Enzymic Properties of a New Mechanism-Based Substrate for Dihydrofolate Reductase†

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Received December 7, 1988; Revised Manuscript Received April 3, 1989

ABSTRACT: The synthesis and enzymic characterization of the primary member, 8-methylpterin, of a new class of substrates for vertebrate dihydrofolate reductases (DHFRs) are reported. The compound was designed, on the basis of theoretical calculations and chemical arguments, as a proposed analogue of the protonated form of folate suggested to be the catalytically active species in the initial enzymic reduction of folate. Under complete reaction conditions at pH's between 5.0 and 7.4, 8-methylpterin was found to be rapidly reduced enzymically with a reductant (NADPH) stoichiometry of 1 to 8-methyl-7,8-dihydropterin, which was subsequently reduced more slowly and incompletely to a product suggested to be 8-methyl-5,6,7,8-tetrahydropterin. The pH activity maximum for the initial reduction was found to be 5.8–6.0 for chicken, bovine, and human DHFRs. The K_m's for the initial reduction for all three enzymes were highly pH dependent, varying from ~30 μM at pH 5.0 to ~200 μM at pH 7.4 for the chicken and bovine enzymes and from ~130 μM at pH 5.0 to >300 μM at pH 7.0 for human DHFR. The specific activities for the initial reduction at the pH maximum and at neutral pH were 130% and 85% (chicken), 65% and 40% (bovine), and 180% and 90% (human) those for dihydrofolate reduction. Under assay conditions 8-methyl-7,8-dihydropterin was found to have negligible substrate activity at neutral pH, but measurable activity at pH's 5.8 and 5.0.

Dihydrofolate reductase (DHFR)[1] (5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase) catalyzes the reduction of 7,8-dihydrofolate (H_2folate) by NADPH to form 5,6,7,8-tetrahydrofolate (H_4folate) and also, under some circumstances, of folate by 2 equiv of NADPH to form H_4folate (Blakley, 1969, 1984). Because of the essential role of the tetrahydrofolate cofactors in purine and pyrimidine biosynthesis, DHFR is the target for a large class of cytotoxic drugs, the "anti-folates". The enzyme has been the subject of intensive physicochemical study (Gready, 1980; Blakley, 1984), especially structural studies of binary and ternary substrate and inhibitor complexes of *Escherichia coli*, *Lactobacillus casei*, chicken liver, mouse L1210, and human enzymes (Baker et al., 1981; Bolin et al., 1982; Volz et al., 1982; Champness et al., 1986; Kraut & Matthews, 1987; Stammers et al., 1987; Oefner et al., 1988) and enzyme kinetic and mechanistic studies with both vertebrate and bacterial enzymes (Williams & Morrison, 1981; Stone & Morrison, 1982, 1984; Morrison & Stone, 1986; Penner & Freiden, 1987; Fierke et al., 1987; Morrison, 1989). More recently, these studies have been extended to mutant enzymes produced by site-directed mutagenesis (Howell et al., 1986, 1987; London et al., 1986; Mayer et al., 1986; Chen et al., 1987; Taira & Benkovic, 1988; Benkovic et al., 1988; Prendergast et al., 1988; Appleman et al., 1988), and a number of molecular modeling and mechanics studies using the X-ray structures as a starting point have been discussed (Benkovic et al., 1988) or reported (Singh, 1988). These studies have provided much information on the kinetic sequence, rate-limiting steps, and pH dependence for both wild-type and mutant enzymes, especially for dihydrofolate reduction by *E. coli* DHFR (Morrison, 1989). In particular, they have addressed two main questions: the source of the proton involved in the reduction (Freisheim & Matthews, 1984) and the underlying reason for the upside-down binding of the inhibitor heterocyclic ring in the enzyme active site (Charlton et al., 1979; Taira et al., 1987). However, several unanswered questions regarding the molecular catalytic mechanism remain.

In previous work we addressed one aspect of the catalytic mechanism, namely, the nature of the presumed protonated-activated forms of folate and H_2folate, by performing ab initio molecular orbital calculations (Gready, 1985a). We concluded that the most likely species, energetically, were the N8- and N5-protonated forms, suggested originally by Huennekens and Scrimgeour (1964) on the basis of classical organic chemistry arguments. Earlier we had hypothesized that enzyme evolutionary structural constraints at the active site may have precluded development of a side chain capable of directly protonating both the oxidized (folate) and dihydro (H_2folate) substrates and instead led to a less obvious mechanism for facilitating the reduction (Gready, 1979). The accumulated experimental evidence, especially the pH dependence of the H_2folate enzymic reduction for wild-type and relevant mutants (see references above), has been interpreted to suggest that the conserved acidic group adjacent to the pyrimidine moiety of the pteridine ring (Bolin et al., 1982) is the proton donor. However, the identities of the catalytically active protonated forms of the folate and H_2folate substrates, or whether there

† This research was supported by grants from the National Health and Medical Research Council and the Sydney University Cancer Research Fund.
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[1] Abbreviations: DHFR, dihydrofolate reductase; NADPH, nicotinamide adenine dinucleotide phosphate (reduced); NADP$^+$, nicotinamide adenine dinucleotide phosphate (oxidized); H_2folate, 7,8-dihydrofolate; H_4folate, 5,6,7,8-tetrahydrofolate; 8-Me-Pt, 8-methylpterin; 8-Me-H_2Pt, 8-methyl-7,8-dihydropterin; 8-Me-H_4Pt, 8-methyl-5,6,7,8-tetrahydropterin; DTT, dithiothreitol; $\epsilon(R)_{340}$, $\epsilon(R)$, and $\epsilon(R-)$, total (without NADP$^+$ recycling) reaction extinction at 340 nm; $\epsilon(R+)$, reaction extinction at 340 nm with NADP$^+$ recycling.

