Mechanism of Mammalian Cobalamin-Dependent Methionine Biosynthesis*

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ABSTRACT: A kinetic study is reported of the interactions among *S*-adenosylmethionine (SAM), N^5-methyltetrahydrofolate (Me-FH_4), methionine, and a cobalamin-containing transmethylase isolated from porcine kidney. Methionine is an uncompetitive inhibitor of its own synthesis with respect to both SAM and Me-FH_4. *S*-Adenosylhomocysteine (SAH) inhibits methionine synthesis noncompetitively with respect to SAM, and tetrahydrofolate (FH_4) exhibits a similar behavior toward Me-FH_4. Kinetic evidence is presented for the reaction of SAM and Me-FH_4 with distinct enzyme forms. The enzyme is inactivated by reaction with propyl iodide in the dark, and regains catalytic activity upon exposure to visible light. The propylation reaction is prevented by SAM, but not by Me-FH_4. The addition of Me-FH_4 to a propylation mixture containing SAM reverses the protective effect of SAM, and propylation occurs. The observed effect is not due to contaminating FH_4 in the Me-FH_4 preparation. A reaction model is proposed which accommodates the data presented and explains the role of SAM in the cobalamin-dependent transmethylation reaction. The model is consistent with the concept of methionine biosynthesis as a central control point for folate-dependent reactions in the cell.

It is now well established (Sakami and Ukstins, 1961; Mangum and Scrimgeour, 1962; Loughlin *et al.*, 1964) that the terminal reaction in methionine biosynthesis in animal liver extracts is described by reaction 1. Cell-free extracts

$$\text{Me-FH}_4{}^1 + \text{homocysteine} \xrightarrow{\text{SAM}} \text{methionine} + \text{FH}_4 \quad (1)$$

which catalyze this reaction have been prepared from mammalian liver (Nakao and Greenberg, 1959; Stevens and Sakami, 1959), chicken liver (Doctor *et al.*, 1957), and *Escherichia coli* methionine cobalamin auxotrophs (Helleiner and Woods, 1956; Hatch *et al.*, 1959). Takeyama and Buchanan (1961) reported that the bacterial enzyme contained some form of bound vitamin B_{12}. Dickerman *et al.* (1964) and Loughlin *et al.* (1964) presented evidence which supported the same conclusion for enzymes extracted from chicken liver and hog liver, respectively. The similarity of optimum reaction conditions displayed by all preparations described suggests that the enzymes which catalyze this reaction may be very similar throughout nature.

Taylor and Weissbach (1967a) reported the first extensive purification of Me-FH_4–homocysteine transmethylase, in which *E. coli* was used as a source of enzyme activity. These workers showed that under appropriate conditions the enzyme accumulated label upon incubation with methyl-labeled Me-FH_4, SAM, or methyl iodide (Taylor and Weissbach, 1968). Extraction of the protein with hot alcohol yielded a labeled compound which was identified as methyl-B_{12}. Rudiger and Jaenicke (1968) working with a highly purified enzyme preparation from *E. coli*, presented kinetic evidence for an enzyme-bound, methylated intermediate, presumably methyl-B_{12}. We have recently reported the formation of enzyme-bound methyl-B_{12} upon incubation of the extensively purified mammalian enzyme with SAM or Me-FH_4 (Burke *et al.*, 1970). Taylor and Weissbach (1969b) have formulated a scheme for the biosynthesis of methionine from *E. coli* in which SAM activates the inactive, cobalamin-containing Me-FH_4–homocysteine transmethylase activity through a prior alkylation of the cobalt. The observation that methyl iodide or *S*-adenosylethionine can substitute to a limited extent for SAM in the activation of *E. coli* methionine synthetase (Taylor and Weissbach, 1966, 1967c) supports this proposal. Rudiger and Jaenicke (1970) have reported the interconversion of methylated and unmethylated forms of the *E. coli* enzyme, and Taylor and Hanna (1970b) described spectral differences for the two species.

The present communication describes a detailed kinetic analysis of the reactions catalyzed by the mammalian enzyme. Initial velocity and product inhibition patterns, as well as the behavior of the enzyme toward the irreversible inhibitor propyl iodide, are used to derive a kinetic mechanism. The data are consistent with a Ping-Pong mechanism (Cleland, 1963) in which SAM and Me-FH_4 react with distinct enzyme forms, each of which gives rise to enzyme-bound methyl-B_{12}. The interaction of the enzyme with propyl iodide in the presence of SAM and Me-FH_4 suggests an obligatory order of tramsmethylation and is consistent with a preliminary reaction involving SAM. A scheme is presented to satisfy the kinetic requirement for at least two distinct enzyme precursors of enzyme-bound methyl-B_{12}.

