# Mammalian Folate Metabolism. Regulation of Folate Interconversion Enzymes†

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ABSTRACT: Mechanisms of regulation of five of the enzymes central to the folate pathway in bovine liver have been examined. These enzymes, 10-formyltetrahydrofolate synthetase, serine transhydroxymethylase, dihydrofolate reductase, methylenetetrahydrofolate dehydrogenase, and methenyltetrahydrofolate cyclohydrolase were purified and their kinetic constants determined. None of the tested metabolites, associated with the pathway, affected the one-carbon addition reactions catalyzed by the first two enzymes. The other three enzymes, however, were subject to inhibition by folic acid and its reduced natural derivatives. The data suggested that the net activity of any particular branch of the pathway and even the *direction* of metabolite flux are determined to some extent at least by the relative levels of folic acid and its derivatives.

The major metabolic pathways of folic acid and its reduced derivatives have been substantially established in a variety of prokaryotic and eukaryotic organisms (Stokstad and Koch, 1967; Blakley, 1969). In the mammal the major reactions (Figure 1) are seen to constitute a series of interlocking pathways in which: (i) folic acid is reduced to dihydrofolate and then to tetrahydrofolate; (ii) the latter compound is converted to a number of one-carbon carrier derivatives, *viz.* methylenetetrahydrofolate, 10-formyltetrahydrofolate, 5-formyltetrahydrofolate, and formiminotetrahydrofolate, all of which can be converted to methenyltetrahydrofolate; (iii) these derivatives can be either utilized in purine biosynthesis, in the formation of TMP or, after reduction of methylenetetrahydrofolate, in the methylation of homocysteine to methionine. In each case reduced folate is recycled into the intracellular pool. The overall pathway is confined to the cellular cytoplasm although certain enzymes are known to be present within different organelles. The mitochondrion, for instance, has a high content of serine transhydroxymethylase (Fujioka, 1969). It is evident that regulatory mechanisms must be critical in determining the flux of metabolites through this pathway as it contains a number of highly unstable tetrahydrofolate derivatives essential for associated biosynthetic reactions. It has already been shown that a number of the enzymes are subject to feed-back inhibition and also induction and repression in a wide variety of bacterial and some mammalian species (Silber and Mansouri, 1971). This study was concerned with the assessment of various metabolites associated with the folic acid pathway as effectors of a number of the enzymes central to this pathway in bovine liver. The enzymes studied were dihydrofolate (folate) reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3), 10-formyltetrahydrofolate synthetase (formate:tetrahydrofolate ligase (ADP), EC 6.3.4.3), serine transhydroxymethylase (L-serine:tetrahydrofolate 10-hydroxymethyltransferase, EC 2.1.2.1), methylenetetrahydrofolate dehydrogenase (5,10-methylenetetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.5, and methenyltetrahydrofolate cyclohydrolase (5,10-methenyltetrahydrofolate 5-hydrolase (decyclizing), EC 3.5.4.9).

## Experimental Procedure

### *Materials*

The following chemicals were obtained from regular commercial sources: folate, dihydrofolate, L(±)-tetrahydrofolate, formiminoglutamic acid, *N*-formylglutamic acid, and other *N*-formyl amino acids, menadione, and trinitrobenzenesulfonic acid from Sigma Chemical Co; glycine, serine, methionine, and homocysteine thiolactone from Schwarz-Mann; purine and pyrimidine nucleotides, $NADP^+$, and NADPH from P-L Laboratories; L-[$^{14}$C]serine (uniformly labeled), 153 mCi/mmol, [3-$^{14}$C]serine, 48 mCi/mmol, and methyl [5-$^{14}$C]tetrahydrofolate, 61 mCi/mmol, from Amersham; L(±)-5-formyltetrahydrofolate was obtained from Cyanamid-DHA (Australia), courtesy of Dr. Alan Hellestrand, and purified by the method described by Greenberg *et al.* (1965); L(±)-10-formyltetrahydrofolate and L(±)-methenyltetrahydrofolate were synthesized according to previously published procedures (Rowe, 1968); L(±)-methylenetetrahydrofolate was synthesized according to Osborne *et al.* (1960) and L(±)-5-methyltetrahydrofolate according to Keresztesy and Donaldson (1961). This latter derivative was converted from the barium to the sodium salt prior to use. 5-Aminoimidazole-4-carboxamide ribonucleotide was a gift from Dr. J. Gots. The formyl derivative of this compound was prepared according to Flaks *et al.* (1957). α-*N*-Formylglycinamide ribonucleotide was a gift from Dr. J. M. Buchanan. The deformylated derivative was prepared according to Hartman and Buchanan (1958).

### *Methods*

Proteins were measured by the technique of Lowry *et al.* (1951).

*Enzyme Assays.* 1. DIHYDROFOLATE REDUCTASE. The cuvet contained $1.42 \times 10^{-5}$ M dihydrofolate, $1.0 \times 10^{-4}$ M NADPH, 5.0 mM β-mercaptoethanol, and 50 mM potassium phosphate buffer, pH 6.25, in a final volume of 1.0 ml. The assay was commenced by the addition of the enzyme and followed at 340 mμ in a Gilford 2400 or Cary 17 recording spectrophotometer at 25°. Appropriate blanks were run with cruder enzyme fractions to allow for any NADPH oxidase present. This was the standard assay employed but assays were also

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carried out in the presence of 0.6 M KCl in 50 mM phosphate buffer at pH 6.0 and in the presence of 4.0 M urea in 50 mM phosphate buffer, pH 5.75. Under these conditions there was a fivefold activation of the enzyme.

The assay for folate reductase activity was carried out under slightly different conditions. The cuvet contained $1.0 \times 10^{-5}$ M folate, $1.0 \times 10^{-4}$ M NADPH, and 5.0 mM $\beta$-mercaptoethanol in 50 mM sodium acetate buffer, pH 4.0. No activation of folate reductase was achieved with urea or monovalent cations.

