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A Nonradioactive Assay for N^5-Methyltetrahydrofolate–Homocysteine Methyltransferase (Methionine Synthase) Based on *o*-Phthaldialdehyde Derivatization of Methionine and Fluorescence Detection

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The enzyme N^5-methyltetrahydrofolate–homocysteine methyltransferase (methionine synthase, EC 2.1.1.13) catalyzes the conversion of homocysteine to methionine in the presence of a reducing system. N^5-Methyltetrahydrofolate serves as a methyl donor in this reaction. An assay for the enzyme is described, which is based on methionine quantitation by *o*-phthaldialdehyde (OPA) derivatization and reversed-phase liquid chromatography. The enzymatic reaction is linear for at least 120 min under reducing conditions (125 mM 2-mercaptoethanol) and running the assay below an oil layer. This reducing system does not interfere with formation of the methionine–OPA adduct, which is separated from interfering compounds and an internal standard (norvaline) by a mobile phase adjusted to pH 5.0. The inclusion of internal standard increases the precision of the assay and corrects for the variable fluorescence yield due to occasional inaccurate pH adjustment before the derivatization step. Norvaline was suitable for this purpose because it elutes close to methionine and is not a natural amino acid present in biological extracts. This nonradioactive assay for methionine synthase was evaluated by comparison with a conventional method based on isolation of radioactive methionine by anion-exchange chromatography and by determination of enzyme activity in extract from cultured cells and liver.

The cobalamin-dependent enzyme N^5-methyltetrahydrofolate–homocysteine methyltransferase (methionine synthase, EC 2.1.1.13) catalyses the transfer of a methyl group from N^5-methyltetrahydrofolate to homocysteine. The products are methionine and tetrahydrofolate. The enzyme operates at the point of convergence of folate metabolism and the transmethylation/transsulfuration pathway (1).

The interest in methionine synthase is motivated not only by its centrality in metabolic regulation (1,2) but also by the involvement of the enzyme reaction in several processes related to human disease (3–6).

Most studies on methionine synthase are based on the assay developed by Weissbach *et al.* (7) which measures the formation of [^{14}C]methionine from N^5-[*methyl*-^{14}C]methyltetrahydrofolate, after the separation of product and substrate on an anion-exchange column. In one modification (8), radioactive methionine is quantitated following conversion to methylthiocyanate by cyanogen bromide, and in another (9) N^5-[*methyl*-^{14}C]methyltetrahydrofolate is adsorbed to methionine-coated charcoal. A reducing system is required for maximal activity and this is obtained by inclusion of NADPH (7), $FADH_2$ (9–11), 2-mercaptoethanol, (8,9,12–16), glutathione (17), or dithiothreitol (18–20) in the assay mixture. The incubation is often performed under an anaerobic atmosphere (7,11,13,14,16).

Use of N^5-[*methyl*-^{14}C]methyltetrahydrofolate as a substrate in the methionine synthase assays may cause some problems since this reagent is unstable, expensive, and not always available from the manufacturers. In addition, there is the general problem of disposal of radioactive material. One assay for methionine synthase based on measurement of unlabeled methionine has been published, but a cumbersome microbiological method was used (10).

Precolumn derivatization followed by separation on a reversed-phase column and fluorescence detection has become increasingly popular during the past few years as a method for the determination of amino acids including methionine (21,22). This method is sensitive

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and precise, but instability of the *o*-phthaldialdehyde (OPA)[1] adducts (23) is a major drawback since the chromatography step must be carried out within a short and constant time after derivatization to obtain reproducible results. This limits the general utility of the reaction and severely restricts the sample output. However, during the recent years several autosamplers for HPLC have been introduced on the market. They carry out complex sample handling prior to injection and open up the possibility of using the OPA reaction in enzymological studies.

The present paper describes the adaption of an OPA method for determination of methionine in a nonradioactive assay for methionine synthase. 2-Mercaptoethanol was selected as the reducing agent because it is a constituent of the OPA reagent (22). The mobile phase was optimized for quantitation of methionine, and norvaline was used as an internal standard to correct for instability of reagents (22) or adducts (23). The precolumn derivatization and the injection were carried out automatically using a Gilson 232 HPLC autosampler. The performance of this assay compares favorably with that of the conventional assay.