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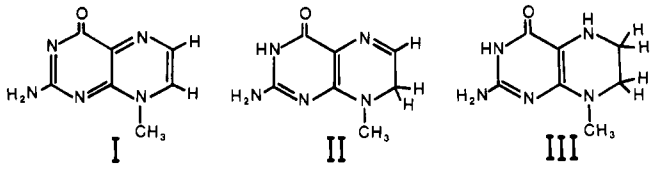

FIGURE 1: Structures for 8-methylpterin (8-Me-Pt, I) and its 7,8-dihydro (8-Me-H_2Pt, II) and 5,6,7,8-tetrahydro (8-Me-H_4Pt, III) forms.

might be initial transient forms protonated on the pyrimidine ring, for example, on O4 (Gready, 1985a), that rearrange to pyrazine ring protonated forms, has not been determined. The X-ray structures provide no clear pathway for proton "hopping" from the pyrimidine ring to N8 or N5 of the pyrazine ring (Freisheim & Matthews, 1984). Also in our earlier study (Gready, 1985a), we suggested an alternative possible role for the acidic residue, if it were negatively charged, in stabilization of the pyrazine ring protonated forms by a mechanism involving π-electron coupling effects across the bicyclic ring system (Gready, 1987). It is noteworthy that comprehensive studies on the enzymic reduction of folate, comparable with those for H_2folate, have not been done, and there has been little recent discussion on the mechanistic requirements for folate reduction (Morrison, 1989). However, it has long been known (Blakley, 1969) that the pH dependence for the DHFR reduction of folate differs greatly from that for H_2folate reduction: folate is a very poor substrate at neutral pH, although it binds well and may be used as an inhibitor (Stone & Morrison, 1986), and for most species of DHFR it exhibits a pH maximum below 5 (Blakley, 1969).

In the present paper we report initial experimental results for the simplest member, 8-methylpterin (Figure 1, I), of a new class of folate-analogue substrates based on the N8-protonated-activated form of folate deduced previously (Gready, 1985a). These compounds can properly be described as mechanism-based, although they are not transition-state analogues. The chemically most significant consequence of 8-substitution of the pterin nucleus is that it forces it into a less stable tautomeric form analogous to N8(*H*)-pterin (Gready, 1985b). This effective destabilization of the N-heterobicyclic ring system results directly in higher basicities for the 8-substituted pterins, including 8-methylpterin (Brown & Jacobsen, 1961; Pfleiderer et al., 1968). The magnitude of this destabilization and the basicities of the relevant tautomerically equivalent protonated froms of pterin [i.e., N8-protonation of the most stable N3(*H*) tautomer] and N8-(*H*)-pterin (i.e., N3-protonation) may be deduced from the results of the theoretical calculations reported previously (Gready, 1985a,b); this scheme is summarized in Figure 2. The predicted basic pK_a for 8-methylpterin (5.2) obtained by using the linear correlation between the theoretical protonation energies and experimental pK_a's (Gready, 1985b) and assuming a neglible perturbation from the methyl group compares well with the value determined experimentally of 5.32 (Pfleiderer et al., 1968). Also, the predicted pK_a of 2.0 for N8-protonation of pterin (Gready, 1985a,b) is consistent with the only available experimental data for forced protonation of this type, for the sterically restricted 3,N^2,N^2-trimethylpterins (pK_a = 1.2–1.7; Rokos & Pfleiderer, 1971); note that the normal protonation site for pterins and folate is N1.

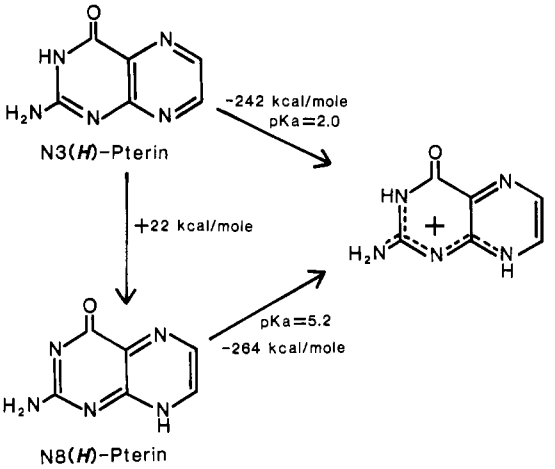

FIGURE 2: Calculated relative energies and pK_a's (Gready, 1985b) for the N3(*H*) and N8(*H*) tautomers of pterin and their common N8-protonated form.

Experimental Procedures

Materials

Enzymic Assays. H_2folate was prepared from folic acid (Blakley, 1960) and stored at −20 °C in sealed glass ampules under nitrogen. NADPH was purchased from Boehringer, and chicken and bovine liver DHFRs and glucose-6-phosphate dehydrogenase (yeast and Leuconostoc) were obtained from Sigma. Some pure chicken liver DHFR was also a gift from Dr. J. F. Morrison. Recombinant human DHFR (Prendergast et al., 1988) was a gift from Dr. J. H. Freisheim; it was stored at −70 °C. Buffer components and other chemicals were of the highest purity available commercially.

Syntheses. 8-Methylpterin (8-Me-Pt) was synthesized by the method of Brown and Jacobsen (1961), with some modification of reaction conditions (unpublished results), starting from 2-amino-4,6-dihydroxypyrimidine (Aldrich). It was purified by recrystallization as the hydrochloride salt from a solution of methanol containing a few drops of methanolic HCl, which had been filtered through activated charcoal. (Anal. Calcd for $C_7H_8N_5O{\cdot}HCl{\cdot}0.05H_2O$: C, 39.2; H, 3.8; N, 32.6; Cl, 16.5. Found: C, 39.5; H, 3.8; N, 32.3; C, 16.1.) 8-Methyl-7,8-dihydropterin (8-Me-H_2Pt, II) was prepared by the method of Pfleiderer and Mengel (1971) involving borohydride reduction of 8-Me-Pt to 8-methyl-5,6,7,8-tetrahydropterin (8-Me-H_4Pt, III) followed by aerial back-oxidation to the dihydro form. It was purified by recrystallization from water and stored at −20 °C in sealed glass ampules under nitrogen. Highly pure 8-Me-H_4Pt was synthesized by catalytic hydrogenation by the procedure of Viscontini (1971) with some modification of reaction conditions (unpublished results) and stored at −20 °C under nitrogen as the dihydrochloride salt. (Anal. Calcd for $C_7H_{11}N_5O{\cdot}2.1HCl{\cdot}0.3H_2O$: C, 31.9; H, 5.3; N, 26.6; Cl, 28.3. Found: C, 31.9; H, 5.1; N, 26.4; Cl, 28.0.)

