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Inhibition of Pig Liver Methylenetetrahydrofolate Reductase by Dihydrofolate: Some Mechanistic and Regulatory Implications†

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ABSTRACT: Methylenetetrahydrofolate reductase has been purified 3400-fold from pig liver. The enzyme exhibits ping-pong bi–bi kinetics during catalysis of NADPH–methylenetetrahydrofolate oxidoreduction. Dihydrofolate is an inhibitor of this reaction and is competitive with respect to methylenetetrahydrofolate. The K_i for dihydrofolate is 6 μM. The inhibition of NADPH–methylenetetrahydrofolate reductase activity is greatest when dihydrofolate is present, and similar concentrations of tetrahydrofolate, methyltetrahydrofolate, folic acid, methenyltetrahydrofolate, or 5-methyl-5,6-dihydrofolate are 2.5–10-fold less effective. These observations suggest that the enzyme-catalyzed reduction of methylenetetrahydrofolate may proceed by tautomerization to form an N^5-methyldihydrofolate derivative. However, the formation of N^5-methyl-7,8-dihydrofolate is precluded by our failure to see loss of tritium from C-6 of the pteridine ring of methylenetetrahydrofolate during reduction. Proton magnetic resonance spectroscopy studies on the methyltetrahydrofolate formed during enzymatic reduction of methylenetetrahydrofolate in deuterium oxide demonstrate that two atoms of deuterium are incorporated into the methyl group. We suggest that inhibition of NADPH–methylenetetrahydrofolate oxidoreductase activity by dihydrofolate may be important in the regulation of the flux of methylenetetrahydrofolate in vivo. Both thymidylate synthetase and methylenetetrahydrofolate reductase are found in the cytoplasm, as is dihydrofolate reductase. When thymidylate biosynthesis is occurring, the resulting increase in steady-state levels of dihydropteroylglutamate derivatives may serve to inhibit methylenetetrahydrofolate reductase, thus sparing methylenetetrahydropteroylglutamates for purine and pyrimidine biosynthesis.

Methylenetetrahydrofolate reductase (EC 1.1.99.15) catalyzes the reaction

$$\text{NADPH} + \text{H}^+ + \text{methylene–H}_4\text{PtGlu}_1 \rightarrow \text{NADP}^+ + \text{methyl–H}_4\text{PtGlu}_1 \quad (1)$$

This reaction, which is effectively irreversible in vivo (Katzen & Buchanan, 1965), commits one carbon units to the pathways of *S*-adenosylmethionine-dependent methylation in mammalian systems. The mammalian enzyme was first identified by Donaldson & Keresztesy (1962), who showed that FAD was required for maximal activity. It was purified about 900-fold by Kutzbach & Stokstad (1971). They demonstrated that the enzyme was inhibited by *S*-adenosylmethionine, a typical example of feedback inhibition by the final common product of a reaction pathway.

Methylenetetrahydrofolate reductase is one of five mammalian enzymes which are known to use methylenetetrahydrofolate as substrate. This substrate is essential for the biosynthesis of dTMP, catalyzed by thymidylate synthetase, for purine biosynthesis, which requires the conversion of methylenetetrahydrofolate to methenyl- and N^{10}-formyltetrahydrofolate, and for *S*-adenosylmethionine-dependent methylation reactions. Thus, the potential exists for competition between these pathways for methylenetetrahydrofolate.

The present studies were initiated to look for possible regulatory mechanisms which might exist to assure an equitable distribution of substrate between methylenetetrahydrofolate reductase and other competing pathways.

Experimental Procedure

Purification of Methylenetetrahydrofolate Reductase. The enzyme used in these experiments has been purified from pig liver by a new method. One kilogram of frozen pig liver was thawed and homogenized in a Waring blender in 2 L of 50 mM phosphate, pH 5.9, 0.3 mM in EDTA. The homogenate was adjusted to pH 5.9 and centrifuged at 20000*g* for 30 minutes. The pH of the supernatant was adjusted to pH 7.2, and 71 g of DEAE-52 (previously equilibrated with 50 mM phosphate buffer, pH 7.2, and sucked dry by aspiration on a Büchner funnel) was added. The suspension was stirred for 30 min, filtered on a Büchner funnel, and washed with 1 L of 50 mM phosphate buffer. The DEAE cake was then extracted twice with 500-mL volumes of 0.3 M phosphate buffer, pH 7.2, and the eluates were collected by filtration, combined, and applied directly to a 1.5 × 18 cm column of Affi-Gel Blue (Bio-Rad Laboratories). The column was rinsed with 100 mL of 0.3 M phosphate buffer, pH 7.2, and then eluted with a linear gradient of 0–10 mM NADPH. Fractions with maximal methylenetetrahydrofolate reductase activity were pooled, and the enzyme was collected by precipitation with 80% ammonium sulfate.

