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Characterization of Nonradioactive Assays for Cobalamin-Dependent and Cobalamin-Independent Methionine Synthase Enzymes

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Methionine synthase enzymes catalyze methyl group transfer from 5-methyltetrahydrofolate to homocysteine to give methionine and tetrahydrofolate. Assays for this enzyme activity usually monitor transfer of a ^{14}C-methyl group from the N^5-position of methyltetrahydrofolate to homocysteine to produce ^{14}C-methionine that must be purified by anion-exchange chromatography. Alternatively, tetrahydrofolate may be derivatized with a formylating agent under acidic conditions to produce methenyltetrahydrofolate. We report optimization of this reaction for assay of cobalamin-dependent methionine synthase to give an economical method for determining enzyme activity that does not require the use of radioactive compounds. By heating for 10 min in 1 N hydrochloric acid containing 12% formic acid, the enzymatic product tetrahydrofolate is converted into methenyltetrahydrofolate, which absorbs light at 350 nm, while residual substrate 5-methyltetrahydrofolate does not contribute to the absorbance at 350 nm. The assay allows the derivatized product to be characterized *in situ* with a minimal increase in volume upon acidification. The results of the spectrophotometric assay given here have been compared with the radioactive assay to confirm the validity of the derivatization under the assay conditions. We also report the extension of this assay method for use in activity measurements of cobalamin-independent methionine synthase. © 1995 Academic Press, Inc.

Methionine synthase enzymes can be divided into two broad classes of proteins, which lack homology in their primary amino acid sequences (1,2). One class, found in prokaryotes and in mammalian cells, uses a methylcobalamin prosthetic group to catalyze methyl transfer from N^5-methyltetrahydrofolate (CH_3—H_4folate) to homocysteine to give tetrahydrofolate (H_4folate) and methionine, as given in reactions 1 and 2.

$$CH_3\text{—}H_4\text{folate} + E\cdot\text{cob(I)alamin} \rightarrow H_4\text{folate} + E\cdot CH_3\text{—cob(III)alamin} \quad [1]$$

$$E\cdot CH_3\text{—cob(III)alamin} + \text{Homocysteine} \rightarrow E\cdot\text{cob(I)alamin} + \text{Methionine} \quad [2]$$

This activity is also dependent upon the presence of adenosylmethionine and a reducing system to provide electrons for activation and is inhibited in the presence of oxygen. Typically, the assay is conducted in the presence of dithiothreitol or 2-mercaptoethanol and aquo- or cyanocobalamin, which carry out the reduction of oxygen to hydrogen peroxide (3,4). A group of cobalamin-independent enzymes, found in prokaryotes, yeasts, and plants, catalyzes the same reaction, but these enzymes require divalent cations and/or phosphate ions and operate aerobically (1,5). Both enzymes can catalyze methionine and H_4folate biosynthesis using folate polyglutamates with three or more glutamyl residues, but only the cobalamin-dependent enzyme operates efficiently with CH_3—H_4PteGlu$_1$ (6). In this paper, we report the development of an assay optimized for the cobalamin-dependent class of enzymes and its extension to the cobalamin-independent methionine synthase enzyme class.

Both forms of methionine synthase are routinely assayed by following the production of radiolabeled methionine from ^{14}C- or ^{3}H-labeled CH_3—H_4folate, where the label is initially in the methyl group that will be transferred to homocysteine to form methionine (5,7).

