A Filter Assay for Thymidylate Synthetase Using 5-Fluoro-2′-deoxyuridylate as an Active Site Titrant†

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ABSTRACT: A filtration assay is described for quantitation of thymidylate synthetase which utilizes radioactive 5-fluoro-2′-deoxyuridylate as an active site titrant. In the presence of 5,10-methylenetetrahydrofolate, this nucleotide forms a stoichiometric complex with the enzyme which may be isolated by filtration through nitrocellulose filter membranes. The cofactor requirement results in a high specificity for thymidylate synthetase. The assay is rapid, simple to perform, and applicable over a wide range of protein concentration. Both bacterial and mammalian thymidylate synthetase can be assayed by this procedure.

Thymidylate synthetase (EC 2.1.1.b) catalyzes the reductive methylation of deoxyuridylate to thymidylate with concomitant conversion of 5,10-methylenetetrahydrofolic acid to 7,8-dihydrofolic acid. The assays most commonly used for this enzyme depend on the increase in absorbance ($\Delta\epsilon_{340}$ = 6400) at 340 nm which accompanies 7,8-FAH_2[1] production, or the release of tritium from [5-^{3}H]dUMP accompanying thymidylate formation. While the spectrophotometric assay is most convenient, it suffers from limitations of sensitivity and, especially in crude preparations, from spurious oxidation of the cofactor with resultant high blanks. The radioisotope assay is more sensitive, but is somewhat inconvenient to perform and may also give anomalous results because of interfering substances and cofactor oxidation. As with all kinetic assays, quantitation of the amount of enzyme is indirect, and may not be accurate for comparison of enzyme levels in different tissues or in crude homogenates possessing inhibitory substances.

It has been known for some time that FdUMP is a potent inhibitor of thymidylate synthetase (Cohen *et al.*, 1958). In a recent communication (Santi and McHenry, 1972) and in the accompanying paper (Santi *et al.*, 1974) evidence is presented which demonstrates that, in the presence of CH_2FAH_4, this inhibitor forms a covalent bond with the enzyme. Using [^{3}H]FdUMP of high specific activity, enzyme-[^{3}H]FdUMP complexes may be formed and readily isolated on nitrocellulose filter membranes. Since the dissociation constant of the complex is lower than practical concentrations of the enzyme (Santi *et al.*, 1974) binding is stoichiometric and the inhibitor is, in effect, an active site titrant. The assay is highly sensitive, rapid to perform, and exhibits a high or absolute specificity for thymidylate synthetase. Unlike kinetic assays, the binding assay is independent of cofactor concentration over a wide range and may be used for quantitation of the FdUMP binding sites in crude homogenates. The method has been found useful for studies of the mechanism of interaction of this drug with thymidylate synthetase as demonstrated in the accompanying report (Santi *et al.*, 1974).

Experimental Section

Materials. A cell-free extract from amethopterin resistant *Lactobacillus casei* (Crusberg *et al.*, 1970) was obtained from the New England Enzyme Center. For most of the studies described in this report, thymidylate synthetase was purified to *ca.* 60% homogeneity by the method of Leary and Kisliuk (1971). Studies of stoichiometry utilized the highly purified preparation described in the accompanying paper (Santi *et al.*, 1974); this preparation was over 90% homogeneous on disk gel electrophoresis and showed a specific activity of 3.7 units/mg using the method of Kalckar (1947) or 6.1 units/mg using ϵ_{278} 1.07×10^5 for protein determinations. Cytoplasmic extracts from rat liver hepatoma tissue culture cells were obtained by the procedure of Rousseau *et al.* (1972). *dl*,L-FAH_4 was prepared by catalytic reduction of folic acid (Hatefi *et al.*, 1960) and purified by DEAE-cellulose chromatography (Scrimgeour and Vitols, 1966). The concentration of *dl*,L-FAH_4 was determined spectrophotometrically (Blakley, 1960); the *l*,L diastereomer represented 86% of the spectrophotometrically calculated value (*i.e.*, 43% of total) as determined by the thymidylate synthetase catalyzed conversion to 7,8-FAH_2 in the presence of excess deoxyuridylate. Solutions of CH_2FAH_4 (15 mM, pH 7.4) were stored under vacuum at −15° in 0.3 M β-mercaptoethanol and 75 mM formaldehyde. [6-^{3}H]FdUMP (6.5 Ci/mmol) was prepared from the nucleoside (New England Nuclear) with carrot phosphotransferase (Harvey *et al.*, 1970). Nitrocellulose filters (Bac-T-Flex, 2.4-cm disks) were obtained from Schleicher and Schuell.