Methods

Pterins. The following extinction coefficients were used to standardize concentrations: NADPH, at pH 7.0, 6200 M^{-1} cm^{-1} at 340 nm; H_2folate, at pH 7.0, 28 400 M^{-1} cm^{-1} at 282 nm (Blakley, 1960, 1969); 8-Me-Pt, at pH 2.3, 9830 and 13 250 M^{-1} cm^{-1} at 388 and 261 nm, respectively (this work); 8-Me-H_2Pt, at pH 5.0, 9330 M^{-1} cm^{-1} at 282 nm (Pfleiderer & Mengel, 1971); 8-Me-H_4Pt, at pH 2.3, 15 490 M^{-1} cm^{-1} at 271 nm (Pfleiderer & Mengel, 1971). Solutions of NADPH and H_2folate (in water at pH 7), 8-Me-H_2Pt [in 10 mM dithiothreitol (DTT) at pH 7], and 8-Me-H_4Pt (4 mM HCl, pH 2.3) were made up freshly each day and kept on ice, while 8-Me-Pt was made up as 1–5 mM stock solutions in 4 mM HCl and stored as frozen aliquots. The concentration of the 8-Me-H_2Pt solutions (0.5–1 mM) was limited by poor solubility.

Enzymes. For the initial rate assays the three DHFRs were freshly diluted to concentrations of 25, 8.4, and 17 μM, respectively, for chicken (Sigma), bovine, and human enzymes, calculated by using molecular weights of 21 650, 21 450, and 21 450, respectively (Blakley, 1984). For the preliminary chicken DHFR (Morrison) tests undiluted enzyme of 10 μM was used. The activities were checked by H_2folate assay at pH 7.4 and 30 °C in the buffer described below using saturating concentrations of H_2folate (50 μM) and NADPH (60 μM): the specific activities of the enzymes under these conditions were 5 (Sigma) and 20 (Morrison) for chicken and 9 and 21 for bovine and human enzymes, calculated by using a reaction extinction coefficient of 11 800 M^{-1} cm^{-1} (Stone & Morrison, 1982). The diluted chicken and bovine enzymes were stable for several hours, but the human enzyme showed some loss of activity after 2–3 h. Undiluted DHFR enzymes were used for the complete reaction and spectral difference experiments.

Enzyme Assays. Complete reaction and initial rate assays were performed at 30 °C with a Shimadzu UV-160 recording spectrophotometer using deaerated solutions: the rate of enzyme-dependent decrease in the absorbance at 340 nm was followed. The buffer was that of Ellis and Morrison (1982); it contained 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 25 mM tris(hydroxymethyl)aminomethane (Tris), 25 mM ethanolamine, and 100 mM NaCl. Over the pH range for which initial velocities were measured (5.0–8.2), the ionic strength of this buffer remained essentially constant at I = 0.15 M (Ellis & Morrison, 1982).

In determining the reaction extinction and reaction stoichiometry for the three enzymes, several types of assay and control experiments were performed at pH's 7.4, 6.6, 5.8, and 5.0. For all enzyme experiments total assay volumes of 2.0 (no $NADP^+$ recycling) or 2.4 mL (with $NADP^+$ recycling) were used with concentrations of 62.5 μM 8-Me-Pt, 150 or 250 μM (pH 5.0 only) NADPH, 10 mM DTT, and ~0.8 μM DHFR for all three enzymes: 1 cm quartz split cells (2 × 0.4 cm compartments) for assay and blank were used to give an equivalent 8-Me-Pt concentration for a 1 cm path length of 50 μM. For determining the total reaction extinction, the 8-Me-Pt and NADPH components were added separately to the assay and blank compartments, with DHFR added to the assay NADPH compartment. After a zero-time OD reading (~0) was taken, the assay cuvette was mixed to start the reaction and the OD change followed until the reading stabilized (pH's 7.4 and 6.6; ~2 h) or maximized (pH's 5.8 and 5.0; 15–30 min): the total change is the sum of enzymic and nonenzymic reaction contributions in the assay with partial compensation for NADPH decay provided by the blank. Checks on the rate of the nonenzymic reaction and the stabilities of 8-Me-Pt, 8-Me-H_2Pt, 8-Me-H_4Pt, and NADPH under the reaction conditions were also done. To determine the reaction stoichiometry, the enzyme reaction experiments were repeated with recycling of the product $NADP^+$ using 10 mM glucose 6-phosphate and ~20 units of glucose-6-phosphate dehydrogenase; a control experiment to check possible contamination of NADPH by $NADP^+$ indicated negligible oxidation. As 8-Me-Pt and 8-Me-H_4Pt have ionizations within the test pH range, spectra and extinction coefficients at 340 nm were measured in the assay buffers at pH 5.0–8.2 in the presence of 10 mM DTT and, for 8-Me-Pt, in the absence of DTT. Similar experiments were performed for 8-Me-H_2Pt, which has no ionization in the pH range, in the presence of 1.5 and 10 mM DTT.

The spectrum of the reaction product of the chicken and human DHFR reduction of 8-Me-Pt with NADPH at pH 7.4 and of chicken DHFR at pH 5.8 was obtained by the density difference method from a modified recycling experiment in which 8-Me-Pt was omitted from the blank cuvette. Complete spectral scans were taken at t = 0 before mixing the split-cell assay cuvette and then at increasing time intervals until reaction completion.

Initial rate assays for 8-Me-Pt as variable substrate were performed for the three DHFRs by addition of 6 μL of NADPH and 1 (human), 2 (chicken), or 4 (bovine) μL of DHFR to prewarmed buffer containing 8-Me-Pt to give final concentrations of 60 μM NADPH and 17 (human), 49 (chicken), or 34 (bovine) nM DHFR in a total volume of 1 mL. 8-Me-Pt concentration ranges for chicken and bovine DHFRs were, pH 5.0–5.8, 100–14.3 μM; pH 6.0–7.0, 200–33.3 μM; pH 7.4 and 7.8, 200–40 μM; and pH 8.2, 400–66.7 μM; and for human DHFR were pH 5.0–6.0, 200–33.3 μM; pH 6.2–6.6, 400–57.1 μM; pH 6.8, 500–100 μM; and pH 7.0 and 7.4, 1000–166.7 μM. Initial rate assays for NADPH as variable substrate (saturating but not inhibiting concentrations of 8-Me-Pt) were performed for chicken (1 μL) and human (1 μL) DHFRs at a number of pH's: chicken, pH's 5.0, 5.8, 6.6, and 7.4 with 200 μM (800 μM at pH 7.4) 8-Me-Pt and 20–1.7 μM NADPH; human, pH's 5.0, 5.8, and 6.6 with 400 μM (800 μM at pH 6.6) and 20–1.7 μM NADPH. To account for nonenzymic reaction and NADPH decay, assays below pH 7.4 were done with a blank containing all components except DHFR. H_2folate activity was also assayed at the test pH's. Tests of the substrate activity of 8-Me-H_2Pt were performed at a number of pH values between 7.4 and 5.0 for the three DHFRs in assays containing 50–300 μM 8-Me-H_2Pt, 60 μM NADPH, 1–4 mM DTT, and up to 34 (human), 100 (chicken), or 50 (bovine) nM of enzyme: all assays were performed with blanks.