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The assay for each enzyme has the advantage of high sensitivity, dependent upon the specific radioactivity of the CH_3—H_4folate used. The assay conditions are substantially different for the two classes of enzyme, but in each case the labeled methionine can be readily separated from unreacted CH_3—H_4folate by passage over an anion-exchange column that binds the folates but allows for passage of methionine (5,7). ^{14}C-Methyltetrahydrofolate monoglutamate (CH_3—$H_4PteGlu_1$) is commercially available, facilitating assay of the cobalamin-dependent enzyme. However, methyltetrahydrofolate polyglutamates are not currently available from commercial sources, and the triglutamate derivative routinely used for assay of the cobalamin-independent enzyme must be synthesized from $PteGlu_3$. Both enzymatic and chemical syntheses of the substrate have employed reduction of $PteGlu_3$ to $H_4PteGlu_3$, followed by condensation of H_4folate with radiolabeled formaldehyde to produce labeled N^5,N^{10}-methylenetetrahydrofolate (CH_2—H_4folate) and then reduction of CH_2—H_4folate to CH_3—H_4folate. Synthesis of $H_4PteGlu_3$ may be accomplished by enzymatic reduction of $PteGlu_3$ (8) or by chemical reduction (5). In the latter case, only 50% of the product is the correct diastereomer, and the cost of the product is proportionately increased and the assay sensitivity proportionately reduced.

The drawbacks to the radioactive assay are many. Among these are the expense of the radioactive substrate and the generation of radioactive waste. During the formation of CH_2—$H_4PteGlu_3$ from formaldehyde and $H_4PteGlu_3$, the condensation reaction is usually driven by an excess of formaldehyde, but this is not a practical approach when radiolabeled formaldehyde is being used, and the yields of product are much lower. Although the enzymatic or chemical synthesis of CH_3—H_4folate is relatively straightforward (8), handling and purification of the radioactive materials are expensive and time-consuming. In addition, the radioactive assay requires a chromatographic workup of each sample to separate the radiolabeled substrate and product.

Nonradioactive assay methods for methionine synthase have been reported for each of the products, methionine and H_4folate. Methionine has been quantitated (9) following derivatization with o-pthalaldehyde, a commonly used reagent for derivatizing amino acids. The derivatized product was quantitated by fluorescence detection, providing a sensitive assay, but the method requires HPLC purification of the derivatized product to distinguish it from other amino acids present. H_4folate has also been quantitated to provide an assay for methionine synthase (10,11) and dihydrofolate reductase (12), both of which produce this product. The H_4folate, but presumably not CH_3—H_4folate or dihydrofolate (H_2folate), was converted to methenyltetrahydrofolate (CH^+=H_4folate) by heating in formic acid (reaction 3).

$$H_4\text{folate} + HCOOH \rightarrow CH^+{=}H_4\text{folate} + 2\ H_2O \quad [3]$$

CH^+=H_4folate has a strong absorbance at 350 nm, with an extinction coefficient of 26,500 M^{-1} cm^{-1} (13), providing the basis for detection of H_4folate after derivatization. Despite citations describing the conversion of H_4folate to CH^+=H_4folate to measure enzyme activities of methionine synthase and dihydrofolate reductase (10–12), the assay procedure has not been characterized, nor have the results been compared with other, more widely used assays.

MATERIALS AND METHODS

Materials. (6*R*,*S*)-5-Methyltetrahydrofolate (calcium salt), (6*R*,*S*)-tetrahydrofolate (dihydrochloride), and pteroyltriglutamate ($PteGlu_3$) were purchased from Schircks Laboratories (Jona, Switzerland). [*Methyl*-^{14}C]CH_3—H_4folate, provided as the barium salt, was purchased from Amersham. *S*-Adenosylmethionine, dithiothreitol, aquocobalamin, and L-homocysteine thiolactone were purchased from Sigma Chemical Co.

Preparation of reagents. Solutions of (6*R*,*S*)-tetrahydrofolate (H_4folate) were prepared by dissolving the solid dihydrochloride salt in 100 mM potassium phosphate buffer containing 10 mM dithiothreitol. The concentration was determined by measuring the absorbance at 297 nm (ϵ = 29,100 M^{-1} (14)) after subtracting the contribution of the buffer solution.

(6*R*,*S*)-N^5-Methyltetrahydrofolate (CH_3—H_4folate) was prepared as a 4.2 mM solution in 8 mM sodium ascorbate to protect it from oxidation. [*Methyl*-^{3}H]-CH_3—$H_4PteGlu_3$ was synthesized as described previously (15) and diluted to 22,000 dpm/nmol for use in the radioactive assay. Aquocobalamin was prepared as a 5 mM stock in a 1:1 (v/v) solution of ethanol and water for storage. This stock was diluted to 500 μM with water for use in the assay. *S*-Adenosylmethionine (iodide salt) was stored at 3.8 mM in 1 mM HCl for stability.