Methods. Standard initial velocity assays of thymidylate synthetase were performed at 30° as previously described (Santi and Sakai, 1971). One unit of enzyme will catalyze the formation of 1 μmol of 7,8-FAH_2 in 1 min by the spectrophotometric assay (Wahba and Friedkin, 1962). Protein concentration was determined by the absorption at 260 and 280 nm (Kalckar, 1947) unless otherwise stated.

Formation of Complex. The standard assay mixture contained 50 mM *N*-methylmorpholine-HCl (pH 7.4), 25 mM $MgCl_2$, 1 mM EDTA, 75 mM 2-mercaptoethanol, 6.5 mM formaldehyde, 50 μg/ml of bovine serum albumin, 0.17 mM FAH_4, [6-^{3}H]FdUMP, and thymidylate synthetase. The reaction was initiated by addition of enzyme; CH_2FAH_4 was

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[1] Abbreviations used are: FdUMP, 5-fluoro-2′-deoxyuridylic acid; FAH_4, *dl*,L-tetrahydrofolic acid; 7,8-FAH_2, 7,8-dihydrofolic acid; CH_2FAH_4, *dl*,L-5,10-methylenetetrahydrofolic acid.

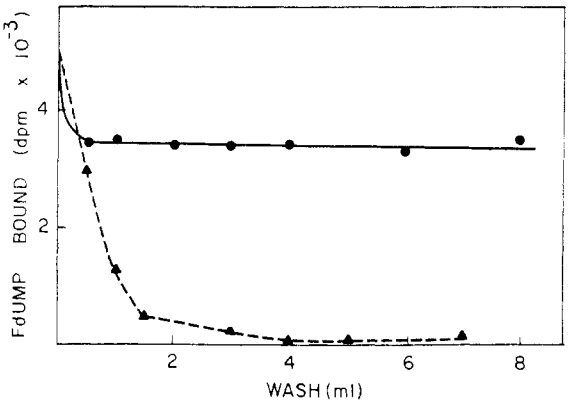

FIGURE 1: Radioactivity retained on filters upon washing [^{3}H]-FdUMP (▲) and the enzyme–[^{3}H]FdUMP complex (●). Each sample contained 172 μM CH_2FAH_4, 95 nM [^{3}H]FdUMP, and enzyme (3.37×10^{-3} unit/ml where indicated). Aliquots (100 μl; 1.36×10^5 dpm) were applied to each filter.

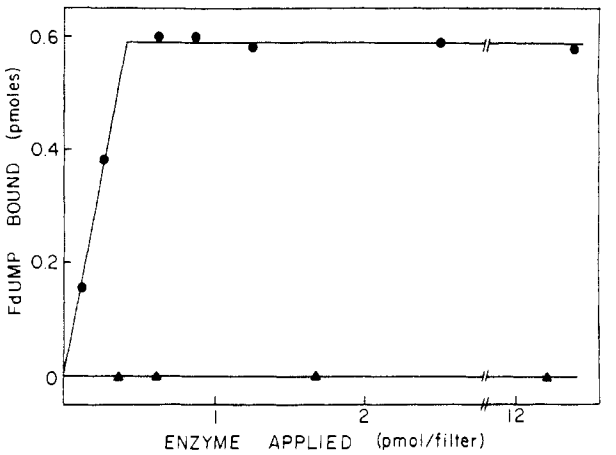

FIGURE 2: Titration of [^{3}H]FdUMP (8.2 nM) with thymidylate synthetase in the presence (●) and absence (▲) of CH_2FAH_4 (172 μM).

omitted in controls. The total volume was normally 0.4 ml; 50 μl was removed for determination of [^{3}H]FdUMP concentration after 1-hr incubation and 100-μl duplicates were assayed as described below. Although 1-hr incubation was found to be sufficient for even the most dilute solutions used, an additional 100-μl aliquot was often assayed after 2 hr to ensure that equilibrium had been obtained.

Filtration Assay. Nitrocellulose membranes were soaked before use in 25 mM potassium phosphate (pH 7.4) and 50 mM $MgCl_2$; filters which were not wetted within 2 min were discarded. The filter disks were placed on a 10-piece filter manifold (Hoeffer Scientific) and a gentle vacuum was applied (*ca.* 2 ml/min filtration rate) to remove excess moisture. Without removing the vacuum, 100-μl aliquots of the reaction mixture were applied to each disk and allowed to permeate the membrane. The filters were washed at the same filtration rate with seven 0.5-ml portions of a solution containing 25 mM phosphate buffer (pH 7.4)–50 mM $MgCl_2$. The damp filters were dissolved in 10 ml of Bray's solution (Bray, 1960) and counted in a Nuclear-Chicago Isocap 300 liquid scintillation counter. Counting efficiencies (45–55%) were determined by the dual channel ratio method.