MATERIALS AND METHODS

Chemicals

(±)-L-N^5-Methyltetrahydrofolate (barium salt), cyanocobalamin, L-norvaline, L-methionine, *S*-adenosyl-L-methionine (iodide salt), and *o*-phthaldialdehyde were from Sigma Chemical Co. (St. Louis, MO). 2-Mercaptoethanol (p.a.) and methanol (gradient grade) were from Merck (Darmstadt, FRG). DL-Homocysteine and bis(3,5,5-trimethylhexyl)phthalate were obtained from Fluka Chemie AG (Bucks, Switzerland). Bio-Rad AG 1-X8 resin, 200–400 mesh, chloride form, were from Bio-Rad Laboratories (Richmond, CA). Columns (0.46 × 25 cm) for reversed-phase liquid chromatography were packed with 5 μm ODS Hypersil from Shandon Southern Products (Chesire, UK) as described previously (24). Flo-Scint II scintillation fluid was obtained from Packard Instruments B.V. (Groeningen, The Netherlands).

Stock solutions of DL-homocysteine and unlabeled (±)-L-N^5-methyltetrahydrofolate were prepared immediately before use.

[*methyl*-^{14}C]Methionine (55 mCi/mmol) was purchased from NEN Research Products (Boston, MA) and (±)-L-N^5-[*methyl*-^{14}C]methyltetrahydrofolate (54 mCi/mmol; barium salt) was from Amersham (Buckinghamshire, England). (±)-L-N^5-[*methyl*-^{14}C]Methyltetrahydrofolate was dissolved in 10 mM ascorbic acid and stored as 200-μl aliquots under nitrogen at −80°C until use.

The OPA reagent was prepared as follows: 1 ml of OPA solution (56 mM in methanol) was mixed with 9 ml of sodium borate buffer (0.1 M, pH 9.5), and the mixture was supplemented with 40 μl of 2-mercaptoethanol immediately before use.

Instruments

The Gilson Model 232-401 automatic sample processor and injector was manufactured by Gilson Medical Electronics S.A. (Villiers le Bel, France). It was programmed to carry out the sample derivatization and the sample injection. The injector was equipped with a 500-μl sample loop. The ODS Hypersil column was mounted in a column heater which was set at 40°C. The fluorescence was detected with a SFM 25 fluorescence detector from Kontron (Zürich, Switzerland). The excitation and emission wavelengths were adjusted to 336 and 450 nm, respectively. The signal was recorded with a SP 4290 integrator from Spectra Physics (San Jose, CA). A Flo One Model A-100 HPLC scintillation detector (Radiometric Instruments & Chemical Co. Inc., Tampa, FL) was connected in series with the fluorescence detector, and the signal was recorded by an Epson LX-400 printer. Both the integrator and the printer were started by a signal from the Gilson autosampler.

Preparation of Liver and Cell Extracts

Rat liver. Outbred Mol:Wist rats were sacrificed, and the livers were removed and immediately homogenized (1:5, w/v) in ice-cold 50 mM potassium phosphate buffer, pH 7.4, containing 0.1 M NaCl (homogenization buffer), using a Potter S homogenizer (B. Braun Melsungen AG, Germany). The homogenate was centrifuged at 1000*g* for 10 min at 4°C, and the supernatant further centrifuged at 24,000*g* for 30 min at 4°C and then at 100,000*g* for 1 h at 4°C. The cytosol fraction thus obtained was stored at −80°C until use. Immediately before the methionine synthase assay the liver extract was passed through a Sephadex G-25 column (0.6 × 8 cm) equilibrated and eluted with 50 mM potassium phosphate buffer, pH 7.4.