Units of enzyme activity were expressed as nanomoles of substrate reduced per minute per milligram of protein based on the extinction coefficients ($\epsilon$ 340 m$\mu$) of $12.3 \times 10^3$ for the dihydrofolate reductase reaction (Hillcoat *et al.*, 1967) and $18.4 \times 10^3$ for the folate reductase reaction (Hillcoat and Blakley, 1966).

2. FORMYLTETRAHYDROFOLATE SYNTHETASE. Two assay systems were employed for this enzyme. Assay 1 was based on that described by Bertino *et al.* (1962), in which the formation of 10-formyltetrahydrofolate is measured by its acid conversion to methenyltetrahydrofolate. Maximal activity was achieved in the presence of 0.1 M KCl with more highly purified enzyme.

Assay 2 was based on the release of phosphate from ATP. This technique was employed for comparison with the first assay and more importantly to enable assessment to be made of the possible effect of various derivatives of tetrahydrofolate on the enzyme. The spectrophotometric assay was insensitive in the presence of added methenyltetrahydrofolate and 5-formyltetrahydrofolate (which are converted to methenyltetrahydrofolate at an acid pH) because of the high blank readings.

Because tetrahydrofolate and its derivatives interfere with the production of the phosphomolybdate complex used in most phosphate estimations, as well as producing an intense blue color of their own, they were removed with Dowex 50 ($H^+$) prior to the phosphate estimation. Methenyltetrahydrofolate has a relatively low affinity for the Dowex resin, and when this derivative (or 5-formyltetrahydrofolate) was used as a potential effector, larger quantities of the resin were required. With very crude preparations there was a relatively high blank reading due to the action of ATPases and, accordingly, formate, not ATP, was deleted from the blank. Sodium fluoride could not be used as an ATPase inhibitor as it was, as might be expected, a potent inhibitor of the formyltetrahydrofolate synthetase.

Routinely, the incubation mixture was identical with that used for assay 1 except that the sodium formate was deleted from the blank. The reaction was terminated by the addition of ice-cold $Cl_3CCOOH$. Dowex 50 ($H^+$) equivalent to one-third of the final assay volume was added, the protein and resin were removed by centrifugation, and the $P_i$ estimated according to Marsh (1959). When large quantities of methenyltetrahydrofolate were present, resin equivalent to one-half the final assay volume was added. This assay was sensitive in the 1.0–10-$\mu$g $P_i$ range and the results correlated extremely well with assay 1.

3. SERINE TRANSHYDROXYMETHYLASE. Two assays were also used for this enzyme for similar reasons as applied to the assays for the formyltetrahydrofolate synthetase. Assay 1 was a spectrophotometric assay based on coupling the $NADP^+$-dependent oxidation of the methylenetetrahydrofolate to methenyltetrahydrofolate by the enzyme methylenetetrahydrofolate dehydrogenase. This assay was an adaptation of that described by Nakano *et al.* (1968). Activity was expressed as nanomoles of methylenetetrahydrofolate produced per minute per milligram of protein based on an extinction coefficient ($\epsilon$) of $7.1 \times 10^3$ (Ramasastri and Blakley, 1964).

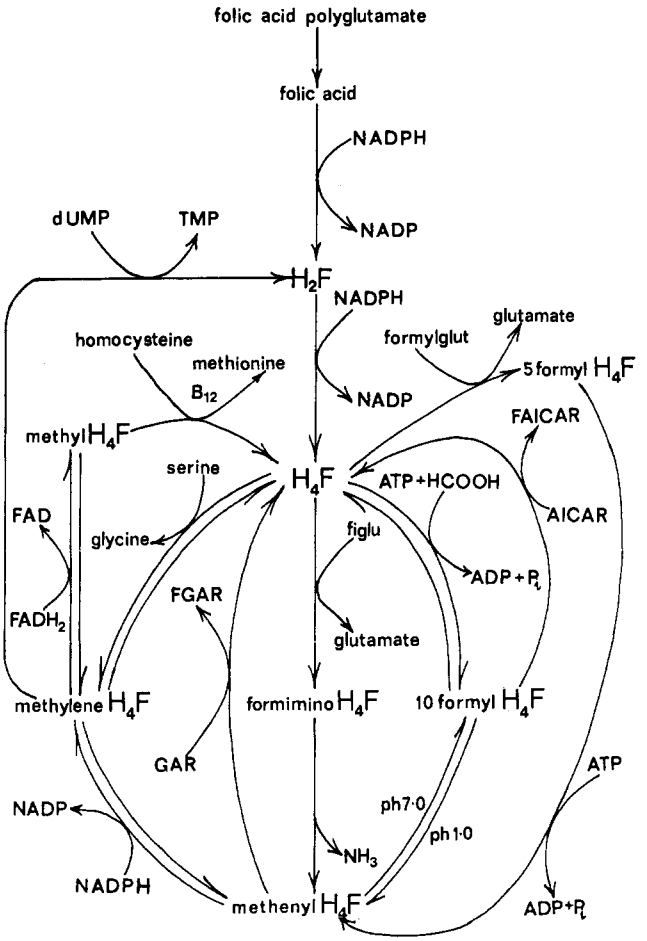


FIGURE 1: Pathways of mammalian folate metabolism based on Stockstad and Koch (1967) and Blakley (1969). Abbreviations used are dihydrofolate ($H_2F$), tetrahydrofolate ($H_4F$), formiminoglutamic acid (figlu), 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), 5-formamidoimidazole-4-carboxamide ribonucleotide (FAICAR), $\beta$-glycinamide ribonucleotide (GAR), and $\alpha$-*N*-formylglycinamide ribonucleotide (FGAR).