Data Fitting. The kinetic results, which gave linear double-reciprocal plots, were fitted to the Michaelis–Menten equation by using a microcomputer nonlinear regression program (Duggleby, 1984) to calculate the K_m and V_{max} values.

Results

Pterin Stabilities and Nonenzymic Reactions. Initial spectral checks indicated that 8-Me-Pt was stable in the assay buffers at 30 °C for 30–60 min and in 10 mM DTT for 1–2 h between pH's 5 and 7.4. In the presence of DTT, and also mercaptoethanol, there were significant spectral changes: the extent of these changes depended on the concentration of DTT and mercaptoethanol; the changes were instantaneous and showed no time dependence and were reversible on dilution of the samples.

8-Me-H_2Pt in water at pH 7 and 0 °C showed spectral changes after 2–3 h and significant deterioration under assay conditions, especially at lower pH's: it was found to be stable at 0 °C for several hours in the presence of 10 mM DTT and under initial rate assay conditions (1–4 mM DTT). DTT caused some spectral changes for 8-Me-H_2Pt compared with the values reported (Pfleiderer & Mengel, 1971; Pfleiderer, 1985) for the neutral species: pH 5.0, 236 nm (ϵ = 22 910 M^{-1} cm^{-1}), 282 nm (9330), 330 nm (4370) with ratio of peaks of 2.5:1.0:0.47. The changes consisted of decreases in the extinctions of the two short-wavelength bands and a small increase in the extinction of the broad long λ band: it should be noted that the strong absorbance of DTT at 234 nm (Cleland,1964) is likely to introduce some errors in the extinctions reported below due to variations in blanking. In 1.5 mM DTT the values were as follows: pH 5.0, 235 nm

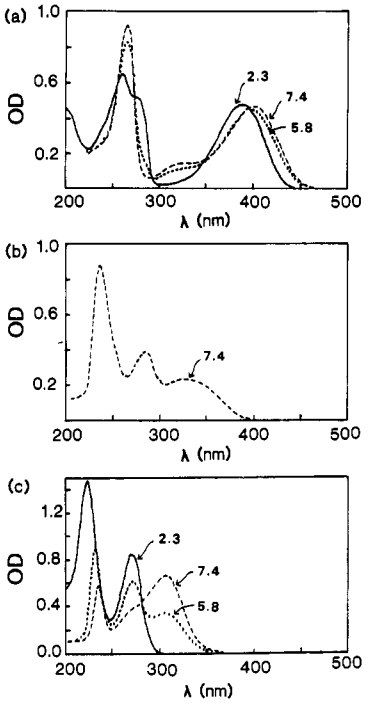

FIGURE 3: pH dependence of pterin spectra. All ϵ expressed as M^{-1} cm^{-1}. (a) 50 μM 8-Me-Pt at pH 2.3 in 4 mM HCl (cation), ϵ_{261} = 13 300, ϵ_{388} = 9800, ratio 1.35:1.0; at pH 5.8 assay buffer, ϵ_{264} = 16 600, ϵ_{397} = 9000, ratio 1.84:1.0; at pH 7.4 assay buffer (neutral), ϵ_{265} = 17 700, ϵ_{400} = 9000, ratio 1.97:1.0. (b) 50 μM 8-Me-H_2Pt in pH 7.4 assay buffer with 10 mM DTT (neutral), ϵ_{236} = 17 600, ϵ_{286} = 7800, ϵ_{330} = 4700, ratio 2.26:1.0:0.60. (c) 55 μM 8-Me-H_4Pt at pH 2.3 in 4 mM HCl (cation), ϵ_{223} = 27 100, ϵ_{271} = 15 400, ratio 1.76:1.0; at pH 5.8 assay buffer with 10 mM DTT, ϵ_{232} = 16 300, ϵ_{271} = 11 300, ϵ_{306} = 6300, ratio 1.44:1.0:0.56; at pH 7.4 assay buffer with 10 mM DTT (neutral), ϵ_{235} = 10 700, [ϵ_{270} = 6900], ϵ_{306} = 12 000, ratio 0.89:[0.58]:1.0.

(23 100), 284.5 nm (9100), 336 nm (4700) with ratio 2.6:1.0:0.52; pH 5.8, 235 nm (22 200), 285 nm (8500), 336.5 nm (4600) with ratio 2.6:1.0:0.54; and pH 7.4, 235.5 nm (22 300), 285 nm (8400), 336.5 nm (4600) with ratio 2.6:1.0:0.55. In 10 mM DTT the values were as follows: pH 5.0, 235.5 nm (18 700), 285.5 nm (7900), 333.5 nm (5000) with ratio 2.4:1.0:0.63; and pH 7.4, 236 nm (17 600), 285.5 nm (7800), 329.5 nm (4700) with ratio 2.3:1.0:0.60 (Figure 3b). 8-Me-H_2Pt in 10 mM DTT showed minor spectral changes (shift of broad long-wavelength band by +5–10 nm) and some increases in absorbance at 340 nm under the complete reaction conditions (<+1, +3%, and +15% per hour at pH's 8.2, 7.4, and 6.6, respectively, and +18% and +6% per 30 min at pH's 5.8 and 5.0, respectively. As there was no appearance of long-wavelength absorbance at ~400 nm, 8-Me-H_2Pt was apparently not decaying to 8-Me-Pt at the test pH's.