Assays of Methylenetetrahydrofolate Reductase. NADPH–menadione oxidoreductase activity was measured

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Table I: Purification of Pig Liver Methylenetetrahydrofolate Reductase

step	methyl-H_4PtGlu_1–menadione oxidoreductase act. (units)	mg of protein	sp act. (units/mg)	yield (%)	rel purification
crude homogenate	90.0	300 000	0.0003	100	1×
pH 5.9 supernatant	79.4	66 200	0.0012	88	4×
eluate from DEAE-52 adsorption	79.1	3 670	0.0216	88	72×
eluate from Affi-Gel Blue column	41.7	41	1.017	46	3390×

by the method of Kutzbach & Stokstad (1971), except that 125 μM NADPH was used in the assays and the NADPH oxidation was followed at 340 nm at 25 °C. The activity was expressed in units of micromoles of NADPH oxidized per minute. Methyltetrahydrofolate–menadione oxidoreductase activity was measured by a modification of the conditions previously described (Kutzbach & Stokstad, 1971). Our incubation mixtures contained 0.42 mM *dl*-[*methyl*-^{14}C]-tetrahydrofolate (2000 dpm/nmol) and 5 μM FAD. Incubations were performed at 37 °C. Otherwise, conditions were as described by Kutzbach & Stokstad (1971). Activity was expressed in units of micromoles of methyltetrahydrofolate oxidized per minute. The NADPH–methylenetetrahydrofolate reductase activity was measured at 25 °C under nitrogen in 50 mM phosphate buffer, pH 6.7, 0.3 mM in EDTA, 2 μM in FAD, and 50 mM in β-mercaptoethanol. The buffer was bubbled with nitrogen in a cuvette and covered with parafilm. NADPH, enzyme, and finally methylenetetrahydrofolate were introduced into the cuvette with Hamilton gas-tight syringes equipped with repeating dispensers. Fresh methylenetetrahydrofolate was prepared each day by dissolving Sigma *dl*-tetrahydrofolate anaerobically in 0.05 M ammonium carbonate buffer, pH 9.2, 50 mM in formaldehyde. Preincubation of the enzyme in a solution containing 2 μM FAD and 100 μM NADPH was necessary in order to obtain maximal activity in assays. Units of activity were expressed as micromoles of NADPH oxidized per minute.

Preparation of Substrates and Inhibitors. Microcrystalline dihydrofolate was prepared by the method of Blakley (1960) and stored as a suspension in 1 mM HCl at 4 °C. Aliquots of the suspension were diluted into 0.1 M Tris-HCl buffer, pH 7.3, containing 1% β-mercaptoethanol just prior to use, and the concentration was determined from the absorbance at 282 nm by using an extinction coefficient of 28 400 M^{-1} cm^{-1} (Blakley, 1969). *dl*-Tetrahydrofolate was prepared enzymatically from dihydrofolate (Mathews & Huennekens, 1960) by using dihydrofolate reductase from trimethoprim-resistant *Escherichia coli* (Poe et al., 1979). The tetrahydrofolate was converted to methylenetetrahydrofolate by addition of 10 mM formaldehyde, and after 10 min the pH was raised to 8.6 by addition of ammonium carbonate. Methylenetetrahydrofolate was purified by chromatography on DEAE-52, and elution was effected by means of a linear gradient of 0 to 1 M NaCl in 0.01 M ammonium carbonate buffer, pH 9.2, 1 mM in formaldehyde. As previously described by Kaufman et al. (1963), chromatography leads to partial separation of the *l* and *d* diastereoisomers of methylenetetrahydrofolate. Methylenetetrahydrofolate concentrations were determined by measurement of the absorbance at 297 nm, using an extinction coefficient of 32 000 M^{-1} cm^{-1} (Blakley, 1969). *dl*-Methenyltetrahydrofolate was prepared by the method of Tatum et al. (1977) and diluted into assay mixtures at the start of each assay from a stock in 0.1 N HCl–0.1 M β-mercaptoethanol. *dl*-Methyldihydrofolate was prepared by oxidation of methyltetrahydrofolate by dichlorophenolindophenol as described by Donaldson & Keresztesy (1962). *l*-[6-^{3}H]-Tetrahydrofolate (20 960 dpm/nmol) was prepared by the method of Pastore & Friedkin (1962) and converted to the *d*-[*methylene*-^{14}C,6-^{3}H]tetrahydrofolate derivative by incubation with [^{14}C]formaldehyde (930 dpm/nmol). The double-labeled methylenetetrahydrofolate was purified by chromatography as described above, except that formaldehyde was omitted from the buffer.

