthetase ternary complex formed with purified enzyme was concentrated, denatured, and subjected to electrophoresis with the first gel. After fixation in 10% trichloroacetic acid, the gel was sliced into 1-mm sections. Fractions were extracted and radioactivity was measured.

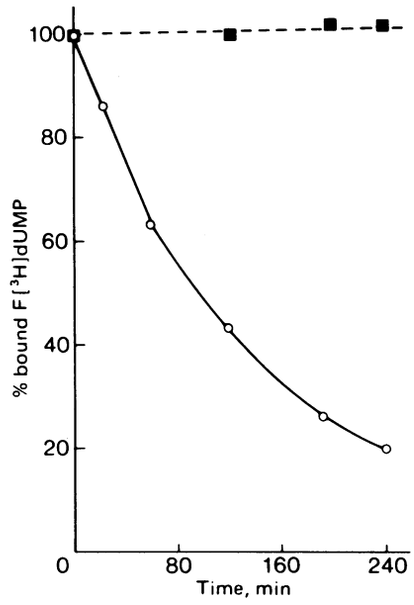

FIG. 2. Effect of 6 M urea on CCRF-CEM and *L. casei* thymidylate synthetase ternary complexes. The complexes were prepared and then incubated at 30°C in 6 M urea containing 20 mM dithiothreitol. Aliquots were filtered periodically through nitrocellulose discs to determine protein-bound radioactivity. F[^{3}H]dUMP–5,10-CH_2H_4PteGlu–enzyme ternary complex from CCRF-CEM (○) and from *L. casei* (■).

Molecular Weight Estimate and Subunit Determination. Results obtained with $NaDodSO_4$ gel electrophoresis and Sephadex G-100 chromatography indicated that the human enzyme was composed of two equivalent subunits of 33,000 daltons each (66,000 for the enzyme M_r). These results will be documented elsewhere.

Effect of Trichloroacetic Acid and Urea on FdUMP–5,10-CH_2H_4PteGlu–Enzyme Ternary Complex. When the ternary complex was treated with cold 10% trichloroacetic acid, 96% of the F[^{3}H]dUMP coprecipitated with the protein. The tritium remained protein bound (as demonstrated by filtration through nitrocellulose discs) when the precipitate was redissolved. However, the ternary complex was disrupted by 6 M urea (Fig. 2). Half of the FdUMP dissociated in 90 min. The remaining nucleotide was removed more slowly, but was 95% dissociated in 20 hr. FdUMP remained bound to the untreated CCRF-CEM ternary complex and to the urea-treated *L. casei* ternary complex.

Stoichiometry of FdUMP Binding to CCRF-CEM Thymidylate Synthetase. When saturating amounts of F[^{3}H]dUMP (200 nM) and 5,10-CH_2H_4PteGlu (150 μM) were incubated with 32 ng of the human enzyme (2 nM), 0.84 pmol of FdUMP was bound, corresponding to 1.7 mol of FdUMP per mol of enzyme.

Amino Acid Composition. The amino acid composition of CCRF-CEM thymidylate synthetase is given in Table 2.

DISCUSSION

These results represent purification to homogeneity of thymidylate synthetase from a human source. This target enzyme for cancer chemotherapy has been isolated in good yield from a crude ammonium sulfate fraction in a one-step purification.

Table 2. Amino acid composition of CCRF-CEM thymidylate synthetase and comparison with *L. casei* and T2 bacteriophage-induced thymidylate synthetases

Amino acid	Residues/mole of protein			
	CCRF-CEM	*L. casei*		T2 phage
Ala	32.2	35.5*	36.0†	28.0‡
Arg	33.9	24.2	19.6	26.3
Asp	49.6	62.8	63.0	61.8
Cys	9.4	3.6	4.2	9.9
Glu	47.1	48.9	49.0	55.5
Gly	60.0	31.0	32.6	39.9
His	13.2	34.2	—	14.2
Ile	23.8	24.7	24.0	35.0
Leu	51.9	58.4	58.2	62.1
Lys	26.8	35.2	30.8	44.8
Met	9.7	9.3	10.4	8.7
Phe	25.9	31.2	31.0	22.9
Pro	31.1	33.5	29.4	27.2
Ser	29.3	22.2	22.6	22.7
Thr	24.7	27.2	25.2	22.4
Trp	—	12.0§	—	13.5
Tyr	18.0	22.0	23.4	29.4
Val	31.2	28.8	24.0	33.9

* Ref. 27.
† Ref. 28.
‡ Ref. 7.
§ Ref. 29.