Spectral checks of 8-Me-H_4Pt indicated that it is stable for up to 6 h in 4 mM HCl at 0 °C, but deteriorates under assay conditions, especially at higher pH's. Under complete reaction conditions (15 min–2 h), 8-Me-H_4Pt in 10 mM DTT showed some spectral changes, especially at higher pH's: however, the measured ϵ_{340}'s for both the neutral (~1600 M^{-1} cm^{-1}) and cationic (~100 M^{-1} cm^{-1}) species are small. DTT caused some spectral changes compared with the values reported in the absence of DTT: pH 3.0 for the cation, 223 nm (ϵ = 26 920 M^{-1} cm^{-1}), 271 nm (15 490) with peak ratio 1.7:1.0; and pH 9.0 for the neutral form, 227 nm (21 380), [265 nm] (5120), 306 nm (12 590) with ratios 1.7:[0.41]:1.0 (Pfleiderer & Mengel, 1971; Pfleiderer, 1985). In 10 mM DTT the most noticeable effect was the quenching of absorbance of the short λ peak for the neutral form especially at pH 7.4 (see Figure 3c): this effect was negligible at pH 7.4 in 1 mM DTT (ratio of 228- and 307-nm peaks 1.8:1.0).

NADPH decay under the complete reaction conditions was, respectively, pH 7.4, 3%/h; pH 6.6, 7%/h; pH 5.8, 12%/30 min. Investigation of the reaction of 8-Me-Pt and NADPH under the assay conditions showed a concentration-dependent nonenzymic reaction that increased with decreasing pH, but was negligible at pH 7.4 and above; hence, all initial rate enzymic assays below pH 7.4 were performed with a blank. Investigation of the reaction of 8-Me-H_2Pt with NADPH under assay conditions showed negligible nonenzymic reaction.

pH Dependence of Pterin Spectra. The effects of varying pH on the spectra of 8-Me-Pt (pK_a = 5.32; Pfleiderer et al., 1968) and 8-Me-H_4Pt (pK_a = 5.70; Pfleiderer & Mengel, 1971) are shown in Figure 3, together with the spectrum of 8-Me-H_2Pt (pK_a = 3.08; Pfleiderer & Mengel, 1971): ionizations of only the first two compounds are expected to affect ϵ_{340}'s in the test pH range. The results indicate a decrease in the ϵ_{340} for 8-Me-Pt as the pH is lowered [2900 (7.4), 2800 (6.6), 2600 (5.8), and 2300 (5.0) M^{-1} cm^{-1}], no change for 8-Me-H_2Pt (4400 M^{-1} cm^{-1}), and a decrease for 8-Me-H_4Pt [1600 (7.4), 1600 (6.6), 400 (5.8), and 100 (5.0) M^{-1} cm^{-1}]. The ϵ_{340} for 8-Me-H_2Pt and 8-Me-H_4Pt was not affected by the concentration of DTT (~1 or 10 mM). The ϵ_{340} for 8-Me-Pt (50 μM) in the presence of 10 mM DTT was slightly increased [4000 (7.4), 4000 (6.6), 5.8 (3900), and 3200 (5.0) M^{-1} cm^{-1}].

Note that both neutral and cationic 8-Me-Pt possess long-wavelength bands at 400 and 388 nm, respectively, which are characteristic of 8-substituted pterins (Pfleiderer et al., 1968) but not of pterins unsubstituted in the 8-position (Pfleiderer, 1985) or of folate (Poe, 1977). Note, also, that simple pterins unsubstituted in the 8-position (e.g., 6-R-pterins) and folate do not have ionizations in the test pH range (Pfleiderer, 1985; Poe, 1977). The spectra and pK_a's of 8-Me-H_2Pt and 8-Me-H_4Pt are similar to those for other 7,8-dihydro- and 5,6,7,8-tetrahydropterins (Pfleiderer, 1985).

Theoretical Reaction Extinctions. From the above values we can calculate total theoretical reaction extinctions at 340 nm, $\epsilon(R)_{340}$, for a number of possible enzymic reductions. For the one-step reduction of 8-Me-Pt to 8-Me-H_2Pt $\epsilon(R)_{340}$'s of 4700 (5800) at pH 7.4, 4600 (5800) at pH 6.6, 4400 (5700) at pH 5.8, and 4100 (5000) M^{-1} cm^{-1} at pH 5.0 are predicted for 8-Me-Pt in the absence (and presence) of 10 mM DTT, respectively. For the two-step reduction of 8-Me-Pt to 8-Me-H_4Pt the corresponding $\epsilon(R)$'s are 13 700 (14 800) at pH 7.4, 14 600 (15 900) at pH 5.8, and 14 600 (15 500) M^{-1} cm^{-1} at pH 5.0. For the one-step reduction of 8-Me-H_2Pt to 8-Me-H_4Pt, the predicted $\epsilon(R)_{340}$'s are 9600 at pH 7.4, 10 200 at pH 5.8 and 10 500 M^{-1} cm^{-1} at pH 5.0. These may be compared with theoretical $\epsilon(R)_{340}$ values for folate reduction by DHFR (i.e., two-step) of 18 480 M^{-1} cm^{-1} (Hillcoat & Blakley, 1966) and for enzymic H_2folate reduction of 12 800 M^{-1} cm^{-1} (Hillcoat et al., 1967). Experimental determination of the latter quantity yields values of 12 270 ± 320 M^{-1} cm^{-1} (Hillcoat et al., 1967) and 11 800 ± 500 M^{-1} cm^{-1} (Stone & Morrison, 1982). Consideration of the spectra for NADPH and $NADP^+$ and those for the predicted pterin substrate(s) and potential product(s) shown in Figure 3 does not suggest a more suitable assay wavelength for the reaction.

Reaction Stoichiometry and Extinction. Reaction extinction results for the three enzymes at pH's 7.4, 6.6, and 5.8 in the absence and presence of $NADP^+$ recycling are shown in Table I: the $\epsilon(R-)$ value at 1240 s for the chicken DHFR reaction

