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Substrate Flux through Methylenetetrahydrofolate Dehydrogenase: Predicted Effects of the Concentration of Methylenetetrahydrofolate on Its Partitioning into Pathways Leading to Nucleotide Biosynthesis or Methionine Regeneration†

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ABSTRACT: Folic acid exists in mammalian cells with a poly-γ-glutamate tail that may regulate the flux of folates through the various cellular pathways. The substrate polyglutamate specificity of methylenetetrahydrofolate dehydrogenase from pig liver has been examined by using a competitive method and measuring apparent tritium kinetic isotope effects on V_{max}/K_m for methylenetetrahydrofolate. This competitive method yields very accurate ratios of K_m values for alternate substrates of an enzyme and may also be applied to reactions with no isotope effect. In combination with published data from our own and other laboratories, the kinetic parameters of methylenetetrahydrofolate dehydrogenase were used to calculate the initial velocities of pig liver methylenetetrahydrofolate dehydrogenase, thymidylate synthase, and methylenetetrahydrofolate reductase, at physiological concentrations of substrates and enzymes. These calculations suggest that the cellular concentration of methylenetetrahydrofolate may regulate the flux of this metabolite into the pathways leading to nucleotide biosynthesis and methionine regeneration. An increase in the cellular level of methylenetetrahydrofolate would permit more one-carbon units to be directed toward nucleotide biosynthesis.

Most cellular folates in mammals possess four to seven glutamyl residues linked in amide bonds involving the γ-carboxyl group. Several research groups have examined the polyglutamate specificites of folate-dependent enzymes from a variety of sources [e.g., MacKenzie and Baugh (1980), Matthews et al. (1985, 1987), and Allegra et al. (1985a); reviewed by McGuire and Coward (1984)]. While a regulatory role for the polyglutamate chain in determining the flux of folates in the cell has been proposed (Baggot & Krumdieck, 1979), no concrete model has been described. In particular, if the polyglutamate chain distribution changes only slowly (Eto & Krumdieck, 1982), alterations in chain length are unlikely to play a significant role in cellular regulation.

We present a model that predicts how changes in the cellular concentration of methylenetetrahydrofolate will alter the flux of this substrate into the pathways leading to thymidylate/purine biosynthesis or methionine regeneration (Figure 1). Our calculations indicate that increasing levels of methylenetetrahydrofolate result in an increased flux of this metabolite into pathways leading both to thymidylate synthesis and to de novo purine biosynthesis, as compared to the flux into the pathway for methionine regeneration.

This model is based on calculations of the initial velocities of pig liver methylenetetrahydrofolate reductase, pig liver methylenetetrahydrofolate dehydrogenase, and fetal pig liver thymidylate synthase and takes into account the polyglutamate specificities of these three enzymes for their polyglutamate substrates. The cellular concentrations of the pertinent enzymes and cosubstrates in pig liver were also considered. While most of the kinetic parameters needed for this calculation have been published, the reported K_m values for methylenetetrahydrofolate dehydrogenase were inconsistent. MacKenzie and Baugh (1980) observed changes in K_m for

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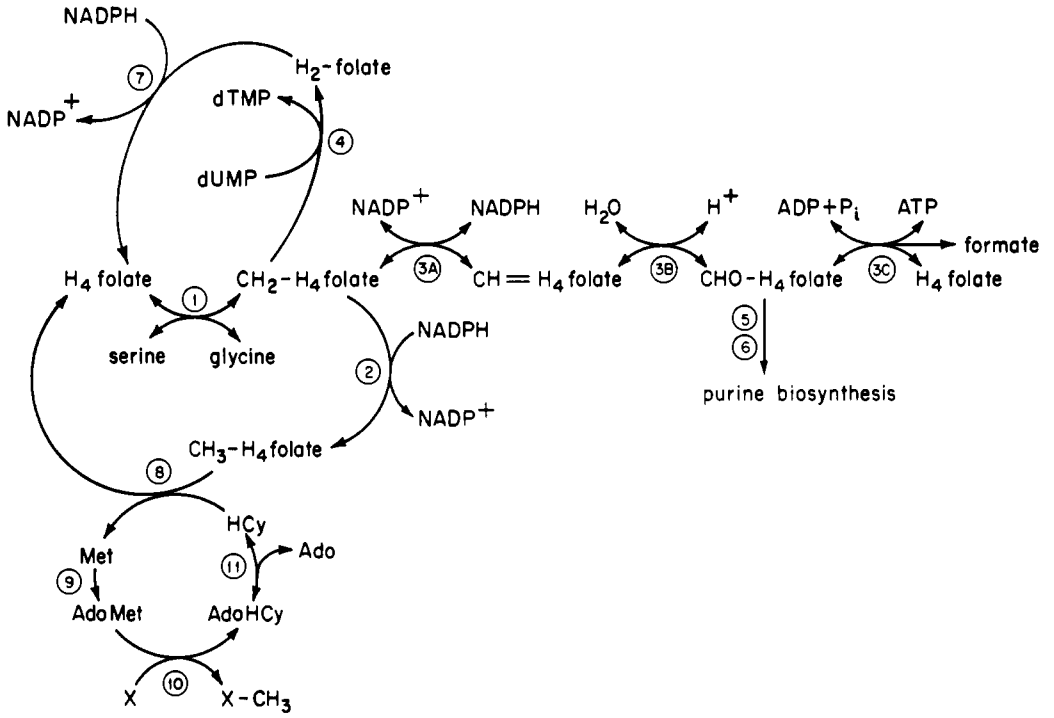

FIGURE 1: Outline of the major folate-dependent pathways in mammalian cells. The enzymes involved are (1) serine hydroxymethyltransferase, (2) methylenetetrahydrofolate reductase, (3) the trifunctional enzyme with methylenetetrahydrofolate dehydrogenase (3A), methenyltetrahydrofolate cyclohydrolase (3B), and formyltetrahydrofolate synthetase (3C) activities, (4) thymidylate synthase, (5) glycinamide ribonucleotide transformylase, (6) aminoimidazolecarboxamide ribonucleotide transformylase, (7) dihydrofolate reductase, (8) methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase), (9) adenosylmethionine synthetase, (10) adenosylmethionine-dependent methyltransferases, and (11) adenosylhomocysteine hydrolase.

folylpolyglutamate substrates, while Ross et al. saw no significant differences (1984). In this study we describe a competitive method for measurement of K_m values using apparent isotope effects on V/K_B for methylenetetrahydrofolate to probe changes in K_m for the folylpolyglutamate substrates of methylenetetrahydrofolate dehydrogenase more accurately.

Experimental Procedures

Pig liver trifunctional enzyme (methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase) was purified as described by Tan et al. (1977); after chromatography on phosphocellulose, the protein was stored as a suspension in 55% ammonium sulfate containing 0.5 mM $NADP^+$. Prior to experiments, protein was diluted into or dialyzed against 60 mM potassium phosphate, pH 7.2, containing 20% (w/v) glycerol. Assays were performed as described before, except that the 2-mercaptoethanol concentration was maintained at 20 mM (Ross et al., 1984).