Cultured cells. Cells were grown at 37°C in an atmosphere of 5% CO_2 in air and a relative humidity of 95%. Stock culture of chemically transformed C3H/10T1/2 Cl 16 (25) mouse embryo fibroblasts was maintained as described previously (26). The cells were seeded in tissue culture dishes (10 cm, Falcon, Becton Dickinson & Co., NJ) in basal medium Eagle (BME, Flow Laboratories, Irvine, Scotland) supplemented with 10% heat-inactivated fetal calf serum (Biological Industries, Beth Haemek, Israel). Late in exponential growth phase the medium was removed and the cells

[1] Abbreviations used: OPA, *o*-phthaldialdehyde; BME, basal medium Eagle; PBS, phosphate-buffered saline; DTT, dithiothreitol; CV, coefficient of variation.

gently washed twice with ice-cold phosphate-buffered saline (PBS). The cells were then harvested either by adding 200 μl of homogenization buffer to each dish and gently scraping the cells off the dish with a rubber policeman, or by trypsinization. The trypsinized cells were centrifuged and washed twice with ice-cold PBS. After resuspension in homogenization buffer, the cells were equilibrated with nitrogen at 400 psi for 10 min at 4°C in a Parr cell disruption bomb (Parr Instrument Co., USA) and ruptured on sudden release of pressure. The cell homogenate was then centrifuged as described above and the cytosol fraction obtained stored at −80°C until use.

Stock culture of human promyelocytic leukemia HL-60 cells was maintained in suspension culture in RPMI 1640 medium (Flow Laboratories) supplemented with 10% heat-inactivated fetal calf serum. The cells were seeded at a density of 10^5 cells/ml in tissue culture flasks (175 cm^2, Nunc, Denmark) in RPMI 1640 medium as described above and harvested by centrifugation late in exponential growth phase.

After resuspension in homogenization buffer, the cells were disrupted and centrifuged, as described above. The cytosol fraction was stored at −80°C until use.

Assay Mixture and Incubation Conditions

The incubation mixture (final volume of 100 μl) contained 400 μM DL-homocysteine; 500 μM (±)-L-N^5-[*methyl*-^{14}C]methyltetrahydrofolate (2 μCi/μmol); 50 μM cyanocobalamin; 300 μM *S*-adenosylmethionine; 125 mM 2-mercaptoethanol; 20 μM L-norvaline; 50 mM potassium phosphate buffer, pH 7.4; and 50 μl of liver or cell extract in 1.5-ml polyethylene tubes. Assay blank was obtained by mixing 50 μl of liver or cell extract with 50 μl of 50 mM potassium phosphate buffer, pH 7.4. The incubation was started by addition of the extract and the incubation mixture was then immediately flushed with nitrogen and overlayered with 50 μl of bis(3,5,5-trimethylcyclohexyl)phthalate, when not otherwise indicated. The incubation was carried out at 37°C in the dark. The incubation was stopped by adding 10 μl of 4 N perchloric acid. The acid was then neutralized by adding 10 μl of 4 N KOH containing 3.3 M potassium bicarbonate, and the precipitate was removed by centrifugation.

Determination of Methionine with the OPA Method

The neutralized supernatants were transferred to the sample tray of the Gilson autosampler. The autosampler was programmed to mix 90 μl of sample (including blank samples) with 175 μl of the OPA reagent, and after 2 min at 23°C, 220 μl of this mixture was injected into the ODS Hypersil column equilibrated with 30% methanol in 50 mM sodium phosphate buffer, pH 5.0. The column was routinely eluted with a methanol gradient in this phosphate buffer. The methanol gradient was linear from 30 to 55% (12 min after injection) and from 55 to 80% (15 min after injection). The flow rate was 2 ml/min. The retention times of the OPA adduct for methionine and norvaline were 13.5 and 15.5 min, respectively.

The radioactive OPA–methionine adduct was recorded on-line using the Flo-One scintillation detector. The effluent from the fluorescence detector was mixed with scintillation fluid delivered at a rate of 6 ml/min.

Quantitation of [^{14}C]Methionine by Anion-Exchange Chromatography

Alternatively, the [^{14}C]methionine formed was quantitated as described by others (16). Briefly, the incubation was terminated by adding 400 μl of ice-cold water to the incubation mixture (100 μl), and the resulting solution immediately applied to a Bio-Rad AG 1-X8 column (1.0 × 4.4 cm) equilibrated with water. One milliliter of water was added to the column and the eluate discarded. [^{14}C]Methionine was then eluted with 2.0 ml of water and collected into scintillation vials. After addition of 5 ml of scintillation fluid, the radioactivity was determined in a Packard Tri-Carb liquid scintillation counter.

Determination of Protein

Protein was determined by the method of Bradford (27), using bovine γ-globulin as standard.