Table I: Complete Reaction Extinctions for Chicken, Human, and Bovine DHFRs with 8-Me-Pt and NADPH as Substrates, with and without $NADP^+$ Recycling[a]

	pH 7.4			pH 6.6			pH 5.8		
DHFR	time (s)	$\epsilon(R-)_{340}$ (M^{-1} cm^{-1})	$\epsilon(R+)_{340}$ (M^{-1} cm^{-1})	time (s)	$\epsilon(R-)_{340}$ (M^{-1} cm^{-1})	$\epsilon(R+)_{340}$ (M^{-1} cm^{-1})	time (s)	$\epsilon(R-)_{340}$ (M^{-1} cm^{-1})	$\epsilon(R+)_{340}$ (M^{-1} cm^{-1})
chicken	6000	7620 (7200)[b]	1960	6000	7540 (7300)[b]	2720	2000	8620	2500
	1400[c]	6760	900	1000[c]	6900	1120	420[c]	7140	1460
		5860[d] (6180)[b]			5780[d] (6520)[b]			5680[d]	
human	6000	7140 (6960)[b]	580	6000	8620 (8600)[b]	1020	2600	7780 (9160)[b]	1800
	2900[c]	6560	300	1000[c]	7060	540	600[c]	6700	900
		6260[d] (6420)[b]			6520[d] (6940)[b]			5800[d] (7620)[b]	
bovine	6000	7600					2000	7400	
	2900[c]	6940					600[c]	6780	

[a] $\epsilon(R-)$, without recycling; $\epsilon(R+)$, with recycling. [b] Duplicates for $\epsilon(R-)$ only. [c] Approximate end of suggested first reaction step; see Figure 4. [d] $\epsilon(R-) - \epsilon(R+)$.

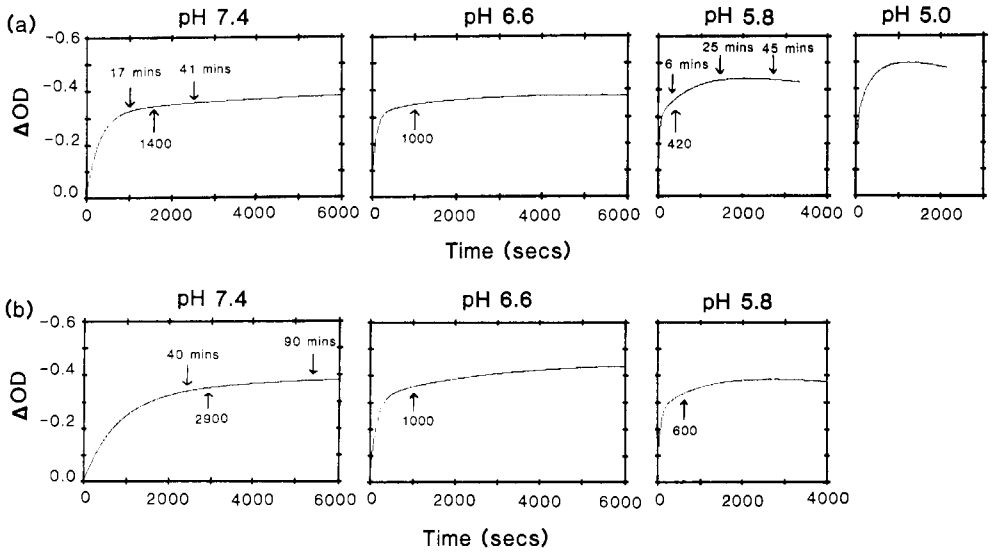

FIGURE 4: Time courses without $NADP^+$ recycling at 340 nm for the enzymic reaction of 8-Me-Pt [50 μM equiv (62.5 μM, 0.8-cm path)] with NADPH [120 μM equiv for pH 7.4, 6.6, and 5.8, 200 μM equiv for pH 5.0]. Marked times refer to those quoted in Table I or Figure 5. (a) Chicken DHFR at pH's 7.4, 6.6, 5.8, and 5.0. (b) Human DHFR at pH's 7.4, 6.6, and 5.8.

at pH 5.0 (Figure 4a) was 9900 M^{-1} cm^{-1}. Sample time traces for chicken and human enzymes at pH's 7.4, 6.6, 5.8, and 5.0 (chicken only) are shown in Figure 4. The use of a split-cell blank in these experiments is essential to prevent nonenzymic reaction in the blank: experiments under the same assay conditions with an ordinary blank gave reaction extinctions of 5300–5700 M^{-1} cm^{-1} at pH 7.4. At the lower pH's, increased decay of assay components, as reported above in *Pterin Stabilities*, and less efficient recycling would be expected to introduce greater errors in the $\epsilon(R-)$ and $\epsilon(R+)$ determinations.

Consideration of the shape of the time traces as a function of pH suggests an initial rapid reaction, increasingly rapid as the pH is lowered, followed by a slower reaction that is more evident at pH's 5.8 and 5.0. The slow approach to reaction completion evident in the time course for human DHFR at pH 7.4 is consistent with the high K_m value discussed below. The approximate positions for the end of the suggested first reaction step are marked in Figure 4 (time in seconds): the results in Table I indicate an average reaction extinction of 6850 ± 150 M^{-1} cm^{-1} for this step and a reaction stoichiometry of approximately 1 [$\epsilon(R-) - \epsilon(R+)$, average of 6000 M^{-1} cm^{-1} compared with a theoretical value of 6200]. It is clear from Table I, however, that there is considerable variation in the $\epsilon(R+)$ values, in fact, a steady increase as the pH is lowered, and that the sign of $\epsilon(R+)$ is not consistent with the small negative value predicted above for a one-step reaction of 8-Me-Pt to 8-Me-H_2Pt (−400 to −500 M^{-1} cm^{-1} for pH 7.4 to 5.8 in 10 mM DTT). The positive $\epsilon(R+)$ values are consistent with, but considerably less than, the value for the two-step reaction of 8-Me-Pt to 8-Me-H_4Pt predicted above to be 2400 and 3300 M^{-1} cm^{-1} at pH's 7.4 and 5.8, respectively; however, the observed stoichiometry is not consistent with a two-step reaction.

Reaction Products for 8-Me-Pt Enzymic Reduction. Results of the spectral density difference experiment for the chicken DHFR enzymic reduction with recycling of $NADP^+$ of 8-Me-Pt at pH's 7.4 and 5.8 are shown in Figure 5: the sample times for the spectra correspond to those marked on the time courses in Figure 4. The spectra should be compared with those shown in Figure 3 and with data given in *Pterin Stabilities*.