Preparation of Substrates. For quantitating enzyme activity in stock solutions (6-*ambo*)-CH_2-H_4PteGlu$_1$[1] was generated in the assay by nonenzymatic condensation of formaldehyde and tetrahydrofolate. (6-*ambo*)-H_4folate was prepared by catalytic hydrogenation of folate as described before [Blakley, 1957; as modified by Tan et al. (1977)]. For kinetic analyses, (6*R*)-CH_2-H_4PteGlu$_n$ was generated by nonenzymatic condensation of formaldehyde with (6*S*)-H_4PteGlu$_n$, which was synthesized as described previously (Matthews et al., 1982).

Pteroylpolyglutamates were synthesized by a solid-phase method previously described (Krumdieck & Baugh, 1969, 1980), and their purity was checked by HPLC analysis (Matthews, 1986). (6*R*)-CH_2-H_4PteGlu$_n$ was prepared by using a modification of the procedure described before (Matthews et al., 1982). Ten micromoles of PteGlu$_n$ was incubated in 100 mL of 10 mM Tris-HCl, pH 7.2, containing 20 mM 2-mercaptoethanol, 0.4 mM NADPH, and 5 units of dihydrofolate reductase from *Lactobacillus casei*. The reaction mixture was maintained in the dark at room temperature, under nitrogen. Formaldehyde (150 μmol, 40 000–60 000 dpm/nmol of ^3H or 10 000–15 000 dpm/nmol of ^{14}C) was added, and the solution was incubated for an additional 15 min. The resulting CH_2-H_4PteGlu$_n$ was purified quickly by chromatography on a 0.9 × 7 cm column of DEAE-52, equilibrated with 10 mM $(NH_4)_2CO_3$, pH 9.2. Elution was effected with a 75-mL gradient of 0–0.7 M NaCl in the same buffer.

Determination of the Apparent Tritium Isotope Effect on V/K for CH_2-H_4PteGlu$_n$. Assays contained 100 mM potassium phosphate, pH 7.2, 2–180 μM $NADP^+$, 100 μM (6*S*)-CH_2-H_4PteGlu$_n$ (labeled with ^{14}C or ^3H in the methylene group), 0.004–0.006 unit of methylenetetrahydrofolate dehydrogenase/100 μL of reaction mix, and sometimes included a system to regenerate $NADP^+$ (see below). The assay mixture was prepared containing everything except enzyme; aliquots were removed to serve as reagent blanks, and enzyme was added to initiate the reaction. Aliquots (100 μL) were taken as the reaction proceeded and were heat-quenched in 400 μL of dimedone (3 mg/mL in sodium acetate, pH 4.5), cooled on ice, and extracted with 3 mL of toluene. All samples were taken within 5 min. CH_2-H_4folate and free formaldehyde

[1] Abbreviations: PteGlu$_n$, pteroylpolyglutamate with *n* glutamyl residues; H_2PteGlu$_n$, dihydropteroylpolyglutamate with *n* glutamyl residues; H_4PteGlu$_n$, tetrahydropteroylpolyglutamate with *n* glutamyl residues; CH_2-H_4PteGlu$_n$, methylenetetrahydropteroylpolyglutamate with *n* glutamyl residues; CH_3-H_4PteGlu$_n$, methyltetrahydropteroylpolyglutamate with *n* glutamyl residues; H_4folate, tetrahydrofolate; CH_2-H_4folate, methylenetetrahydrofolate; CH_3-H_4folate, methyltetrahydrofolate; CH^+-H_4folate, methenyltetrahydrofolate; AdoMet, *S*-adenosylmethionine; AdoHCy, *S*-adenosylhomocysteine; HPLC, high-performance liquid chromatography; $^T(V/K)$, tritium isotope effect on V/K.

both react with dimedone to form a complex that is extractable in toluene, while radioactive products remain in the aqueous phase. Aliquots of the toluene phase were counted and deconvoluted as described in the next section. The isotope effect was calculated by using the expression

$$^{T}\left\{\frac{V}{K}\right\} = \frac{\log (1 - x)}{\log (1 - y)} \quad (1)$$

where y is the fractional conversion of tritiated substrate at a given time, x is the fractional conversion of ^{14}C-labeled substrate at the same time, and $^{T}(V/K)$ is the tritium isotope effect on V/K (Melander & Saunders, 1980). Average values, standard errors, and propagation of error were determined in accordance with Bevington (1969).

Isotopes were counted in a Beckman LS 7500 spectrometer, and quench was determined by measurement of the Compton edge of an external cesium standard. Sufficient counts were collected to ensure no greater than a 1% standard error. Where double-labeled compounds were being counted, the spectrometer was operated in the automatic quench compensation mode, in which the ^{3}H and ^{14}C windows were shifted to optimize counting efficiency while minimizing spillover. Corrections for ^{3}H and ^{14}C spillover and efficiency determinations were made by comparison with a series of quenched ^{3}H and ^{14}C standards. The standard curves were fit with cubic equations and the dpm of ^{3}H and ^{14}C in samples were determined by analytical solution of the following equations, where α is the counting efficiency for ^{3}H in channel 1, β is the counting efficiency for ^{14}C in channel 1, γ is the counting efficiency for ^{3}H in channel 2, and δ is the counting efficiency for ^{14}C in channel 2 (Beckman, (1976):

$$\text{dpm } ^{14}C = \frac{\alpha\text{cpm2} - \gamma\text{cpm1}}{\alpha\delta - \beta\gamma} \quad (2)$$

$$\text{dpm } ^{3}H = \frac{\delta\text{cpm1} - \beta\text{cpm2}}{\alpha\delta - \beta\gamma} \quad (3)$$

Recycling systems were included to maintain a constant concentration of $NADP^+$ and to remove NADPH, a potential product inhibitor of the reaction. The trapping system consisted of malate dehydrogenase from porcine heart (500 units/mL of reaction mix) and 2 mM oxaloacetate or glutathione reductase from baker's yeast (4 units/mL of reaction mix) and 3.25 mM oxidized glutathione.

Labeled CH_2-$H_4PteGlu_n$ dissociates into free formaldehyde and tetrahydrofolate. Every experiment using this substrate included a control where the substrate was reacted with a large excess of methylenetetrahydrofolate dehydrogenase (about 0.04 unit of enzyme/100 μL of assay mix) to ascertain the degree of dissociation that had occurred during storage of this compound. The amount of unreacted radioactivity was assumed to be free formaldehyde that had dissociated from the tetrahydrofolate, and results were corrected accordingly. Substrate used in these experiments contained 80–95% reactive radiolabel (no more than 20% of the formaldehyde had dissociated). There was no isotope efect observed on dissociation of formaldehyde from tetrahydrofolate.

