BBA 66440

MAMMALIAN METHYLENETETRAHYDROFOLATE REDUCTASE

PARTIAL PURIFICATION, PROPERTIES, AND INHIBITION BY *S*-ADENOSYLMETHIONINE

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(Received June 23rd, 1971)

SUMMARY

1. 5,10-Methylenetetrahydrofolate reductase (5-methyltetrahydrofolate:NAD oxidoreductase, EC 1.1.1.68) has been purified several hundred-fold from both rat and pig liver.

2. A spectrophotometric assay for the purified enzyme is given and its optimum conditions have been studied.

3. The enzyme is a flavoprotein specific for FAD.

4. The enzyme is specific for NADPH as electron donor and the (+) diastereoisomer of 5,10-CH_2=H_4PteGlu as its physiological substrate. In addition, the enzyme exhibits a strong NADPH-specific diaphorase (reduced-NAD:lipoamide oxidoreductase, EC 1.6.4.3) or menadione reductase (reduced-NAD(P):(acceptor) oxidoreductase, EC 1.6.99.2) activity.

5. Apparent K_m values of the pig liver enzyme are: In the reduction of menadione: 49 μM for NADPH, 85 μM for NADH; in the reduction of methylenetetrahydrofolate: 33 μM for NADPH and 310 μM for NADH, and 21 μM for methylenetetrahydrofolate.

6. All activities of the enzyme are strongly inhibited by *S*-adenosylmethionine. This inhibition is partially reversed by *S*-adenosylhomocysteine. The menadione reductase property of the enzyme is also inhibited by the folate substrates.

INTRODUCTION

The enzymatic reduction of methylenetetrahydrofolate to 5-methyltetrahydrofolate is the first step in the *de novo* biosynthesis of methyl groups. The mammalian enzyme was originally reported by DONALDSON AND KERESZTESY[1,2] to be an oxidase catalyzing the conversion of prefolic A, isolated from horse liver, to tetrahydrofolate

Abbreviation: PCMB, *p*-chloromercuribenzoate.

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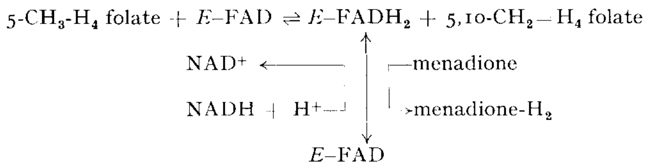
in the presence of menadione or a number of other artificial electron acceptors. The preparation was purified approx. 20-fold from pig liver and shown to require the addition of FAD for maximum activity. Prefolic A was later found to be identical with 5-methyltetrahydrofolate, which had been obtained as an intermediate in methionine biosynthesis in *Escherichia coli*[3] and in pigs[4]. DONALDSON AND KERESZTESY established that their enzyme preparation would also catalyze the reduction of methylenetetrahydrofolate to methyltetrahydrofolate. To account for these observations they proposed the following scheme:



(1)

In accordance with this scheme the enzyme also showed menadione reductase (reduced-NAD(P):(acceptor) oxidoreductase, EC 1.6.99.2) activity.

Methylenetetrahydrofolate reductase has also been purified 100-fold from extracts of derepressed *E. coli* 113-3 by KATZEN AND BUCHANAN[5]. The purified enzyme catalyzes the reduction of methylenetetrahydrofolate with free $FADH_2$ according to Eqn. 2:

$$5,10\text{-}CH_2{=}H_4\ \text{folate} + FADH_2 \rightarrow 5\text{-}CH_3\text{–}H_4\ \text{folate} + FAD \qquad (2)$$

Reduction with NADH requires the addition of a flavin reductase which is removed during the purification procedure. No information was obtained as to whether the *E. coli* methylenetetrahydrofolate reductase itself contains a flavin as a prosthetic group.

The *E. coli* enzyme has been shown to be under repressive control by methionine[5]. An analogous feedback-type regulation of the mammalian enzyme has been repeatedly proposed in order to explain the effect of methionine on disorders of folic acid-mediated metabolism in vitamin B_{12} deficiency[6–9]. In previous work we were unable to find evidence for an effect of dietary methionine on the activity level of the reductase in rat liver[10]. However, an investigation of *in vitro* effects of methionine derivatives on the enzyme activity in crude liver homogenates showed strong and specific inhibition by *S*-adenosylmethionine which could be partially reversed by *S*-adenosylhomocysteine[11]. The present publication confirms this inhibition with methylenetetrahydrofolate reductase purified several hundred-fold from both rat and pig liver. Properties of the purified enzyme include its specificity for NADPH and requirement for FAD as a firmly bound cofactor. The reaction catalyzed by the enzyme from mammalian liver is described by Eqn. 3:

$$5,10\text{-}CH_2{=}H_4\ \text{folate} + NADPH + H^+ \xrightarrow{(E\text{–}FAD)} 5\text{-}CH_3\text{–}H_4\ \text{folate} + NADP^+ \qquad (3)$$

This gives the enzyme the systematic name: 5-methyltetrahydrofolate:NADP oxidoreductase, EC 1.1.1.68). (A preliminary account of some of these findings has been presented[12].)

EXPERIMENTAL PROCEDURE

Materials

NAD^+, NADH, $NADP^+$, NADPH, FAD, ATP, glutamate dehydrogenase, and diaphorase* were purchased from Boehringer Mannheim. D,L- and L-homocysteine, adenosine, menadione, dimedon, FMN and folic acid were obtained from General Biochemicals. Other reagents were commercial products of analytical grade.

S-Adenosylmethionine was purchased from Calbiochem and purified according to the procedure of SHAPIRO AND EHNINGER[13]. *S*-Adenosylhomocysteine was prepared by enzymatic synthesis according to DE LA HABA AND CANTONI[14] and purified as described by DUERRE[15]. (±)Tetrahydrofolic acid was prepared by catalytic hydrogenation of folic acid in aqueous solution according to RAMASASTRI AND BLAKELEY[16]. To prepare 5,10-methylenetetrahydrofolic acid, two equivalents of formaldehyde were added after completion of the hydrogenation. The material was stored with the platinum catalyst under an atmosphere of H_2 in the dark until used. (+)5,10-methylenetetrahydrofolic acid was obtained by chromatographic separation of the diastereoisomeric mixture on DEAE-cellulose as described by KAUFMAN *et al.*[17]. Fractions containing the desired isomer were lyophilized and the residue dissolved in 0.01 M 2-mercaptoethanol. Preparation of 5-methyltetrahydrofolate and the [^{14}C]derivative was done by a modification of published procedures[18,19] as described by STAVRIANOPOULOS AND JAENICKE[20]. [^{14}C]Formaldehyde was a product of New England Nuclear Corp. Partially purified methyltetrahydrofolate:homocysteine methyltransferase (B_{12} enzyme) was prepared from extracts of *E. coli* 113-3 according to HATCH *et al.*[21]. After purification by DEAE-cellulose chromatography followed by a hydroxylapatite column, the preparation had a specific activity of 490 nmoles/h/mg when assayed in a radioactive assay system essentially as described by DICKERMAN *et al.*[7].