This assay, however, possibly provided an overestimate of the true activity of serine transhydroxymethylase present in cruder enzyme preparations because of the presence of the enzyme methenyltetrahydrofolate cyclohydrolase which would rapidly convert the final end product methenyltetrahydrofolate to 10-formyltetrahydrofolate. In addition, it could not be used to assess potential effectors of the enzyme when they might, as proved to be the case, affect the coupled dehydrogenase enzyme. The split coupled assay described by Schirch and Mason (1962) could not be employed, because the bovine enzyme was not inhibited by the potassium bicarbonate used to terminate the reaction with rabbit liver enzyme. Consequently, an alternative assay procedure was developed which measured directly the formation of glycine from serine. For assay 2 the reaction mixture consisted of 0.4 mM L(±)-tetrahydrofolate, 10.0 mM serine (containing 0.09 $\mu$Ci of uniformly labeled [$^{14}$C]serine (154 mCi/mmol)), 12 mM $\beta$-mercaptoethanol, and enzyme in 50 mM Tris-Cl buffer, pH 7.5, to a final volume of 0.5 ml. After incubation for 10 min at 25° the reaction was terminated by placing the tube in a boiling water bath for 30 sec and then into an ice bath. Carrier glycine was

TABLE I: Purification of Serine Transhydroxymethylase.

| Step | Vol (ml) | Total Protein (g) | Total Act.[a] (Units) | Sp Act.[a] (units/mg) | % Yield |
|---|---|---|---|---|---|
| Homogenate supernatant | 950 | 45.6 | 275 | 6 | 100 |
| First $(NH_4)_2SO_4$ fraction | 76 | 4.71 | 110 | 23 | 40 |
| Second $(NH_4)_2SO_4$ fraction | 40 | 2.76 | 97 | 31 | 35 |
| Cation exchange eluate | 59 | 0.33 | 77 | 230 | 28 |
| Final $(NH_4)_2SO_4$ fraction | 9 | 0.18 | 72 | 400 | 27 |

[a] Units expressed as micromoles per minute.

added to a final concentration of 2.0 mM. Following centrifugation a 10-$\mu$l sample of the supernatant solution was spotted on a 0.5-mm cellulose thin layer plate, sprayed with 50 mM sodium borate buffer, pH 9.2, and electrophoresed for 1 hr at 40 V/cm on a Savant high-voltage flat-plate electrophoresis tank. The plates were dried at 110° and sprayed with 2.0 mg % trinitrobenzenesulfonic acid in acetone to visualize the amino acids. Under these conditions glycine moves toward the cathode while serine remains at the origin. The amino acid spots were scraped into vials containing 2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene–toluene:Triton X-100 (2:1) scintillation fluid and counted at 75% efficiency in a Packard Tricarb scintillation spectrometer. Two blanks were run with each assay. One of these blanks omitted tetrahydrofolate while the other was a control assay which contained [3-$^{14}$C]serine instead of the uniform labeled amino acid. In this control assay, the glycine formed will have no radioactive label, the label being incorporated into methylenetetrahydrofolate. This control is required in order to account for any possible cross-contamination of the radioactive glycine by radiolabeled methylenetetrahydrofolate. This was not in fact a problem as the electrophoretic system efficiently resolved the amino acids and the folate derivative. In calculating the conversion of serine to glycine it was assumed that the serine radioactive labeling was, in fact, uniform and that there was therefore a loss of one-third of the radioactivity from serine.

The quantity of enzyme was usually adjusted to allow some 10–15% conversion of serine to glycine. The assay was highly reproducible and was linear with respect to time and the quantity of enzyme up to a point where some 40% conversion had occurred, and correlated well with the spectrophotometric assay.

4. METHYLENETETRAHYDROFOLATE DEHYDROGENASE. This assay was based on that described by Dalal and Gots (1967). Potassium chloride, 0.1 M, was required for the maximal activation of more highly purified enzyme but this was without effect on the crude enzyme preparations.

5. METHENYLTETRAHYDROFOLATE CYCLOHYDROLASE. As might have been anticipated by the pH-dependent nonenzymatic interconversion of the two derivatives involved in this reaction, *viz.* methenyltetrahydrofolate and 10-formyltetrahydrofolate, different assay conditions were necessary to assay the forward (ring cleavage) reaction and the reverse (ring closure) reaction.

The forward reaction was based on the decrease in optical density at 355 m$\mu$ due to the conversion of methenyltetrahydrofolate to 10-formyltetrahydrofolate. The assay mixture consisted of $5.0 \times 10^{-5}$ M L(±)-methenyltetrahydrofolate–0.1 M *N*-tris(hydroxymethyl)methyl-2-aminomethanesulfonic acid buffer, pH 7.3, and enzyme in a final volume of 1.0 ml. The blank cuvet excluded the enzyme and the reaction, commenced by the addition of the substrate, was followed at 355 m$\mu$ in either a Unicam SP 800 or Cary 17 spectrophotometer at 25°.

The reverse reaction was based on the increase in optical density at 355 m$\mu$ due to the formation of methenyltetrahydrofolate. The assay mixture consisted of $5.0 \times 10^{-5}$ M 10-formyltetrahydrofolate–0.1 M 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 5.0, and enzyme in a final volume of 1.0 ml. As before, substrate was added to start the reaction.

Activity was based on $\epsilon$ 355 m$\mu$ of $25 \times 10^3$ for methenyltetrahydrofolate and was expressed as nanomoles produced or removed per minute per milligram of protein.

*Enzyme Purification Procedures.* 1. DIHYDROFOLATE REDUCTASE was purified by standard methods of homogenization, centrifugation, molecular sieve, and anion-exchange chromatography (Rowe and Russel, 1973). This procedure yielded a single protein band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. This enzyme was stable, losing little activity on storage for 12 months at −20° in the presence of $1.0 \times 10^{-6}$ M $NADP^+$.