At pH 7.4 the initial product spectrum (17 min) shows peaks at 239, 285, and 318 nm with peak ratio of 1.8:1.0:0.53; for

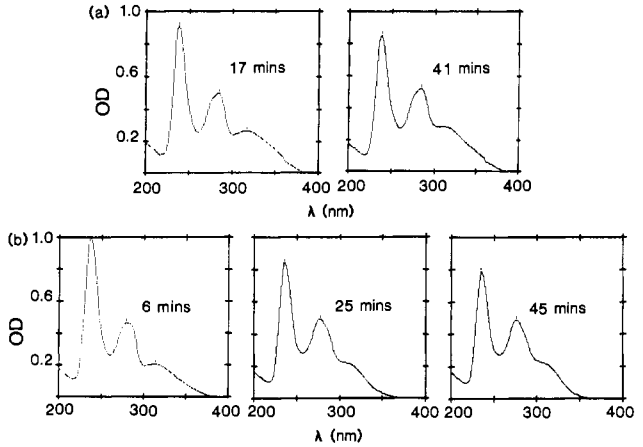

FIGURE 5: Development of product spectra for chicken DHFR enzymic reaction with 8-Me-Pt [50 μM equiv (62.5 μM, 0.8-cm path)] and NADPH (120 μM equiv) (all ϵ expressed as M^{-1} cm^{-1}): (a) at pH 7.4 with 10 mM DTT for 17 min, ϵ_{239} = 18 200, ϵ_{285} = 9900, ϵ_{318} = 5300, ratio 1.8:1.0:0.53, and for 41 min, ϵ_{239} = 17 000, ϵ_{285} = 10 400, ϵ_{314} = 5700, ratio 1.6:1.0:0.55; (b) at pH 5.8 with 10 mM DTT for 6 min, ϵ_{237} = 20 000, ϵ_{281} = 9200, ϵ_{314} = 4100, ratio 2.2:1.0:0.44, for 25 min, ϵ_{236} = 16 800, ϵ_{277} = 9900, [ϵ_{309} = 4600], ratio 1.7:1.0:[0.47], and for 45 min, ϵ_{235} = 15 800, ϵ_{276} = 9800, [ϵ_{306} = 4800], ratio 1.6:1.0:[0.49].

the human enzyme the corresponding experiment yielded a similar spectrum (40 min) with peaks at 238, 285, and 320 nm with extinctions 20 200, 9700, and 4900 M^{-1} cm^{-1} and peak ratio 2.1:1.0:0.50. At a later time (41 min) the long λ band had shifted to 314 nm (310 nm for human at 90 min), and its extinction had increased slightly. In general, the product spectrum resembles that for 8-Me-H_2Pt and not that for 8-Me-Pt or 8-Me-H_4Pt at pH 7.4.

At pH 5.8 the initial product spectrum (6 min) shows peaks at 237, 281, and 314 nm with peak ratio of 2.2:1.0:0.44. As indicated by the traces at 25 and 45 min, the spectrum changes by a shift of the long λ peak to become a shoulder at ~306 nm with a slight increase in extinction and a shift of the middle peak to shorter λ (276 nm). As indicated by the spectra in Figure 3, these spectra are consistent with initial development of the 8-Me-H_2Pt spectrum, which develops an increasing contribution from 8-Me-H_4Pt.

pH Profiles for 8-Me-Pt Enzymic Reaction. pH profiles for chicken and human enzymes for the enzymic reaction of 8-Me-Pt obtained from the initial rate results with 8-Me-Pt as variable substrate are shown in Figure 6; an incomplete profile only for the bovine enzyme was obtained. Reaction extinctions of 4700–4100 M^{-1} cm^{-1} corresponding to the theoretical values calculated above for the one-step reaction between pH's 8.2 and 5.0 in the absence of DTT were used in calculating the specific activities. Substrate inhibition at high concentrations was found for the chicken and bovine reactions: this phenomenon has been found previously for pterin substrates of DHFR (J. F. Morrison, private communication). For pH values over 7 this necessitated choosing an 8-Me-Pt concentration range slightly lower than normal for the V_{max}/K_m experiments. The K_m values for the chicken DHFR reaction varied over the pH range from 30 ± 2 μM at pH 5.0 to 50 ± 3 μM at pH 6.6 and 217 ± 25 μM at pH 7.4. Initial tests with the purer chicken DHFR indicated a slightly lower K_m of 170 ± 20 μM at pH 7.4, with a specific activity of 14 ± 1 or 70% of the maximal rate for H_2folate reduction under these conditions. The K_m values for the bovine DHFR reaction varied from ~30 μM at pH 5.0 to ~200 μM at pH 7.4. The K_m values for the human enzyme pH profile were significantly higher, varying from 130 ± 7 μM at pH

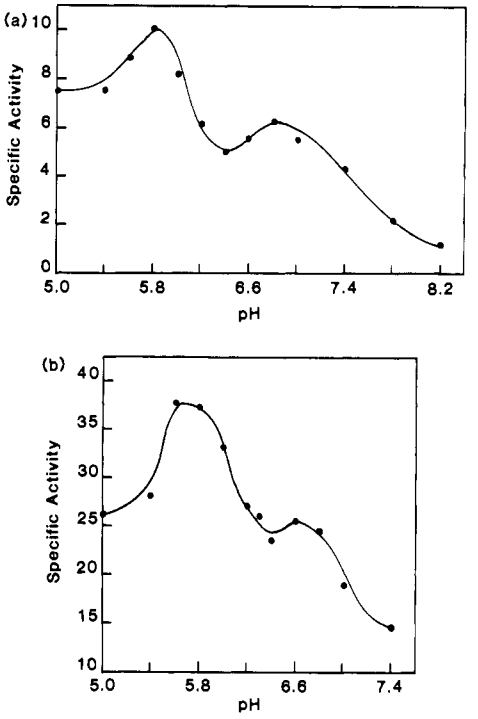

FIGURE 6: pH profiles for enzymic reaction with 8-Me-Pt: specific activity in mol of reaction (mg of protein)$^{-1}$ min^{-1}: (a) chicken DHFR; (b) human DHFR.

5.0 to 153 ± 2 μM at pH 6.0 and 310 ± 40 μM at pH 6.8. At higher pH values the K_m values increased abruptly, resulting in larger errors for the V_{max} values. Variation of K_m with varying pH has previously been reported for 7,8-dihydropterin substrates with chicken liver DHFR (Morrison & Stone, 1986). The initial rate results with NADPH as variable substrate gave pH-independent K_m values of 3.0 ± 0.5 μM for both chicken and human enzymes: this value is similar to those previously reported for dihydrofolate reduction (Freisheim & Matthews, 1984).