Assays

During purification methylenetetrahydrofolate reductase was routinely assayed in the reverse direction with menadione as electron acceptor (Assay A)[2]. With highly purified enzyme, a spectrophotometric assay in the forward direction was used according to Eqn. 3 (Assay B). The menadione reductase property of the enzyme was measured by Assay C.

Assay A. The incubation mixture contained in a final volume of 0.6 ml: 0.55 mM (±)-5-$^{14}CH_3$-H_4PteGlu (110-250 disint./min per nmole), 8 mM FAD, 8 mM ascorbate (as stabilizing agent in the methyltetrahydrofolate stock solution), 166 mM potassium phosphate buffer (pH 6.3), 1.6 mM EDTA, approx. 3.5 mM menadione and an amount of enzyme to produce approx. 30 nmoles of formaldehyde. The mixture was kept in ice and menadione (0.2 ml of a saturated solution in water at 100°) was added to start the reaction. The samples were then incubated for 1 h at 30° in stoppered tubes, and the reaction stopped by the addition of 0.3 ml dimedon reagent (3 mg/ml dimedon in 1 M acetate buffer (pH 4.5) (ref. 5)), and the tubes heated for 5 min at 95° (ref. 22). After cooling in ice, the formaldehyde–dimedon condensation product was extracted into 3 ml of toluene by vigorous agitation with a Vortex mixer for 15 sec. The phases

* Lipoamide dehydrogenase (reduced-NAD:lipoamide oxidoreductase, EC 1.6.4.3) from pig heart.

were separated by centrifugation, and 1 ml of the toluene phase counted in a scintillation counter. Blanks were obtained from samples where the enzyme had been added at the end of the incubation period. Amounts of protein exceeding 1 mg, as had to be used in the assay of crude fractions, interfered with the recovery of formaldehyde. Appropriate corrections were taken from a correction curve obtained with pure formaldehyde and H_4PteGlu under the conditions of the assay. 97% of the theoretical radioactivity was recovered from 5-$^{14}CH_3$-H_4PteGlu when the incubation was performed with an excess of purified enzyme.

Assay B. The assay mixture in a cuvette with 1 cm light path contained in a total volume of 2.5 ml: 40 mM potassium phosphate (pH 7.2), 2 μM FAD, 160 μM NADPH, 10 mM 2-mercaptoethanol, 1 mM ($\pm$)-5,10-CH_2=H_4PteGlu (as a mixture of $\pm H_4$PteGlu and formaldehyde, see *Materials*) and enzyme. The reaction was started after a preincubation period of 5 min* with the addition of either the folate substrate or in some cases NADPH, and initial rates recorded at 366 nm in a Gilford 2000 recording spectrophotometer with automatic cuvette changer at room temperature. Observations were done at 366 nm in order to minimize any effect of absorption changes due to decomposition of the folate substrate. Blank cuvettes without NADPH (or without enzyme) showed only a small increase in absorbance compared to the reaction rate. In most of the experiments automatic compensation for this blank was made by using the blank compensator module of the photometer.

Assay C. The assay of the menadione reductase property of the enzyme was similar to Assay B, except that folate and 2-mercaptoethanol were omitted and the reaction started by adding 0.2 ml of a fresh saturated solution of menadione in 20% methanol corresponding to 0.16 mM in the final mixture. Menadione was not a limiting factor under these conditions. Reduced menadione is rapidly oxidized nonenzymatically at pH 7 (ref. 23).

Protein was determined by the biuret method in the modification of BEISENHERZ *et al.*[24]. If higher sensitivity was needed, the Folin method according to LOWRY *et al.*[25] was used. Protein in column fractions was estimated from the absorbance at 280 and 260 nm, measured in a Beckman DB photometer, and calculated according to LAYNE[26].

Purification procedure

Identical procedures were used for the preparations from rat and pig liver. The procedure is described for a preparation starting with 4 kg of pig liver and is summarized in Table I. With rat liver, 0.5 or 1 kg were processed at one time. Because of the limiting capacity of the centrifuge, Steps 1–3 had to be done in several separate runs when more than 500 g of starting material were used. All steps were performed at 0–4°. Potassium phosphate buffer (pH 7.2) was used throughout.

(1) Extraction. 4 kg of pig liver (fresh or frozen) were minced in a meat grinder and homogenized in portions of 500 g with 1 l 0.05 M potassium phosphate buffer (pH 7.2) (1.5 l in the case of rat liver) in a Waring blendor for 1 min. The homogenate was adjusted to pH 6 with 4 M acetic acid and centrifuged for 25 min at 15 000 $\times$ *g*.

(2) $(NH_4)_2SO_4$ precipitation. Solid $(NH_4)_2SO_4$ was added to the supernatant

* This preincubation period was found necessary to obtain reproducible maximal rates.

to 45% saturation, the precipitate separated by centrifugation for 30 min at 15 000 $\times$ *g* and dissolved in 2.4 l of the extraction buffer.

(3) Acid precipitation. The $(NH_4)_2SO_4$ fraction was brought to pH 4.5 by the addition of 4 M acetic acid and after stirring for 10 min centrifuged for another 10 min. The fairly solid precipitate was homogenized with 800 ml of extraction buffer for a few sec at low speed in a Waring blendor, and the resulting turbid suspension neutralized to pH 7.2 with 4 M ammonia. This fraction was kept overnight at 0° and then centrifuged to yield a clear and yellow supernatant. The precipitate was discarded.