Materials

N^5-Methyltetrahydrofolate–homocysteine transmethylase was purified from hog kidneys by protamine sulfate precipitation, ammonium sulfate fractionation, and chromatography on Sephadex G-200 and DEAE-Sephadex. The most highly purified preparations have specific activities of up to 12,000 n-

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[1] Abbreviations used are: Me-FH_4, N^5-methyltetrahydrofolate; FH_4, tetrahydrofolate; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; Hom, homocysteine.

moles of Met synthesized per mg of protein per 15 min. These preparations have a B_{12a}-like spectrum, an apparent molecular weight of approximately 140,000 and are heterogeneous on disc gel electrophoresis. Experimental details will be reported in a separate communication. It should be emphasized that a number of experiments reported in this paper have been repeated with less pure enzyme preparations prepared by the method of Loughlin *et al.* (1964) and more highly purified preparations and the results were essentially independent of the stage of purification. In addition to properties described by Burke *et al.* (1970) a property of this enzyme which is of some importance to the present study is the rate of Me-FH_4–mercaptoethanol methyl transferase activity. This has been determined to be <0.1% of the Me-FH_4–homocysteine transfer under all conditions used in this paper. The K_m values for the substrates are: SAM, 2×10^{-7} M; Me-FH_4, 5×10^{-5} M; and Hom, $\sim 10^{-4}$ M. Me-FH_4-*methyl*-^{14}C and SAM-*methyl*-*t* were purchased from Amersham-Searle. Unlabeled SAM was a product of Mann Research Laboratories. Unlabeled Me-FH_4 was prepared by the method of Keresztesy and Donaldson (1961). Homocysteine thiolactone was a product of General Biochemicals, Inc. *S*-Adenosylhomocysteine was purchased from Boehringer-Mannheim Corp. FMN and dithiothreitol were obtained from Calbiochem. Other chemicals were the best commercially available.

Methods

Methyl group transfer from Me-FH_4 to homocysteine was assayed by the method of Weissbach *et al.* (1963) using small columns of AG 1-X8 Cl anion-exchange resin to separate methionine from Me-FH_4. SAM–homocysteine transmethylation was measured essentially by the method of Taylor and Weissbach (1969b), using CG-50 cation-exchange resin equilibrated with 0.1 M ammonium acetate buffer (pH 5.0). The flavin-reducing system was prepared fresh daily by passing a stream of hydrogen through a solution of 1 mg of FMN/ml of H_2O, in which was suspended 4 mg of PtO_2/ml of H_2O. Assays were run at 37° in the dark under a positive pressure of hydrogen and were initiated by the addition of reduced flavin. Under all conditions used, the amount of product formed was proportional to the time of incubation for at least 20 min and the lag was less than 1 min. Radioactivity in methionine was determined by scintillation counting with an efficiency of approximately 55% for ^{14}C and 13% for ^{3}H. Kinetic figures were plotted using an unweighted least-squares analysis program on an Underwood-Olivetti Programma 101 calculator. When the tracer was the variable component, nonenzymic blanks were run on a point-for-point basis and the unweighted, least-squares line was plotted to obtain the blank values. Each point on the kinetic plots represents the gross observed counts less the blank values. At all values of substrate the blank values did not exceed 15% of the gross radioactivity. The schematic method of King and Altman (1956) as extended by Wong and Hanes (1962) was used to derive the rate laws. Propylation of the enzyme was carried out and analyzed by the method of Taylor and Weissbach (1967b).

Results

The analysis of product inhibition and initial velocity patterns constituted the general experimental strategy for the examination of the interrelationships among Me-FH_4, SAM, and the B_{12} enzyme. Cleland (1963) has observed that the interaction between two substrates of an enzyme-catalyzed reaction, or between a substrate and a product, can be predicted by inspection of plots of reciprocal velocity *vs.* reciprocal substrate concentration in the presence of fixed concentrations of the product, or of the second substrate. For the case of product inhibition, increasing levels of product may raise the slope of the plot, or its intercept, or both. If only the slope is affected, the inhibition is competitive, and the substrate and product are envisioned as reacting with the same enzyme form. If the slope is unaffected, a series of parallel lines results, and the inhibition is called uncompetitive. The substrate is then said to combine with a different enzyme form from that which reacts with the product, and the two forms are separated by one or more irreversible steps. For the purposes of this type of analysis, the addition of a substrate from saturating concentrations, or the release of a product at zero concentration are considered to be irreversible steps. If both the slope and the intercept are affected, the inhibition is noncompetitive, and the substrate and product react with different enzyme forms; these forms, however, are connected by a series of reversible steps through which the two forms can interact.

The interaction between Me-FH_4 and its reaction product, tetrahydrofolate, was analyzed by saturating the enzyme with SAM and homocysteine, while varying the concentration of labeled Me-FH_4 in the presence of several fixed levels of FH_4. Methionine is initially absent. The results, shown in Figure 1a, indicate that FH_4 is a noncompetitive inhibitor of Me-FH_4. The effect on both the slopes of the lines and their intercepts is a linear function of FH_4 concentration. We may conclude that FH_4 and Me-FH_4 react with different, but reversibly connected enzyme forms.