2. FORMYLTETRAHYDROFOLATE SYNTHETASE was extremely unstable when partially purified, restricting extended purification. A 40-fold purification was achieved by fractionation between 33 and 55% saturation with ammonium sulfate followed by anion exchange chromatography on DEAE-cellulose. This latter step was associated with the loss of some 60–70% of the applied activity, although a twofold increase in specificity activity was achieved. More importantly, the enzyme was separated from other folate pathway enzymes, cyclohydrolase, dihydrofolate reductase, serine transhydroxymethylase, methylenetetrahydrofolate dehydrogenase, and formiminotransferase. At this stage, the enzyme was extremely unstable, all activity being lost within 72 hr at 4°. Stability could not be achieved by the addition of substrates, glycerol, monovalent or divalent cations, EDTA, thiols, or bovine serum albumin. In contrast to this, the ammonium sulfate fraction remained stable for at least 12 months at 4° and there was little loss of activity from the liver itself in 50 mM Tris-Cl buffer containing 50% (v/v) glycerol at −20° over the same time period.

3. SERINE TRANSHYDROXYMETHYLASE. The pruification of this enzyme was modified from the procedure described by Schirch and Gross (1968) for the enzyme from rabbit liver. Table I summarizes the purification of the enzyme from 575 g of fresh beef liver. The tissue was homogenized in 10 mM potassium phosphate buffer, pH 7.3, containing 1.0 mM EDTA. The supernatant solution following centrifugation was heated to 55° prior to ammonium sulfate fractionation between 30 and 55% saturation. This solution was then refractionated between 33 and 55% ammonium sulfate saturation, dialyzed, and chromatographed on a CM-25 Sephadex column. Two enzyme peaks were eluted from this column, a minor one representing some 10% of the enzyme activity which emerged with the bulk of the applied protein and a late peak associated with a yellow protein band which emerged

later. Both enzyme peaks were collected and the enzyme was precipitated by 60% saturation with ammonium sulfate and redissolved in 5.0 mM potassium phosphate buffer containing 1.0 mM EDTA and $1.0 \times 10^{-4}$ M pyridoxal phosphate. Stored at −20° the activity remained stable for at least 12 months. This procedure resulted in an 80–100-fold purification of the second cation-exchange column peak compared with the original homogenate supernatant solution. There was very little, if any, overall purification of the enzyme from the lead peak of the column. On rechromatographing this latter peak on the cation-exchange column, activity was again eluted with the lead peak which contained all of the applied protein. Pyridoxal phosphate was required to maintain enzyme stability following the second ammonium sulfate fractionation.

4. METHYLENETETRAHYDROFOLATE DEHYDROGENASE. Purification of this enzyme was again hindered by instability after partial purification. The activity was stable for months in beef liver stored at −20° in the Tris-glycerol buffer. An overall 40- to 50-fold purification could be achieved by ammonium sulfate fractionation and anion exchange chromatography on DEAE-cellulose. The enzyme was now extremely labile, all activity being lost in about 96 hr. The enzyme was also activated by 0.1 M potassium chloride, which was not the case with the crude enzyme. Attempts to stabilize the enzyme by any of the usual methods such as the addition of substrates, glycerol, or albumin were unsuccessful.

5. METHENYLTETRAHYDROFOLATE CYCLOHYDROLASE. This enzyme was again relatively stable in beef liver stored at −20° in Tris-glycerol buffer but became quite unstable after even mild purification. Purification was carried out at 4° by a modfication of the method described by Lombrozo and Greenberg (1967) (Table II). Following ammonium sulfate fractionation the enzyme was stable at −20° virtually indefinitely but all activity was lost within 48 hr of elution from the anion exchange column.

TABLE II: Purification of Methenyltetrahydrofolate Cyclohydrolase.

| Step | Vol (ml) | Total Protein (g) | Total Act.[a] (Units) | Sp Act.[b] (Units/mg) | % Yield |
|---|---|---|---|---|---|
| Homogenate supernatant | 280 | 19.6 | 814.8 | 42 | 100 |
| Protamine sulfate | 146 | 6.57 | 639.5 | 97 | 78.5 |
| First $(NH_4)_2SO_4$ fraction | 40 | 3.12 | 793.6 | 496 | 97.4 |
| Second $(NH_4)_2SO_4$ fraction | 33.5 | 1.71 | 584.2 | 521 | 71.5 |
| DEAE eluate | 50 | 0.085 | 137 | 1720 | 16.8 |

[a] Units expressed as micromoles/minute. [b] Units expressed as nanomoles/minute.

## Results

*Enzyme Properties.* 1. DIHYDROFOLATE REDUCTASE. This highly purified enzyme exhibited two pH optima, at pH 4.25 (50 mM sodium acetate buffer) and at pH 6.25 (50 mM potassium phosphate buffer) for the NADPH-dependent reduction of dihydrofolate. The maximum velocity was approximately 70% higher at the lower pH optimum. There was a single optimum at pH 4.0 for the NADPH-dependent reduction of folate to dihydrofolate. NADH could not replace NADPH in either reaction. The turnover numbers were relatively low for this enzyme, being 21.8 for the reduction of dihydrofolate at 6.25 and 4.84 for the reduction of folate. In the presence of 0.6 M potassium chloride, the pH–activity profile for the reduction of dihydrofolate was altered to a single peak pattern with an optimum at pH 6.0. There was additionally a fivefold activation of the enzyme. There was a similar response to the addition of 4.0 M urea except that the pH optimum was shifted to pH 5.5.

The $K_m$ values for dihydrofolate ($6.0 \times 10^{-6}$ M) and NADPH ($1.5 \times 10^{-5}$ M) were similar at both pH optima and were little altered by urea or salt activation.

2. FORMYLTETRAHYDROFOLATE SYNTHETASE displayed a relatively broad pH activity profile between pH 4.25 and 8.5, with a maximun at pH 7.5. The $K_m$ values were determined at 37° in the presence of 0.1 M potassium chloride which was required for maximal activity. Under these conditions $K_m$ for formate was 2.0 mM, $K_m$ for ATP was $2.3 \times 10^{-4}$ M, and $K_m$ for L(±)-tetrahydrofolate was 1.0 mM. There was an absolute requirement for divalent cations, magnesium being the most effective.