(4) DEAE-cellulose chromatography. The supernatant from the previous step was diluted with 0.05 M buffer to 1.4 l in order to lower the ionic strength sufficiently to allow adsorption on the column. A 3.9 cm $\times$ 60 cm column was prepared from DEAE-cellulose DE-22 (Whatman) and equilibrated with 0.05 M buffer. The sample was applied at a flow rate of 200 ml/h followed by (a) 1 l of buffer, (b) 1 l of 0.15 M KCl in buffer, (c) a linear gradient composed of 1 l each 0.15 and 0.5 M KCl in buffer, and (d) 0.5 M KCl in buffer. Fractions of 20 ml each were collected from the beginning of the gradient elution (Fig. 1) and assayed for total protein and enzymatic activity. The latter could be done most conveniently by making use of the menadione reductase

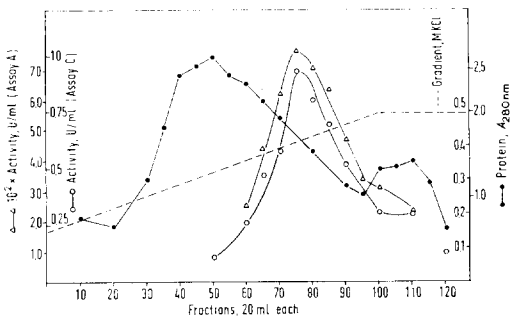


Fig. 1. Chromatograpic purification of methylenetetrahydrofolate reductase from pig liver on DEAE-cellulose. For conditions see experimental section. The broken line represents the KCl concentration entering the column.

property of the enzyme, since, as Fig. 1 shows, the peaks of activity obtained with Assays A and C coincided completely.

(5) DEAE-Sephadex chromatography. The combined active fractions from the preceding step (900 ml) were diluted with 500 ml of 0.05 M buffer and applied to a short column containing 100 ml of Sephadex A-50 equilibrated with 0.05 M buffer. Some inactive protein was not absorbed and was eluted with 0.05 M buffer. Active enzyme was then eluted in one peak with 0.6 M KCl in buffer.

(6) Concentration. Small volumes of enzyme were concentrated by immersion of a dialysis bag filled with polyoximethylene into the enzyme solution. For larger volumes a Diaflo ultrafiltration device was used. The final product was dialyzed against 0.05 M potassium phosphate buffer (pH 7.2).

RESULTS

Purification

Summaries of purifications of methylenetetrahydrofolate reductase from pig liver and rat liver are given in Tables I and II, respectively. Up to the acid precipitation step, the method was adopted in modified form from the work of DONALDSON AND KERESZTESY[2]. The pH-6 supernatant after the extraction step represents approx. a 2.5-fold purification over a pH-7 high-speed supernatant, so that the total purification is about 900-fold. The unexplained increase in total activity at the acid step leads to an apparent high overall yield.

TABLE I

PURIFICATION OF METHYLENETETRAHYDROFOLATE REDUCTASE FROM PIG LIVER

Step	*Volume (ml)*	*Protein (mg)*	*Activity* units***	*Specific activity (units/mg × 10^3)*	*Relative purification*	*Recovery (%)*
1. Extract (pH 6.0)	6600	190 000	77.3	0.41	- -	100
2. $(NH_4)_2SO_4$, 0–45% satn.	2900	62 000	92.0	1.48	3.6	118
3. pH 4.5 precipitate	950	11 000	97.5	8.85	21.5	125
4. DEAE-cellulose column	970	1 400	63.5	45.0	110.0	82
5. DEAE-Sephadex column	70	420	66.5	153.0	374.0	86

* By assay A.
** 1 unit equals 1 μmole/min.

Attempts at further purification with hydroxylapatite chromatography and/or gel filtration on Sephadex G-200 were unsuccessful. In the latter case some inactive protein was separated but at the expense of a large loss in total activity. The position of methylenetetrahydrofolate reductase activity in the eluate corresponded to a molecular weight in the range 150 000–200 000. The specific activity of the purified enzyme decreased by about 10% during frozen storage for 4 months. When measured in the reducing direction according to Assay B, the specific activity of the purest fractions obtained from pig liver was 25–30% of that of the purified enzyme obtained from *E. coli*[5]. Information on the degree of purity of this preparation was not reported.

TABLE II

PURIFICATION OF METHYLENETETRAHYDROFOLATE REDUCTASE FROM RAT LIVER

Step	*Volume (ml)*	*Protein (mg)*	*Activity* units***	*Specific activity (units/mg × 10^3)*	*Relative purification*	*Recovery (%)*
1. Extract (pH 6.0)	2750	47 000	9.7	0.21	- -	100
2. $(NH_4)_2SO_4$, 0–45% satn.	1220	18 000	12.5	0.70	3.3	129
3. pH 4.5 precipitate	475	2 860	8.3	2.9	13.8	86
4. DEAE-cellulose column	330	300	5.2	17.5	83	54
5. DEAE-Sephadex column	73	53	5.7	108	515	59
6. Concentrated	6	52	4.0	78	370	41

* By assay A.
** 1 unit equals 1 μmole/min.

Reactions catalyzed by the purified enyzme

The upper part of Table III shows the reactions that were found to be catalyzed by the purified mammalian methylenetetrahydrofolate reductase. Reaction II is used in the routine Assay A which can be made sensitive enough for crude extracts by using substrate of appropriate high specific radioactivity. A direct assay in the "forward" direction according to Reaction I is very difficult because the low activities found in most organisms and tissues do not allow a spectrophotometric approach.

TABLE III

REACTIONS CATALYZED BY MAMMALIAN METHYLENETETRAHYDROFOLATE REDUCTASE AND THEIR RELATIVE ACTIVITIES

I. 5,10-CH_2=H_4Folate + NADPH + H^+ → 5-CH_3-H_4Folate + $NADP^+$
II. 5-CH_3-H_4Folate + Menadione → 5,10-CH_2=H_4Folate + Menadione H_2

III. NADPH + H^+ + Menadione → $NADP^+$ + Menadione-H_2

The activities were determined according to Assays A ,B and C. The number of independent assays on different preparations is shown in brackets.

Source	*Ratio III/I*	*Ratio II/I*	*Ratio III/II*
Rat liver	16 (1)	1.5–3.0 (2)	6–10 (2)
Pig liver	10–16 (8)	0.8–1.6 (3)	7-16 (8)

It has been one of the aims of the purification work to obtain a specific activity high enough to allow a less tedious direct spectrophotometric assay on the basis of NADPH consumption. Table IV shows the requirements of this assay: NADPH is a better substrate than NADH and there is no stimulation by the addition of a flavin

TABLE IV

REQUIREMENTS OF THE SPECTROPHOTOMETRIC ASSAY FOR METHYLENETETRAHYDROFOLATE REDUCTASE

The complete assay system was the same as described in the *Experimental Procedure Assay B*. Each assay contained 350 μg of enzyme purified from rat liver with a specific activity of 24 munits/mg (Assay A). Where indicated, 0.1 mM FAD and 10 units of diaphorase (Boehringer) were added.