The effect of increasing methionine concentration on Me-FH_4–homocysteine transmethylase is shown in Figure 1b. Me-FH_4 is the variable substrate, SAM and homocysteine are saturating, and FH_4 is initially absent. The pattern of parallel lines dictates the intervention of at least one irreversible step between the addition of Me-FH_4 and the release of methionine from the enzyme.

The results of Figure 1 are consistent with an overall ordered addition, Ping-Pong reaction sequence in which release of FH_4 precedes binding of homocysteine with the formation of an enzyme-bound methylated intermediate.

As previously reported (Burke *et al.*, 1970) the Me-FH_4–homocysteine transmethylase purified from hog kidney also catalyzes methyl group transfer from SAM to homocysteine.

$$\text{SAM} + \text{homocysteine} \longrightarrow \text{methionine} + \text{}$$
$$S\text{-adenosylhomocysteine} \quad (2)$$

In order to assess the importance of reaction 2 to the activation of Me-FH_4–homocysteine transmethylase by SAM, a product inhibition study was undertaken in which label transfer from Me-FH_4-*methyl*-^{14}C to homocysteine was measured as a function of the concentration of SAM in the presence of the products of reaction 2.

In this set of experiments, methyl-labeled Me-FH_4 and homocysteine were held at saturating levels, and SAM was varied in the presence of fixed concentrations of SAH. Methionine is initially absent. The results, as indicated in Table I, showed a noncompetitive inhibition of SAM by SAH similar to the kinetic relationship of Me-FH_4 and FH_4, since both the slope and intercept of the plots were increased by the addition of SAH.

The behavior of methionine toward SAM was investigated by varying the concentration of SAM in the presence of fixed levels of methionine, when labeled Me-FH_4 and homocysteine

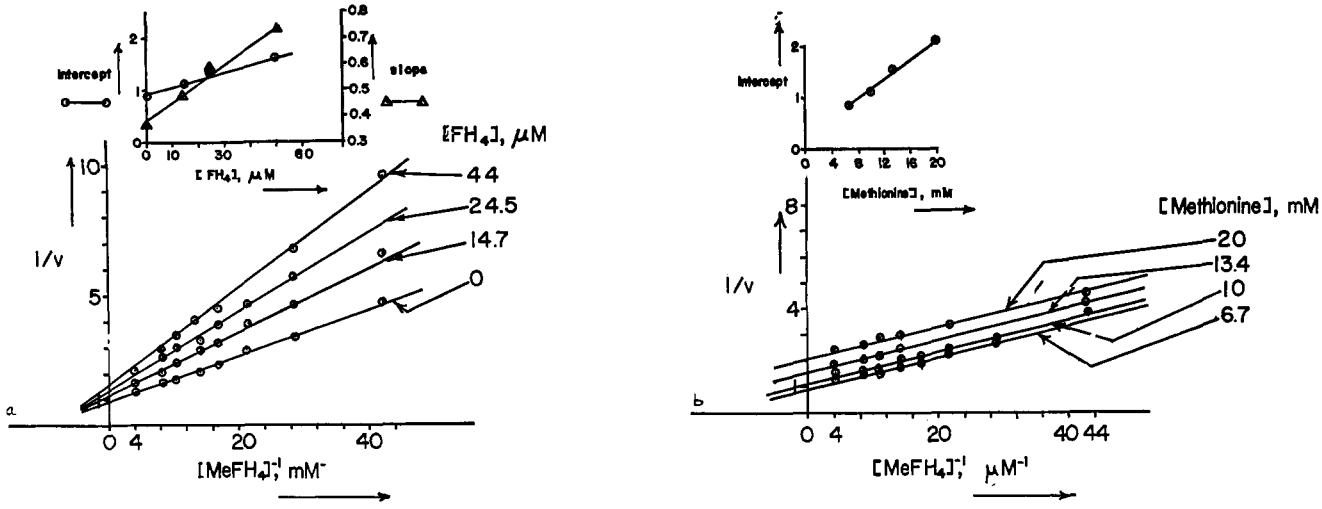

FIGURE 1: Product inhibition of Me-FH_4–homocysteine transmethylase activity as a function of Me-FH_4 concentration. Incubation mixtures, (1.0 ml) contained enzyme, specific activity 2000, 0.06 mg; SAM, 50 nmoles; homocysteine, 10 μmoles; dithiothreitol, 10 μmoles; potassium phosphate buffer (pH 7.4), 100 μmoles; $FMNH_2$, 195 nmoles; and PtO_2, 0.4 mg. Me-FH_4-*methyl*-^{14}C (1 μCi/μmole) and FH_4 or methionine were added as indicated. V = counts per minute as Met $\times 10^{-4}$. Incubations were for 15 min at 37° in the dark. Methionine was determined as described in Methods.

TABLE I: Product Inhibition Patterns for the Transmethylase.