3. SERINE TRANSHYDROXYMETHYLASE. This enzyme had a mol wt of 180,000 by the criteria of sucrose gradient centrifugation and Sephadex G-150 chromatography, a value which approximates the figure obtained by Fujioka (1969) for rabbit liver enzyme rather than the figure of 331,000 obtained by Schirch and Mason (1963). There was a broad pH optimum, maximal at pH 7.5, the activity profile again closely resembling that described by Nakano *et al.* (1968) for the rat liver cytosol enzyme. Pyridoxal phosphate was required for enzyme stabilization following the second precipitation with ammonium sulfate during the course of purification. The enzyme was strongly inhibited by L-canaline (a structural analog of L-ornithine), a potent inhibitor of pyridoxal-dependent enzymes (Rahiala *et al.*, 1965). The $K_m$ for serine was $5.0 \times 10^{-4}$ M and for L(±)-tetrahydrofolate it was $7.0 \times 10^{-5}$ M. The molecular weight determination and the kinetic constants were identical for both species of enzyme eluted from the cation exchange column.

4. METHYLENETETRAHYDROFOLATE DEHYDROGENASE exhibited a pH–activity profile very similar to that described for the calf thymus enzyme by Yeh and Greenberg (1965) with an optimum at pH 7.0. The partially purified enzyme, eluted from the anion exchange column, required 0.1 M potassium chloride for maximal activity, but this was not required for the cruder more stable enzyme. It was inhibited strongly by divalent cations such as $Ca^{2+}$ and $Ba^{2+}$. $K_m$ values were $1.8 \times 10^{-4}$ M for L(±)-methylenetetrahydrofolate and $2.0 \times 10^{-5}$ M for $NADP^+$, closely resembling those for calf thymus (Yeh and Greenberg, 1965) and for yeast enzyme (Ramasastri and Blakley, 1962). $NAD^+$ could not be utilized by this enzyme.

5. METHENYLTETRAHYDROFOLATE CYCLOHYDROLASE. Chromatography of this enzyme (from step 5 of the purification procedure), on Sephadex G-150 in 50 mM potassium phosphate buffer, pH 6.8, containing 10 mM β-mercaptoethanol and 25% glycerol, revealed the presence of two species of enzyme, one corresponding to a mol wt of 60,000 and the other to a mol wt of 30,000. Both species exhibited forward (ring cleavage) and reverse (ring closure) reactions and were sensitive to the various effectors tested (*vide infra*). The lower molecular weight entity was, however, far more labile, losing

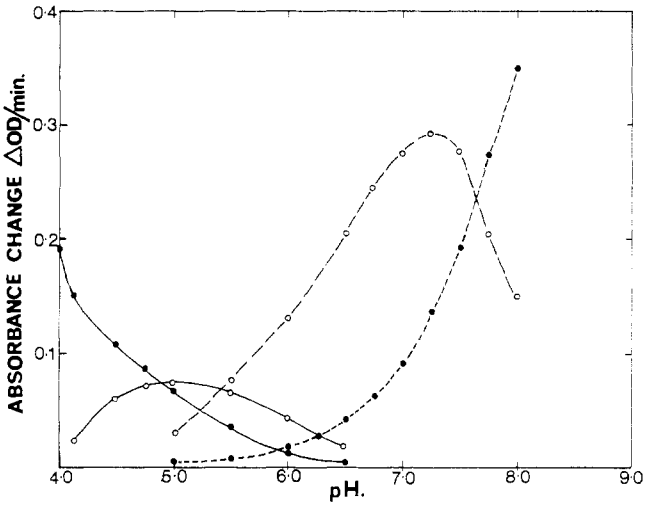


FIGURE 2: Plots of the rates of interconversion of 10-formyltetrahydrofolate and methenyltetrahydrofolate by nonenzymatic and enzymatic mechanisms as a function of pH. The following buffers were used: 50 mM sodium acetate (pH 4.0–5.0), 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (pH 5.0–6.75), 50 mM lithium maleate (pH 6.0–7.0), and 0.1 M *N*-tris(hydroxymethyl)methyl-2-aminomethanesulfonic acid (pH 7.0–8.0). Substrate concentrations were those used for the standard assays. The forward nonenzymatic (●- - ●) and enzymatic (O- -O) reactions were based on the decrease in optical density at 355 mμ. The reverse nonenzymatic (●—●) and enzymatic (O—O) reactions were based on the increase in optical density at 355 mμ.

all activity within 24–48 hr at −15° while the high molecular weight entity retained its activity for several weeks under the same conditions. The different pH optima for the forward and reverse reactions were predictable on the basis of the pH dependency on the equivalent nonenzymatic reactions (Figure 2). The more rapid forward reaction had a pH optimum at 7.3 with a rapid drop in activity above this. The reaction was still quite significant as low as pH 5.0. The slower reverse reaction had an optimum at pH 5.0 with a range from 4.0 to 7.0. A crossover point was seen at pH 5.5. The buffer used in the assays was critical in terms of yielding optimal activity values. This has been demonstrated repeatedly for the nonenzymatic reactions (Robinson and Jencks, 1967). $K_m$ values were $4.0 \times 10^{-5}$ M for L(±)-methenyltetrahydrofolate and $1.5 \times 10^{-5}$ M for L(±)-10-formyltetrahydrofolate.

*Effector Studies.* From a functional point of view the enzymes can be considered as forming two groups. Firstly, there were those catalyzing one-carbon addition reactions, namely serine transhydroxymethylase and 10-formyltetrahydrofolate synthetase, and secondly those catalyzing the interconversion reactions, dihydrofolate reductase, methylenetetrahydrofolate dehydrogenase, and methenyltetrahydrofolate cyclohydrolase.