Omission or addition	ΔA_{366nm}/10 *min*
Complete	0.055
−formaldehyde	0
−H_4PteGlu	0
−NADPH, + NADH	0.005
+FAD, + diaphorase	0.055
−CH_2=H_4PteGlu, + menadione	0.90

reductase. 5-Methyltetrahydrofolate was identified as the product of the reaction by its conversion to methionine (see Table V) and by chromatographic identification.

The lower line of Table IV shows that a much faster oxidation of NADPH is observed when methylenetetrahydrofolate is replaced by menadione. A menadione reductase activity (Reaction III, Table III) of the enzyme is reasonable since it is

simply the sum of Reactions I and II as has already been implied in Scheme (1) from the work of DONALDSON AND KERESZTESY[2]. Evidence that all the menadione reductase activity found in purified methylenetetrahydrofolate reductase is a property of this enzyme can be summarized as follows:

(a) A fairly constant ratio of activities III/II after the acid step. (b) Coincidence of enzyme activities in the elution peaks from DEAE-cellulose (Fig. 1). (c) Similar degree of heat inactivation: 5 min at 50° inactivated I to 67% and III to 76%. (d) Similar degree of inhibition by *p*-chloromercuribenzoate (PCMB): 40 μM inhibited I 60% and III 83%. (e) Same high specificity for NADPH (Table VII). (f) Inhibition of menadione reductase activity by 5-methyltetrahydrofolate (Fig. 2) and methylenetetrahydrofolate. (g) Inhibition of menadione reductase by *S*-adenosylmethionine (Fig. 4b).

The ratios of the three activities are shown in Table III. Although some variation occurred between assays on different preparations, the ratios are rather similar for preparations from rat and pig livers.

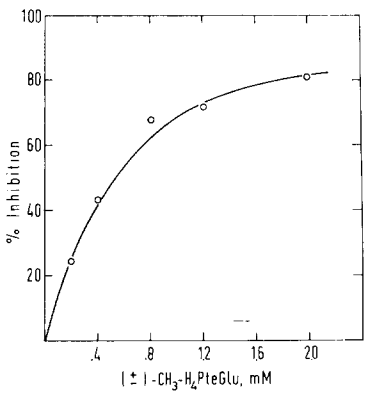


Fig. 2. 5-Methyltetrahydrofolate inhibition of menadione reductase activity of purified methylenetetrahydrofolate reductase from pig liver. Assay C was used with the addition of the appropriate amounts of 5-CH_3–H_4PteGlu dissolved in water from which O_2 had been removed by boiling followed by gassing with N_2. 28 μg of enzyme of specific activity 1.5 were used in each assay.

Conditions of assays

The optimum conditions for the spectrophotometric Assays B and C have been determined. Figs. 3a and b give pH–activity curves for methylenetetrahydrofolate reductase and menadione reductase, respectively. The pH optimum of the folate-specific reaction was found at 6.6–6.7 in agreement with the value found by DONALDSON AND KERESZTESY[1] for the backward reaction. The pH optimum of menadione reductase was less sharp, with a maximum at around pH 7.1. Thus it appears that the pH optimum of 6.6 reflects some property of either the folate binding site or the folate substrate itself, and determines the observed optimum when the interaction with the folate limits the dehydrogenase activity. During investigation of the pH optimum, it was observed that 60–80% lower rates were obtained in Tris or borate buffer, even if phosphate was added to a concentration of 4 mM. Increasing the phophate concentration above 40 mM did not result in higher rates. Activation by

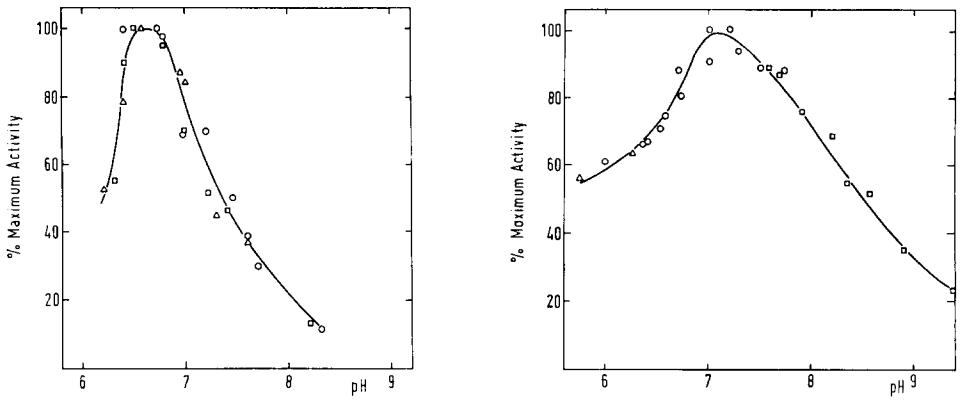


Fig. 3.a. Effect of pH on the activity of methylenetetrahydrofolate reductase from pig liver. The different symbols represent results from three sets of experiments normalized on the basis of the maximum activity observed in each set. Experiments were carried out according to Assay B in 40 mM potassium citrate–phosphate buffer. The pH of the solution at the end of the reaction was measured in the cuvettes with a glass combination electrode. b. Effect of pH on the menadione reductase activity of methylenetetrahydrofolate reductase from pig liver. Experiments were carried out according to Assay C. Buffer concentration was 40 mM in all assays (total concentration of buffering anions). △, potassium-citrate–phosphate buffer; ○, potassium phosphate buffer; □, sodium pyrophosphate–HCl buffer. pH was measured as under a.

phosphate has also been observed with another FAD containing oxidoreductase, lipoamide dehydrogenase[34].

The rate of Reaction I at pH 7.2 increases slowly between 25 and 45° with an energy of activation of approx. 5 kcal. A sharp inactivation was found above 45°. The bacterial enzyme was also found to be very heat sensitive[5].

Both spectrophotometric assays (B and C) gave a linear response to increasing amounts of enzyme in the range used in our experiments. The amount of NADPH utilized was strictly proportional to the amount of folate added. The rate of reaction in the spectrophotometric assay was nearly constant over the first 1–3 min, but later decreased faster than could be explained by utilization of substrate. This decrease may be caused by inactivation of the enzyme by menadione or excess formaldehyde, as supported by experiments in which the concentrations of these compounds were varied.