Determination of the Primary Tritium Isotope Effect on V/K of CH_2-$H_4PteGlu_n$. Assays contained 100 mM potassium phosphate buffer, pH 7.2, 180 μM $NADP^+$, and 100 μM CH_2-$H_4PteGlu_1$ or CH_2-$H_4PteGlu_5$ (labeled with ^{14}C or ^{3}H in the methylene group). Assays were initiated by addition of methylenetetrahydrofolate dehydrogenase (0.004–0.006 unit/100 μL of reaction mix). Aliquots were removed as the reaction proceeded. A portion of each aliquot was heat-quenched in dimedone and worked up as described previously; this sample was used to determine the extent of conversion of the ^{14}C-labeled folate substrate, which was taken as the extent of conversion for protiated substrate at the time point. An identical sample was applied to an Ultrasphere ODS HPLC column that had been equilibrated in 5 mM tetrabutylammonium phosphate buffer (Pic A, Waters). Samples were eluted with a 0–30% propanol gradient. Those fractions containing tritium-labeled NADPH were counted and analyzed; NADPH separated cleanly from the other radioactive fractions and was identified spectrophotometrically (Green et al., 1986). Fractions were collected and counted in Safety-Solve (Research Products International). A total of 95–100% of the counts applied to the HPLC eluted from the column. Deconvolution of counts was performed as described above.

Determination of Relative K_m Values for Methylenetetrahydropteroylpolyglutamates Using Competition Experiments. Assays containing 100 mM potassium phosphate, pH 7.2, 180 μM $NADP^+$, 50 μM [*methylene*-^{14}C]CH_2-$H_4PteGlu_1$, 50 μM [*methylene*-^{3}H]CH_2-$H_4PteGlu_n$ (where n = 1, 2, 4, and 5), and 0.004–0.006 unit of methylenetetrahydrofolate dehydrogenase/100 μL of reaction mix. Folate substrate was added to initiate the reaction. Duplicate aliquots of substrate were added to tubes containing dimedone as blanks. An aliquot of substrate was also added to a 100-μL reaction mix containing a large excess of enzyme (0.04 unit) as a measure of substrate dissociation. Aliquots were taken as the reaction proceeded and heat-quenched as described before. The apparent isotope effect was analyzed as described above.

Calculation of the Concentration of Enzyme in Pig Liver. The concentration of methylenetetrahydrofolate dehydrogenase in pig liver was estimated from the data of MacKenzie and Tan (1980) to be 3.3 μM, assuming a subunit molecular weight of 100 000 (Tan et al., 1977) and a pig liver density of 1.08 g/mL. The concentration of methylenetetrahydrofolate reductase in pig liver (38.4 nM) was calculated similarly from the data of Daubner and Matthews (1982); the concentration of active sites was assumed to be equivalent to the concentration of enzyme-bound flavin.

Calculation of the Cellular Velocities of Methylenetetrahydrofolate Dehydrogenase, Methylenetetrahydrofolate Reductase, and Thymidylate Synthase. For each enzyme the velocity was first calculated for each polyglutamyl chain length of substrate, neglecting the presence of substrates of differing polyglutamyl chain length. To correct for the presence of competing substrates, we then estimated a velocity for each given total folate concentration. The kinetic parameters that varied with folylpolyglutamyl chain length were weighted according to the relative concentration of that polyglutamyl chain length in the cell (Cichowicz & Shane, 1987a). Octaglutamates and nonaglutamates were included with heptaglutamates. These weighted kinetic parameters were used to calculate a total velocity for each enzyme at total methylenetetrahydrofolate concentrations of 0.5, 5, and 15 μM. Next the velocities calculated for each polyglutamyl chain length of substrate (neglecting the other substrates) were multiplied by a factor to correct for the presence of the other substrates. This factor was equal to the total weighted velocity divided by the sum of the individual velocities calculated for each folate of a particular polyglutamyl chain length. This method both considers the contributions of each substrate polyglutamate to the total velocity and reflects the competition between the various substrates.

Methylenetetrahydrofolate dehydrogenase proceeds by an ordered bi-bi sequential mechanism whereby $NADP^+$ binds first and CH^+-H_4folate is released last (Cohen & MacKenzie,

1978). In calculating the velocity for this enzyme we used the following equation for an ordered bi-bi mechanism (Segel, 1977):

$$\text{velocity} = \frac{V_{max}[A][B]}{K_{iA}K_{mB} + K_{mA}[B] + K_{mB}[A] + [A][B]} \quad (4)$$

This calculation also required values for the turnover number [1700 min^{-1}, from Tan et al. (1977)], the K_m and K_d for $NADP^+$ (10.4 μM and 30 μM; Ross et al., 1984), and the values for K_m for CH_2-H_4PteGlu$_n$ (given in Table I). The concentration of active sites of methylenetetrahydrofolate dehydrogenase in pig liver is 3.3 μM. Since the measured K_m values for CH_2-H_4PteGlu$_2$, CH_2-H_4PteGlu$_4$, and CH_2-H_4PteGlu$_5$ were all about 1.9 μM, we assumed the K_m values for CH_2-H_4PteGlu$_3$, CH_2-H_4PteGlu$_6$, and CH_2-H_4PteGlu$_7$ were also 1.9 μM. The intracellular concentration of $NADP^+$ was assumed to be 2.3 μM, calculated from an [$NADP^+$]/[NADPH] ratio of 0.011 (Krebs et al., 1973) and an intracellular NADPH concentration of 200 μM (Conway et al., 1983).

Since methylenetetrahydrofolate reductase exhibits parallel line kinetics with monoglutamyl and polyglutamyl substrates (Vanoni et al., 1983; Matthews & Baugh, 1980), we used the following equation in calculating the velocity of this enzyme (Segel, 1977):

$$\text{velocity} = \frac{V_{max}[A][B]}{K_{mA}[B] + K_{mB}[A] + [A][B]} \quad (5)$$

The kinetic constants for pig liver methylenetetrahydrofolate reductase with monoglutamyl and polyglutamyl substrates are published (Matthews & Baugh, 1980). The turnover number for this enzyme is 1700 min^{-1} (Daubner & Matthews, 1982). The concentration of this enzyme in pig liver (38.4 nM) was calculated as described previously.

For thymidylate synthase velocities were calculated by using eq 4 for an ordered bi-bi sequential mechanism (Lu et al., 1984) and V_{max} and K_m values measured by these investigators. These values were determined for enzyme from fetal pig liver. Thymidylate synthase activities in normal adult liver are too low to measure, so thymidylate synthase activity will not contribute significantly to the flux of methylenetetrahydrofolate in this tissue. The intracellular dUMP concentration was estimated to be 19 μM on the basis of Jackson's determination of 13 nmol of dUMP/10^9 cells (1978) and by assuming 0.68 mL/10^9 cells.