Each of the partially purified enzymes was tested against a range of metabolites associated with the folate pathway which included homocysteine, methionine, glycine, serine, formiminoglutamic acid, formylglutamic acid, *S*-adenosylmethionine, formate, dUMP, TMP, $NAD^+$, $NADP^+$, deformylated and α-*N*-formylglycinamide ribonucleotide, 5-aminoimidazole-4-carboxamide ribonucleotide and its formyl derivative, AMP, ADP, ATP, GMP, GDP, GTP, IMP, IDP, ITP, CTP, UTP, folate, dihydrofolate, tetrahydrofolate, and methenyl, 5-methyl, 5-formyl, and 10-formyl derivatives of tetrahydrofolate. None of the *nonfolate* compounds had any effect on any of the reactions considered unless they served as either the substrate or the product of a particular reaction. The same results with regard to folic acid and its reduced derivatives were achieved in respect to the reactions catalyzed by serine transhydroxymethylase and formyltetrahydrofolate synthetase in the one-carbon addition reactions. The competitive inhibition of 10-formyltetrahydrofolate synthetase by ADP with respect to ATP has been shown previously with other species (Bertino *et al.*, 1962; Whiteley *et al.*, 1959), and this was confirmed with the bovine enzyme, the $K_i$ for ADP being $1.0 \times 10^{-4}$ M.

TABLE III: Inhibition of Dihydrofolate Reductase by Folate Derivatives.[a]

| Inhibitor | $K_i$ (M) | |
|---|---|---|
| | No Urea | With Urea |
| Methylenetetrahydrofolate | $1.2 \times 10^{-5}$ | $3.4 \times 10^{-5}$ |
| Methenyltetrahydrofolate | $2.2 \times 10^{-5}$ | $4.4 \times 10^{-5}$ |
| 10-Formyltetrahydrofolate | $2.4 \times 10^{-5}$ | $4.75 \times 10^{-5}$ |
| 5-Formyltetrahydrofolate | $12.0 \times 10^{-5}$ | $25 \times 10^{-5}$ |

[a] Assays were performed at pH 6.25 (no urea) and at pH 5.5 (with 4.0 M urea). $K_i$ values for methenyltetrahydrofolate were determined at pH 5.0 in 50 mM sodium acetate buffer.

The other three enzymes were sensitive to inhibition by folate and its reduced derivatives.

Dihydrofolate reductase was tested both in the native and the urea-activated states under the appropriate optimum assay conditions. With respect to the "native" enzyme equivalent inhibition was seen at both pH optima. In view of the nonenzymatic pH-mediated interconversion of methenyltetrahydrofolate and 10-formyltetrahydrofolate, the critical assessment of the effect of these derivatives was made at different appropriate pH values. The enzyme was sensitive to inhibition by methylenetetrahydrofolate, methenyltetrahydrofolate, 10-formyltetrahydrofolate and 5-formyltetrahydrofolate in decreasing order of effectiveness, while 5-methyltetrahydrofolate had no effect whatsoever. Inhibition was competitive with respect to dihydrofolate and Table III lists the various $K_i$ values for both the native and the urea-activated enzyme. It can be seen that the order of effectiveness of inhibitors is the same in the presence of 4.0 M urea but that the sensitivity is decreased by a factor of two. Similar values were obtained with the potassium chloride activated enzyme. The inhibitors were additive in their effect, and showed no evidence of cooperativity.

Methylenetetrahydrofolate dehydrogenase was strongly inhibited by folate, dihydrofolate, tetrahydrofolate, 5-methyltetrahydrofolate, 5-formyltetrahydrofolate and 10-formyltetrahydrofolate. Inhibition was noncompetitive with respect to methylenetetrahydrofolate as is illustrated in Figure 3 for the inhibition by tetrahydrofolate. The $K_i$ for each of these inhibitors is shown in Table IV. Again no cooperativity was demonstrated, the inhibitors being additive in their effect.

As shown in Table V, folate, dihydrofolate, tetrahydrofolate, 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, and methylenetetrahydrofolate were all inhibitors of methenyltetrahydrofolate cyclohydrolase when assayed in the forward direction. It would appear, however, that methylenetetrahydrofolate was the most potent inhibitor, while folate and 5-methyltetrahydrofolate were relatively ineffective in-

TABLE IV: Inhibition of Methylenetetrahydrofolate Dehydrogenase by Folate and Its Derivatives.[a]

| Inhibitor | $K_i$ (M) |
|---|---|
| Folate | $9.0 \times 10^{-5}$ |
| Dihydrofolate | $2.4 \times 10^{-4}$ |
| Tetrahydrofolate | $3.6 \times 10^{-4}$ |
| 5-Methyltetrahydrofolate | $2.0 \times 10^{-4}$ |
| 5-Formyltetrahydrofolate | $2.0 \times 10^{-4}$ |
| 10-Formyltetrahydrofolate | $3.0 \times 10^{-4}$ |

[a] Assays were performed at 25° and at the pH optimum of 7.0 with 0.1 M potassium chloride to achieve maximum activation.

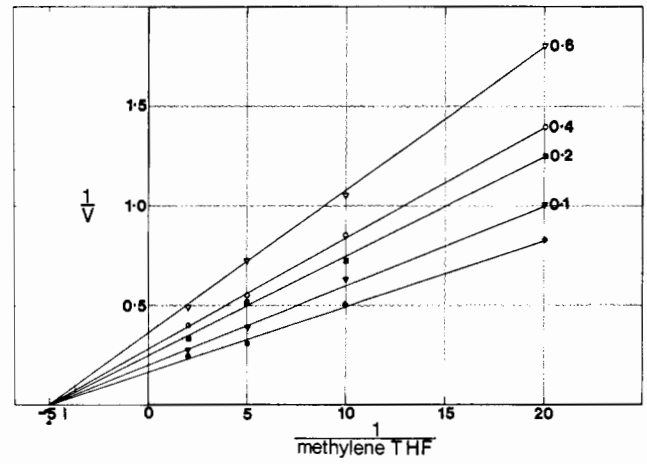


FIGURE 3: Lineweaver–Burk plot of the kinetics of inhibition of methylenetetrahydrofolate dehydrogenase by tetrahydrofolate. The numbers on the inhibitor plots refer to the concentration of the inhibitor in mmol/l. and methylenetetrahydrofolate concentration was also millimolar.

hibitors. The various inhibitors were again additive in their effect. Equivalent competitive inhibition was demonstrated in the reverse direction.