Cofactor requirement

DONALDSON AND KERESZTESY[2] demonstrated a requirement for FAD with the partially purified enzyme from pig liver. With the preparation from rat liver, however, we could not find a requirement for FAD in any of the three assays. Attempts to separate the assumed cofactor from the apoenzyme by $(NH_4)_2SO_4$ precipitation at low pH caused a complete loss of activity. A 1.7-fold stimulation by FAD was obtained with a preparation which had been dialyzed 48 h against 1 M KBr followed by dialysis against potassium phosphate buffer[28]. This preparation, however, had lost 50% of its activity. FMN was inactive.

The enzyme from pig liver, in contrast, required the addition of FAD for full activity after purification through the acid precipitation step. In the absence of FAD the purified enzyme had only about 25% of its maximal activity in all three assays.

Complete reactivation required only 4 μM FAD, FMN being inactive. Half maximal activation was found at 40 nM FAD. The added FAD did not bind strongly to the enzyme but could easily be removed by dialysis, gel filtration or even sedimentation in a sucrose gradient. Complete separation of the pig liver enzyme from its cofactor was achieved by 12 h dialysis against 1 M KBr, but again 30% of the activity was lost. An attempt was made to determine the FAD binding capacity of the most purified preparation, but the purity of the enzyme was still too low for an accurate estimation.

No evidence could be found for the participation of heavy metals in the catalytic process. There was no significant inhibition by addition of or by 12 h dialysis against 1 mM EDTA, α,α'-dipyridyl and *o*-phenanthroline. It is concluded that methylenetetrahydrofolate reductase is a metal-free flavoprotein.

Specificity

Three aspects of the specificity of the enzyme have been investigated: (1) Electron donor specificity for the reduction of methylenetetrahydrofolate and menadione, (2) electron acceptor specificity for the oxidation of NADPH, other than methylenetetrahydrofolate, and (3) specificity for the isomers of 5,10-methylenetetrahydrofolic acid.

(1) In the standard spectrophotometric assay NADPH was found to be a much better substrate than NADH (Table IV). However, the work of KATZEN AND BUCHANAN[5] on the reduction of methylenetetrahydrofolate to 5-methyltetrahydrofolate in *E. coli* suggested the involvement of two enzymes in the overall oxidoreduction between NADH and 5,10-CH_2=H_4PteGlu with free FAD/$FADH_2$ as intermediate carrier. Thus it was important to test this possibility, although the absence of a requirement for FAD with the purified enzyme from rat liver would argue against this. No significant reaction was observed when $FADH_2$ was used instead of NADPH in the spectrophotometric assay and the assay was performed at 450 nm in Thunberg cuvettes under anaerobic conditions. Therefore the two-step assay system described by KATZEN AND BUCHANAN[5] was used to determine reduction of methylenetetrahydrofolate, *i.e.*, conversion of the reaction product methyltetrahydrofolate to methionine with methyltransferase from *E. coli* followed by the microbiological assay of methionine. Results in Table V show that NADPH is the most potent electron donor, but NADH and $FADH_2$ also give about 25% of maximum product formation. $FMNH_2$ was inactive as was enzyme in the presence of Pt and H_2.

The apparent discrepancy between the spectrophotometric assay and these results with respect to NADH can be explained on the basis of the apparent K_m values for NADH and NADPH. An 80 times higher concentration of NADH has been used in the two-step system in comparison with the spectrophotometric assay. This is also the case in the experiments of DONALDSON AND KERESZTESY[2] who found NADPH and NADH equally active. $FADH_2$, prepared either chemically or enzymatically with diaphorase *in situ*, is a little more active than NADH but still is only 25–30% as effective as NADPH. It can be concluded that NADPH is the physiological electron donor of the mammalian enzyme.

The experiment described in Table V also provides clear evidence that 5-CH_3–H_4PteGlu is the product of the reaction, since it can serve as precursor in the syn-

TABLE V

ELECTRON DONOR SPECIFICITY OF MAMMALIAN METHYLENETETRAHYDROFOLATE REDUCTASE

The incubation procedure was similar to the "Two step" assay described by KATZEN AND BUCHANAN[5]. Incubations were carried out in 0.8 cm × 10 cm test tubes equipped with a tight rubber cap and a sidearm carrying a threeway stopcock. The first incubation mixture contained in a total volume of 0.5 ml: 5 mM of a 1:1 mixture of (±)H_4PteGlu and formaldehyde, 200 mM potassium phosphate buffer (pH 7.2), 50 mM 2-mercaptoethanol, 300 μg reductase purified from rat liver (specific activity 38 munits/mg) and the electron donor (4 mM of NADPH or NADH, 1 mM of catalytically reduced $FADH_2$ or FMN_2, and 0.2 mM FAD + 250 μg of purified diaphorase, respectively). The tubes containing all components except the flavins were made anaerobic by 10 cycles of evacuation with a vacuum pump and refilling with pure H_2 while kept in ice. The flavins were added through the rubber cap with a syringe and the reaction started by immersion of the tubes into a 30° water bath. After 1 h the reaction was stopped by heating the tubes at 95° for 3 min to inactivate the enzyme. To convert methyltetrahydrofolate formed during the first incubation into methionine, the following additions were made (total volume of 1.0 ml): 0.25 mM *S*-adenosylmethionine, 5 mM L-homocysteine, 0.1 mM $FADH_2$ and 1.8 mg of purified methyltransferase catalyzing the formation of 375 nmoles of methionine per 3 h per mg. The tubes were again made anaerobic before $FADH_2$ was added with a syringe. Incubation was for 3 h at 35° and the reaction was terminated by heating at 95° for 3 min. Methionine formed was assayed microbiologically with *Leuconostoc mesenteroides* ATCC 8042 with Bacto-methionine assay medium by the method given by the supplier (Difco) except that a total volume of 2 ml was used to increase the sensitivity. L-Methionine was used as standard and the growth evaluated by reading the turbidity at 640 nm. Results with each donor were corrected for a blank with the enzyme omitted.

Expt. No.	*Donor*	*Methionine formed (nmoles)*
1	NADPH	300
	NADH	66
	NADH + FAD + diaphorase	86
2	NADPH	200
	NADH	46
	$FADH_2$	53
	Enzyme only	5
	$FMNH_2$	5

TABLE VI

RELATIVE ELECTRON ACCEPTOR ACTIVITIES WITH RESPECT TO NADPH OXIDATION BY METHYLENETETRAHYDROFOLATE REDUCTASE

Acceptor activities were compared under the conditions of Assay C with 20 μM FAD present in all assays except the one with O_2. In the absence of FAD, added as cofactor, the rates with the other acceptors were about 90% lower. Pig liver enzyme purified to 150 munits/mg (Assay A) was used in varying amounts in order to get significant readings. Blanks without enzyme were subtracted.