Donor of Labeled Methyl Group	Variable Reactant	Inhibitor	Pattern Obsd	Conditions	
				Saturating	Absent
Me-FH_4	Me-FH_4	FH_4	Noncompetitive	SAM, homocysteine	Methionine, SAH
Me-FH_4	Me-FH_4	Methionine	Uncompetitive	SAM, homocysteine	FH_4, SAH
Me-FH_4	SAM	SAH	Noncompetitive	Me-FH_4, homocysteine	FH_4, methionine
Me-FH_4	SAM	Methionine	Uncompetitive	Me-FH_4, homocysteine	FH_4, SAH
SAM	SAM	Methionine	Uncompetitive	Homocysteine	FH_4, SAH, Me-FH_4
Me-FH_4	Homocysteine	FH_4	Competitive	Me-FH_4, SAM	Methionine, SAH
SAM	Homocysteine	SAH	Competitive	Me-FH_4, SAM	Methionine, FH_4

are saturating, and SAH is initially absent. Increasing methionine raises the intercepts of the lines as a linear function of methionine concentration, but has no effect on the slope of the lines (Table I), which are comparable to the results observed when Me-FH_4 was the substrate.

As described previously (Burke *et al.*, 1970), the hog kidney tramsmethylase is stabilized during purification by the maintenance of high levels (0.01 M) of homocysteine. For this reason, homocysteine is always present in assay incubations. In fact, we have found only a limited range of concentrations of homocysteine over which the velocity is proportional to added homocysteine. This complicates the direct examination of the effect of varying homocysteine on the interaction of the other reactants with the enzyme. If, however, Me-B_{12} is an intermediate methyl carrier, generated upon the release of SAH or FH_4, and demethylated by homocysteine, one should observe competitive inhibition of homocysteine by either FH_4 or SAH. Because the high levels of homocysteine already present give rise to curved double-reciprocal plots when $1/v$ is plotted against 1/[exogenous homocysteine], the analysis was undertaken by examining the effect of increasing SAH or FH_4 on the concentration of homocysteine required to saturate the enzyme. If SAH and FH_4 compete with homocysteine for the same enzyme form, an increase in inhibitor concentration would result in an increase in the concentration of homocysteine required to reach apparent saturation. The results of the analysis are presented as plots of reciprocal velocity *vs.* inhibitor concentration, at varying levels of homocysteine (Figure 2). As can be seen, 10 mM homocysteine is essentially saturating in the absence of inhibitor, and both FH_4 and SAH compete with homocysteine. Furthermore, the slope of the lines decreases as a linear function of reciprocal homocysteine concentration.

Taylor and Weissbach (1968) have clearly demonstrated that Me-FH_4 and SAM are both capable of transferring methyl groups to generate enzyme-bound methyl-B_{12} and these results have been confirmed in kidney enzyme preparations (Burke *et al.*, 1970). Furthermore, Stavrianopoulos and Jaenicke (1967) have purified the bacterial enzyme to near homogeneity and their data suggest that the enzyme contains only one B_{12} prosthetic group per enzyme molecule. These observations are explicable on the basis of an initial reaction of SAM and Me-FH_4 with enzyme forms which are unique for these compounds (also *cf.* discussion in Taylor and Weissbach, 1969b). The release of SAH or FH_4 could generate identical methyl-B_{12} enzyme forms. This suggestion is sup-

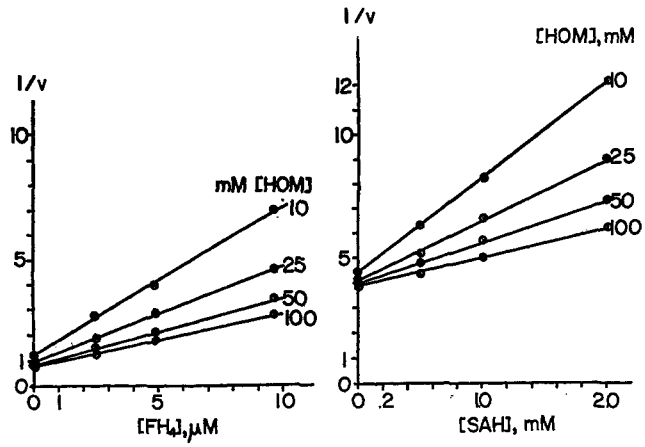

FIGURE 2: Effect of increasing homocysteine on product inhibition patterns. (A) Inhibition of Me-FH_4–homocysteine transmethylase activity by FH_4 at varying levels of homocysteine. Incubation mixtures (1.0 ml) contained homocysteine and FH_4 as indicated, plus enzyme, specific activity 2000, 0.06 mg; Me-FH_4-*methyl*-^{14}C, 1 μCi/μmole, 0.23 μmole; SAM, 50 nmoles; DTT, 10 μmoles; potassium phosphate buffer (pH 7.4), 100 μmoles; $FMNH_2$, 195 nmoles; and PtO_2, 0.4 mg. v = counts per minute as Met $\times 10^{-4}$. Incubations were for 15 min at 37° in the dark. Methionine was determined as described in Methods. (B) Inhibition of SAM–homocysteine transmethylase activity by SAH at varying levels of homocysteine. Incubation mixtures (1.0 ml) contained homocysteine and SAH as indicated, plus enzyme, Me-FH_4–homocysteine transmethylase activity 2000, 0.5 mg; SAM-*methyl-t* 1000 μCi/μmole, 20 nmoles; dithiothreitol, 10 μmoles; potassium phosphate buffer (pH 7.4), 100 μmoles; $FMNH_2$, 195 nmoles; and PtO_2, 0.4 mg. v = counts per minute as Met $\times 10^{-5}$. Incubation was for 15 min at 37° in the dark. Label transfer to methionine was determined as described under Methods.