The relative concentrations of total pig liver polyglutamates have been determined by Cichowicz and Shane (1987a). They are 0.4% for diglutamate, 0.4% for triglutamate, 1.7% for tetraglutamate, 15.9% for pentaglutamate, 49.7% for hexaglutamate, 24.5% for heptaglutamate, 6.3% for octaglutamate, and 1.2% for nonaglutamate (percent of the total folate concentration). These authors estimate that the total folate concentration in pig liver is 30 μM, and Kashanis and Cooper (1985) estimate that 17% of intracellular folate is methylenetetrahydrofolate. These values were used in the flux and velocity calculations. We have assumed that the relative proportions of the different polyglutamate chain lengths are not different for CH_2-H_4PteGlu$_n$ and for the total folate pool and that they do not change with transient fluctuations in total methylenetetrahydrofolate concentration. We estimated the concentration of methylenetetrahydrofolate in quiescent cells to be at most 5 μM. This concentration, however, includes both bound and free cofactor and mitochondrial folates; the concentration of unbound, cytosolic methylenetetrahydrofolate is certainly substantially lower. For purposes of illustration we have performed flux and velocity calculations for 0.5, 5, and 15 μM total methylenetetrahydrofolate.

Results

Rationale for the Development of a Competitive Method To Measure Relative K_m Values for Methylenetetrahydropteroylpolyglutamate Substrates of Methylenetetrahydrofolate Dehydrogenase. We have developed a competitive method that permitted us to determine accurately relative differences in K_m for pairs of folate substrates. This method was based on the measurement of apparent isotope effects on V/K for the folate substrate. Equal concentrations of [*methylene*-^{14}C]CH_2-H_4PteGlu$_1$ and [*methylene*-^{3}H]CH_2-H_4PteGlu$_n$ were combined and incubated with methylenetetrahydrofolate dehydrogenase and $NADP^+$. Analyses of these data were dependent on the following information: (a) Because the reaction catalyzed by methylenetetrahydrofolate dehydrogenase involves oxidation of the labeled methylene group, the tritium isotope effect[2] $^T(V/K_B)$ associated with this oxidation must be known for each substrate. This tritium isotope effect was found to be the same for folate substrates with one and five glutamyl residues. (b) V_{max} for each substrate must be known. Our previous studies had shown that V_{max} is the same for folate substrates with 1, 4, and 5 glutamyl residues (Ross et al., 1984). If the tritium-labeled species differs from the ^{14}C-labeled methylenetetrahydrofolate only in the number of residues on the polyglutamate tail, measurement of an apparent tritium isotope effect on the reaction allows a direct comparison of the K_m values of the two folate substrates (see below).

Measurement of the Tritium Kinetic Isotope Effect of Double-Labeled CH_2-H_4PteGlu$_1$. The labeled substrate, methylenetetrahydrofolate, was prepared by nonenzymatic condensation of labeled formaldehyde with tetrahydrofolate. The double-labeled CH_2-H_4PteGlu$_1$, which consisted of a mixture of [*methylene*-^{3}H]CH_2-H_4PteGlu$_1$ and [*methylene*-^{14}C]CH_2-H_4PteGlu*1*, contained tritium that was distributed randomly in the *R* and *S* positions of CH_2-H_4PteGlu$_1$. Pig liver methylenetetrahydrofolate dehydrogenase stereospecifically removes only one of these, probably H_R by analogy with the chicken liver enzyme (Sliecker & Benkovic, 1984). The tritium isotope effect obtained from monitoring the radioactivity remaining in the substrate is a mixture of secondary and primary isotope effects, since 50% of the ^{3}H will be in the *R* position of the methylene group and its removal will be associated with a primary kinetic isotope effect and 50% of the ^{3}H will be in the *S* position and will remain in the methenyltetrahydrofolate product, giving rise to a secondary kinetic isotope effect. In order to measure the primary tritium isotope effect directly, we separated reaction mixtures initially containing methylenetetrahydrofolate dehydrogenase, $NADP^+$, and double-labeled CH_2-H_4PteGlu$_1$ by HPLC. The tritiated NADPH produced in the reaction was separated from the other components of the reaction and counted (see Figure 2A). The primary isotope effect for double-labeled CH_2-H_4PteGlu$_1$ was 3.19 ± 0.28.

An alternative and more convenient measure of the $^T(V/K)$ for CH_2-H_4PteGlu$_1$ involved monitoring the radioactivity remaining in the double-labeled substrate. The isotope effect

[2] Because tritium and ^{14}C are present in tracer concentrations and the bulk of the substrate contains only hydrogen, labeled substrate is always present at nonsaturating concentrations (V/K regime) regardless of the concentration of the bulk substrate. $^T(V/K_B)$ represents the ratio of the rate constants, k_H/k_T, characterizing product formation under conditions where the velocity is limited by the concentration of tracer isotope (*B*).

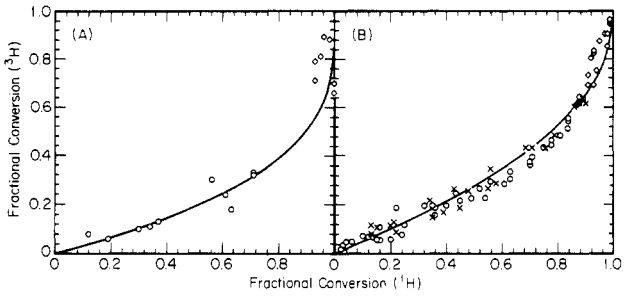

FIGURE 2: Determination of the tritium isotope effect on V/K for CH_2-H_4folate. Assays containing 100 mM potassium phosphate, pH 7.2, 180 μM $NADP^+$, and 100 μM double-labeled CH_2-$H_4PteGlu_1$ were initiated with the addition of methylenetetrahydrofolate dehydrogenase. Aliquots were removed and heat-quenched with dimedone. The fractional conversion of tritiated substrate was plotted as a function of the fractional conversion of protiated substrate, which was labeled with ^{14}C. Because of the high relative systematic error, only those points with a fractional conversion (protiated substrate) less than 0.90 were included in the analysis. Those data left out of the analysis are represented by open diamonds (◇). (A) Measurement of the primary tritium kinetic isotope effect on V/K_B. CH_2-$H_4PteGlu_1$ was used as the double-labeled substrate. Identical aliquots were removed; one was heat-quenched in dimedone, and the other was applied to an HPLC column. The tritiated counts associated with the product NADPH as separated by HPLC were used to determine the fractional conversion for tritiated substrate, while the fractional conversion of protiated substrate was determined from the sample subjected to the heat quench. A primary isotope effect of 3.19 ± 0.28 was determined. (B) Measurement of the apparent tritium kinetic isotope effect on V/K_B. Assays included an $NADP^+$-regenerating system and either double-labeled CH_2-$H_4PteGlu_1$ (○) or CH_2-$H_4PteGlu_5$ (×). Isotope effects of 2.18 ± 0.09 and 2.11 ± 0.10 were measured for CH_2-$H_4PteGlu_1$ and CH_2-$H_4PteGlu_5$, respectively; if all points from both experiments are averaged together, one obtains a $^T(V/K)$ of 2.16 ± 0.07.

obtained in this way is an average of the primary and secondary isotope effects, as explained above:

$$\text{apparent } {}^T(V/K_B) = \frac{\text{primary } {}^T(V/K_B) + \text{secondary } {}^T(V/K_B)}{2} \quad (6)$$

The apparent $^T(V/K_B)$ measured in this way was 2.16 ± 0.07 as shown in Figure 2B. Using eq 6 and the value for the primary $^T(V/K_B)$ of 3.19 ± 0.28, one obtains a secondary isotope effect of unity (1.13 ± 0.29), which therefore can be neglected.