Acceptor	*Concn. (mM)*	*Relative activity (%)*
Menadione	0.16	100
Dichlorophenolindophenol	0.2	100
K-Ferricyanide	1.4	50
Cytochrome *c*	18	13
FAD, FMN	0.2	≤ 5
O_2	air	≤ 1
Methylenetetrahydrofolate	0.5	7

thesis of methionine. The activity of the enzyme calculated from this assay is comparable to that in Assay B.

(2) With respect to electron acceptors, the enzyme reacts with a large variety of compounds as has been observed with most flavoenzymes. DONALDSON AND KERESZTESY[1,2] have already presented evidence that naphtho- and benzoquinones, as well as indigo disulfonates, would act as oxidants in the oxidation of 5-CH_3-H_4PteGlu. We have assayed some other commonly used electron acceptors. As shown in Table VI, ferricyanide, dichlorophenolindophenol and cytochrome *c* are better electron acceptors than the folate substrate in the reaction with NADPH. FAD and FMN have little acceptor activity, and the enzyme is not oxidized by air at a measurable rate. Since, however, the rate of the menadione:NADPH oxidoreduction is 10–15 times that of the methylenetetrahydrofolate:NADPH reaction, the absolute rate of the oxidation of NADPH with FAD as acceptor is at least equal to the reduction of methylenetetrahydrofolate with $FADH_2$.

(3) All enzymes of tetrahydrofolate metabolism investigated so far are specific for the same one diastereoisomer. This generality has been corroborated by the use of the chromatographically separated isomers of (±)-L-5,10-CH_2-H_4PteGlu as substrates. Only the isomer emerging second from the DEAE-cellulose column (+ rotatory according to RAMASASTRI AND BLAKELEY[29]) reacted in Assay B and yielded a stoichiometric amount (95%) of NADPH if supplemented with excess formaldehyde, whereas the diastereoisomer mixture yielded always ≤50%. No inhibition was observed by the inactive isomer since the active (+) isomer, the diastereoisomeric mixture, and a 1:1 mixture of the isolated (+) and (−) isomer gave essentially the same reaction rate:

Isomer	ΔA_{366}/5 *min*
±	0.30
+	0.31
1:1 mixture of + and −	0.32
−	0.002

TABLE VII

APPARENT K_m-VALUES FOR SUBSTRATES OF THE METHYLENETETRAHYDROFOLATE REDUCTASE AND MENADIONE REDUCTASE REACTIONS

The K_m values were calculated by unweighted least-square fits to the S/v_{max} *vs.S* linearization of the Michaelis-Menten equation with an IBM digital computer.

Source of enzyme	*Reaction**	*pH*	*Concn. of 2nd substrate (mM)*	*Substrate*	*Substrates*		K_m *(app.)* ± % S.D. *(μM)*	*Relative* v_{max}
					No. of concns. tested	*Range of concn. (μM)*		
Rat	III	7.2	0.16	NADPH	6	16–224	59 ± 2.5%	1.0
			0.16	NADH	5	120–1200	576 ± 7.7%	0.4
Pig	III	6.8	0.16	NADPH	7	14–201	49 ± 2.3%	1.0
			0.16	NADH	6	140–840	855 ± 9.6%	0.5
Pig	I	6.7	1.0	NADPH	6	13–192	33 ± 6.6%	1.0
			1.0	NADH	5	220–1100	313 ± 5.0%	0.5
			0.16	(+)CH_2-H_4PteGlu	9	11–285	21 ± 4.7%	1.0
			0.16	(+)CH_2-H_4PteGlu	7	5–240	25 ± 3.6%	1.0

* III = menadione reductase, I = methylenetetrahydrofolate reductase.

Kinetic constants

Apparent K_m's of substrates have been determined from saturation curves for NADPH and NADH in the menadione reductase reaction with enzyme from rat and pig livers. The methylenetetrahydrofolate reductase has been studied with the pig liver enzyme only and the constants were determined for NADPH, NADH and the folate substrate. Results are summarized in Table VII. Substrate inhibition occurred with NADH at >1 mM. For methyltetrahydrofolate in the back reaction with menadione, K_m values between 70 and 150 μM have previously been obtained[11] at pH 6.6 with less pure rat liver enzyme. A higher value for K_m of methyltetrahydrofolate as compared to the methylene derivative should favour the reduction of the latter under physiological conditions.

Reversibility

The overall equilibrium of the reduction of 5,10-methylenetetrahydrofolate is far on the side of methyltetrahydrofolate formation. With the *E. coli* enzyme, KATZEN AND BUCHANAN[5] obtained equilibrium constants of $K_{eq} = 2.4 \cdot 10^3$ and $3.4 \cdot 10^3$ for the reduction of 5,10-CH_2=H_4PteGlu with $FADH_2$:

$$K_{eq} = \frac{CH_3\text{–}H_4PteGlu \times FAD}{CH_2{=}H_4PteGlu \times FADH_2}$$

They stated that this value should have the right order of magnitude, a more precise determination being impossible because of experimental difficulties. On the basis of their values, the equilibrium constant for the reduction of CH_2=H_4PteGlu by NADPH can be easily calculated:

$$K_{eq} = \frac{CH_3\text{–}H_4PteGlu \times NADP^+}{CH_2{=}H_4PteGlu \times NADPH} = 10^7 \text{ at pH } 7.0$$

The K_{eq} for the oxidation of methyltetrahydrofolate with menadione is calculated to be:

$$K_{eq} = \frac{CH_2{=}H_4PteGlu \times \text{menadione-}H_2}{CH_3\text{–}H_4PteGlu \times \text{menadione}} = 8 \cdot 10^3$$

These calculations are in agreement with the observation that reduction of CH_2=H_4PteGlu with NADPH and oxidation of CH_3–H_4PteGlu with menadione go to completion.

An experiment testing the possibility of oxidation of methyltetrahydrofolate by $NADP^+$ is shown in Table VIII. The oxidation of methyltetrahydrofolate by FAD in the absence of menadione was only 3.6% compared to menadione, in agreement with the activity of FAD in the oxidation of NADPH (Table VI). Addition of $NADP^+$ did not stimulate but rather inhibited the reaction. If accumulation of NADPH was prevented by the addition of the glutamate dehydrogenase system, a small stimulation could then be observed. The oxidation rate under these conditions amounts to about 1% of the forward reaction (Assay B). However, these are completely unphysiological conditions, since in the cytoplasm of the cell the ratio NADPH/$NADP^+$ has always been found to be greater than 1 (ref. 30).