RESULTS AND DISCUSSION

Incubation Conditions

Methionine synthase requires a reducing system for activity (2), and we chose 125 mM 2-mercaptoethanol, especially since this thiol is also a component of and is compatible with the OPA reagent (22). The presence of a significant amount of DTT during the derivatization may interfere with formation of the 2-mercaptoethanol–OPA adducts.

The methionine synthase reaction was linear for about 30 min when run in microvials (500 μl) under aerobic conditions. About the same initial velocity was observed but the reaction proceeded at a constant rate for at least 120 min when the reaction was carried out below an oil layer (Fig. 1). The oil layer probably functions as a shield against oxygen exposure and represents an alternative to anaerobic conditions obtained by flushing the tubes with nitrogen (7,15) or hydrogen (10,11). Flushing is inconvenient, especially when multiple samples are taken from the tubes during time-course studies.

Others (11,12,28,29) have performed the incubation under aerobic conditions in the presence of 2-mercaptoethanol as the reducing agent. We observed that under these conditions, the reaction leveled off after a variable time period, dependent on sample volume and

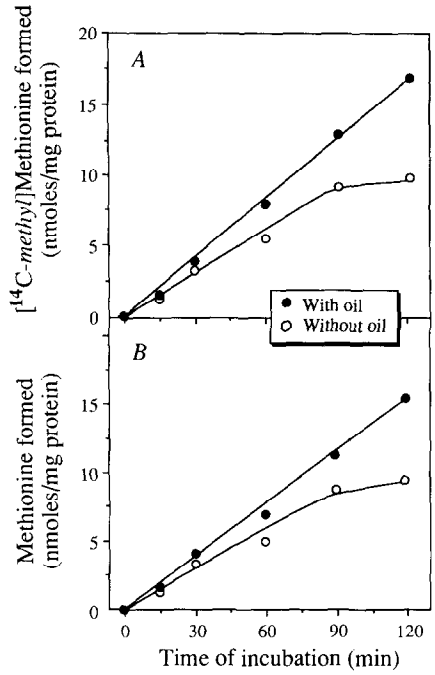

FIG. 1. Time course of the methionine synthase reaction incubated with and without an oil layer. Extract from liver (15 mg protein/ml) was assayed for methionine synthase using N^5-[*methyl*-^{14}C]methyltetrahydrofolate as substrate. The assay was run in 500 μl polyethylene tubes, either under an aerobic atmosphere or below a layer (150 μl) of bis(3,5,5-trimethylhexyl)phthalate. Methionine formed was derivatized with OPA, separated by HPLC, and the methionine was quantified both by on-line scintillation counting (A) and by fluorescence monitoring (B).

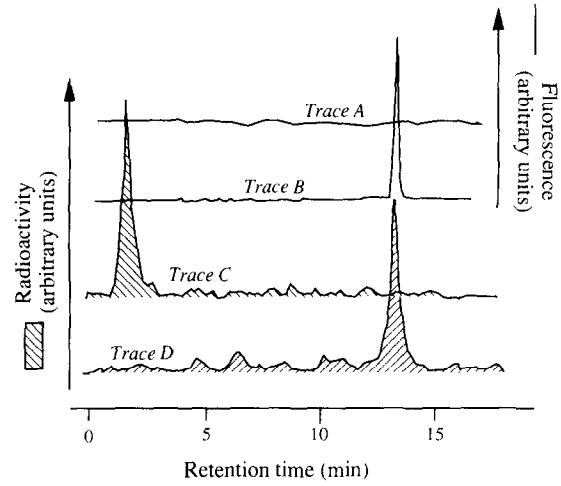

FIG. 2. Derivatization of [^{14}C]methionine with OPA and chromatographic behavior of the radioactive adduct. [^{14}C]Methionine (10 μM) was derivatized with OPA (Traces B and D) or incubated under the same conditions without OPA reagent (Traces A and C). The upper two traces (A and B) show the fluorescence profiles, and the cross-hatched traces (C and D) the corresponding radioactive profiles.

diameter of the incubation tube (data not shown), probably reflecting different surface area relative to the incubation volume.