Interestingly, the apparent isotope effect for CH_2-$H_4PteGlu_1$ was independent of whether or not the NADPH was trapped by an $NADP^+$-regenerating system (data not shown). We had predicted that the trapping system would be necessary to remove product NADPH to enable the reaction to approach completion. We found that even the untrapped system (in which an excess of $NADP^+$ was used relative to the concentration of CH_2-$H_2PteGlu_1$) approached complete conversion to products. To explain this, we postulated that the CH^+-H_4folate was converted quickly enough to CHO-H_4folate, via both enzymatic and nonenzymatic reactions, to be removed as a product, thus making the reaction essentially irreversible.

Measurement of the Apparent Tritium Isotope Effect for Double-Labeled CH_2-$H_4PteGlu_5$. We measured the apparent $^T(V/K_B)$ associated with oxidation of double-labeled CH_2-$H_4PteGlu_1$ and repeated the measurement with CH_2-$H_4PteGlu_5$, since these chain lengths showed the largest differences in affinity for the enzyme as assessed by measurements of K_i values for $PteGlu_n$ inhibitors (Ross et al., 1984) and $H_2PteGlu_n$ inhibitors (Matthews et al., 1985). When the radioactivity remaining in the substrate was monitored and analyzed, the apparent $^T(V/K_B)$ of double-labeled CH_2-$H_4PteGlu_5$ was 2.11 ± 0.10, as shown in Figure 2B. This value was independent of the concentration of $NADP^+$ and independent of the inclusion of an $NADP^+$-regenerating system (data not shown).

Description of the Results Obtained by Using the Competitive Method To Measure Relative K_m Values for CH_2-$H_4PteGlu_n$, where n = 1, 2, 4, and 5. Methylenetetrahydrofolate dehydrogenase catalyzes an ordered bi-bi mechanism according to eq 7 Under the conditions of these competition

$$\text{velocity} = \frac{V_{max}}{\frac{K_{mB}}{[B]}\left\{1 + \frac{K_{iA}}{[A]}\right\} + 1 + \frac{K_{mA}}{[A]}} \quad (7)$$

experiments the concentration of $NADP^+$ was saturating and the conversion of monoglutamate and polyglutamate substrates was monitored by using tracer labels, so $[B] \ll K_{mB}$ for each isotopically labeled substrate. With these assumptions, the initial velocity of [*methylene*-^{14}C]CH_2-$H_4PteGlu_1$ reduces to

$$^{14}\text{velocity}_1 = \frac{^{14}V_{max1}}{^{14}K_{mB1}}[^{14}B_1] \quad (8)$$

V/K isotope effects are defined as the ratio of the rate constant for product formation from bulk substrate to the rate constant for product formation from the labeled substrate (Melander & Saunders, 1980). Under conditions where the label is present in tracer amounts in substrate B, eq 9 defines the rate constant for reaction of [*methylene*-^{14}C]CH_2-$H_4PteGlu_1$

$$^{14}k_1 = \frac{^{14}\text{velocity}_1}{[^{14}B_1]} = \frac{^{14}V_{max1}}{^{14}K_{mB1}} \quad (9)$$

and the rate constant for reaction of [*methylene*-^{3}H]CH_2-$H_4PteGlu_n$ is defined by

$$^3k_n = \frac{^3\text{velocity}_n}{[^3B_n]} = \frac{^3V_{maxn}}{^3K_{mBn}} \quad (10)$$

The measured isotope effect obtained by using a mixture of [*methylene*-^{14}C]CH_2-$H_4PteGlu_1$ and [*methylene*-^{3}H]CH_2-$H_4PteGlu_n$ may be expressed as

$$^T\left(\frac{V}{K}\right)_{measd} = \frac{^{14}k_1}{^3k_n} = \frac{\frac{^{14}V_{max1}}{^{14}K_{mB1}}}{\frac{^3V_{maxn}}{^3K_{mBn}}} \quad (11)$$

Since there is no detectable kinetic isotope effect on V/K_B associated with the introduction of the ^{14}C label, the ratio of $^{14}V_{max1}/^{14}K_{mB1}$ is equal to the ratio of $^1V_{max1}/^1K_{mB1}$. Making this substitution and multiplying the numerator and denominator by $^1V_{maxn}/^1K_{mBn}$, we obtain

$$^T\left(\frac{V}{K}\right)_{measd} = \frac{^{14}k_1}{^3k_n} = \frac{\frac{^1V_{maxn}}{^1K_{mBn}}\frac{^1V_{max1}}{^1K_{mB1}}}{\frac{^3V_{maxn}}{^3K_{mBn}}\frac{^1V_{maxn}}{^1K_{mBn}}} \quad (12)$$

The same derivations for the isotope effect for double-labeled CH_2-$H_4PteGlu_n$ yield the expression

$$^T\left(\frac{V}{K}\right)_n = \frac{^{14}k_n}{^3k_n} = \frac{\frac{^{14}V_{maxn}}{^{14}K_{mBn}}}{\frac{^3V_{maxn}}{^3K_{mBn}}} = \frac{\frac{^1V_{maxn}}{^1K_{mBn}}}{\frac{^3V_{maxn}}{^3K_{mBn}}} \quad (13)$$

Table I: Summary and Comparison of K_m Ratios Using Steady-State and Competitive Methods[a]

ref	$K_m(CH_2\text{-}H_4PteGlu_1)/K_m(CH_2\text{-}H_4PteGlu_1)$	$K_m(CH_2\text{-}H_4PteGlu_2)/K_m(CH_2\text{-}H_4PteGlu_1)$	$K_m(CH_2\text{-}H_4PteGlu_4)/K_m(CH_2\text{-}H_4PteGlu_1)$	$K_m(CH_2\text{-}H_4PteGlu_5)/K_m(CH_2\text{-}H_4PteGlu_1)$
Ross et al. (1984)[b]	ND	ND	1.00 ± 0.33	0.61 ± 0.22
this work[c]	1.00 ± 0.04	0.37 ± 0.01	0.33 ± 0.01	0.39 ± 0.03

[a] Propagation of error in the calculations was performed according to the method of Bevington (1969). Values are given with their standard errors. [b] The K_m values for $CH_2\text{-}H_4PteGlu_1$, $CH_2\text{-}H_4PteGlu_4$, and $CH_2\text{-}H_4PteGlu_5$ were calculated to be 5.20 ± 0.63, 5.21 ± 1.60, and 3.17 ± 1.05 respectively; these data were analyzed by using an adaptation of a program written by Dr. W. W. Cleland to calculate V_{max}, K_{iA}, K_{mA}, and K_{mB} for a bi-bi ordered reaction. [c] These values were calculated by using eq 15 and apparent $^T(V/K)$ values of 2.16 ± 0.07 ($CH_2\text{-}H_4PteGlu_1$), 0.80 ± 0.01 ($CH_2\text{-}H_4PteGlu_2$), 0.71 ± 0.01 ($CH_2\text{-}H_4PteGlu_4$), and 0.85 ± 0.05 ($CH_2\text{-}H_4PteGlu_5$). The K_m values for $CH_2\text{-}H_4PteGlu_1$, $CH_2\text{-}H_4PteGlu_2$, $CH_2\text{-}H_4PteGlu_4$, and $CH_2\text{-}H_4PteGlu_5$ were calculated to be 5.21 ± 0.65, 1.93 ± 0.24, 1.71 ± 0.21, and 2.05 ± 0.28, respectively.