## Discussion

Previous studies have demonstrated that in a variety of bacterial and mammalian cells, different folate enzymes are regulated by folate derivatives and other key intermediates throughout the pathway. This work has recently been reviewed by Silber and Mansouri (1971) who also discussed the broader areas of repression and induction of folate-dependent enzyme activity in a wide range of cell types. The data available on the mammalian enzymes are still limited and no overall pattern has been established for the regulation of a series of these interrelated enzymes in any one tissue.

Schirch and Ropp (1967) have demonstrated that serine transhydroxymethylase from rabbit liver was competitively inhibited by 5-methyltetrahydrofolate and by 5-formyltetrahydrofolate, while 10-formyltetrahydrofolate and *p*-aminobenzoyl glutamate had no effect.

Methylenetetrahydrofolate reductase (5-methyltetrahydrofolate:$NAD^+$ oxidoreductase, EC 1.1.1.68) from both rat and pig liver is subject to feedback inhibition by *S*-adenosylmethionine, an effect which is partially reversed by *S*-adenosylhomocysteine (Kutzbach and Stokstad, 1971). This control has a number of features which would suggest that it is allosteric in type. Methionine, an effective repressor of the *Escherichia coli* enzyme (Katzen and Buchanan, 1965) does not have any effect on the level of activity of the rat liver enzyme (Kutzbach *et al.*, 1967).

An impressive body of data has been compiled on the enzyme homocysteinemethionine–5-methyltetrahydrofolate methyltransferase. Methionine represses the enzyme in both chicken liver (Dickerman *et al.*, 1964) and rat liver (Kutzbach *et al.*, 1967) while cyanocobalamin apparently induces the enzyme in tissue culture (Mangum *et al.*, 1969). The terms induction and repression, however, are used somewhat loosely as other factors such as stabilization may well be involved.

Tabor and Wyngarden (1959) have demonstrated that formiminotetrahydrofolate cyclodeaminase from hog liver is inhibited by tetrahydrofolate.

As might be anticipated, 10-formyltetrahydrofolate synthetase is subject to competitive inhibition of ATP by ADP. This has been demonstrated with the human red cell enzyme (Bertino *et al.*, 1962) as well as with the enzyme from bacterial sources (Whiteley *et al.*, 1959).

The present study was concerned with a systematic examination of five of the folate pathway enzymes in beef liver cytosol. It would appear that the enzymes responsible for the reduction of folate to tetrahydrofolate *via* dihydrofolate and for the interconversion of methylenetetrahydrofolate, methenyltetrahydrofolate, and 10-formyltetrahydrofolate are susceptible to feedback inhibition by folate and its reduced natural derivatives. Neither of the enzymes responsible for the formation of the one-carbon derivatives of tetrahydrofolate, serine transhydroxymethylase and 10-formyltetrahydrofolate synthetase, are subject to this type of regulation. Preliminary studies in our laboratory would suggest that the role of regulators in the formiminotransferase–cyclodeaminase branch of the pathway follows the same pattern.

Dihydrofolate reductase is an enzyme with a broad substrate specificity (Blakley, 1969), as is borne out by its ability to reduce a wide variety of folate derivatives including the γ-glutamyl peptide forms and by its sensitivity to inhibition by a wide variety of pteridine structural analogs. In this light it is of interest to note the failure of 5-methyltetrahydrofolate to inhibit the reduction of either folate or dihydrofolate. This is not unexpected when it is considered that approximately 70% of mammalian liver cell folate is in the form of 5-methyltetrahydrofolate and its polyglutamyl peptides (Noronha and

TABLE V: Inhibition of Methenyltetrahydrofolate Cyclohydrolase by Folate and Its Derivatives.[a]

| Inhibitor | $K_i$ (M) | |
|---|---|---|
| | Forward | Reverse |
| Folate | $3.0 \times 10^{-5}$ | $2.9 \times 10^{-5}$ |
| Dihydrofolate | $8.8 \times 10^{-6}$ | $7.5 \times 10^{-6}$ |
| Tetrahydrofolate | $7.0 \times 10^{-6}$ | $7.0 \times 10^{-6}$ |
| 5-Methyltetrahydrofolate | $3.0 \times 10^{-5}$ | $4.2 \times 10^{-5}$ |
| 5-Formyltetrahydrofolate | $8.6 \times 10^{-6}$ | $6.2 \times 10^{-6}$ |
| Methylenetetrahydrofolate | $2.12 \times 10^{-6}$ | $2.3 \times 10^{-6}$ |

[a] Inhibition constants were determined for both the forward (ring cleavage) and reverse (ring closure) reactions.

Silverman, 1962). Inhibition by this derivative could lead to a situation where there would be a limited input of reduced folate into the pathway, a situation which could only be relieved by recycling of 5-methyltetrahydrofolate *via* the enzyme homocysteine methyltransferase. The feedback inhibition of the reductase by the other tetrahydrofolate derivatives would be an effective means of regulating their own biosynthesis at an early step in the pathway. As evidenced by low $K_i$ values for methylene-, methenyl-, and 10-formyltetrahydrofolate, the enzyme is relatively sensitive to these derivatives. It is considerably less sensitive to 5-formyltetrahydrofolate which may correlate with the observation that this compound and its polyglutamate derivatives are reported to be the second most prevalent folate species in mammalian liver (Noronha and Silverman, 1962).

The $K_i$ values for inhibitors of methylenetetrahydrofolate dehydrogenase indicate that this enzyme is inhibited by concentrations of effector considerably greater than those producing equivalent effects on dihydrofolate reductase. The $K_i$ values, however, fall into the same range as the $K_m$ for methylenetetrahydrofolate. Ramasastri and Blakley (1964) could not demonstrate any inhibition of the bakers yeast enzyme by dihydrofolate, 10-formyltetrahydrofolate, or 5-methyltetrahydrofolate. Baker *et al.* (1964), however, described the inhibition of the avian liver enzyme by a wide variety of folate analogs suggesting that species difference, associated with evolutionary development, may account for the effects we have demonstrated. In contrast to the findings of Dalal and Gots (1966) with the enzyme from *S. typhimurium*, purine nucleotides did not inhibit the bovine enzyme.