With H_4MTX on hand, the entire purification, including preparation of the affinity column, can be completed in 48 hr.

Homogeneous CCRF-CEM thymidylate synthetase has a specific activity (3.8 units/mg of protein) 200 times greater than that reported as the minimum specific activity of thymidylate synthetase derived from human leukemic blast cells (9) and is, in fact, greater than the specific activity of thymidylate synthetase isolated from any mammalian tissue so far reported. Pure CCRF-CEM thymidylate synthetase has a specific activity comparable to the *L. casei* enzyme (14, 29), but lower than that of the enzyme isolated from *E. coli* (12) or the T2 bacteriophage-induced thymidylate synthetase (7). The turnover number of the enzyme at 37°C is calculated as 250/min.

H_4MTX acts as a specific affinity ligand for purification of thymidylate synthetase in the presence of dUMP through formation of a tightly bound complex which retains the enzyme on the column in the presence of the high-ionic-strength buffers required to wash out all contaminating proteins (12). When dUMP is eliminated, the enzyme elutes from the column. As was noted by workers using a methotrexate affinity column (9), the enzyme elutes more readily at room temperature than in the cold. We found, however, that with methotrexate as the affinity ligand, thymidylate synthetase did not remain bound when the column was washed with high-ionic-strength buffers containing dUMP.

Polyacrylamide was used as the ligand support because its hydrophilic character reduces nonspecific protein binding (30). In addition, commercial availability of aminoethyl polyacrylamide permits a much more facile preparation of the affinity column with a one-step procedure, in contrast to the Sepharose column (12). Because of the known instability of H_4MTX, each column was used within a day of preparation and for only one purification. This presented little practical difficulty because of the ease of preparing new columns.

The ratio of 1.7 mol of FdUMP bound per mol of enzyme molecule is very similar to that found for the *L. casei* (6, 31, 32) and T2 phage-induced enzymes (7).

The effects of protein denaturants were examined to determine the nature of the tight-binding FdUMP–5,10-CH_2H_4PteGlu–enzyme ternary complex. The CCRF-CEM complex migrates intact in $NaDodSO_4$ denaturing gels and survives precipitation by trichloroacetic acid; these results indicate that the complex is covalently bound, similarly to the *L. casei* enzyme (3–6). However, this conclusion is difficult to reconcile with the disruption of these bonds with 6 M urea. It has been reported (7) that 6 M urea likewise dissociates 55% of the T2 phage enzyme ternary complex in 1 hr. In contrast, urea has no effect on the *L. casei* complex (3–5) (Fig. 2).

We have also observed (unpublished data) an enzyme-mediated dissociation of the thymidylate synthetase ternary complex from CCRF-CEM with concomitant recovery of enzymatic activity. These results, similar to what has been found for the *L. casei* enzyme (14), will be reported in detail elsewhere.

The amino acid composition of CCRF-CEM thymidylate synthetase shows greater similarity to the T2 phage enzyme than to the *L. casei* enzyme (Table 2). The human enzyme has considerably more residues of cysteine, glycine, and arginine and fewer of histidine than the bacterial enzyme. The 2.5-fold more cysteine residues in the human enzyme are especially interesting because a cysteine residue is catalytically active (13, 31, 32) and binds FdUMP (28, 33) in the *L. casei* enzyme. For the CCRF-CEM thymidylate synthetase, activation and protection of enzyme activity by sulfhydryl reagents indicate, but do not yet establish, that a thiol-containing residue is directly involved in catalysis. Histidine (34) and arginine (35) have also been implicated in the *L. casei* enzyme catalysis.

We hope that the facile purification of high-activity human thymidylate synthetase described in this report will stimulate further investigations into the mechanisms of action and inhibition of this important enzyme from human sources.