Nuclear Magnetic Resonance Studies. *dl*-Methylenetetrahydrofolate was reduced enzymatically to methyltetrahydrofolate in either H_2O or D_2O, using the assay conditions described above. For reactions in D_2O, all components were prepared in D_2O, the enzyme was precipitated in 80% ammonium sulfate, and the precipitate was redissolved in D_2O. The reaction mixture was incubated until NADPH oxidation had stopped (an excess of NADPH was present), usually ~30 min, and was then applied to a 1.5 × 25 cm DEAE-52 column equilibrated with 0.05 M ammonium acetate buffer, pH 7.0, and eluted with a gradient of 0.05–1.0 M ammonium acetate. The methyltetrahydrofolate isolated was free of contaminants as judged by its ultraviolet spectrum. The fractions containing methyltetrahydrofolate were lyophilized to dryness, and the residue was dissolved in D_2O and relyophilized twice. The residue was then dissolved in 0.1 N NaOD for spectroscopy. A control experiment was performed in which 200 μM *dl*-methyltetrahydrofolate which has previously been purified by chromatography on DEAE-52 was incubated in a reaction mixture containing 25 μM NADPH, 100 μM $NADP^+$, 20 μM *dl*-methylenetetrahydrofolate, and enzyme in buffered D_2O. After incubation for 1 h, the methyltetrahydrofolate was reisolated by chromatography and prepared for spectroscopy as described above. Nuclear magnetic resonance spectra were recorded on a Jeol PFT-100 at 25 °C using a 10-s delay between pulses. Peak positions were determined with reference to the low-field aromatic proton doublet at 7.75 and 7.66 ppm relative to tetramethylsilane. The position of the low-field doublet was determined in a separate experiment in which tetramethylammonium carbonate (3.2 ppm relative to tetramethylsilane) was included as an internal standard. In 0.1 N NaOD, the N^5-methyl protons appear as a sharp singlet at 2.54 ppm (S. J. Benkovic, personal communication). Integrations of the N^5-methyl protons were calibrated by comparison with the four aromatic proton resonances.

Results

Enzyme Purification. The results of the enzyme purification procedure are summarized in Table I. The data shown are for 1 kg of pig liver homogenate. The spectrum of the enzyme after purification as outlined shows elements characteristic of flavin absorption, and addition of NADPH leads to absorbance changes which are characteristic of the bleaching of a flavoprotein. However, there is still significant contamination with colored impurities and the protein is heterogeneous on disc gel electrophoresis. The approximate content of enzyme-bound FAD in the solutions used for assays was determined from the absorbance changes at 450 nm on addition of 50 μM NADPH by using an estimated change in extinction

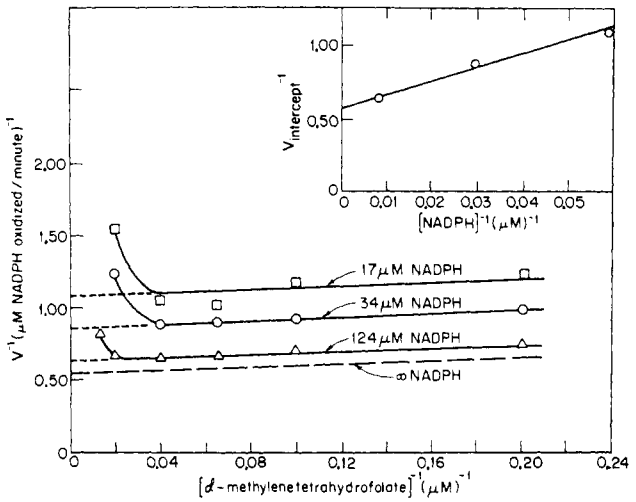

FIGURE 1: Kinetics of the NADPH–methylenetetrahydrofolate reductase reaction. The velocities are all expressed for addition of 10 μL of 260 nM enzyme-bound FAD to a 2.0-mL assay volume, although lower concentrations of enzyme were actually used at low substrate concentrations.

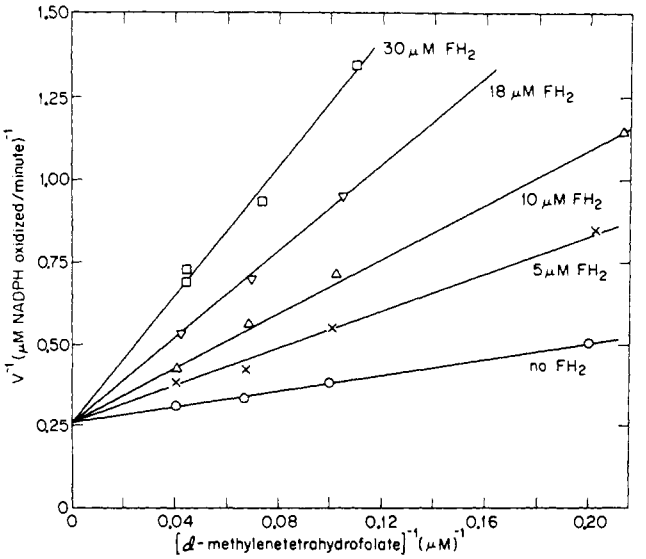

FIGURE 2: Effect of dihydrofolate on the NADPH–methylenetetrahydrofolate oxidoreductase reaction. The velocities are expressed for addition of 25 μL of 260 nM enzyme-bound FAD to a 2.0-mL assay volume. The NADPH concentration was 124 μM. The data have been corrected for nonenzymatic blank rates.

coefficient of 9000 M^{-1} cm^{-1} for flavin bleaching.

In some cases the Affi-Gel Blue column was eluted with 2 M NaCl. In these cases, recoveries of activity were 73% of the activity applied to the column, but the specific activity of the eluted enzyme was lower, ~0.5 unit/mg. Enzyme eluted with 2 M NaCl required preincubation with 100 μM NADPH before maximal activity could be observed in either the methyltetrahydrofolate–menadione or NADPH–methylenetetrahydrofolate oxidoreductase activities.