Inspection of the pH profiles for the chicken and human enzymes in Figure 6, and for bovine enzyme (not shown), indicates a pH maximum at 5.8–6.0 in all cases, a smaller maximum or shoulder around 6.6–6.8, which is the region in which the K_m values start to increase steeply, and a steep decrease in activity at alkaline pH's. The relative activities with respect to H_2folate reduction at the pH maximum and neutral pH for the three enzymes are, respectively, 130% and 85% (pH 7.4) for chicken, 65% and 40% (pH 7.4) for bovine, and 180% and 90% (pH 7.0) for human.

Substrate Activity for 8-Me-H_2Pt. Under the assay conditions, there was negligible enzymic reaction for the three enzymes with 8-Me-H_2Pt at pH 7.4. For the chicken and human enzymes (bovine not tested) measurable and reproducible enzymic reaction with 8-Me-H_2Pt was observed at lower pH's (5.8 and 5.0) with increased rates at pH 5.0; however, this activity did not obey standard kinetics with respect to concentration of 8-Me-H_2Pt and was difficult to quantify. There appeared to be at least two reasons for this. First, the reaction rate varied with DTT concentration, which under assay conditions was 1–4 mM: because of the limited solubility of 8-Me-H_2Pt, it was not possible to prepare sufficiently concentrated stock solutions to allow the DTT to be diluted out in the assay mixtures. Second, 8-Me-H_2Pt appeared to be also a significant inhibitor of the enzymic reaction: initial tests indicated it inhibits the enzymic reactions of both H_2folate and 8-Me-Pt at the concentrations tested (50–300 μM) (unpublished results).

Discussion

The finding of substrate activity for 8-Me-Pt has a number of implications for the further elucidation of the reaction mechanism of DHFR. If this reaction is a good model for the mechanism of the enzymic reduction of fully oxidized pterin of folate substrates, and if the initial product is indeed the 7,8-dihydro form as the present results suggest, then the finding is significant because no intermediate dihydro product has previously been observed in the DHFR reduction of folate (Gready, 1980) or short-chain analogues such as 6-methylpterin: only the final two-step reduction product in the tetrahydro form is observed. Our current studies with a number of 8-substituted pterins with varying substituents in the 6- and/or 7- and 8-positions have established a range of DHFR enzymic activity for this class of tautomerically activated pterins (unpublished results). Further experiments with identification of product distributions by HPLC and with more stable 7,8-dihydro derivatives are being undertaken to characterize the suggested second-step enzymic reduction of this class of compounds to the tetrahydro forms.

The natural questions that arise from these initial studies are, what is the mode of binding of the pterin ring of the oxidized and dihydro forms of 8-Me-Pt in the DHFR active site and are they significantly different? One interpretation of the finding of negligible or poor substrate activity for 8-Me-H_2Pt over the pH range studied, coupled with the fact that it inhibits the 8-Me-Pt and H_2folate enzymic reductions, is that this compound binds in a suboptimum orientation in the active site or that a proportion of it binds in an unproductive orientation, for example, the upside-down orientation.

In the present discussion it is also useful to draw attention to the similarities in the chemistries of 8-Me-Pt and the quinonoid–dihydropterin substrates of dihydropteridine reductase (Armarego et al., 1984). The N8(*H*) tautomeric form of pterin differs formally from the most stable N8(*H*) tautomer of quinonoid–dihydropterin (Armarego et al., 1984) only in the degree of saturation of the C6–C7 bond, and it also closely resembles it structurally (Gready, 1985b,c). In addition, the preferred protonated forms have similar basicities of ~5.5 (Pfleiderer et al., 1968; Bailey & Ayling, 1983) and theoretical protonation energies and possess structurally similar resonance-delocalized extended guanidinium substructures (Gready, 1985b,c; Williams & Gready, 1989). This N3-protonated form of the quinonoid–dihydropterin has been suggested to be the catalytically active form for the dihydropteridine reductase enzymic reaction (Armarego et al., 1984; Gready, 1985c). Quinonoid–dihydropterins have also been shown to react nonenzymically with NADH or NADPH (Armarego et al., 1984).

Given these similarities, one possibility that should be considered is that the initial products of the 8-Me-Pt reductions are not the 7,8-dihydro compound, or possibly not even the same dihydro product for the enzymic and nonenzymic reductions. If the mechanism of the reduction were to be directly analogous to that for the quinonoid–dihydropterins, then the expected dihydro product would be the 5,8 form not the 7,8 one. 5,8-Dihydrpterins have been observed only transiently during electrochemical reductions and have not been characterized (Kwee & Lund, 1973). As for 8-substituted quinonoid–dihydropterins (Randles & Armarego, 1985; Armarego et al., 1986), 8-methyl-5,8-dihydropterin would be expected to rearrange rapidly (Kwee & Lund, 1973) to the 7,8-dihydro tautomer: our previous theoretical calculations suggested that 5,8-dihydropterin would be ~11 kcal mol^{-1} less stable than 7,8-dihydropterin (Gready, 1985b). On mechanistic grounds it is unlikely that the reduction product would be 8-methylquinonoid–dihydropterin. However, in the present experiments we have not seen a transient initial reduction product in the enzymic density difference spectra, but only an initial product with a spectrum consistent with that of 8-Me-H_2Pt.

Further work is required to map the structure–activity relationships for the oxidized and reduced forms of the 8-substituted pterin class to determine their mode(s) of binding in the DHFR active site and the stereochemistry of the hydride ion transfers. A further interesting question with respect to the enzymic activity of 8-Me-Pt is the nature of the rate-limiting step: for the H_2folate reaction the rate-limiting step has been reported to be H_4folate dissociation, not catalytic events involving the protonation or hydride ion transfer steps, at least at lower pH's (Fierke et al., 1987). The present results for the pH dependence of the 8-Me-Pt enzymic reaction are consistent with a mechanism whereby the protonated form is more active. These further studies are necessary to establish whether the 8-Me-Pt and 8-Me-H_2Pt enzymic reactions are valid models for the normal DHFR reductions and whether the underlying reason for the enzymic activity of 8-Me-Pt is its increased basicity and the nature of its protonated form, as predicted by theory (Gready, 1985a,b).

Acknowledgments

We thank Drs. F. Huennekens, T. Duffy, J. Whiteley, W. Armarego, and J. Morrison for initial help or advice with the syntheses and enzyme assays, Drs. J. Freisheim and J. Morrison for gifts of purified enzymes, and I. Torville and P. Pilling for technical assistance.

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