Inhibitors

A number of commonly used inhibitors have been tested in the two spectro-

TABLE VIII

ATTEMPT TO DEMONSTRATE THE REVERSAL OF THE METHYLENETETRAHYDROFOLATE REDUCTASE REACTION

Conditions of assays were as in Assay A, except that menadione was omitted and the pH was 7.0 (optimum pH of glutamate dehydrogenase). Additions were 2 μmoles of $NADP^+$ and the glutamate dehydrogenase system consisting of 10 μmoles each of ketoglutarate and NH_4Cl and 0.5 mg of crystalline beef liver glutamate dehydrogenase (20 I.U.). A blank without enzyme has been substracted.

Additions to assay system	*Enzyme added (μg)*	*Counts/min*	*Formaldehyde (nmoles)*	*nmoles/mg enzyme*	*%*
+ FAD, + menadione	6	850	12	2050	100
− FAD, + menadione	6	350	5	850	41
+ FAD, − menadione	600	3100	45	75	3.6
+ FAD, + $NADP^+$	600	2200	32	53	2.6
+ FAD, + $NADP^+$, + glutamate dehydrogenase system	600	4000	58	97	4.7

photometric Assays B and C. The enzyme was not inhibited by iodoacetamide and *N*-ethylmaleimide at concentrations up to 1 mM. 40 μM *p*-chloromercuribenzoate inhibited methylenetetrahydrofolate reductase 60% and menadione reductase 83%. 1 mM mersalyl inhibited both activities 80%. These inhibitions by typical sulfhydryl group reagents were rapidly reversed (up to 90%) by 2-met. Thus, the enzyme appears to have sulfhydryl groups which are essential for activity, but their blocking does not lead to rapid irreversible denaturation. Up to 50 μM of the vitamin K antagonist dicoumarol inhibited only the menadione reductase activity of the enzyme. 50% inhibition required 20μM dicoumarol, which is much more than is necessary for inhibition of "specific" quinone reductases[31].

Inhibition by S-adenosylmethionine

The inhibition of methylenetetrahydrofolate reductase by *S*-adenosylmethionine which was shown in crude extracts[11] has been confirmed with the purified enzyme and a more reliable assay in the forward reducing direction. Furthermore, it could be shown that *S*-adenosylmethionine is an equally strong inhibitor of the menadione reductase property of the enzyme. The inhibition of both activities was partially released by *S*-adenosylhomocysteine. *S*-Adenosylhomocysteine added alone, however, was not an activator of the enzyme. Figs. 4a and 4b show double reciprocal plots[27] of the fractional inhibition *versus* the *S*-adenosylmethionine concentration for both activities in the presence and absence of *S*-adenosylhomocysteine. It is seen that the action of *S*-adenosylhomocysteine is strictly competitive to *S*-adenosylmethionine. Half maximal inhibition is obtained at 2.8 μM *S*-adenosylmethionine in the absence of *S*-adenosylhomocysteine and at 5 μM in the presence of *S*-adenosylhomocysteine for methylenetetrahydrofolate reductase and at 2 μM (−*S*-adenosylhomocysteine) and 7.7 μM (+*S*-adenosylhomocysteine) for the menadione reductase activity of the enzyme. Experiments in which the substrate concentration was varied at different levels of the inhibitor indicated a mixed type of inhibition. A more thorough kinetic treatment of the inhibition by *S*-adenosylmethionine and its reversal by *S*-adenosylhomocysteine does not seem justifiable because of the unusually slow action of the

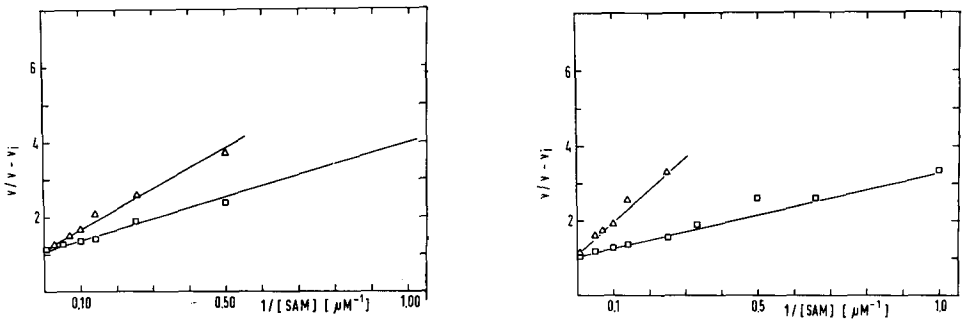


Fig. 4. Double reciprocal plots of the inhibition of the pig liver enzyme by *S*-adenosylmethionine (for reference to the type of plot used see ref. 27). a. Methylenetetrahydrofolate reductase activity (Assay B) at pH 6.7. *S*-adenosylhomocysteine and *S*-adenosylmethionine were present during the preincubation period and the reaction was started by the addition of 5,10-CH_2 = H_4PteGlu. b. Menadione reductase activity at pH 7.6. After preincubation in the presence of the inhibitor, the reaction was started by the addition of menadione. □, without *S*-adenosylhomocysteine; △, with 2 μM *S*-adenosylhomocysteine.

inhibitor as shown in Fig. 5. When *S*-adenosylmethionine was added to an on-going reaction, approx. 5 min were needed to reach a slower constant reaction rate. Similarly, when *S*-adenosylhomocysteine was added subsequently, several minutes were needed for reactivation. These observations would suggest either a chemical reaction between the modifiers and the enzyme, *e.g.*, a methylation, or a slow conformational change. However, the first possibility seems unlikely since inhibition was completely reversed by removal of *S*-adenosylmethionine with activated charcoal. Because of this time dependence, samples were preincubated for 5 min with the inhibitor before the reaction was started with either the folate substrate or menadione.

The inhibition by *S*-adenosylmethionine shows certain characteristics of allosteric inhibition[32]:

(1) There is no structural resemblance of the inhibitor to any of the substrates, especially in the menadione reductase reaction. (2) The inhibitor and its structural

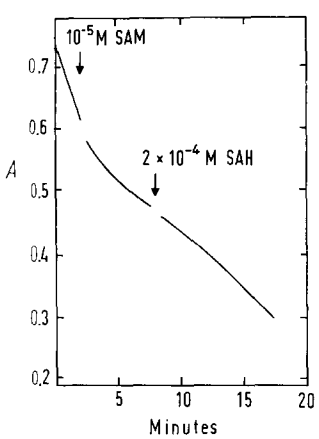


Fig. 5. Time course of inhibition by *S*-adenosylmethionine (SAM) and reactivation by *S*-adenosylhomocysteine (SAH) of rat liver menadione reductase activity in Assay C.