Kinetics of the NADPH–Methylenetetrahydrofolate Oxidoreductase Reaction. Preliminary studies on the NADPH–methylenetetrahydrofolate oxidoreductase reaction showed that equivalent results were obtained for both K_m and V_{max} with enzymatically prepared, purified *d*-methylenetetrahydrofolate or with *dl*-methylenetetrahydrofolate prepared from Sigma *dl*-tetrahydrofolate without further purification, provided that care was taken to exclude oxygen during preparation of the substrate and provided that the K_m was calculated on the basis of the *d*-methylenetetrahydrofolate concentration. These results are in agreement with the results reported by Kutzbach & Stokstad (1971) and suggest that the *l* diastereoisomer, which is not a substrate, also does not bind to the enzyme.

Figure 1 shows the kinetics of the NADPH–methylenetetrahydrofolate oxidoreductase reaction. The kinetics are consistent with a ping-pong bi–bi mechanism in which the enzyme is alternately reduced by NADPH and oxidized by methylenetetrahydrofolate. The K_m for NADPH is 16 μM, and that for *d*-methylenetetrahydrofolate, which varies somewhat from one preparation of enzyme to another, is ~2–7 μM. On the basis of an estimated flavin concentration of 1.3 nM in the assay mixtures, the turnover number of the enzyme is ~1400/(min mol of FAD) under V_{max} conditions. Marked inhibition of the assays is seen at high concentrations of methylenetetrahydrofolate. This inhibition is not due to the formaldehyde present since excess substrate inhibition is also seen when a substrate stock containing 10 mM rather than 50 mM formaldehyde is used. Under these conditions, virtually all the formaldehyde is present as methylenetetrahydrofolate.

The ratio of V_{max} in the methylenetetrahydrofolate reductase reaction to V_{max} in the NADPH–menadione oxidoreductase reaction was 0.28 for the enzyme preparation used in these experiments. This value is substantially higher than the ratio of 0.06–0.10 previously reported (Kutzbach & Stokstad, 1971). The turnover number for the enzyme in the NADPH–menadione oxidoreductase reaction under V_{max} conditions is ~5000/(min mol of FAD).

The turnover number for the enzyme under the standard conditions for the methyltetrahydrofolate–menadione oxidoreductase reaction is ~2000/(min mol of FAD) measured at 37 °C and ~1000/(min mol of FAD) measured at 25 °C. Comparison of V_{max} for the NADPH–menadione oxidoreductase reaction (5000/min) with those for the NADPH–methylenetetrahydrofolate oxidoreductase (1400/min) and methyltetrahydrofolate–menadione oxidoreductase (1000/min) reactions indicates that reactions of the enzyme with the tetrahydrofolate derivatives must be largely rate limiting in the latter instances.

Although our data do not eliminate the possibility of an ordered bi–bi mechanism, the catalysis of electron transfer from either methyltetrahydrofolate or NADPH to menadione is also compatible with a ping-pong or binary complex mechanism.

Inhibition by Dihydrofolate. In the course of these kinetic studies it was observed that exposure of the assay mixture or the substrate, methylenetetrahydrofolate, to air resulted in marked inhibition of the NADPH–methylenetetrahydrofolate oxidoreductase reaction. The activity could not be restored by addition of more substrate. If methylenetetrahydrofolate was added to an aerobic solution of the assay buffer, rapid oxidation to a spectrum resembling dihydrofolate was observed. These results suggested that dihydrofolate might be an inhibitor of this reaction. Figure 2 shows the effect of dihydrofolate on the methylenetetrahydrofolate reductase reaction. The results indicate that dihydrofolate is competitive with respect to methylenetetrahydrofolate. A replot of the slopes at fixed levels of dihydrofolate vs. the concentration of dihydrofolate is linear and gives a value for the K_i of 6 μM. We have also examined the effect of dihydrofolate on the methylenetetrahydrofolate reductase reaction as NADPH is varied. Dihydrofolate is uncompetitive with respect to NADPH, and the K_i obtained from the intercept replot is 6.5 μM, in good agreement with the slope replot of the data in