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1. Heidelberger, C. (1975) in *Antineoplastic and Immunosuppressive Agents*, eds. Sartorelli, A. C. & Johns, A. G. (Springer, New York), Part II, pp. 193–231.
2. Danenberg, P. V. (1977) *Biochim. Biophys. Acta* **473**, 73–92.
3. Langenbach, R. J., Danenberg, P. V. & Heidelberger, C. (1972) *Biochem. Biophys. Res. Commun.* **48**, 1565–1571.
4. Santi, D. V. & McHenry, C. S. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 1855–1857.
5. Danenberg, P. V., Langenbach, R. J. & Heidelberger, C. (1974) *Biochemistry* **13**, 926–933.
6. Santi, D. V., McHenry, C. S. & Sommer, H. (1974) *Biochemistry* **13**, 471–481.
7. Galivan, J., Maley, G. F. & Maley, F. (1974) *Biochemistry* **13**, 2282–2289.
8. Capco, G. R., Krupp, J. R. & Mathews, C. K. (1973) *Arch. Biochem. Biophys.* **158**, 726–735.
9. Dolnick, B. J. & Cheng, Y.-C. (1977) *J. Biol. Chem.* **252**, 7697–7703.
10. Horinishi, H. & Greenberg, D. M. (1972) *Biochim. Biophys. Acta* **258**, 741–752.
11. Bonney, R. J. & Maley, F. (1975) *Cancer Res.* **35**, 1950–1956.
12. Slavik, K., Rode, W. & Slavikova, V. (1976) *Biochemistry* **15**, 4222–4227.
13. Dunlap, R. B., Harding, N. G. L. & Huennekens, F. M. (1971) *Biochemistry* **10**, 88–97.
14. Danenberg, P. V. & Danenberg, K. D. (1978) *Biochemistry* **17**, 4018–4024.

15. Moran, R. G., Spears, C. P. & Heidelberger, C. (1979) *Proc. Natl. Acad. Sci. USA*, in press.
16. Hatefi, Y., Talbert, P. T., Osborn, M. J. & Huennekens, F. M. (1960) *Biochem. Prep.* **7**, 89–92.
17. Foley, G. E., Lazarus, H., Farber, S., Uzman, B. G., Boone, B. A. & McCarthy, R. E. (1965) *Cancer* **18**, 522–529.
18. Wahba, A. J. & Friedkin, M. (1961) *J. Biol. Chem.* **236**, PC11–PC12.
19. Santi, D. V., McHenry, C. S. & Perriard, E. R. (1974) *Biochemistry* **13**, 467–470.
20. Kihara, H. K. & Kuno, H. (1968) *Anal. Biochem.* **24**, 96–105.
21. Bensadoun, A. & Weinstein, D. (1976) *Anal. Biochem.* **70**, 241–250.
22. Hartree, E. F. (1972) *Anal. Biochem.* **48**, 422–427.
23. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
24. Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 59–62.
25. Simpson, R. J., Neuberger, M. R. & Liu, T.-Y. (1976) *J. Biol. Chem.* **251**, 1936–1940.
26. Aull, J. L., Lyons, J. A. & Dunlap, R. B. (1974) *Microchem. J.* **19**, 210–218.
27. Dunlap, R. B., Harding, N. G. L. & Huennekens, F. M. (1971) *Ann. N. Y. Acad. Sci.* **186**, 153–165.
28. Danenberg, P. V. & Heidelberger, C. (1976) *Biochemistry* **15**, 1331–1337.
29. Lyon, J. A., Pollard, A. L., Loeble, R. B. & Dunlap, R. B. (1975) *Cancer Biochem. Biophys.* **1**, 121–128.
30. Inman, J. K. (1974) *Methods Enzymol.* **34**, 30–58.
31. Plese, P. C. & Dunlap, R. B. (1977) *J. Biol. Chem.* **252**, 6139–6144.
32. Galivan, J., Noonan, J. & Maley, F. (1977) *Arch. Biochem. Biophys.* **184**, 336–345.
33. Bellisario, R. L., Maley, G. F., Galivan, J. H. & Maley, F. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1848–1852.
34. Langenbach, R. J., Danenberg, P. V., Fridland, A., Cleland, W. W. & Heidelberger, C. (1972) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **31**, 419 (abstr.).
35. Cipollo, K. L. & Dunlap, R. B. (1978) *Biochem. Biophys. Res. Commun.* **81**, 1139–1144.