TABLE II: Conditions for Propylation of Partially Purified N^5-Methyltetrahydrofolate–Homocysteine Transmethylase.[a]

Propylation Mixture	Methionine Formed (μmoles/mg) Light	Dark	% Propylation
Complete system 37°	2.1	0.25	86
Complete system 0°	1.8	1.66	7
Complete system 37°			
+Homocysteine (10 μmoles)	2.4	0.33	85
+Me-FH_4 (195 nmoles)	1.9	0.3	84
+SAM (50 nmoles)	1.9	2.0	0
+SAM (50 nmoles), homocysteine (10 μmoles), and (Me-FH_4 (195 nmoles)	1.8	0.5	73
−$FMNH_2$–PtO_2	1.6	1.45	12
−Dithiothreitol	0	0.1	0 complete inactivation
−Dithiothreitol			
+Homocysteine (10 μmoles)	0.57	0.15	75 partial inactivation

[a] Propylation mixtures contained 28–31 μg of transmethylase, specific activity 8000, or 2.0 μmoles of Met synthesized/mg of enzyme under the above conditions. The complete system contains enzyme; $FMNH_2$, 195 nmoles; PtO_2, 0.4 mg; dithiothreitol, 10 μmoles; potassium phosphate buffer (pH 7.4), 100 μmoles; albumin, 1 mg; and propyl iodide, 8 μmoles added as 20 μl of an ethanolic solution in a total volume of 1.0 ml. Incubation was for 15 min in the dark at 37°. Aliquots were withdrawn after propylation and were analyzed for extent of propylation as described in Methods.

ported by the observed kinetic interaction of SAM and Me-FH_4 with the mammalian enzyme. When the mammalian enzyme was incubated with varying levels of either Me-FH_4 or SAM, in the presence of several fixed concentrations of the other substrate, plots of $1/v$ *vs.* 1/[S] generated a series of parallel lines. The intercept of the lines was a linear function of the reciprocal concentration of the fixed substrate. These results indicate that methyl groups from Me-FH_4 and SAM are initially transferred to distinct enzyme forms.

The relationships among SAM, Me-FH_4, and the enzyme were further investigated with the aid of propyl iodide inactivation studies. The reaction of propyl iodide with Me-FH_4–homocysteine transmethylase, first reported by Brot *et al.* (1965), yields a modified enzyme which is catalytically inactive until it is exposed to visible light. Using this property in their examination of an extensively purified bacterial enzyme, Taylor and Weissbach (1967b) reported that very low levels of SAM present in the propylation mixture could prevent propylation from occurring. Me-FH_4 was a weak inhibitor of propylation but its effectiveness in this regard was markedly improved by the addition of catalytic amounts of SAM. When stoichiometric amounts of homocysteine were present the protective effect of SAM was reversed, allowing propylation to occur (Taylor *et al.*, 1968).

The conditions required for the propylation of the mamalian enzyme, shown in Table II, are rather different from those described for the bacterial enzyme. Albumin was added to prevent denaturation of the very dilute enzyme solution by the gas stream. Although SAM prevents propylation of the mammalian enzyme, Me-FH_4 does not. The concentration of homocysteine introduced into the propylation mixture by the enzyme solution was estimated at about 10^{-4} M (Sedlak and Lindsay, 1968). Clearly, this amount of homocysteine does not prevent SAM from inhibiting propylation. With the bacterial enzyme, in contrast, levels of 6×10^{-8} M homocysteine (approximately stoichiometric with the enzyme) were sufficient to reverse the effect of SAM (Taylor *et al.*, 1968).

The effect of adding SAM to a propylation mixture is shown in Figure 3. As reported for the bacterial enzyme, levels of SAM approximating its K_m for substrate level transmethylation are sufficient to inhibit propylation. Me-FH_4, however, has no effect on propylation in the absence of SAM (Figure 4). In the presence of sufficient SAM to give a partial inhibition of propylation, Me-FH_4 not only fails to further inhibit propylation, it clearly reverses the effect of SAM. Control experiments indicated that this effect was not due to homocysteine nor to 2-mercaptoethanol, which is present in Me-FH_4 preparations.