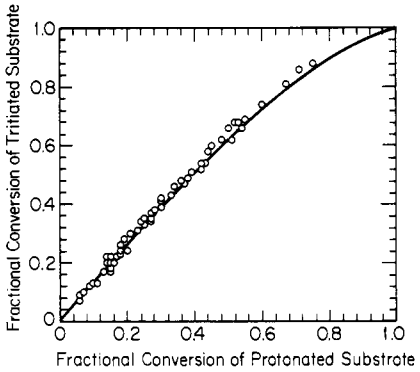

FIGURE 3: Measurement of the apparent tritium isotope effect on V/K using [*methylene*-^{14}C]$CH_2\text{-}H_4PteGlu_1$ and [*methylene*-^{3}H]$CH_2\text{-}H_4PteGlu_4$. Assays containing 100 mM potassium phosphate, pH 7.2, 180 μM $NADP^+$, 50 μM [*methylene*-^{14}C]$CH_2\text{-}H_4PteGlu_1$, 50 μM [*methylene*-^{3}H]$CH_2\text{-}H_4PteGlu_4$, and methylenetetrahydrofolate dehydrogenase were initiated with the addition of folate substrates. Samples were taken with time and heat-quenched as discussed under Experimental Procedures. The fractional conversion of tritiated substrate ($CH_2\text{-}H_4PteGlu_1$) is plotted vs the fractional conversion of protiated substrate ($CH_2\text{-}H_4PteGlu_1$). The data yielded an apparent isotope effect of 0.71 ± 0.01.

Because V_{max} is independent of the substrate polyglutamyl chain length (Ross et al., 1984), we may rearrange eq 12 to obtain

$$^T\left(\frac{V}{K}\right)_{measd} = \frac{^{14}k_1}{^3k_n} = {}^T\left(\frac{V}{K}\right)_n \frac{^1K_{mBn}}{^1K_{mB1}} \qquad (14)$$

Finally, since the isotope effect $^T(V/K_B)$ is the same for each folylpolyglutamate substrate, we may substitute 2.16 ± 0.07 (Figure 2B):

$$^T\left(\frac{V}{K}\right)_{measd} = \frac{^{14}k_1}{^3k_n} = (2.16 \pm 0.07)\frac{^1K_{mBn}}{^1K_{mB1}} \qquad (15)$$

Measurement of an apparent isotope effect would thus be a direct comparison of the K_m values of the two substrates.

Figure 3 shows a representative plot for the measured $^T(V/K_B)$ isotope effect using [*methylene*-^{14}C]$CH_2\text{-}H_4PteGlu_1$ and [*methylene*-^{3}H]$CH_2\text{-}H_4PteGlu_4$ where the observed isotope efect is 0.71 ± 0.01. Table I provides a summary of data for several different competition experiments with substrates containing two, four and five glutamyl residues. Because this method most accurately measures ratios of K_m values, we have reported the values as such and offer the values calculated from the steady-state kinetic data from Ross et al. for comparison (1984). The measured K_m for $CH_2\text{-}H_4PteGlu_1$ is 5.2 ± 0.63 μM (Table I). Therefore, the K_m value for folylpolyglutamate substrates is estimated to be about 1.9 ± 0.3 μM.

Controls containing 50 μM [*methylene*-^{3}H]$CH_2\text{-}H_4PteGlu_4$ and 50 μM [*methylene*-^{14}C]$CH_2\text{-}H_4PteGlu_1$ were incubated for varying amounts of time in 100 mM phosphate buffer, pH

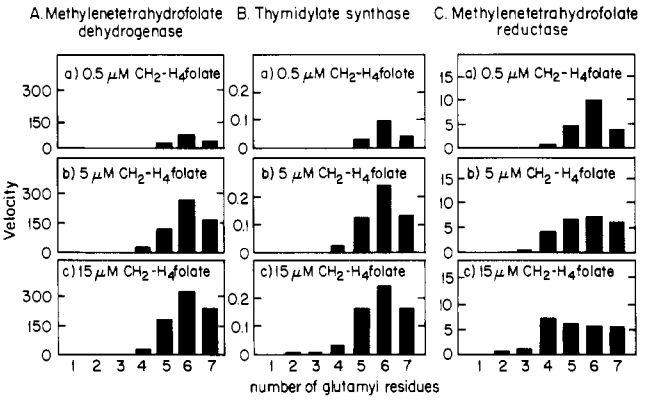

FIGURE 4: Plots of initial velocity versus number of glutamyl residues in the substrate for three methylenetetrahydrofolate-dependent enzymes. For each enzyme, the velocities have been calculated as described in the text, for total methylenetetrahydrofolate concentrations of 0.5, 5, and 15 μM. For methylenetetrahydrofolate dehydrogenase (A) and methylenetetrahydrofolate reductase (C), velocity is expressed as μM min^{-1}. For thymidylate synthase (B), velocity is expressed as μmol min^{-1}.

7.2; samples were separated by HPLC as described above. Such controls revealed that while $CH_2\text{-}H_4PteGlu_n$ did dissociate into formaldehyde and H_4folate (15% dissociation occurred in 5 min), the labeled formaldehyde did not reassociate with the $H_4PteGlu_n$ to result in transfer of label between $CH_2\text{-}H_4PteGlu_1$ and $CH_2\text{-}H_4PteGlu_4$ (data not shown).

Calculation of Flux of Methylenetetrahydrofolate through the Purine and Methionine Biosynthetic Pathways. Using measured kinetic constants of methylenetetrahydrofolate reductase (Matthews & Baugh, 1980), thymidylate synthase (Lu et al., 1984), and methylenetetrahydrofolate dehydrogenase from pig liver (Ross et al., 1984; this paper) and estimated physiological concentrations of the reactants, we endeavored to predict the flux of methylenetetrahydropteroylpolyglutamates through these pathways in pig liver. The calculations for the velocities of methylenetetrahydrofolate dehydrogenase, methylenetetrahydrofolate reductase, and thymidylate synthase in pig liver are discussed under Experimental Procedures.