Derivatization and Chromatography

Figure 2 shows that the derivatization of [^{14}C]methionine with OPA resulted in a sharp radioactive peak which cochromatographed with the fluorescent peak. Essentially no radioactivity eluted corresponding to the retention time of underivatized methionine (void volume), suggesting a quantitative conversion to the OPA adduct. Furthermore, a symmetrical, radioactive peak with essentially no tailing was observed, suggesting essentially no decomposition of the derivative during chromatography.

Almost complete conversion of methionine to the OPA derivative and stability during chromatography (Fig. 2) may contribute to high precision of the methionine assay. It is also a prerequisite for the validation of the fluorescent assay by simultaneous scintillation counting. We therefore used (±)-L-N^5-[*methyl*-^{14}C]-methyltetrahydrofolate as substrate, and the formation of methionine determined as the OPA adduct was routinely recorded both as radioactivity with the on-line HPLC scintillation detector and as fluorescence.

The incubation was terminated by addition of perchloric acid, which was then neutralized by addition of KOH/$KHCO_3$. The presence of 0.1 M sodium borate buffer in the OPA reagent brings the final pH of the derivatization mixture to about pH 9 which is the optimal pH for the formation and stability of the OPA adducts (30). Figure 3 shows that the fluorescence yield from methionine and norvaline decreases markedly at

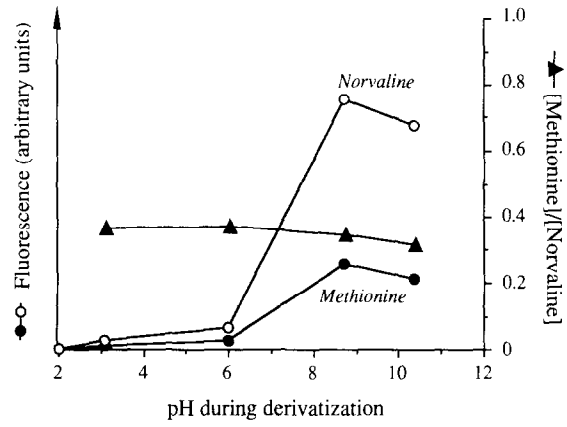

FIG. 3. Yield of fluorescent adducts of methionine and norvaline derivatized with OPA at various pH. Methionine (5.7 μM) and norvaline (20 μM) were derivatized with OPA. The pH of the derivatization mixture was adjusted to below pH 8.9 by addition of concentrated perchloric acid and to pH above 8.9 by adding KOH. The ratio between the areas of the methionine and norvaline peaks is also given (right axis).