Methenyltetrahydrofolate cyclohydrolase is extremely sensitive to the folate effectors tested. The *in vivo* function of this enzyme is difficult to evaluate in the light of *in vitro* studies which would suggest that it merely acts as an "overdrive" for the pH-mediated nonenzymatic interconversion of the methenyl- and 10-formyltetrahydrofolate derivatives. It is accordingly even more difficult to evaluate the overall role of inhibitors with regard to this particular reaction. That the enzyme is of signal importance in the mammal has been emphasized by the description of the severe biological sequelae resulting from a partial deficiency of the enzyme in a human mutation (Arakawa, 1970).

Another major unexplored question relates to the form in which most folate derivatives exist within the mammalian cell, that is, as polyglutamyl peptides. Many of the folate-dependent enzymes, admittedly largely of bacterial origin, can utilize the polyglutamate derivatives as substrates although it is not clear what limitations are created by variability in the size of the peptide. As a corollary to this it is not known to what extent polyglutamate derivatives might compete for the active site with oligoglutamate derivatives, thus perhaps creating another potential regulatory mechanism (Baugh and Krumdiek, 1971).

Another critical regulatory mechanism for folate-dependent enzymes is related to their susceptibility to monovalent cation activation (Suelter, 1970). No clear-cut mechanistic explanation for this phenomenon is as yet available although there is a wide variation in the different types of reaction catalyzed.

It is evident that, as far as the bovine liver enzymes we have studied are concerned, the net activity of any one enzyme and hence the resultant flux of metabolite through any particular branch of the folate pathway are the end results of a wide variety of interreacting factors. Not the least of these, it would appear, is the regulation by the various folate derivatives throughout the pathway.

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# $^{18}O$ Studies on the Oxidative and Nonoxidative Pentose Phosphate Pathways in Wild-Type and Mutant *Escherichia coli* Cells†

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ABSTRACT: The experiments described were carried out in order to determine the role of the oxidative and the nonoxidative pathways for the synthesis of pentose phosphate in *Escherichia coli*. The experimental approach involved growth of the organism on [1-$^{18}O$]glucose or [2-$^{18}O$]fructose and isolation of the ribonucleosides. Mass spectrometry of the nucleosides gave the $^{18}O$ content of each of the oxygen atoms in the ribose moiety. Incubations were carried out with wild-type *E. coli* K10 which utilizes both pathways, a mutant lacking transketolase (*tkt*$^-$) which utilizes the oxidative pathway exclusively, and a mutant lacking glucose-6-phosphate dehydrogenase (*zwf*$^-$) which utilizes the nonoxidative pathway exclusively. Growth of the *tkt*$^-$ mutant on [1-$^{18}O$]-glucose yielded unlabeled pentose phosphate showing that there was no equilibration between the C-1 oxygen and C-6 oxygen of glucose *via* the trioses. Growth of the *zwf*$^-$ mutant on [1-$^{18}O$]glucose yielded pentose phosphate containing 48% of the $^{18}O$ in the 5′-oxygen atom, suggesting that the nonoxidative pathway in this organism consists mainly of a single transketolase reaction. From growth of this mutant on [2-$^{18}O$]fructose it was concluded that 35% of the label of the intermediate [2-$^{18}O$]dihydroxyacetone phosphate exchanged with water during the reaction. The results of growth of wild-type *E. coli* K10 on [1-$^{18}O$]glucose and [2-$^{18}O$]fructose were analyzed with respect to two models; one which proposes that both pathways operate simultaneously for the synthesis of pentose phosphate, and a second model which proposes that only the oxidative pathway is utilized for the synthesis of pentose phosphate. The contributions of both pathways were also studied in *E. coli* K12 W6, which is relaxed in RNA synthesis, both during growth as well as during starvation for methionine when the cells synthesize RNA but not protein or DNA.

The synthesis of pentose phosphate from glucose supplies the ribose and deoxyribose necessary for RNA and DNA synthesis as well as the NADPH required for various biosynthetic processes. Ribose phosphate can be formed from glucose by two routes, the oxidative pathway and the nonoxidative pathway. In the oxidative pathway, glucose 6-phosphate is oxidized by $NADP^+$ to gluconate 6-phosphate and the latter is oxidized by $NADP^+$ to $CO_2$ and ribulose 5-phosphate. In this conversion 1 mol of hexose is converted to 1 mol of pentose phosphate, 1 mol of $CO_2$, and 2 mol of NADPH. This pathway is essentially irreversible.

The nonoxidative pathway branches from the glycolytic pathway at fructose 6-phosphate and glyceraldehyde 3-phosphate. The action of transketolase on these two substrates yields xylulose 5-phosphate and erythrose 4-phosphate. If erythrose 4-phosphate reacts with another molecule of fructose 6-phosphate in a reaction catalyzed by transaldolase, sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate are produced which, if acted upon by transketolase, give rise to xylulose 5-phosphate and ribose 5-phosphate. In this series of reactions, 2.5 mol of hexose phosphate gives rise to 3 mol of pentose phosphate. These reactions are shown in Figure 3.

Though there are other pathways which could give rise to pentose phosphate, it has been shown (Caprioli and Rittenberg, 1969) that, quantitatively, they do not play an important role in ribose phosphate synthesis in *E. coli*. Since both the oxidative and nonoxidative pathways can operate simultaneously, there has been much interest in evaluating the contribution of each pathway to ribose phosphate synthesis. A major function of the oxidative pathway, in addition to supplying pentose phosphate, is the production of NADPH.

The contribution of the oxidative pathway to pentose phosphate synthesis has been measured by the radiorespirometric method (Wang and Krackov, 1962) in which the ratio of the radioactivity of $^{14}CO_2$ produced by cells grown on [1-$^{14}C$]glucose to that of cells grown on [6-$^{14}C$]glucose is mea-

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