Scheme I

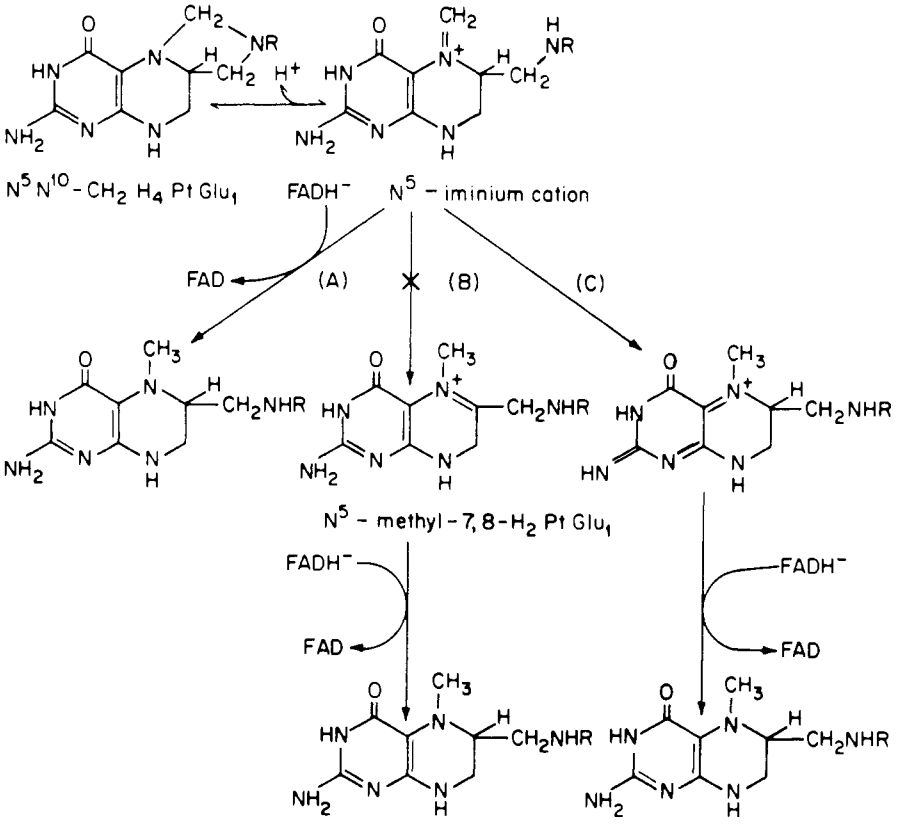

Figure 2. The data are consistent with simple competition of dihydrofolate for the methylenetetrahydrofolate binding site on reduced enzyme. Although inhibition of the enzyme by *S*-adenosylmethionine is known to occur slowly (Kutzbach & Stokstad, 1971), requiring 5 min after addition of inhibitor for maximal effect, the inhibition exerted by dihydrofolate occurs immediately on addition of this compound, and preincubation of the enzyme with dihydrofolate does not increase the level of inhibition seen. The K_i values obtained for dihydrofolate inhibition are the same whether *d*-methylenetetrahydrofolate or *dl*-methylenetetrahydrofolate is used as substrate.

Dihydrofolate is not a substrate for methylenetetrahydrofolate reductase. This was established by incubating 40 μM NADPH and 20 μM dihydrofolate in an anaerobic cuvette and monitoring the mixture spectrophotometrically after addition of enzyme sufficient to reduce methylenetetrahydrofolate at a rate of 0.01 μmol/min. No spectral changes were observed during a 30-min period.

The effect of dihydrofolate on the methyltetrahydrofolate–menadione reductase reaction was also examined at 25 °C. It was found that dihydrofolate was competitive with respect to methyltetrahydrofolate in the presence of saturating menadione (3.5 mM). However, in this case the K_i for dihydrofolate was ~33 μM, suggesting that dihydrofolate binds the oxidized enzyme less tightly than it binds the reduced enzyme.

Figure 3 compares the inhibition exerted by dihydrofolate with the effects of other folate derivatives on the NADPH–methylenetetrahydrofolate oxidoreductase reaction. The concentration of dihydrofolate required for 50% inhibition under these conditions is 2.5–10-fold lower than the comparable concentrations of other folate derivatives. In particular, derivatives at the tetrahydrofolate level of oxidation inhibit more weakly than dihydrofolate. One possible explanation of the inhibition specificity shown in Figure 3 would be that reduction of methylenetetrahydrofolate proceeds through an N^5-methyldihydrofolate intermediate. Two such pathways are shown as pathways B and C in Scheme I. The experiments described below were designed to assess this possibility.

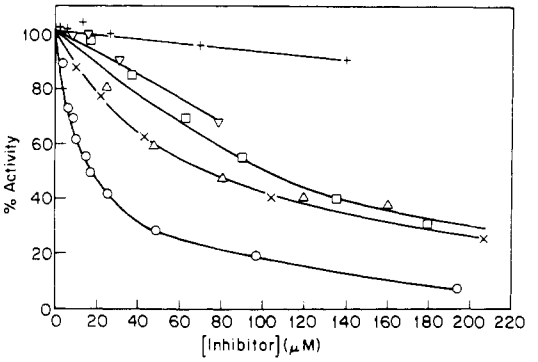

FIGURE 3: Inhibition of the NADPH–methylenetetrahydrofolate oxidoreductase reaction exerted by folate derivatives. Conditions were as in Figure 2, with a *d*-methylenetetrahydrofolate concentration of 15 μM. The data shown have been corrected for blank rates measured in the absence of enzyme. Data points are for inhibition by dihydrofolate (○), *dl*-methyltetrahydrofolate (×), folic acid (△), *dl*-tetrahydrofolate (□), *dl*-methenyltetrahydrofolate (▽), or *dl*-N^5-methyl-5,6-dihydrofolate (+).

Enzymatic Reduction of [6-^{3}H]Methylenetetrahydrofolate. *d*-[*methylene*-^{14}C,6-^{3}H]Tetrahydrofolate was prepared by the method of Pastore & Friedkin (1962) and then purified by chromatography as described under Experimental Procedure. The specific activities of both the tritium label and the ^{14}C label were constant across the peak fractions.