Results

Figure 1 shows that when [6-^{3}H]FdUMP is applied to nitrocellulose filter membranes, in the presence of all components except enzyme, washing with as little as 2 ml of buffer is sufficient to remove almost all radioactivity. We have performed experiments which demonstrate that after application of as much as 2×10^5 dpm of [^{3}H]FdUMP and washing with 3 ml of buffer, less than 0.2% of the radioactivity is retained. In contrast, incubation of [^{3}H]FdUMP with thymidylate synthetase and CH_2FAH_4 results in the retention of substantial amounts of radioactivity after extensive washing. This experiment demonstrates that an enzyme–[^{3}H]FdUMP complex may be trapped on nitrocellulose membranes under conditions where free [^{3}H]FdUMP is readily removed by washing. When varying amounts (10–500 μl) of a solution containing the preformed complex are filtered, the retained radioactivity is strictly linear to the amount applied. This demonstrates that, within the range examined, the assay is not sensitive to the volume filtered, and that the retained radioactivity reflects the protein-bound [^{3}H]FdUMP present in solution prior to filtration.

In the presence of constant levels of CH_2FAH_4 and [^{3}H]-FdUMP increasing amounts of enzyme results in proportional increase in the amount of radioactivity retained until apparent saturation of the radioactive ligand is reached (Figure 2). When CH_2FAH_4 was omitted, there was no retention of radioactivity at the highest concentration of the enzyme used. From the bound radioactivity at saturation ($[FdUMP]_b$) and the total added to the filter ($[FdUMP]_t$), the filtration efficiency may be calculated (eq 1). This value represents the probability

$$\text{filtration efficiency} = [FdUMP]_b/[FdUMP]_t \qquad (1)$$

that the protein–ligand complex will survive the filtration and washing procedure (Yarus and Berg, 1967) and is used to convert the amount of filter-bound complex to the amount of complex actually present in solution prior to filtration. For routine determinations of this parameter, we recommend that $1–2 \times 10^4$ dpm of [^{3}H]FdUMP be used per assay, and that the saturation level be determined using a minimum of two concentrations of enzyme. We routinely obtain filtration efficiencies ranging from 0.5 to 0.9, depending on the lot of filters and the purity of components used. The filtration efficiency has been found to be constant within an experiment.

Figure 3 shows that the amount of complex formed is proportional to the FdUMP added until saturation of the sites is obtained. The filtration efficiency for this experiment (0.76) may be calculated from the region corresponding to concentrations of FdUMP where the enzyme is present in excess. At saturating concentrations of [^{3}H]FdUMP, all the enzyme is bound and, after correction for filtration efficiency, we calculate that 1.7 mol of nucleotide are bound per mol of enzyme. This value is in complete agreement with that obtained by independent methods (Santi *et al.*, 1974) and is in accord with the proposal that thymidylate synthetase consists of two identical subunits (Dunlap *et al.*, 1971; Loeble and Dunlap, 1972). Thus, as long as free FdUMP can be detected in the reaction mixture (*i.e.*, conditions of excess FdUMP) and the capacity of the filter for protein is not exceeded, the bound radioactivity can be taken as a direct measurement of the amount of enzyme in solution. An example of the utility of this feature is given in Figure 4 which shows an analysis of a Sephadex G-100 purification of thymidylate synthetase assayed by both the spectrophotometric assay and the FdUMP binding assay.

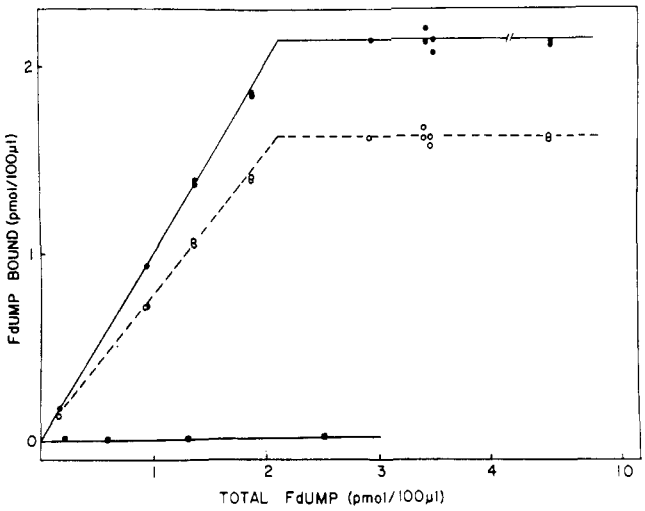

FIGURE 3: Titration of thymidylate synthetase with [^{3}H]FdUMP in the presence (●) or absence (■) of CH_2FAH_4 (172 µM). The dashed line represents the curve obtained before the appropriate correction for filtration efficiency was made. The thymidylate synthetase concentration was 12.7 and 6.7 pmol/ml, respectively, in the curves with and without CH_2FAH_4. The enzyme preparation used was that described in the accompanying paper (Santi *et al.*, 1974).

