An Isotopic Assay for Thymidylate Synthetase*

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ABSTRACT: The assay for thymidylate synthetase by the release of tritiated water from deoxyuridine 5′-monophosphate-5-*t* has been simplified. After the enzyme reaction was terminated by the addition of trichloroacetic acid, the residual substrate was adsorbed onto charcoal. An aliquot of the supernatant fluid was removed for counting. This method made it possible for thymidylate synthetase to be easily measured in crude homogenates of mouse ascites tumor cells.

A sensitive isotopic method for the measurement of thymidylate synthetase was based on the release of tritium, as tritiated water, from deoxyuridine 5′-monophosphate specifically labeled in position 5 of the pyrimidine ring when thymidylate is formed (Smith and Greenberg, 1964). These investigators collected the tritiated water released in the methylation reaction in a cold trap to remove the product from the remaining substrate.

A new method is described here which depends upon the removal of the remaining substrate by absorption onto charcoal and assay of the supernatant fluid by liquid scintillation counting. The method is rapid, extremely sensitive, and has been successfully applied to a number of different types of human and mouse cells.

Methods

Isolation of Mouse Ascites Tumor Cells. The mouse ascites tumor cells upon removal from the peritoneum were placed in 5 ml of EDTA (1 mg/ml) at pH 7.5, in 5% dextrose, until an equal volume of ascites fluid was collected. The cells were placed in an ice bath immediately upon removal from the mouse and kept at 0–4° throughout the isolation procedure (Roberts *et al.*, 1965). A 50% suspension of the isolated cells was prepared by the addition of one volume of 0.01 M Tris, pH 7.0, containing 0.25 M sucrose equivalent to the volume of the cell pellet.[1] The cells were disrupted by sonic oscillation and the cellular debris collected by centrifugation at 30,000*g* for 25 min. The supernatant fluid was stored in aliquots at −20°. Approximately 30% of the thymidylate synthetase activity was slowly lost over a 3-month period with storage at −20°.

The final conditions for the method are in many ways similar to the requirements observed by other investigators (Wahba and Friedkin, 1962; Reyes and Heidleberger, 1965) for the assay of thymidylate synthetase.

Reagent A. The stock solution of tetrahydrofolate, 8.6×10^{-4} M; 2-mercaptoethanol, 0.075 M; Tris buffer, 0.235 M, pH 7.5; and formaldehyde, 0.43%, was adjusted to pH 7.5 and prepared fresh each week. This reagent was stored frozen and thawed for a minimum length of time each day.

Reagent B. This solution was prepared fresh just prior to use and contained 250 μl of 1.0 M NaF; 100 μl of 2.8×10^{-3} M deoxyuridine 5′-monophosphate, pH 7.5; 500 μl of 1.0 M Tris, pH 7.5; 120 μl of deoxyuridine 5′-monophosphate-5-*t*, 60 μc, 4.7 mc/mmole; and 500 μl of reagent A. This volume of reagent B was adequate for 100 assays. The tritium-labeled deoxyuridine 5′-monophosphate was divided into aliquots, lyophilized, and stored dry at −10° to keep the blank at a minimum.

Procedure. A 5-μl aliquot of cell homogenate diluted 1:6 with Tris–sucrose solution was incubated for 1 hr at 37° in a 6 mm o.d. × 75 mm tube with 15 μl of reagent B and 20 μl of 1.25% bovine plasma albumin in Tris–sucrose. The reaction was terminated by the addition of 12 μl of 33% trichloroacetic acid. Unlabeled deoxyuridine 5′-monophosphate (5 μl, 5 mg/ml) was added to improve the efficiency of the adsorption of unreacted substrate onto charcoal in the next step. Charcoal (180 μl, 100 mg/ml) was added, and the suspension was centrifuged at 200*g* for 10 min. A 100-μl aliquot of the supernatant fluid was removed and added to 10 ml of 2,5-bis-2-(5-*t*-butylbenzoxazolyl)-thiophene scintillation solution for counting by liquid scintillation.