The results of a typical experiment in which the labeled methylenetetrahydrofolate was reduced to methyltetrahydrofolate enzymatically, using NADPH as reductant, and

Table II: Fate of the Label in [6-^{3}H]Methylenetetrahydrofolate during Oxidoreduction[a]

stage of reaction	dpm in formaldehyde ^{14}C	dpm in formaldehyde ^{3}H	^{3}H/^{14}C ratio
before addn of enzyme	18 117	5423	0.30
at completion of redn to methyltetrahydrofolate	7 853	2516	0.32
at completion of reoxidn to methylenetetrahydrofolate	13 760	4507	0.33

[a] 119 nmol of [*methylene*-^{14}C,6-^{3}H]tetrahydrofolate (20 960 dpm of ^{3}H per nmol; 930 dpm of ^{14}C per nmol) was incubated under nitrogen at 25 °C with 189 nmol of NADPH and methylenetetrahydrofolate reductase in 3 mL of 0.05 M phosphate buffer, pH 7.2, containing 0.3 mM EDTA and 2.5 μM FAD. When the reaction was complete, as judged by termination of NADPH oxidation, 0.2 mL of a saturated solution of menadione in 20% methanol was added and the solution was incubated at 37 °C. At the intervals indicated above, 0.5-mL aliquots were withdrawn and the content of labeled formaldehyde was determined by complexation with dimedone and extraction into toluene. In the initial aliquot, the recovery of labeled formaldehyde was 98% of the expected value, based on the ^{14}C label.

then reoxidized to methylenetetrahydrofolate, using menadione as oxidant, are shown in Table II. If reduction were associated with transfer of label from C-6 of the pteridine ring to the methyl group, then reoxidation of the methyltetrahydrofolate to methylenetetrahydrofolate should be associated with the appearance of at least two-thirds of the label initially at C-6 in the formaldehyde derived from methylenetetrahydrofolate. In this experiment, 66 nmol of methyltetrahydrofolate was formed from 119 nmol of labeled methylenetetrahydrofolate, and then 38 nmol of methylenetetrahydrofolate was re-formed by oxidation of the methyltetrahydrofolate. Thus, we might expect at least 89 000 dpm of tritium label in the formaldehyde isolated at the end of the reaction. No evidence of tritium transfer from C-6 of the pteridine ring into the methyl group of methyltetrahydrofolate was obtained. Furthermore, if an aliquot of the methyltetrahydrofolate produced in such an experiment was purified by chromatography on DEAE-52, as described under Experimental Procedure, the methyltetrahydrofolate had a ^{3}H/^{14}C dpm ratio of 22.5, in good agreement with the initial methylenetetrahydrofolate ratio of 23.1. Thus, tritium was not released from C-6 during reduction of methylene to methyltetrahydrofolate.

Incorporation of Solvent Deuterium into Methyltetrahydrofolate. Methylenetetrahydrofolate reductase (40 units) was incubated with 40 μmol of *dl*-methylenetetrahydrofolate and 50 μmol of NADPH in 400 mL of buffered D_2O under the conditions described under Experimental Procedure. The methyltetrahydrofolate was purified by chromatography and the protonic content of the methyl group determined by nuclear magnetic resonance spectroscopy. The results of these experiments are shown in Table III. The data indicate that reduction of methylenetetrahydrofolate in D_2O by the pig liver enzyme results in the incorporation of two deuterium atoms per methyl group.

If [*methylene*-^{3}H]tetrahydrofolate (11 000 dpm/nmol) is reduced by NADPH in the presence of methylenetetrahydrofolate reductase in buffered H_2O, the content of tritium in formaldehyde can be measured by forming a dimedone–formaldehyde complex which is extracted into toluene. The remaining tritium content in the aqueous phase could either be in the methyltetrahydrofolate product (which is retained on columns of the anion-exchange resin AG1-X8) or in the water (which would not be retained on AG1-X8). Forty-five

Table III: Incorporation of Solvent Deuterium into Methyltetrahydrofolate

conditions	protons at 2.54 ppm
dl-methyltetrahydrofolate in 0.1 N NaOD	2.95
dl-methyltetrahydrofolate incubated with enzyme, NADPH, NADP$^+$, and 10% methylenetetrahydrofolate and then purified by chromatography on DEAE-52 in 0.1 N NaOD	2.84
methyltetrahydrofolate produced by enzymatic redn of *d*-methylenetetrahydrofolate and purified by chromatography on DEAE-52 in 0.1 N NaOD (3 expt)[a]	0.97 (1) 0.96 (2) 0.92 (3)

[a] Doubling the delay time (to 20 s) did not affect the integrated value for protons at 2.54 ppm.

nanomoles of methyltetrahydrofolate was formed as estimated by NADPH oxidation during the reaction, and this was associated with the appearance of 511 600 dpm in the methyltetrahydrofolate product. The specific activity of the product methyltetrahydrofolate was 11 120 dpm/nmol, which is the same as that of the initial methylenetetrahydrofolate. Thus, tritium is not released into solvent during reduction of [*methylene*-^{3}H]tetrahydrofolate in buffered H_2O.

Methylenetetrahydrofolate reductase (1.66 units) was also incubated with [*methylene*-^{3}H]tetrahydrofolate (11 000 dpm/nmol) in the absence of NADPH for 2 h. Aliquots were removed at intervals, the formaldehyde was removed by extraction of the dimedone–formaldehyde complex, and the tritium content of the residual aqueous phases was determined. No appearance of tritium in the aqueous phases was seen.