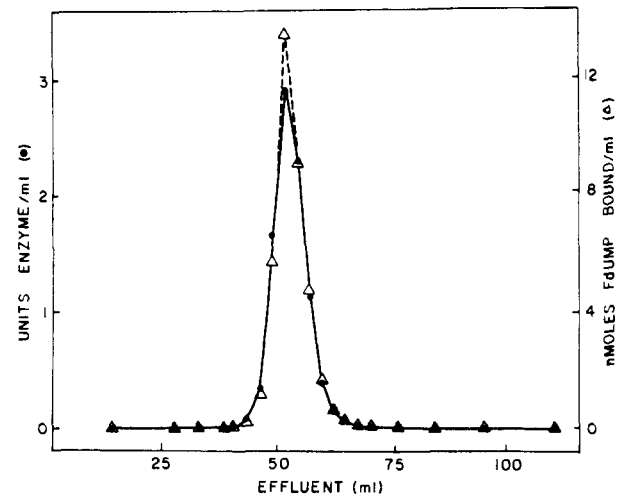

FIGURE 4: Comparison of spectrophotometric (●) and nitrocellulose filtration (△) assays of an effluent from a Sephadex G-100 column purification. Specific activities in the fractions between 41 and 62 ml varied from 0.4 to 2.1 units/mg. The [^{3}H]FdUMP–enzyme complex was formed as described in Methods; precautions were taken to ensure that an excess (*ca.* 30%) of FdUMP over enzyme binding sites was present.

The rate of complex formation follows bimolecular kinetics and is dependent upon the concentration of components. Using the lowest concentrations of enzyme and [^{3}H]FdUMP permissible with accurate detection, there is no change in the amount of filterable complex after 1-hr incubation. We routinely use a 1-hr incubation period, and perform an additional determination after 2 hr to verify that equilibrium has been obtained. Complete kinetic studies of formation and dissociation of the complex are given in the accompanying report (Santi *et al.*, 1974). Under the conditions employed in the assay there is no detectable loss in filterable radioactivity for at least 8 hr. Thus, there is no error introduced in the described assay because of dissociation of the complex or decomposition of the enzyme.

To define limiting conditions for the assay, the effect of a number of variables on the filtration efficiency was investigated. The complex used in these experiments was formed by incubating a saturating amount of enzyme (*ca.* 0.01 unit/ml) with [^{3}H]FdUMP (*ca.* 5 nM) and CH_2FAH_4 (0.1 mM) for 1 hr prior to filtration. In the incubation mixture, it was demonstrated that concentration of the buffer, $MgCl_2$, or 2-mercaptoethanol could be varied from 0 to 100 mM without affecting the efficiency; it is noted that a small amount of mercaptoethanol (*ca.* 3 mM) was introduced with the CH_2FAH_4. Omission of EDTA showed no change in efficiency compared to the 1 mM present in the standard assay. In another experiment it was determined that the CH_2FAH_4 concentration could be varied from 30 to 190 µM with no effect on the efficiency; since extraordinary precautions were not taken to prevent FAH_4 oxidation, the lower value should be regarded as an operational rather than exact limit. For dilute solutions of protein it has become a standard practice to add bovine serum albumin to prevent adsorption to reaction vessels. Introduction of bovine serum albumin (25 µg/100 µl of assay) before or after the incubation period showed minimal effect on the filtration efficiency.

In the wash fluid the pH could be varied between 6.0 and 8.0, the volume between 1 and 10 ml, the flow rate to 8 ml/min, and the buffer concentration between 0 and 100 mM without significant effect on the efficiency. The $MgCl_2$ concentration was optimal between 25 and 75 mM and showed 28% decrease in efficiency at 0 and 100 mM concentration; no further change in efficiency was observed at concentrations up to 500 mM $MgCl_2$.