L-Homocysteine thiolactone (150 mg) was converted to free homocysteine by dissolving the solid in distilled water (5.0 ml) that had been bubbled with nitrogen to remove dissolved oxygen. Thiolactone hydrolysis was initiated by adding 0.2 ml of 0.8 M NaOH and incubating the solution for 6 min at 45°C under an atmosphere of nitrogen. The reaction was terminated by addition of 0.25 ml of 0.4 M HCl, and the pH of the solution was adjusted to 5, where homocysteine is stable. The nominal titer of this homocysteine stock is 100 mM, and the actual concentration of each preparation was determined by titration with 5,5′-dithiobis-2-nitrobenzoic acid (16).

A stock solution for acid quenching and derivatization of H_4folate was prepared by slow addition of 41.6 ml of concentrated HCl (12 N) to 58.4 ml of 88% formic acid.

Sources of enzymes. Recombinant cobalamin-dependent methionine synthase (the *metH* gene product) from an overexpressing strain was purified to homogeneity from *Escherichia coli* as previously described (17). Recombinant cobalamin-independent methionine synthase from *E. coli* (the *metE* gene product) was prepared from an overexpressing strain as described by González *et al.* (2).

Procedure for assay of cobalamin-dependent methionine synthase. The assays are routinely performed in duplicate and the absorbance values averaged. In a total volume of 800 μl, the stock solutions described above are combined in 1 × 10-cm Pyrex test tubes. Glass-distilled water (544 μl, less the volume of enzyme to be added) is combined with the following reagents in the order given: 1 M potassium phosphate buffer, pH 7.2 (80 μl), 500 mM dithiothreitol (40 μl), 3.8 mM *S*-adenosylmethionine iodide (4 μl), and 100 mM L-homocysteine (4 μl). The enzyme solution to be assayed is then added. Each set of assays should be performed with a blank, from which enzyme is initially omitted. If desired, enzyme can be added to the blank after the acid quench. Aquocobalamin (500 μM, 80 μl) is added to each tube to initiate enzyme activation, and the tube is placed in a water bath maintained at 37°C. Along with dithiothreitol to provide electrons, aquocobalamin catalyzes the reduction of oxygen to hydrogen peroxide (3,4) to provide an anaerobic assay environment. From this moment forward, timing is critical to the assay; the time required for activation of methionine synthase must be balanced against slow enzyme inactivation due to buildup of hydrogen peroxide. After exactly 5 min, 250 μM (6*R*,*S*)-CH_3—H_4folate (48 μl) is added to initiate turnover. For routine assays, a fixed incubation time of 10 min instead of the time course described below was employed. The value for the product H_4folate concentration must fall within the linear range of the assay, optimally within an absorbance reading of 0.1 to 1.0 absorbance units at 350 nm. Two hundred microliters of acidic derivatization solution (5 N HCl in aqueous 60% formic acid) is added to quench the turnover, bringing the volume to 1.0 ml, and the tubes are heated at 80°C for 10 min. H_4folate is not stable in acid (18), and therefore the assay should be heated to form methenyltetrahydrofolate (CH^+=H_4folate) as soon as possible. Solutions were cooled to room temperature and the absorbance was measured in a Perkin–Elmer Lambda 4C spectrophotometer. After acid quenching the aerobic solutions are stable for several hours, and measurements are made within 2 h of quenching.

When radioactive assays of cobalamin-dependent methionine synthase were performed, the protocol of Taylor and Weissbach (7), as modified by Drummond *et al.* (19), was employed.