Discussion

The observation that 7,8-dihydrofolate is a better inhibitor of pig liver methylenetetrahydrofolate reductase activity than tetrahydrofolate derivatives suggests the possibility that reduction involves a planar N^5-methyldihydrofolate intermediate. The substrate, methylenetetrahydrofolate, is probably reduced after ring cleavage to form an N^5-iminium cation (Benkovic & Bullard, 1973; Kallen & Jencks, 1966). This could then be tautomerized to form an N^5-methyl derivative at the dihydrofolate level of oxidation, with subsequent reduction of the pteridine ring system. Our experiments eliminate the possibility of formation of N^5-methyl-7,8-dihydrofolate, which would result in the removal of tritium label from the C-6 position of the pteridine ring. However, they do not distinguish between pathways A and C of Scheme I. (Formation of N^5-methyl-5,6-dihydrofolate as an intermediate appears unlikely since this compound is a poor inhibitor of methylenetetrahydrofolate reductase activity and does not itself stimulate NADPH oxidation.)

Kisliuk (1963) had previously examined the source of hydrogen for methionine methyl formation by using a crude extract from *E. coli* which catalyzed the reduction of methylenetetrahydrofolate to methyltetrahydrofolate followed by the transfer of the methyl group to homocysteine. His experiments established that one deuterium atom from the solvent was incorporated into the methionine methyl group and that if deuterium atoms were incorporated into C-6 and -7 of methylenetetrahydrofolate, they were not released into solvent during the reduction and methyl-transfer reactions. Thus, N^5-methyl-7,8-dihydrofolate intermediates were precluded in the *E. coli* system.

We have repeated the classical experiments of Kisliuk (1963) on the pig liver system for several reasons. First, the NADH–methylenetetrahydrofolate oxidoreductase reaction

in *E. coli* is catalyzed by two separable enzymes, a flavin reductase which transfers electrons from NADH to FAD and then a methylenetetrahydrofolate reductase which transfers electrons from reduced flavin to methylenetetrahydrofolate (Katzen & Buchanan, 1965). In the pig liver system these two activities are not separable, although our purest enzyme fractions have specific activities of over 2.0 units/mg, representing a 6700× purification. Secondly, the *E. coli* sequence is required for methionine biosynthesis and the activity of methylenetetrahydrofolate reductase is regulated primarily by methionine, while the pig liver pathway serves only for the regeneration of homocysteine formed in biological methylation reactions and the methylenetetrahydrofolate reductase is regulated by *S*-adenosylmethionine and not by methionine. Thus, the mechanism of reduction catalyzed by methylenetetrahydrofolate reductase in these two systems may not be identical.

Indeed, our results show that two deuterium atoms from solvent are incorporated into the methyl group of methyltetrahydrofolate in the reaction catalyzed by the pig liver enzyme. This could be due either to reversible reduction of the N^5-iminium cation followed by rate-limiting product release (Scheme IA) or to reversible tautomerization of the enzyme-bound iminium cation followed by rate-limiting reduction or product release (Scheme IC). The retention of an average of one hydrogen per methyl group in the product may reflect the relative magnitudes of the rate constants for oxidation of methyltetrahydrofolate vs. product release (Scheme IA) or for tautomerization of the quinonoid intermediate vs. reduction (Scheme IC). In agreement with the ascription of partial retention of hydrogen to a ratio of rate constants is the finding that tritium is fully retained during enzymatic reduction in H_2O within experimental error, presumably reflecting a primary isotope effect on removal of tritium during oxidation (Scheme IA) or tautomerization (Scheme IC) as well as a possible solvent isotope effect on reduction. When substrate level concentrations of enzyme are available, rapid reaction measurements of the rate of oxidation of the enzyme by methylenetetrahydrofolate may distinguish between these two possibilities. Overall reversal of the reaction is excluded as an explanation of our results by the retention of three hydrogens in the N^5-methyl group when methyltetrahydrofolate is incubated in the presence of enzyme and NADPH, $NADP^+$, and methylenetetrahydrofolate. Thus, incorporation of solvent deuterium into the methyl group must precede the dissociation of product methyltetrahydrofolate from the enzyme.

Inhibition of the reduction of methylenetetrahydrofolate by dihydrofolate may also be of physiological importance. The studies of Werkheiser (1961) on the subcellular localization of [2-^{14}C]methotrexate in rat liver cells indicated that dihydrofolate reductase (or at least the species of dihydrofolate reductase which binds methotrexate tightly) is localized in the cytoplasmic fraction. Methylenetetrahydrofolate reductase (Wang et al., 1967; Finklestein et al., 1978), N^5-methyltetrahydrofolate–homocysteine methyltransferase (Panichajakul & Bertino, 1976), and about half of the cellular serine hydroxymethyltransferase (Nakano et al., 1968) are also localized in the cytoplasmic fractions of mammalian tissues. The activity of thymidylate synthetase in adult rat liver cells is too low to permit detection, but following partial hepatectomy, thymidylate synthase activity has been detected in the supernatant of a rat liver homogenate after centrifugation at 30000*g* for 30 min (Maley & Maley, 1960). Subcellular fractionation studies on rat liver after partial hepatectomy indicated that 40% of the thymidylate synthetase activity precipitated with the nuclear fraction, and 50% of the activity was in the cytoplasmic fraction (Brown et al., 1965). Thus, methylenetetrahydrofolate reductase and thymidylate synthetase can be viewed as competing for methylenetetrahydrofolate.