The method appears to be advantageous for the assay of thymidylate synthetase from other sources. For example, enzyme activity could not be detected by the spectrophotometric assay with a crude cytosol preparation from rat liver hepatoma tissue culture cells. This is a common observation with mammalian cells and results from the low levels of the enzyme present and spurious oxidation of FAH_4 from other cellular components. When rat liver hepatoma tissue culture cytosol was added to an excess of [^{3}H]FdUMP in the standard assay, the filter-bound radioactivity was linear up to *ca.* 125 µg of protein (Figure 5). In the absence of CH_2FAH_4, there was no filter-bound radioactivity. Although saturation of FdUMP could not be achieved in this experiment due to

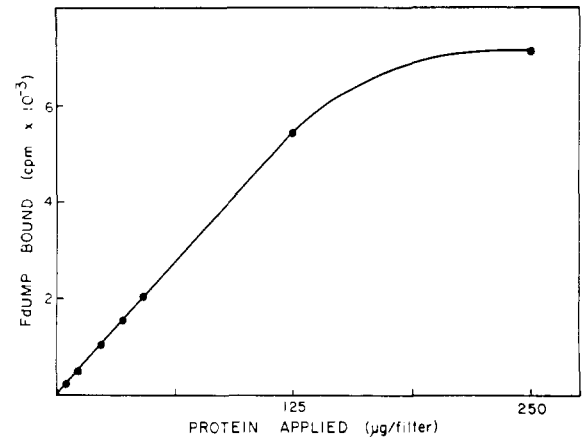

FIGURE 5: [^{3}H]FdUMP–thymidylate synthetase complex retained as a function of the amount of protein applied from cytosol of rat liver hepatoma cells. The solution in which the complex was formed contained 40 nM [^{3}H]FdUMP; 100-µl filtration aliquots were applied containing the amount of protein specified. Protein determinations were performed by the method of Lowry *et al.* (1951).

saturation of the filter with protein, extrapolation of a plot of $1/[FdUMP]_b$ *vs.* 1/[protein] to infinite protein concentration gave a value of 0.50 for the filtration efficiency (Yarus and Berg, 1970). Using eq 1, and the data given in Figure 5, it was calculated that the HTC cytosol preparation contained 14 pmol of thymidylate synthetase binding sites per mg of protein.

Discussion

In the presence of CH_2FAH_4, FdUMP forms a specific stable complex with thymidylate synthetase. Using [^{3}H]-FdUMP of high specific activity, complexes present in solution may be retained on nitrocellulose filter membranes under conditions where the free nucleotide is readily removed and disruption of the complex is negligible. The affinity constant is sufficiently high that, with typical concentrations of components used in such experiments, the limiting reagent (FdUMP or enzyme) is completely bound. Therefore, the ratioactive nucleotide may be used as an active site titrant. For example, using a slight excess of [^{3}H]FdUMP and corrections for filtration efficiency, the assay provides a direct measurement of the concentration of thymidylate synthetase binding sites in solution. As shown here, and further demonstrated in the accompanying paper, we have determined that the enzyme from *L. casei* contains two binding sites for FdUMP. The sensitivity of the assay appears to be limited only by the specific activity of the labeled FdUMP available. In the present case it is about 20-times more sensitive than the spectrophotometric kinetic assay and assuming two binding sites per molecule of enzyme (see Santi *et al.*, 1974) as little as 3×10^{-14} mol of thymidylate synthetase can be quantitated. This method is significantly more rapid than kinetic assays when numerous samples must be analyzed; we routinely perform as many as 150 determinations in an afternoon. For this reason we favor the filtration assay over the spectrophotometric method for detection of enzymic activity in column effluents and other purification procedures.

Another major advantage of the binding assay described here is its high specificity. There are expectedly few proteins which will form isolable complexes with FdUMP at the low concentrations of components used; even if such proteins were to exist, the probability that binding would also require the cofactor, CH_2FAH_4, is negligible. The accuracy of kinetic assays for this enzyme require a knowledge of substrate concentration (an uncertainty with the easily oxidized cofactor), and are often encumbered by interfering substances which complete with or degrade the substrates. In contrast, the binding assay does not depend on a knowledge of the concentration of CH_2FAH_4 or FdUMP, so long as they are in excess, and interfering substances have negligible effects.

The above features make the assay ideal for studies of tissues where thymidylate synthetase levels are low or where substances which interfere with kinetic assays are present. For example, we were unable to determine the initial velocity of 7,8-FAH_2 production in crude homogenates of hepatoma tissue culture cells by the spectrophotometric assay; however, the filter assay described here could be performed without difficulty. Although extensive investigation of this method has only been performed with thymidylate synthetase from *L. casei*, it should be easily adapted to the same enzyme obtained from a variety of sources.

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