Procedure for the assay of cobalamin-independent methionine synthase (MetE). The assay mixture for this enzyme contains 10 mM potassium phosphate buffer, pH 7.2, 50 mM Tris chloride buffer, pH 7.2, 100 μM magnesium sulfate, 10 mM dithiothreitol, 2 mM homocysteine, and 66 μM (6*S*)-CH_3—$H_4PteGlu_3$ in a total volume of 400 μl, including enzyme. After equilibration at 37°C for 5 min, assays were initiated by addition of enzyme and were incubated for an additional 5 min before quenching with 100 μl of 5 N HCl/60% formic acid, as described above. If crude extracts were used as the source of enzyme, acid quenching resulted in considerable protein precipitation, and it was necessary to centrifuge the quenched assay solutions for 5 min in an Eppendorf centrifuge at 11,000*g* before measuring the absorbance at 350 nm.

The (6*S*)-CH_3—$H_4PteGlu_3$ was prepared from 20 μmol of $PteGlu_3$ by a modification of the general procedure of Matthews (8). Instead of purifying the CH_3—$H_4PteGlu_3$ on DEAE-52 cellulose, a Pharmacia Mono-Q FPLC anion-exchange column (HR 10/10) was used. The column was equilibrated with buffer A (50 mM ammonium acetate containing 10 mM 2-mercaptoethanol and adjusted to pH 7.2 with ammonium hydroxide). After equilibration, the CH_3—$H_4PteGlu_3$-containing solution (80 ml) was filtered and loaded on the column using a 50-ml superloop. The column was washed with 100 ml of buffer A at a flow rate of 5 ml/min, and the CH_3—$H_4PteGlu_3$ was eluted with a 300-ml linear gradient of buffer B (buffer A + 1 M NaCl). Five-microliter fractions were collected, and the major peak eluting at ~40% buffer B was identified as CH_3—$H_4PteGlu_3$ by its ultraviolet absorbance spectrum (peak at 292 nm). The pooled fractions containing CH_3—$H_4PteGlu_3$ (~20 ml) were desalted using C18 Sep-pak cartridges (Millipore). The cartridge was washed with 10 ml of methanol, 10 ml of water, and then 10 ml of buffer C (10 mM acetic acid and 10 mM 2-mercaptoethanol in water). Approximately 10 ml of the pooled CH_3—$H_4PteGlu_3$ (no more than 8 μmol) was slowly loaded on the cartridge. The cartridge was washed with 3 ml of buffer C, and the product was eluted with 2.5 ml of buffer D (10 mM acetic acid and 10 mM 2-mercaptoethanol in methanol) followed by 2.5 ml of buffer C. The procedure was repeated using the remainder of the CH_3—$H_4PteGlu_3$. The product fractions were pooled, diluted twofold with water, frozen, and lyophilized. The resulting white powder was dissolved in 10 ml of buffer A, and the concentration was determined from the absorbance at 292 nm using a molar extinction coefficient of 31,700 M^{-1} cm^{-1} (20). A typical preparation resulted in a yield of 15.4 μmol

(77% overall yield). CH_3—$H_4PteGlu_3$ (200–300 μM) dissolved in buffer A was stable to storage at −80°C for at least 1 year.

Procedure for the preparation of crude homogenates of pig liver and for assay of cobalamin-dependent methionine synthase in pig liver homogenates. Diced pig liver (75 g) was mixed with 50 ml of 50 mM potassium phosphate buffer, pH 7.2, containing 62.5 μl of a 2 mg/ml solution of Aprotinin (Sigma) and 0.625 mg of soybean trypsin inhibitor (Sigma). The mixture was homogenized in a Waring blender for two 1-min pulses and then centrifuged for 1 h at 39,200*g*. The supernatant was decanted, filtered through cheesecloth, and then used for assays without further purification. The procedures for assaying using radioactive or nonradioactive methods were identical to those described for the cobalamin-dependent enzyme from *E. coli*. After acid quenching, assay solutions were centrifuged for 5 min in an Eppendorf centrifuge at 11,000*g* before measuring the absorbance at 350 nm. In all cases, a blank reaction was run from which CH_3—H_4folate was omitted and then added after the acid quench, and the values reported for each assay were corrected by subtraction of the appropriate blank.