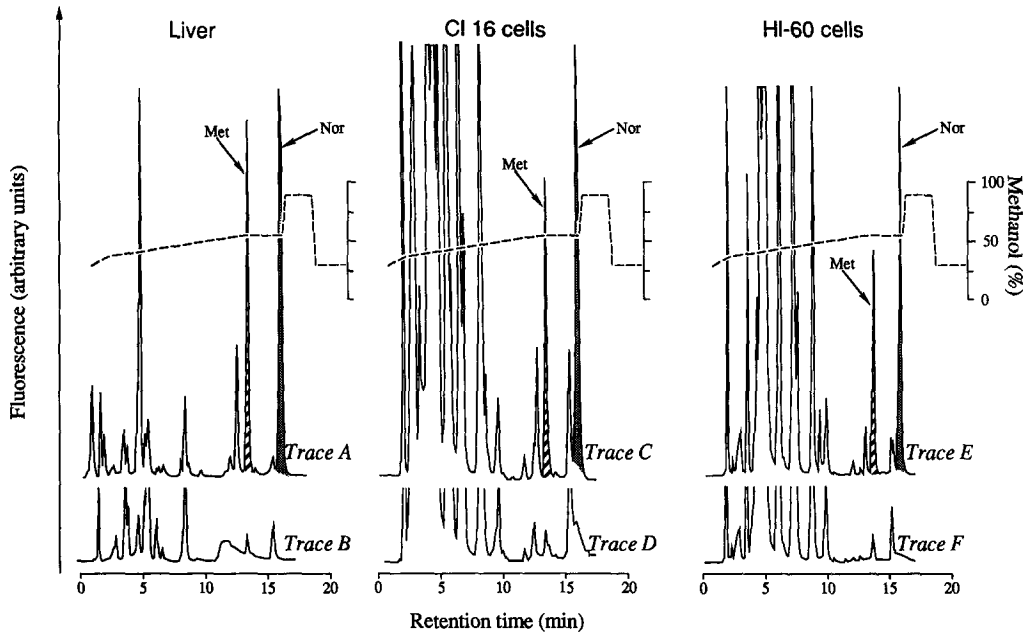

FIG. 4. Chromatographic resolution of methionine and norvaline (internal standard) in various assay mixtures derivatized with OPA. Extracts from liver (16.2 mg/ml), Cl 16 cells (5.6 mg/ml), and Hl-60 cells (4.4 mg/ml) were assayed for methionine synthase for 120 min, and the assay mixture was deproteinized with acid and neutralized and derivatized with OPA, as described under Materials and Methods. Methionine (retention time 13.6 min) is the cross-hatched peak and norvaline (retention time 15.7 min) the shaded peak. The profiles for the corresponding assay blanks are shown as traces B, D, and F. The lines across the upper chromatograms indicate the methanol gradient of the mobile phase.

low pH. Notably, there was a parallel decrease in the OPA adducts of methionine and norvaline, and the ratio remained constant over a large pH range. Thus, inclusion of norvaline as an internal standard in the incubation mixture may correct for error due to inaccurate pH adjustment.

Inclusion of norvaline as an internal standard improves the precision of the assay and reduces the coefficient of variation (CV) from 7.4 to 3.5% (liver), from 7.5 to 2.9% (Cl 16 cells), and from 8.9 to 6.4% (HL-60 cells).

Norvaline was preferred as an internal standard in the methionine synthase assay for additional reasons: First, high concentrations do not affect the enzyme activity, neither is it decomposed under the assay conditions (data not shown), and it can therefore be included in the incubation mixture. Second, norvaline is not a naturally occurring amino acid and is not present in tissue or cell extracts. Third, the OPA–norvaline adduct has ideal chromatographic properties. It elutes in a region of the chromatogram with no interference from fluorescent material derived from cells or tissue extract or constituents in the assay mixture; it is base-line separated from methionine. It has a retention time only 2 min longer than that of methionine (Fig. 4) and therefore does not significantly prolong run time.

The chromatograms of the incubation mixture for liver, mouse Cl 16 fibroblasts, and human HL-60 cells are shown in Fig. 4. The peak corresponding to methionine is low in the assay blanks and appears upon incubation of the extracts with the assay mixture. Methionine is base-line separated from norvaline and other fluorescent peaks (Fig. 4). The pH of the mobile phase was critical for the separation of methionine from interfering material eluting ahead of methionine. At pH 5.0 satisfactory separation was obtained.

Figure 5 shows the radioactivity profile corresponding to the chromatogram shown in Fig. 4, trace E. The substrate, N^5-methyltetrahydrofolate, eluted in the void volume, clearly separated from methionine.

Evaluation of the Assay

A time-course study of the methionine synthase reaction with extracts from three different sources showed that the enzymatic reaction was linear with time for at least 2 h (Fig. 1 and data not shown). We assayed extract from liver, Cl 16 cells, and Hl-60 cells for 7.5, 15, 30, 60, 90 and 120 min and measured simultaneously the radioactivity associated with the OPA adduct and the fluorescence. Linear regression analysis showed that a good correlation ($R < 0.99$) existed between radioactivity and fluorescence throughout the time course (data not shown).

We also validated the assay by determining methionine synthase activity in replicates of extracts from liver and exponentially growing Cl 16 fibroblasts and