Figure 5 shows that tetrahydrofolate is able to reverse the effect of SAM on propylation, presumably by removing the methyl group donated by SAM, thus generating an alkylatable B_{12} species. This raised the possibility that the observed effect of Me-FH_4 was an artifact, due to contaminating FH_4 in the Me-FH_4 preparation. Accordingly, our preparation of Me-FH_4 was purified by the method of Rudiger and Jaenicke (1969a); the FH_4 assay described by Rosenthal *et al.* (1965) showed the isolated Me-FH_4 to be free of FH_4. This FH_4-free

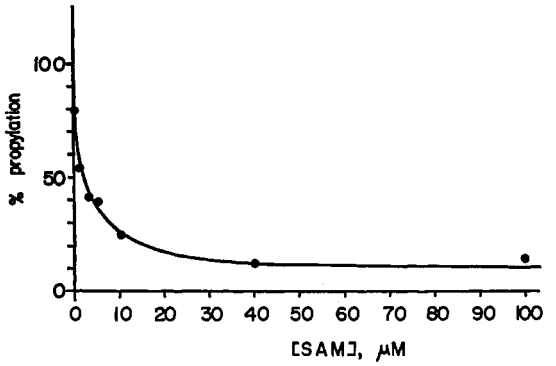

FIGURE 3: Effect of increasing SAM concentration on propylation of Me-FH_4–homocysteine transmethylase. Propylation incubations (1.0 ml) contained enzyme, specific activity 6000, 0.09 mg; DTT, 10 μmoles; potassium phosphate buffer (pH 7.4), 100 μmoles; propyl iodide, 8 μmoles added as 20 μl of an ethanolic solution; $FMNH_2$, 195 nmoles; PtO_2, 0.4 mg; and SAM as indicated. After incubation for 15 min at 37° in the dark, extent of propylation was determined on aliquots of the propylation mixture, as described in Methods.

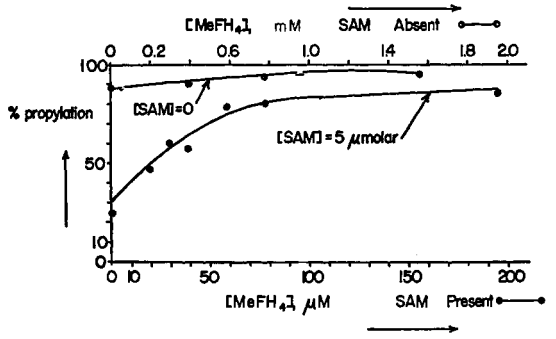

FIGURE 4: Effect of increasing Me-FH_4 concentration on propylation of Me-FH_4–homocysteine transmethylase. Propylation incubations were identical to those used in Figure 3, with the addition of SAM (5 nmoles) and Me-FH_4 as indicated. Extent of propylation was determined as described in Methods.

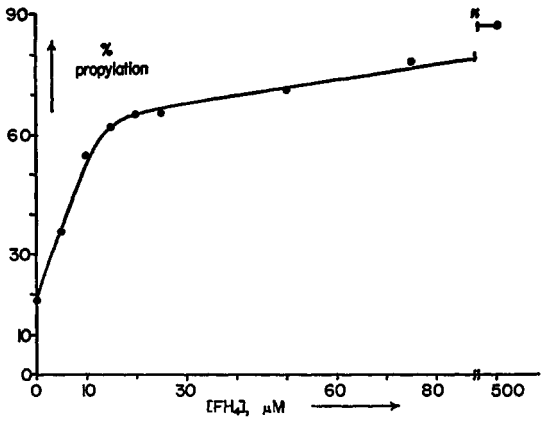

FIGURE 5: Effect of increasing FH_4 concentration on the propylation of Me-FH_4–homocysteine transmethylase, in the presence of SAM. Propylation mixtures are identical to those used in Figure 3, with the addition of SAM (20 nmoles) and FH_4 as indicated. Propylation was determined on aliquots of the propylation mixture as described in Methods.

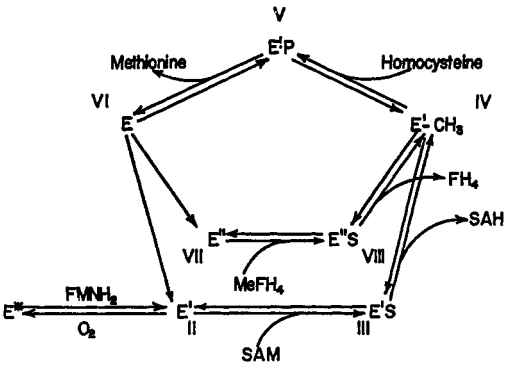

FIGURE 6: Proposed reaction sequence for cobalamin-dependent mammalian Me-FH_4–homocysteine transmethylase.

substrate displayed the same behavior toward SAM-inhibited propylation as that seen in Figure 4.

The propylation experiments require us to conclude that SAM must react with the enzyme prior to the participation of Me-FH_4 and is consistent with the suggestion (Taylor and Weissbach, 1969b) that reaction 2 is responsible for the activation of Me-FH_4–homocysteine transmethylase. Any other reaction scheme would result in the gradual accumulation of propylated enzyme, because the enzyme form which reacts with Me-FH_4 is clearly subject to propylation in the presence of the substrate. Therefore, this form can only be present in negligible amounts prior to the reaction with SAM.

method described by King and Altman (1956) as extended by Wong and Hanes (1962) to the outer loop results in eq 3, in which a, b, c, etc., represent complex collections of constants. When SAH is absent, the equation reduces to eq 4. This expression contains no terms which include both SAM and methionine. Thus, when $1/v$ *vs.* 1/(SAM) is expressed as a double-reciprocal plot, an increase in methionine concentration will affect the intercept, but not the slope of the lines. This predicts that the inhibition should be uncompetitive and this was observed (*cf.* Table I). Methionine is also predicted to be noncompetitive with homocysteine. High levels of endogenous homocysteine precluded a test of this predicition.