The K_m of dihydrofolate reductase from mammalian tumor cell lines for its dihydropteroylglutamate substrates is generally rather independent of the number of glutamyl residues and lies between 20 and 80 μM (Coward et al., 1974). However, the total (monoglutamate and polyglutamate) concentration of folate and dihydrofolate in such tumor cell lines is below 1.2 μM (Jackson et al., 1976), and the level of dihydropteroylglutamate derivatives has been estimated to be (2–6) $\times 10^{-8}$ M in resting cells (Jackson et al., 1973). Under these conditions, dihydrofolate reductase velocity should be directly proportional to the cellular dihydrofolate concentration. Any increase in the rate at which dihydrofolate is produced (i.e., in the rate of thymidylate biosynthesis) will lend to a new, higher steady-state level of dihydropteroylglutamate derivatives. Our results suggest that elevation of cellular levels of dihydropteroylglutamates will lead to inhibition of methylenetetrahydrofolate reductase. In this way, limiting amounts of methylenetetrahydrofolate could be conserved for DNA biosynthesis. Since the majority of intracellular folate derivatives are present as polyglutamates (Noronha & Aboobaker, 1963; Shin et al., 1972; Houlihan & Scott, 1972), these findings have prompted us to examine the inhibition of methylenetetrahydrofolate reductase by dihydropteroylpolyglutamates. These experiments have been performed in collaboration with Dr. Charles Baugh and demonstrate that dihydropteroylhexaglutamate has a K_i value of 1.2×10^{-8} M for inhibition of the NADPH–methylenetetrahydrofolate oxidoreductase reaction (R. G. Matthews and C. M. Baugh, unpublished experiments). It is clear that an assessment of the quantitative significance of inhibition of methylenetetrahydrofolate reductase by dihydropteroylpolyglutamates will also require a knowledge of the K_m values of the reductase for its methylenetetrahydropteroylpolyglutamate substrates and of the range of their concentrations in cells.

Several laboratories (Chello & Bertino, 1973; Halpern et al., 1974) have shown that the conversion of methylenetetrahydrofolate to methionine is blocked in certain lines of transformed cells in tissue culture. These cell lines show an absolute requirement for methionine which cannot be replaced by homocysteine, vitamin B_{12}, and folic acid. Assays of the levels of the two enzymes required for the conversion of methylenetetrahydrofolate to methionine, methylenetetrahydrofolate reductase and N^5-methyltetrahydrofolate–homocysteine methyltransferase, indicate that both enzymes are present in approximately normal amounts in these cell lines (Kamely et al., 1977; Hoffman & Erbe, 1976) and that uptake of labeled methyltetrahydrofolate by these cells is normal and totally dependent on the presence of homocysteine, the methyl acceptor (Hoffman & Erbe, 1976). Our results suggest that these cell lines might be characterized by elevated steady-state levels of dihydropteroylglutamates, sufficient to inhibit methylenetetrahydrofolate reductase activity.

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reductase from trimethoprim-resistant strains.

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Studies on Prolactin. Selective Reduction of the Disulfide Bonds of the Ovine Hormone†

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ABSTRACT: Methods for selective reduction of the disulfide bonds in ovine prolactin are reported. Cleavage of all three disulfide bonds abolishes biological activity and denatures the hormone. Reduction–carbamidomethylation of one or two of the disulfide bridges does not diminish the biological activities in the pigeon crop-sac and mouse mammary gland bioassays. When compared to the native hormone, monomers of these two partially reduced–carbamidomethylated derivatives also show only modest changes in properties measured by exclusion chromatography, circular dichroism, and immunological cross-reactivities. However, cleavage of cystine-4–11 and cystine-191–199, followed by carbamidomethylation, destroys the biological activity of this derivative in a teleost fish bioassay (*Gillichthys* urinary bladder). In contrast, reduction of cystine-4–11 actually increased the teleost potency of this derivative compared to the intact hormone. Since teleost prolactin appears to lack a homologue to the cystine-4–11 disulfide bond in the amino-terminal loop of the ovine hormone, selective reduction of this bond in ovine prolactin may produce a derivative whose properties more closely resemble the fish hormone.

Mammalian pituitary prolactins and somatotropins are single-chain proteins which possess homologous amino acid residues at 55–60% of their sequence positions (Bewley & Li, 1969; Niall et al., 1971; Bewley et al., 1972). Similarities in the sequences of prolactins and somatotropins indicate a separate evolution of these proteins from a common ancestral peptide.

Despite sequence homology, certain conserved structural features distinguish prolactins from somatotropins (and from their closely allied placental homologues, the chorionic somatomammotropins; Bewley et al., 1972). With regard to primary structure, prolactins possess three disulfide bonds (Figure 1) (Li et al., 1970; Li, 1976). In contrast, mammalian growth hormones have two disulfide bonds (Li & Dixon, 1971;

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