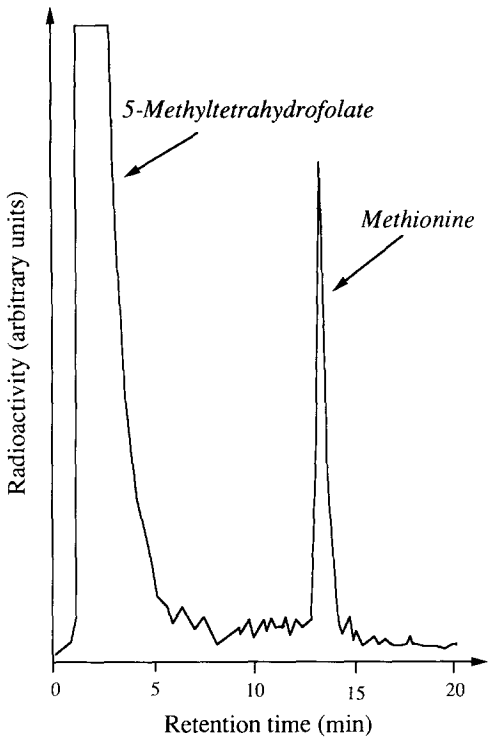

FIG. 5. Radioactive profile demonstrating the separation of the OPA adduct of methionine from N^5-methyltetrahydrofolate. Extract from HL-60 cells was incubated with assay mixture containing N^5-[*methyl*-^{14}C]methyltetrahydrofolate and the incubation mixture derivatized with OPA, as described in the legend to Fig. 4. The trace demonstrates the on-line recording of radioactivity, and the corresponding fluorescence profile is shown as trace E in Fig. 4.

HL-60 cells. The activity obtained with the OPA assay was compared with the values obtained by the conventional assay based on anion-exchange chromatography. These assays gave comparable results, and the CV of the assay based on derivatization with OPA was 2.9–6.4% (Table 1). Furthermore, the specific activities for methionine synthase listed in Table 1 compare with the activity in rat liver (31,32) and fibroblasts (15) reported by others.

Assay Blank

The background of the OPA method was determined by incubating extract from liver or the cultured cells with assay buffer containing no homocysteine and no N^5-methyltetrahydrofolate. Under these conditions, there was a time-dependent formation of trace amounts of methionine which was about or less than 10% of the amount of methionine formed during the enzyme assay (Fig. 4). This may be due to the presence of endogenous methionine and to proteolysis. When the complete assay mixture without liver or cell extract was incubated for 120 min at 37°C, methionine was not detected. Assessment of blank values by omission of one substrate does not seem to be justified, since the liver and cell extract certainly contain trace amounts of free and protein-bound homocysteine (33) and 5-methyltetrahydrofolate (34).

Blank values for the methods based on anion-exchange chromatography and determination of radioactive methionine were determined by assaying boiled enzyme, or by substituting extracts with buffer.

The selection of proper assay blank for the OPA method is verified by comparison of methods (Fig. 1, Table 1) and the correlation analysis described above.

Summary and Conclusion

The adaption of the OPA assay for methionine to measure methionine synthase activity involves the selection of a reducing agent which does not interfere with the formation of the methionine–OPA adduct, the inclusion of an internal standard which corrects for variable fluorescence yield and variations in the injection volumes, and optimalization of the mobile phase to give efficient resolution of the methionine peak. The performance of this assay compares favorably with that of the conventional methionine synthase assays, and carries the advantages of not requiring radiolabeled N^5-methyltetrahydrofolate, which is an unstable and expensive compound. Inconsistent data on specific activities of me-

TABLE 1

Performance and Comparison of Methionine Synthase Assays

	Method for determination of methionine								
	Fluorescence of OPA adduct			Radioactivity of OPA adduct			Anion-exchange chromatography		
Extract	Enzyme activity	SD	CV (%)	Enzyme activity	SD	CV (%)	Enzyme activity	SD	CV (%)
Liver	8.1	0.27	3.5	7.6	0.27	3.4	11.3	0.28	2.5
Cl 16 cells	4.0	0.12	2.9	6.12	0.40	6.6	6.8	0.52	7.7
Hl-60 cells	5.2	0.33	6.4	6.7	0.34	5.1	7.6	0.49	6.5

Note. The methionine synthase activity is the mean of 10 determinations and is expressed in nmol · mg^{-1} · h^{-1}. SD, standard deviation; CV, coefficient of variation.

thionine synthase have been reported (35), and partial oxidation of radioactive N^5-methyltetrahydrofolate may contribute to such errors. It is conceivable that determination of methionine synthase activity in the presence of saturable concentration of substrates and measurement of the formation of the amount of unlabeled product may provide more accurate determination of enzyme activity. This may be particularly important for an assay of potential value in biochemical laboratory diagnosis (4,5,36,37). Finally, the assay can be used in studies of substrate activity of analogues of 5-methyltetrahydrofolate (38).

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