TABLE IX

EFFECTS OF SUBSTRATE AND MODIFIERS ON DESENSITIZATION OF METHYLENETETRAHYDROFOLATE REDUCTASE BY HEAT INACTIVATION

2.4 mg enzyme purified from pig liver were incubated for 5 min at 50° with the addition of substrate or modifiers in the amounts shown in a total volume of 0.6 ml. After cooling, 0.2 ml of albumin-coated charcoal[33] was added, the mixture shaken for 5 min and centrifuged. 0.1 ml of the supernatant was taken for each assay of methylenetetrahydrofolate reductase in Assay B and 0.02 ml for menadione reductase according to Assay C. In assays with *S*-adenosylmethionine the reaction was started after a preincubation of 5 min by the addition of the folate substrate or the menadione, respectively.

Other controls (not shown) showed that treatment of the enzyme with albumin-coated charcoal did not influence its activity and that *S*-adenosylmethionine was completely removed by the charcoal treatment as judged by complete recovery of enzyme activity from a non-heated control with *S*-adenosylmethionine added.

Addition	*CH_3-H_4PteGlu reductase*		*Menadione reductase*	
	Activity (%)	*Inhibition* (%)*	*Activity (%)*	*Inhibition** (%)*
Control (not heated)	100	87	100	70
Heated				
(no additions)	33	30	24	35
+ SAM, 0.17 mM	48	71	58	79
+ SAH, 3 mM	48	50	48	50
+ CH_3-H_4PteGlu, 3 mM	43	14	24	37

* By 0.1 mM *S*-adenosylmethionine.
** By 10 μM *S*-adenosylmethionine.

analogue, *S*-adenosylhomocysteine, compete for the same site. If this were the catalytic site, *S*-adenosylhomocysteine should also inhibit rather than abolish the inhibition caused by *S*-adenosylmethionine. (3) The enzyme can (at least partially) be desensitized against the action of the inhibitor.

Table IX shows the effect of *S*-adenosylmethionine, *S*-adenosylhomocysteine and substrate on heat desensitization of the enzyme. *S*-Adenosylmethionine and *S*-adenosylhomocysteine both gave some protection of the catalytic activity as well as the sensitivity to inhibition. The substrate protected only the catalytic activity. Similar results had been obtained with the unpurified enzyme[11]. In view of the slow interaction of the enzyme and inhibitor, however, further investigations are necessary before a more definitive statement on the type of inhibition can be made. Attempts to demonstrate inhibition of methylenetetrahydrofolate reductase by *S*-adenosylmethionine in crude extracts from *E. coli* 113-3 were unsuccessful.

DISCUSSION

KATZEN AND BUCHANAN[5] have shown that in *E. coli* the overall reduction of methylenetetrahydrofolate to methyltetrahydrofolate by NADH is catalyzed by a sequence of two separable enzymes, NADH:FAD oxidoreductase and $FADH_2$:methylenetetrahydrofolate oxidoreductase. Experimental results presented in this paper give evidence that in mammals a single enzyme catalyzes the reduction of

methylenetetrahydrofolate by NADPH. Although the enzyme was not purified to homogeneity, this conclusion can still be made since the observed rates of reduction of free flavin-coenzyme by NADPH and of methylenetetrahydrofolate by reduced FAD are too small to account for the observed overall rate of reduction of methylenetetrahydrofolate by NADPH. Also, in the case of the rat liver enzyme, the overall reaction did not require the addition of FAD. Thus, the scheme (Eqn. 1) given by DONALDSON AND KERESZTESY[2] to explain their results with the pig liver enzyme has been confirmed, except that it has now been shown that the mammalian enzyme is specific for NADPH.

Methylenetetrahydrofolate reductase is the first enzyme of a pathway leading to the biogenesis of methyl groups from C_1 units of higher oxidation state. The methyl group generated in this first step is transferred to homocysteine yielding methionine by a B_{12}-dependent methyltransferase. Methionine is further activated to *S*-adenosylmethionine from which the methyl group can now be transferred to a large variety of acceptors[35].

DU VIGNEAUD and coworkers[36,37] in their classical studies have shown that very little *de novo* synthesis of methyl groups occurs in rats if an adequate supply of preformed labile methyl groups is present in the diet. In contrast, *de novo* synthesis can satisfy the requirements of the animal if labile methyl groups are missing in the diet. A 4-fold increase in neogenesis of labile methyl groups (as derived from the incorporation of deuterium from ^2H_2O into choline-methyl) has been observed, if rats were given a labile methyl-free diet[38].

These observations indicate that the pathway of methyl group neogenesis must be regulated. In analogy to the general pattern of regulation by feed-back inhibition that has emerged in recent years, the main target should be the first enzyme of the sequence, methylenetetrahydrofolate reductase, which also lies at a branching point of folate metabolism. The inhibition of this enzyme by *S*-adenosylmethionine as described in this paper would fulfill the requirements for metabolic regulation:

(a) The inhibitor, *S*-adenosylmethionine, is a direct intermediate in the pathway and a common precursor of all methyl groups derived from the pathway; (b) the levels of *S*-adenosylmethionine in liver[39,40] cause strong inhibition of the enzyme *in vitro*; (c) the *S*-adenosylmethionine level in liver is dependent on the dietary intake of labile methyl groups, *e.g.*, methionine[41].

The proposed regulatory mechanism could also explain the effect of methionine on the disturbances of folate metabolism caused by vitamin B_{12} deficiency. (For review see ref. 42). In B_{12} deficiency the B_{12}-dependent transmethylase is less active[10]. Because the equilibrium of the methylenetetrahydrofolate reductase reaction favors the side of methyltetrahydrofolate synthesis, this folate compound should accumulate and be unavailable for functions in nucleic acid and protein metabolism. This "methyl trap" could possibly be prevented through inhibition of the reductase reaction by *S*-adenosylmethionine formed from dietary methionine. NORONHA AND SILVERMAN[43] have shown that dietary methionine causes a large reduction in the level of methyltetrahydrofolate in rat liver.

ACKNOWLEDGEMENTS

This work has been supported, in part, by U.S. Public Health Service-National

Institutes of Health Grant AM 08171. The *E. coli* 133-3 culture was kindly donated by Dr. Beverly Guirard, Department of Biochemistry, University of California, Berkeley. We also wish to thank Dr. J. C. Rabinowitz of the same Department for making available facilities for growth of the organism. Thanks are also due to Dr. M. A. Williams for her help in preparing this manuscript.

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