Panels A and C in Figure 4 show how the concentration of total $CH_2\text{-}H_4$folate affects the profiles of velocity for methylenetetrahydrofolate dehydrogenase and methylenetetrahydrofolate reductase as the length of the polyglutamate chain of the substrate increases. Although the velocity increases with increasing concentrations of folate substrate, the shape of the curve for methylenetetrahydrofolate dehydrogenase remains the same, with velocity peaking for $CH_2\text{-}H_4PteGlu_6$. In contrast, the curve for the velocity of methylenetetrahydrofolate reductase peaks at hexaglutamates with low concentrations of folate substrate; it shifts to shorter chain lengths with 5 μM folate substrate concentrations, and the profile peaks at tetraglutamates for 15 μM folate substrate. The

Table II: Summary of the Ratios of the Calculated Initial Velocity of Pig Liver Methylenetetrahydrofolate Dehydrogenase to the Velocity of Pig Liver Methylenetetrahydrofolate Reductase

	0.5 μM CH_2-H_4folate			5 μM CH_2-H_4folate			15 μM CH_2-H_4folate		
	velocity[b]			velocity[b]			velocity[b]		
n[a]	dehydrogenase	reductase	ratio	dehydrogenase	reductase	ratio	dehydrogenase	reductase	ratio
4	1.8	0.78	2.3	13	4.1	3.1	27	7.3	3.7
5	17	4.4	3.8	104	6.7	16	180	6.0	30
6	50	9.8	5.2	250	7.1	36	330	5.7	58
7	26	3.8	6.8	150	6.1	25	230	5.4	43
total	95	19	5.1	520	24	22	770	24	32

[a] n represents the number of glutamyl residues in CH_2-H_4PteGlu$_n$. [b] The velocity of methylenetetrahydrofolate dehydrogenase or methylenetetrahydrofolate reductase represents the initial velocity of that enzyme for that chain length of methylenetetrahydrofolate and in the presence of the indicated total concentration of substrate. Velocity was calculated as described under Experimental Procedures and is in units of μM min^{-1}. The ratio is the velocity of methylenetetrahydrofolate dehydrogenase divided by the velocity of methylenetetrahydrofolate reductase. The velocities for CH_2-H_4PteGlu$_1$, CH_2-H_4PteGlu$_2$, and CH_2-H_4PteGlu$_3$ were not included because these are not present in significant amounts in pig liver (Cichowicz & Shane, 1987a).

velocity patterns for thymidylate synthase and methylenetetrahydrofolate dehydrogenase are very similar (see Figure 4, parts A and B).

The calculated flux ratio for CH_2-H_4PteGlu$_n$ through methylenetetrahydrofolate dehydrogenase versus methylenetetrahydrofolate reductase for each of the three chosen concentrations of folate substrate is given in Table II. The dehydrogenase pathway becomes more favored with increasing concentrations of total folate. Also, at every concentration, it is at the hexaglutamate or heptaglutamate level where one finds the greatest proportion of folate substrate entering the methylenetetrahydrofolate dehydrogenase pathway.

Discussion

Previous measurements of K_m values for polyglutamate substrates of pig liver methylenetetrahydrofolate dehydrogenase have used independent steady-state kinetic measurements to determine the K_m values for each substrate. Each measurement had an associated standard error of about 30%, since the measured K_m values were low relative to the substrate required for accurate determination of initial rates. Furthermore, the standard errors may not fully reflect the uncertainty in the determinations since systematic errors are likely to occur where a substantial amount of the initial substrate is consumed during the measurement. Given this level of uncertainty, it may be very dificult to determine K_m ratios for two different substrates. We have used a competitive method that permitted us to measure K_m ratios for pairs of folate substrates with considerable accuracy (standard error of about 10%). Because this method does not require kinetic measurements under initial rate conditions, the systematic errors inherent in independent measurements of K_m values are also avoided.

The K_m values calculated from the measured ratios will have a standard error that reflects the standard error of the reference K_m value (in this case that for CH_2-H_4PteGlu$_1$), but their magnitude relative to the standard K_m will be well-defined. We believe such competitive measurements are the method of choice for determining comparative K_m values for alternate substrates. An intrinsic isotope effect on the reaction catalyzed is not necessary or even desirable; one needs only an accurate measure of V_{max} for each substrate and a method for separating labeled substrates and products to perform competitive determinations of K_m values.

While this method may be applied to a number of kinetic schemes, the underlying assumptions associated with the kinetic mechanism should first be examined. We have treated the bi-bi ordered case in which B is the labeled substrate; if A is the labeled species, however, the concentration of B must be saturating for eq 8 and 12 to hold true. These expressions also hold for ping-pong bi-bi and random bi-bi rapid equilibrium mechanisms. In the latter case, however, one must assume there is no isotope effect on α, the constant associated with ligand synergism.

We are now in a position to calculate partitioning of methylenetetrahydrofolate between conversion to methyltetrahydrofolate and conversion to methenyltetrahydrofolate (and presumably to 10-formyltetrahydrofolate). This is made possible by these more accurate K_m values for the methylenetetrahydrofolate dehydrogenase and by the recently published data of Cichowicz and Shane (1987a,b) on total folate concentration and polyglutamate chain length distributions of pig liver folate pools.

We believe that these calculations, while they have limitations to be discussed below, provide considerable insight into folate metabolism in vivo. The high cellular concentrations of trifunctional enzyme and serine hydroxymethyltransferase relative to those of methylenetetrahydrofolate reductase and the transformylases have led many investigators to assume that the former enzymes maintain their substrates and products at equilibrium. Our calculations suggest that methylenetetrahydrofolate dehydrogenase operates in the V/K region for both its folate substrate and $NADP^+$ and that, especially at low concentrations of methylenetetrahydrofolate, its activity may limit flux of one-carbon units into the purine biosynthetic pathway. It is interesting to note that bone marrow cells, transformed cells, and embryonic liver cells contain an NAD^+-dependent methylenetetrahydrofolate dehydrogenase as part of a methylenetetraydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase bifunctional protein that is not detected in other cells (Mejia & MacKenzie, 1985). The cytoplasmic NAD^+/NADH ratio in liver is about 1000, while the $NADP^+$/NADPH ratio is 0.011 (Krebs, 1973). The presence of this alternate enzymatic activity in certain rapidly growing cell types is further evidence that the $NADP^+$-dependent methylenetetrahydrofolate dehydrogenase can be rate-limiting for de novo purine biosynthesis.

Our calculations support a model in which the cellular concentration of methylenetetrahydrofolate regulates the flux of methylenetetrahyrofolate into the pathways leading to nucleotide biosynthesis and methionine regeneration. Increasing cellular levels of methylenetetrahydrofolate would result in an increased flux of methylenetetrahydrofolate into pathways leading both to thymidylate synthesis and to de novo purine biosynthesis.