RESULTS

To assay methionine synthase activity on the basis of H_4folate production by conversion to CH^+=H_4folate, several conditions must be met. The H_4folate must be reproducibly derivatized at concentrations that are formed in the assay, while the CH_3—H_4folate substrate must not contribute to the absorbance at the wavelength where the assay is performed. The product, CH^+=H_4folate, must produce a spectral signal that can be easily monitored at the concentrations produced in the assay, and this signal must vary linearly with the amount of H_4folate present. The components of the assay must not interfere with the absorbance measurements. A number of these requirements have been directly assessed using stock solutions of tetrahydrofolate to characterize the chemical reaction.

Figure 1 shows the near ultraviolet absorbance changes associated with the formation of CH^+=H_4folate from H_4folate under the assay conditions used for cobalamin-dependent methionine synthase. The absorbance changes result from the introduction of increasing amounts of H_4folate into the anaerobic assay solution. The spectrum with the least absorbance at 350 nm contains no H_4folate, and it represents the spectrum of the assay components including the degraded aquocobalamin. The remaining spectra result from incremental increases in the amount of H_4folate added to the assay.

When the absorbance at 350 nm is plotted against the concentration of added H_4folate (Fig. 2), it is apparent that the yield of CH^+=H_4folate is about 90%, somewhat better than the value of 80% determined previously by Scrimgeour (21). It is not clear whether the efficiency of the chemical conversion of H_4folate to CH^+=H_4folate is 90%, as suggested by the plot, or whether the tetrahydrofolate is impure. Even as a solid, tetrahydrofolate decomposes slowly unless stored under rigorously anaerobic conditions and is subject to decomposition once the vessel is opened. As indicated in Fig. 2, the yield of CH^+=H_4folate is linearly related to the concentration of H_4folate in the assay buffer over the concentration range from 0 to 43 μM.

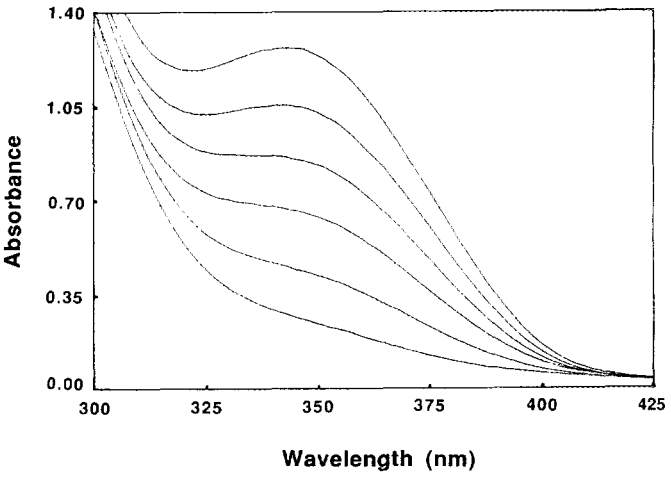

FIG. 1. Absorbance changes associated with the conversion of H_4folate to CH^+=H_4folate under conditions used for assay of cobalamin-dependent methionine synthase (MetH). (6-*R*,*S*)$H_4PteGlu_1$ (purchased from Schircks Laboratories) was added to the assay buffer used for assay of cobalamin-dependent methionine synthase (MetH) to produce final H_4folate concentrations ranging from 0 to 43 μM in increments of 4.3 μM. This assay mixture contains 100 mM potassium phosphate buffer, pH 7.2, 25 mM dithiothreitol, and 50 μM aquocobalamin. After incubation at 37°C for 10 min, the individual assays were quenched with formic acid/HCl as described under Materials and Methods and incubated at 80°C for 5 min, and then the absorbance spectra were measured. Increasing absorbance at 350 nm was observed as the concentration of $H_4PteGlu_1$ in the assay was increased; the spectra shown were obtained for H_4folate concentrations of 0, 8.6, 17.2, 25.8, 34.4, and 43.0 μM.