If, rather than SAH, methionine is absent, the rate equation

$$v = \frac{V}{1 + a(\text{Met}) + b\frac{(\text{Met})}{(\text{Hom})} + c\frac{(\text{Met})(\text{SAH})}{\text{Hom}} + d\frac{(\text{Met})(\text{SAH})}{(\text{SAM})(\text{Hom})} + e\frac{1}{(\text{SAM})} + f\frac{(\text{SAH})}{(\text{SAM})(\text{Hom})} + g\frac{(\text{SAH})}{(\text{Hom})} + h\frac{1}{(\text{Hom})}} \quad (3)$$

Discussion

In order to accommodate the data presented here, the scheme shown in Figure 6 is proposed for the interaction of SAM, Me-FH_4, and homocysteine with the pig kidney transmethylase. It is convenient to examine each loop of the model independently of the other. The application of the schematic

$$v = \frac{V}{1 + a(\text{Met}) + b\frac{(\text{Met})}{(\text{Hom})} + e\frac{1}{(\text{SAM})} + h\frac{1}{(\text{Hom})}} \quad (4)$$

reduces to eq 5. Since SAH never appears independently of homocysteine in this equation, SAH is a competitive inhibitor

$$v = \frac{V}{1 + e\frac{1}{(\mathrm{SAM})} + f\frac{(\mathrm{SAH})}{(\mathrm{SAM})(\mathrm{Hom})} + g\frac{(\mathrm{SAH})}{(\mathrm{Hom})} + h\frac{1}{(\mathrm{Hom})}} \quad (5)$$

of homocysteine. At very high levels of homocysteine, the expression predicts that SAH will cease to be inhibitory. This behavior is shown in Figure 2. When homocysteine concentration and SAH concentration are of the same order of magnitude (1–10 mM), SAH is a noncompetitive inhibitor of SAM (Table I).

The interaction of Me-FH_4 with the kidney transmethylase, shown schematically as the inner loop of Figure 6, is analogous to the relationship of SAM with the enzyme. Therefore, the kinetic data presented in Figure 1a,b are predicted by analogous reasoning. Methionine is inhibitory toward Me-FH_4 in an uncompetitive manner, while FH_4 is a noncompetitive inhibitor of Me-FH_4. The interaction between FH_4 and homocysteine observed in Figure 2 is also predicted.

The present model includes an irreversible isomerization of the enzyme after the release of methionine. If this step is omitted, methionine and Me-FH_4 could react with the same enzyme form, *cf.* holo enzyme form 3 (Blakley, 1969), and competitive inhibition of Me-FH_4 by methionine would be predicted. The failure of this prediction to be realized experimentally requires the insertion of an irreversible step between the enzyme forms involved. The interaction of methionine and SAH with SAM also includes an irreversible step because methionine displays uncompetitive inhibition (*cf.* Table I) with respect to SAM.

The scheme is also compatible with the extreme variability in the requirement for SAM observed by us (Burke *et al.*, 1970) and others (Rudiger and Jaenicke, 1969b; Taylor and Hanna, 1970b). The distribution between the methylated from IV of the enzyme and the unmethylated forms VI, VII, and II, might be strikingly different from one organism to another, and even within an organism under different metabolic conditions. Any extraction procedure might also alter the distribution. Thus, Rudiger and Jaenicke (1969b) have isolated a transmethylase from *E. coli* which is independent of added SAM until it is incubated with homocysteine. Taylor and Weissbach (1969b) have produced such an enzyme *in vitro* by preincubating their preparation with SAM. These preparations contain methyl-B_{12}, and are thus in form IV. Homocysteine generates form VI, which isomerizes to forms II and VII. The distribution between forms II and VII may be influenced by such factors as reducing conditions, the presence of contaminating materials in the incubations and perhaps several other variables. Consistent with this scheme are the observations that after incubation with homocysteine, both preparations are highly dependent on added SAM for catalytic activity. Taylor (1970) reported the formation of a methyl-^{14}C-B_{12} enzyme upon incubation of the *E. coli* apo enzyme with methyl-^{14}C-B_{12}. This enzyme was capable of transferring all of its label to homocysteine without SAM, but SAM was required to maintain a steady rate of transmethylation from Me-FH_4 to homocysteine.

These data can be explained by a reaction model such as that presented in Figure 6 which indicates than an enzyme which started out predominantly in the methylated form would continually generate enzyme form II at some small, finite rate. This form is viewed as a dead end in the absence of SAM; thus the scheme predicts that catalysis by Me-FH_4–homocysteine transmethylase must eventually cease without SAM. Taylor and Weissbach (1969b) and Taylor and Hanna (1970b), have reported such behavior for the bacterial enzyme.