There are, admittedly, weaknesses in our assumptions. In particular, we have neglected the effect of NADPH on methylenetetrahydrofolate dehydrogenase activity. NADPH is

abundant in the cell (200 μM; Conway et al., 1983), while the cellular concentration of substrate $NADP^+$, about 2 μM, is well below its K_m. We have also neglected the effects of AdoMet, an allosteric inhibitor of methylenetetrahydrofolate reductase, and AdoHCy, which relieves this inhibition (Kutzbach & Stokstad, 1971). While the presence of such metabolites may alter the partitioning of methylenetetrahydrofolate at any given concentration, they will not change the general dependence of the flux ratio on methylenetetrahydrofolate concentration.

This model is based on the assumption that the cellular concentration of methylenetetrahydrofolate can be regulated. An increase in the level of methylenetetrahydrofolate could be effected in one of two ways: the cell would have to increase its uptake of exogenous folate, or, more likely, it could increase the intracellular concentration of methylenetetrahydrofolate at the expense of some other folate pool. Since methyltetrahydrofolates are abundant, constituting about 50% of cellular folates in mammalian cells [Kashanis & Cooper, 1985; Allegra et al., 1986; reviewed by Cossins (1984)], this seems the most likely source of additional methylenetetrahydrofolate. Using human breast cancer cells, Allegra et al. (1986) have demonstrated that dramatic changes in the cellular levels of methyltetrahydrofolate correlate with changes in growth rate: while less than 10% of total folate is CH_3-H_4folate during the early log phase, greater than 60% of the total pool is CH_3-H_4folate during the mid and late log phases of cell growth. Our model would have been more directly tested, however, if the levels of CH_2-H_4folate had also been monitored. Our model predicts that higher levels of CH_2-H_4folate will be found in cells that are rapidly dividing than in quiescent cells.

We predict that depletion of methyltetrahydrofolate pools is the result of a decrease in methylenetetrahydrofolate reductase activity, which is controlled allosterically by the cellular ratio of AdoMet to AdoHCy. Inhibitors of adenosylhomocysteine hydrolase have been used to raise the cellular ratio of AdoHCy/AdoMet and to inhibit methylation. It would be interesting to determine whether these inhibitors also affect the cellular levels of methylenetetrahydrofolate and methyltetrahydrofolate, as we would predict.

A change in cellular levels of methyltetrahydrofolates might effect other changes in the cell. Because polyglutamates of methyltetrahydrofolate bind tightly to serine hydroxymethyltransferase in vitro, it has been suggested that this might regulate the activity of serine hydroxymethyltransferase in the cell (Matthews et al., 1982). A depletion in the methyltetrahydrofolate pools might thus result in an increase in serine hydroxymethyltransferase activity. This may act to translate the decrease in cellular methyltetrahydrofolate pools into an increase in methylenetetrahydrofolate pools.

Data from our and other laboratories on the polyglutamate specificities of folate-dependent enzymes suggest that they may be divided into two groups. The first group consists of enzymes with very low K_m values for their folylpolyglutamate substrates, such that these enzymes are essentially saturated at physiological folate concentrations. Included in this group of enzymes are methylenetetrahydrofolate reductase (Matthews & Baugh, 1980), methionine synthase (Matthews et al., 1987), and formyltetrahydrofolate synthetase (MacKenzie & Baugh, 1980; MacKenzie, unpublished data; Strong et al., 1987). The second group of enzymes is characterized by K_m values for folate substrates that are higher than the intracellular concentrations of these substrates, such that the activities of these enzymes are very dependent on the concentration of their folate substrates. This group includes thymidylate synthase (Lu et al., 1984), dihydrofolate reductase (Smith et al., 1979; Blakley et al., 1983), methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase (this paper), aminoimidazolecarboxamide ribonucleotide (AICAR) transformylase (Baggot & Krumdieck, 1979; Allegra et al., 1985b), and glycinamide ribonucleotide (GAR) transformylase (Allegra et al., 1985b). The latter group of enzymes are all involved in supplying one-carbon units for nucleotide biosynthesis. While methylenetetrahydrofolate has been estimated to be about 5 μM in liver (see above), this value includes both free and protein-bound pools, and the concentration of unbound cytosolic methylenetetrahydrofolate is probably much less than 5 μM. Since peripheral cells possess a lower total folate pool size (Richardson et al., 1979), the concentration of methylenetetrahydrofolate in these tissues will be proportionately reduced. Thus, regulation of the intracelluar pool size of CH_2-H_4folate could play an important role in determining the availability of one-carbon units for nucleotide biosynthesis.

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2-Octynoyl Coenzyme A Is a Mechanism-Based Inhibitor of Pig Kidney Medium-Chain Acyl Coenzyme A Dehydrogenase: Isolation of the Target Peptide†

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ABSTRACT: Pig kidney medium-chain acyl-CoA dehydrogenase (EC 1.3.99.3) is irreversibly and stoichiometrically inactivated by [1-^{14}C]-2-octynoyl coenzyme A. The linkage is stable at pH 2-6, but labile under basic conditions. The inhibitor labels a unique tryptic peptide, Ile-Tyr-Gln-Ile-Tyr-Glu-Gly-Thr-Ala-Gln-Ile-Gln-Arg, close to the C-terminus of the protein. The peptide is labeled at Glu-401 with the acyl moiety of the inhibitor but does not contain detectable coenzyme A. Both the inactivation of the dehydrogenase and the appearance of an absorption band at 800 nm show large primary deuterium isotope effects using 4,4′-dideuterio-2-octynoyl-CoA (7.3 and 6.3, respectively). Thus, 2-octynoyl-CoA is a mechanism-based inactivator of the dehydrogenase and is activated by rate-limiting γ-proton abstraction. Glutamate-401 may be the base that abstracts the *pro-R* α-proton during the dehydrogenation of normal substrates.

The short-, medium-, and long-chain acyl-CoA[1] dehydrogenases are immunologically distinct (Ikeda et al., 1985a) flavoproteins that catalyze the first oxidative step of β-oxidation (Beinert, 1963). These enzymes introduce a trans double bond between C-2 and C-3 of their acyl-CoA substrates (Beinert, 1963) and are subsequently reoxidized by electron-transferring flavoprotein (ETF; Crane & Beinert, 1956):

$$dH_{ox}\cdot RCH_2CH_2COSCoA \rightleftharpoons dH_{red}\cdot RCH{=}CHCOSCoA$$

$$dH_{red}\cdot RCH{=}CHCOSCoA + 2ETF_{ox} \rightleftharpoons dH_{ox}\cdot RCH{=}CHCOSCoA + 2ETF_{red}$$

The most thoroughly studied of these flavoproteins is the mammalian medium-chain acyl-CoA dehydrogenase. Recently the gene sequences of both the human and rat liver enzymes have been reported (Kelly et al., 1987; Matsubara et al., 1987, respectively). A deficiency of this enzyme in humans leads to sometimes fatal organic acidurias, and this

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[1] Abbreviations: CoA(SH), coenzyme A; HPLC, high-performance liquid chromatography; ETF, electron-transferring flavoprotein; TFA, trifluoroacetic acid; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; PTH, phenylthiohydantoin; TCA, trichloroacetic acid; dH, dehydrogenase; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane.

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