In Fig. 3, cobalamin-dependent methionine synthase activity was measured using the nonradioactive assay reported here and commercially available unlabeled CH_3—$H_4PteGlu_1$ from Schircks Laboratories or alternatively using the radioactive assay that is commonly used for enzyme assay (7,19) and commercially available [5-*methyl*-^{14}C]CH_3H_4folate (Amersham, Inc.). The labeled CH_3—H_4folate was diluted with unlabeled CH_3—H_4folate to a final specific radioactivity of ~2000 dpm per nanomole. The radioactive assay measures the conversion of [5-*methyl*-^{14}C]CH_3—H_4folate to ^{14}C-labeled methionine and requires separation of the labeled substrate and product by anion-exchange chro-

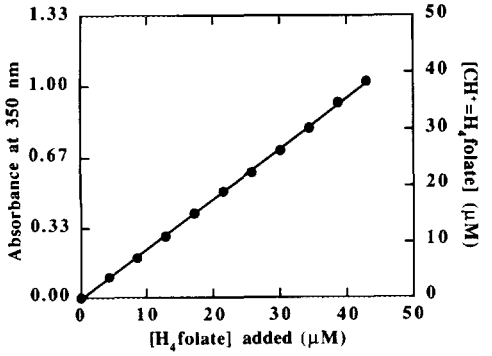

FIG. 2. Yield of $CH^+ = H_4$folate formed in the experiment shown in Fig. 1. The concentration of $CH^+ = H_4$folate formed by acid quenching of the individual assays was determined from the $\Delta\epsilon$ at 350 nm using a molar extinction coefficient of 26,500 M^{-1} cm^{-1} (13).

matography on microcolumns. As shown here, the yield of product methionine determined by the radioactive assay is identical to the yield of product H_4folate determined for the nonradioactive assay by conversion to $CH^+ = H_4$folate. When a similar comparison of the two methods was made using [5-*methyl*-^{14}C]CH_3—H_4folate for both types of assay, the yields of product in the two methods were again identical. These finding suggest that both radioactive and nonradioactive assays determine product formation with about 90% efficiency, with losses in the former case occurring during the chromatographic separation of labeled methyltetrahydrofolate substrate and methionine product.

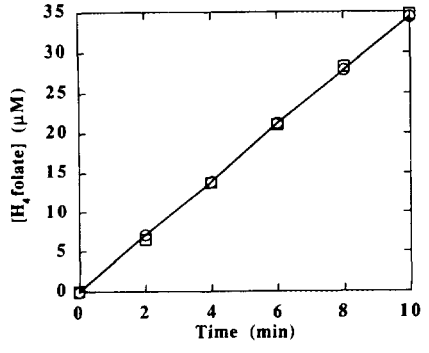

FIG. 3. Comparison of the radioactive (circles) and nonradioactive (squares) assays of cobalamin-dependent methionine synthase (MetH). The radioactive assay measures the conversion of labeled [5-*methyl*-^{14}C]methyltetrahydrofolate (1300 dpm per nanomole) to labeled methionine as described under Materials and Methods. Assay cocktails contained a total volume of 800 μl and were diluted with 200 μl of cold water before separation of the labeled substrate and product by anion-exchange chromatography. The nonradioactive assay measures the absorbance at 350 nm after conversion of H_4folate product to $CH^+ = H_4$folate as described under Materials and Methods. An 800-μl aliquot of the nonradioactive assay was quenched with 200 μl of acid quench solution. The same stock of [5-*methyl*-^{14}C]CH_3—H_4folate was used for both experiments, so that the assays could be compared. The concentrations of product (methionine or $CH^+ = H_4$folate) have been corrected for dilution of the initial 800-μl assay to 1000 μl.