Relatively few experiments have been reported concerning the transmethylation from SAM to homocysteine in the absence of Me-FH_4; the present model predicts that this activity should be similar in character to the Me-FH_4–homocysteine transmethylation. Notably, in the absence of Me-FH_4, the enzyme should accumulate form VII, which is viewed as a dead end in the absence of Me-FH_4. Thus, SAM–homocysteine methyl transfer should not be independent of Me-FH_4–homocysteine methyl transfer. Rather, small amounts of Me-FH_4 should be required to maintain a steady rate of SAM–homocysteine methyl transfer. Unfortunately, the animal enzyme is not active enough to test this possibility; the rate of catalysis of SAM–homocysteine transmethylation is very low, and SAM is unstable at the alkaline pH required to maintain enzyme activity.

The anomalous behavior of the mammalian enzyme toward propyl iodide can be accomodated by the present model. If forms II and VII could be inactivated by propyl iodide, then both SAM and Me-FH_4 would be expected to protect against propylation. The failure of Me-FH_4 to protect can be explained by assuming that propyl iodide cannot react with form VII but only with forms II and VIII. Because propylation is irreversible in the dark, any form VIII enzyme which appeared in the propylation incubation would be inactivated.

We observed that SAM blocks propylation in spite of the presence of endogenous homocysteine. This is probably due to the preferential formation of the nonpropylatable form VII in the presence of SAM. This conclusion is consistent with the catalytic role of SAM in methionine biosynthesis. The addition of Me-FH_4 to a propylation mixture which contains SAM presumably results in rapid transmethylation from the added Me-FH_4 to endogenous homocysteine, *via* enzyme forms VII, VIII, IV, V, and VI. The net effect is a shift in the distribution of the enzyme forms, increasing the effective concentration of propylatable form VIII.

The reversal of the protective effect of SAM by FH_4 is predicted in a straightforward manner. If FH_4 can combine with enzyme form IV, it will generate the propylatable species VIII. Although there is no direct evidence in the animal system for the recombination of FH_4 with the methyl-B_{12} enzyme, Taylor and Weissbach (1969a) have shown the formation of Me-FH_4 from $^{14}CH_3$-B_{12} enzyme from *E. coli* added FH_4. Rudiger and Jaenicke (1969a) have shown in addition that the bacterial enzyme catalyzes reaction 6 which is consistent with

$$FH_4 + \text{methionine} \longrightarrow \text{Me-}FH_4 + \text{homocysteine} \quad (6)$$

the proposal.

It may be noted that in Table II, we show that propyl iodide is capable of inactivating the animal transmethylase in the presence of all the substrates and cofactors necessary for transmethylation. This contrasts with the behavior of the bacterial enzyme for which data presented by Taylor *et al.* (1968) reported that the complete synthesis mixture protects the enzyme from propylation. The explanation for this difference may lie in the fourfold greater concentration of propyl iodide used in the present experiments.

With the exception of methyl-B_{12}, no absolute chemical identification of any proposed intermediate in Me-FH_4–homocysteine transmethylase has been reported. However, observed interactions of Me-FH_4, SAM, and the mammalian B_{12} trans-

methylase are consistent with the proposal that enzyme form VI contains a reduced form of the prosthetic group. This species would be analogous to holo enzyme form 3 in the model proposed by Blakley (1969). Thus, the enzyme forms II and VII could represent stabilized enzyme forms in which the cobalt is coordinated with amino acid residues near the active site. Among the possibilities for such stabilization are coordination with protein sulfhydryl groups or imidazole groups. For example, the coordination with a protein sulfhydryl group would generate mercapto-B_{12}, a form proposed (Taylor and Weissbach, 1969b; Taylor and Hanna, 1970b) as the prosthetic group in bacterial B_{12} transmethylase which reacts with SAM to form the first molecule of enzyme-bound methyl-B_{12}.

We have noted that the model presented in Figure 6 predicts that Me-FH_4–homocysteine transmethylase activity must eventually cease in the absence of SAM. The absolute requirement for SAM which develops in initially SAM-independent preparations upon incubation with homocysteine (Taylor and Weissbach, 1969b; Rudiger and Jaenicke, 1969b), suggests that the enzyme is ordinarily in form II. A rise in SAM concentration enables the production of form VII with the concommitant utilization of Me-FH_4. If the use of SAM is a physiological feature of the enzyme, and not an artifact, then any condition which could affect local concentrations of SAM would affect the activity of Me-FH_4–homocysteine transmethylase. Loughlin *et al.* (1964) have previously proposed that transmethylation from Me-FH_4 to homocysteine may be the major source of tissue FH_4. Our studies are consistent with this proposal; thus the biochemical basis of the known clinical relationships between folic acid and B_{12} may be the biosynthesis of the nonessential amino acid, methionine. The scheme in Figure 6 suggests a number of ways by which this process could be regulated.

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