The charcoal was suspended by mixing with a small magnetic stirrer while aliquots were being removed with a modified Lang–Levy pipet (Lowry *et al.*, 1954). The orifice of the pipet and the opening of the constriction were enlarged to prevent clogging by the charcoal.

The assay was standardized by adding water in place of the charcoal suspension. An aliquot of the solution was removed for counting, and the specific

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[1] Dr. Morris Friedkin in a personal communication suggested the use of 0.25 M sucrose to stabilize the enzyme.

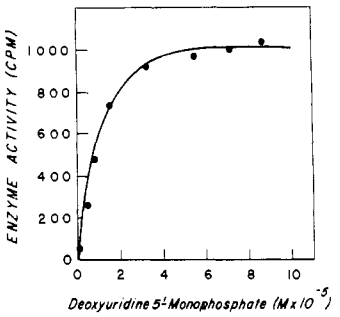

FIGURE 1: Concentration curve with deoxyuridine 5′-monophosphate.

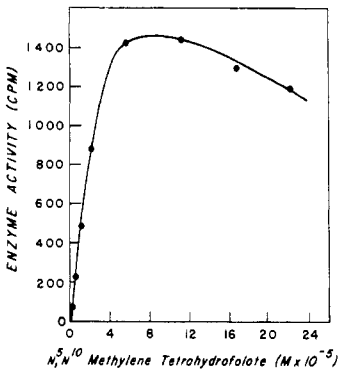

FIGURE 2: Concentration curve with N^5,N^{10}-methylene-tetrahydrofolate.

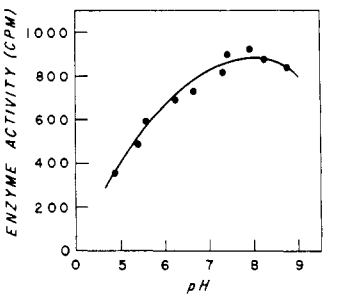

FIGURE 3: pH curve of thymidylate synthetase activity.

activity of the substrate was determined on the basis of the counts per minute per millimicromole of deoxyuridine 5′-monophosphate which was present during the incubation. An unincubated control was used to correct for the presence of nonadsorbable radioactivity in the substrate. Since less than 5% of the substrate was converted to product, the change in the blank as a result of the enzymatic conversion of substrate to product was considered negligible.

Source of Chemicals. Tris and tetrahydrofolate were obtained from Sigma Chemical Co., St. Louis, Mo. Labeled and unlabeled deoxyuridine 5′-monophosphate and bovine plasma albumin were obtained from Calbiochem, Los Angeles, Calif. Activated charcoal, N. F. Powder, was obtained from Merck and Co., Inc., Rahway, N. J. BBOT, 2,5-bis-2-(5-*t*-butylbenzoxazolyl)thiophene, was obtained from Packard Instrument Co., Inc., LaGrange, Ill., and prepared according to their instructions.

Experimental Section

All of the studies to be described were with crude sonicates of L1210 mouse ascites tumor prepared as described above. The optimal level of deoxyuridine 5′-monophosphate was determined from Figure 1.

The concentration curve with N^5,N^{10}-methylene-tetrahydrofolate is shown in Figure 2. The N^5,N^{10}-methylenetetrahydrofolate was prepared as described for reagent A with 1.76×10^{-3} M tetrahydrofolate and with a proportional increase in the concentration of formaldehyde. This reagent was then diluted with modified reagent A lacking tetrahydrofolate and formaldehyde to give the desired concentration.

The variation in enzyme activity with pH is shown in Figure 3. Optimal enzyme activity was observed between pH 7.4 and 8.2. The buffer for the pH curve was 0.01 M Tris, which was adjusted to the final desired pH with citric acid. The assay has been routinely used at pH 7.5 with Tris–HCl buffer.