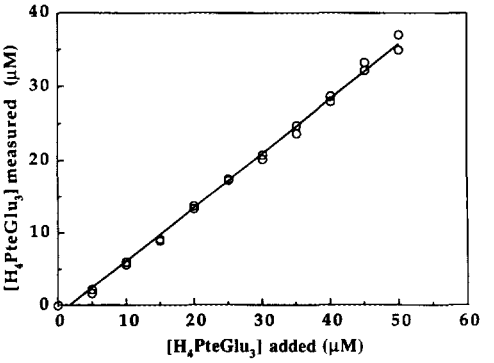

FIG. 4. Standardization of the yield of $CH^+ = H_4PteGlu_3$ formed under assay conditions for cobalamin-independent methionine synthase (MetE). Portions of $H_4PteGlu_3$ were added to the assay buffer used for MetE activity measurements, which contains 50 mM Tris chloride buffer, pH 7.2, 100 μM magnesium sulfate, 10 mM dithiothreitol, and 2 mM homocysteine. After incubation at 37°C for 10 min, the individual assays were quenched with formic acid/HCl as described under Materials and Methods and incubated at 80°C for 5 min, and then the absorbance spectra were measured. The concentration of $CH^+ = H_4PteGlu_3$ formed by acid quenching of the individual assays was determined from the $\Delta\epsilon$ at 350 nm using a molar extinction coefficient of 26,500 M^{-1} cm^{-1} (13).

It is important to recognize that both the yield and the effective linear range of the assay are likely to vary with the experimental conditions prior to derivatization, and assays should contain thiols or ascorbate to stabilize the H_4folate. Standard curves of the type shown in Fig. 2 should be prepared for each set of assay conditions. Furthermore, yields may change as an enzyme is purified from crude extracts, so that standard curves should be run at each stage of a purification.

Figure 4 shows standard curves for $H_4PteGlu_3$ under the conditions used to assay cobalamin-independent methionine synthase (MetE). The $H_4PteGlu_3$ used in these experiments was produced by demethylation of an equivalent amount of CH_3—$H_4PteGlu_3$ in the presence of a large excess of MetE. The slope of the line in Fig. 4 indicates that 74% of the added $H_4PteGlu_3$ is recovered as $CH^+ = H_4PteGlu_3$ under the conditions of the MetE assay. Figure 5 compares the results of measurements of cobalamin-independent methionine synthase activity using the radioactive and nonradioactive assays. The slope of the line indicates that the nonradioactive assay detects $CH^+ = H_4PteGlu_3$ at 85% of the level of the methionine detected in the radioactive assay. These results are in excellent agreement with the assumption that the efficiency of methionine detection in the radioactive assay is ~90% (see above). The K_m for CH_3—$H_4PteGlu_3$ in the MetE assay is reported to be 4.7 μM (5), and enzyme assays are conveniently determined in the presence of 66 μM CH_3—$H_4PteGlu_3$

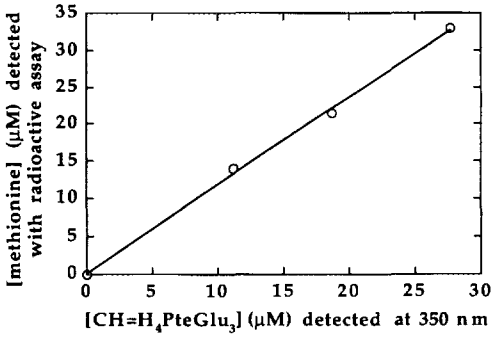

FIG. 5. Comparison of the radioactive and nonradioactive assays of cobalamin-independent methionine synthase (MetE). The radioactive assay measures the conversion of labeled [5-*methyl*-^{3}H]-CH_3—$H_4PteGlu_3$ (22,000 dpm per nanomole) to labeled methionine as described under Materials and Methods. Assay cocktails contained a total volume of 50 μl and were diluted with 950 μl of cold water before separation of the labeled substrate and product by anion-exchange chromatography. The nonradioactive assay measures the absorbance at 350 nm after conversion of $H_4PteGlu_3$ product to CH^+=$H_4PteGlu_3$ as described under Materials and Methods. A 400-μl aliquot of the same assay cocktail used for the radioactive assay was quenched with 100 μl of acid quench solution and heated at 80°C for 5 min, and then the absorbance of CH^+=$H_4PteGlu_3$ was measured at 350 nm. The concentrations of product (methionine or CH^+=$H_4PteGlu_3$) have been corrected for dilution of the initial assay mixtures during the workups.

under conditions where less than 30% of the substrate is consumed (2). The sensitivity of the nonradioactive assay is adequate for determination of the rate of product formation under these conditions. At the specific radioactivity of [*methyl*-^{14}C]CH_3—$H_4PteGlu_1$ normally employed for the radioactive assay (2000 dpm/nmol) the sensitivity of the radioactive and nonradioactive assays is approximately the same; formation of 5 μM product can readily be detected in a fixed-time assay, while rates of product formation as low as 0.2 μM/min can be detected if a series of samples are removed over a period of 10 min (see below).

The nonradioactive assay is also suitable for the detection of cobalamin-dependent methionine synthase in crude extracts of pig liver. Enzyme activity in pig liver homogenates is very low (10.0 nmol CH^+=H_4folate formed per minute per milliliter of homogenate as measured with the nonradioactive assay; 10.3 nmol methionine formed per minute per milliliter of homogenate measured with the radioactive assay). Product formation was shown to be dependent on the addition of AdoMet to the assay, as expected for measurement of cobalamin-dependent methionine synthase activity. Product formation is linear over 12 min and also varies linearly with the amount of enzyme added over the range of homogenate volumes from 20 to 60 μl (data not shown). Thus, the formation of product at a rate of 0.2 nmol per minute can be reliably detected by this assay. The nonradioactive assay appears to be suitable for the measurement of methionine synthase activity during purification of the enzyme from mammalian liver.

DISCUSSION

In this paper, we report the refinement and characterization of a nonradioactive assay for cobalamin-dependent methionine synthase that is based on the conversion of the H_4folate product to CH^+=H_4folate and measurement of the absorbance of the derivatized product at 350 nm. This assay method has also been adapted for measurements of the activity of cobalamin-independent methionine synthase.

The method for conversion of H_4folate to CH^+=H_4folate described in this communication substantially reduces the volume required for each assay determination when compared to the reported methodologies for conversion of H_4folate to CH^+=H_4folate (10,21), providing a corresponding increase in the sensitivity of the assay. Previously, aqueous solutions containing H_4folate were diluted with approximately 9 vol of concentrated formic acid and derivatized by heating to 100°C for 5 min. We have found that the formic acid can be replaced with 1 N HCl containing 12% formic acid without affecting the yield of the conversion. In addition to reducing the amount of formic acid needed for each determination, the assay volume is reduced roughly 10-fold, since hydrochloric acid and formic acid can be added in a combined, concentrated stock. Because the product is quantitated by the absorbance change at 350 nm, minimizing the assay volume effectively increases the sensitivity of the method.

The nonradioactive assay method has the advantages of being substantially cheaper than the radioactive assay method and avoiding the generation of radioactive wastes. The cobalamin-independent methionine synthase activity has been particularly difficult to measure, since the radiolabeled CH_3—$H_4PteGlu_3$ substrate is not commercially available. In addition, the new method is considerably easier to carry out than the radioactive method, and large numbers of samples can be processed at one time, permitting detailed kinetic analyses to be performed.

The sensitivity of the assay method is suitable for measurement of MetH and MetE enzyme activities in crude extracts from *E. coli* strains that overproduce these proteins and for measurement of cobalamin-dependent methionine synthase activity in homogenates of mammalian liver. Thus, it can be used to monitor routine purifications of these enzymes. The method we have described should be suitable for the assay of other enzymes that convert CH_3—H_4folate or methyltetrahydropterin derivatives to H_4folate or tetrahydropterins, such as the corrinoid Fe/S protein methyltransferase (22) or methylenetetrahydrofolate

reductase (15,23). The assay should also be adaptable for measurement of thymidylate synthase activity, since the substrate CH_2—H_4folate can be converted to CH^+=H_4folate by quenching with formic acid/HCl, while the product H_2folate should not be derivatized by this procedure.

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