Mercaptoethanol was required for optimal activity (Figure 4). The 2-mercaptoethanol was added with reagent A in order to take advantage of its presence to stabilize the N^5,N^{10}-methylenetetrahydrofolate. Reyes and Heidleberger (1965) observed that mercaptoethanol stabilized the enzyme. The enzyme reaction was linear for 70 min at both concentrations of mercaptoethanol. The enzyme assay was linear with enzyme dilution when the concentration of the 5-μl aliquot of homogenate was equivalent to 25% (v/v), or less (Figure 5).

When 5 μl of an homogenate of mouse ascites tumor cells, diluted 1:6, was added to 20 μl of a 33% (grams/volume) homogenate of human tissues, the total amount of enzyme activity was greater than the sum of the activity measured for the individual homogenates. Supplementation of the assay of mouse ascites homogenate with 20 μl of 1.25% bovine plasma albumin increased the activity of this fraction, and the sum of the activity of the two preparations now became equivalent to the amount of activity observed when the homogenates were combined. Figure 6 shows the stimulation of thymidylate synthetase activity by bovine plasma albumin.

Supplementation with $MgSO_4$, 6×10^{-4} M, inhibited the reaction. EDTA inhibited the reaction, but with the crude enzyme preparation it was not determined if this agent was acting by inhibiting the enzyme *per se* or by chelating a cation. A slight increase in enzyme activity was observed when sodium fluoride was added to the assay. The stimulation by sodium fluoride was not always observed, but addition of this compound to the assay has been continued.

The conditions described for the assay for thymidy-

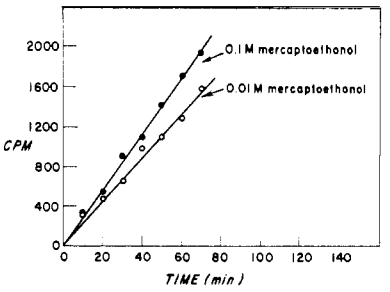

FIGURE 4: 2-Mercaptoethanol stimulation of thymidylate synthetase.

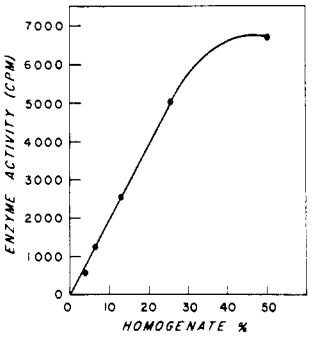

FIGURE 5: Dilution curve of thymidylate synthetase.

late synthetase in homogenates of L1210 leukemic cells have been found applicable to homogenates of three other murine lymphocytic leukemias. The rates of the reaction were linear with time and enzyme dilution for P1534Ja, P288, and P388. The level of enzyme activity of these four strains of tumor cells is presented in Table I.

TABLE I: Thymidylate Synthetase Activity of Mouse Ascites Tumors.

Leukemia	Enzyme Act.[a]
L1210	2.04 ± 0.83
P1534Ja	1.42 ± 0.67
P288	3.17 ± 1.42
P388	2.60 ± 0.65

[a] Millimicromoles of thymidine 5′-monophosphate per hour per milligram of protein. Protein was determined by the method of Lowry *et al.* (1951). Cells from three animals were pooled, and the results with three to five groups of pooled cells were averaged and are presented with the standard deviation.

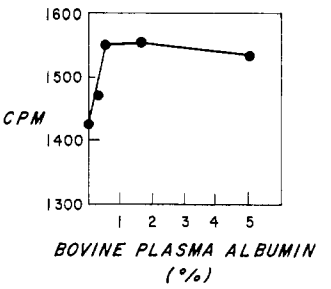

FIGURE 6: Stimulation by bovine plasma albumin.

Discussion

This method for thymidylate synthetase has been applied to homogenates of normal and leukemic human leukocytes (Roberts and Hall, 1966), normal and malignant human tissues, and cell cultures of human leukemic leukocytes and L1210.[2] The removal of the residual substrate by adsorption onto charcoal has made the assay of thymidylate synthetase simple and rapid, and the sensitivity of the isotopic procedure allows the enzyme to be easily measured in crude homogenates.

Acknowledgment

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[2] D. Roberts, unpublished observations.