Paper I

Fully Automated Assay for Cobalamin-Dependent Methylmalonyl CoA Mutase

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We constructed a fully automated assay for the cobalamin-dependent enzyme methylmalonyl coenzyme A (CoA) mutase. The assay involves preincubation of the enzyme with adenosylcobalamin, incubation with substrate, termination of the reaction by adding trichloroacetic acid, filtration to remove precipitated protein, and finally analysis of the filtrate (containing methylmalonyl CoA and the product succinyl CoA) by HPLC. These steps were carried out by an inexpensive programmable autosampler equipped with thermostated sample racks and mobile disposable extraction column racks used here as a sample filtering device. A central element in the developmental work was to measure stability of reagents, enzyme, and product against the storage conditions during unattended analysis and the time table of the program. We evaluated the performance of the method by measuring methylmalonyl CoA mutase activity in rat liver, human fibroblasts, and human glioma cells. The within-run imprecisions (CV) were 2–10% for measuring enzyme activity in 20 replicate samples of a homogenate (test of the automated assay), and 7–12% for measuring enzyme activity in homogenates from 20 culture dishes (test of the total procedure). The method allows the unattended analysis of 56 samples per 24 h. This strategy for automation may be easily adapted for other enzyme assays.

Indexing Terms: *enzyme activity/robotics/sample treatment/chromatography, reversed-phase/cell-culture assays*

Methylmalonyl CoA mutase (EC 5.4.99.2) is a mitochondrial matrix enzyme that catalyzes the isomerization of methylmalonyl CoA to succinyl CoA. It is one of the two known cobalamin-dependent enzymes in mammalian tissue and requires adenosylcobalamin (AdoCbl) as cofactor for activity (*1*).[2]

Cobalamin deficiency decreases the activity of methylmalonyl CoA mutase, leading to accumulation of methylmalonyl CoA. This explains the increase of the cobalamin marker methylmalonic acid in serum (*2*), and the decreased activity of methylmalonyl CoA mutase has been assigned a role in cobalamin neuropathy (*3*). Genetic deficiency of the enzyme or impaired synthesis of AdoCbl causes methylmalonic acidemia, a disorder characterized by the accumulation of methylmalonic acid and other metabolites in body fluids (*1*).

Several spectrophotometric assays for methylmalonyl CoA mutase have been described (*4–6*), and at least two of them (*4, 6*) involve enzyme coupling to NADH-dependent dehydrogenases. Some (*5, 6*) but not all (*4, 7*) of these assays are applicable to crude cell homogenate. Another problem is that the coupling enzymes may not be commercially available (*6*).

The prevailing methods for measurement of methylmalonyl CoA mutase are based on the conversion of radioactive methylmalonyl CoA to succinyl CoA (*8–10*). One gas-chromatographic assay measures [^{14}C]succinate produced from [^{14}C]methylmalonate (*7*). However, the cost of radioactive methylmalonyl CoA is high, it is occasionally not available from commercial sources (*11*), and in-house synthesis is a cumbersome procedure involving handling of radioactive reagents, products, and waste (*11*). Recently, Kikuchi et al. (*12*) published a method based on the separation of unlabeled methylmalonyl CoA and succinyl CoA by HPLC.

The aim of the present work was to construct a fully automated, nonradioactive assay for total and holomethylmalonyl CoA mutase, which allows the unattended analysis of large series of samples from isolated cells or tissues. The assay might be useful in clinical studies evaluating methylmalonyl CoA mutase as a marker of cobalamin deficiency, in diagnosis and screening of methylmalonic acidemias, and in large cell-culture studies of the methylmalonyl CoA mutase activity and its regulation during impaired cobalamin homeostasis.

Materials and Methods

Materials

DL-Methylmalonyl CoA (lithium salt), succinyl CoA (sodium salt), 5′-deoxyAdoCbl, cyanocobalamin, folic acid, L-methionine, mineral oil (M-5904), and thymidine were purchased from Sigma Chemical Co., St. Louis, MO. Nonessential amino acids and trypsin were provided from Bio Whittaker, Walkersville, MD. Glutamine, glacial acetic acid, trichloroacetic acid (TCA), and phosphoric acid were obtained from Merck, Darmstadt, Germany; HPLC-grade methanol was a product of Labscan, Dublin, Ireland.

A calibration solution of methylmalonyl CoA, 100 μmol/L dissolved in 100 mmol/L phosphate buffer, pH 4.0, was stable for several months when kept at −18 °C. Succinyl CoA (for calibration, 100 μmol/L) was dissolved in 200 mmol/L phosphoric acid, pH 1.8, and stored at −18 °C; under these conditions it decomposed at a rate of ~10% per month. The decomposition

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[2] Nonstandard abbreviations: AdoCbl, adenosylcobalamin; TCA, trichloroacetic acid; ODS, octadecylsilyl; and DMEM, Dulbecco's modified Eagle's medium.
Received February 28, 1995; accepted May 10, 1995.

product was recovered as an ultraviolet-absorbing material eluting 0.28 min behind the parent compound.

An HPLC column (0.46 × 10 cm) for reversed-phase liquid chromatography was packed with 3-μm octadecylsilyl (ODS) Hypersil from Shandon Southern Products, Cheshire, UK; it was equipped with a guard column packed with Pelliguard LC-18 from Supelco, Bellefonte, PA.

Empty filtration columns (0.50 × 5.5 cm) were prepared from used (Bond Elut) solid-phase extraction columns purchased from Analytichem International, Varian Associates, Harbor City, CA. The bottom was covered with a glass fiber filter (AP 20) obtained from Millipore, Oslo, Norway, and the top was sealed with sealing caps (Gilson Medical Electronics,Villiers le Bel, France).

Custom-made Dulbecco's modified Eagle's medium (DMEM), delivered as powder, was the same composition as ordinary DMEM except without folic acid and methionine (Gibco BRL, Paisley, Scotland). Heat-inactivated fetal calf serum was purchased from Biological Industries, Kibbutz Beth Haemek, Israel. The disposable culture flasks and 9-cm-diameter petri dishes were delivered from Nunc, Roskilde, Denmark.

Cell Lines, Culture Conditions, and Harvesting

The MRC-5 cell line, isolated from normal lung of a human male fetus, is referred to as lung fibroblasts. These cells were obtained from the ATCC (Rockville, MD). The GaMg cell line was established in 1984 from a glioblastoma multiforme tumor in a 42-year-old woman (*13*). Both cell lines were cultured as monolayers in DMEM supplemented with 0.6 g/L L-glutamine, 1.5 μmol/L cyanocobalamin, 10 μmol/L folic acid, 50 μmol/L methionine, nonessential amino acids (330 μmol/L each), and 100 mL/L heat-inactivated fetal calf serum. The cells were incubated at 37 °C in an atmosphere of 50 mL/L CO_2/950 mL/L air with a relative humidity of 98%.

Samples of cells were harvested on ice in red dim light, combining cells from two 9-cm petri dishes containing a total of ~3.5×10^6 cells (GaMg) or 4.5×10^6 cells (MRC-5). Cells were brought into suspension by treating with trypsin (0.1 g/L) for 5 min, which was followed by trypsin inactivation with fresh medium. The cells were then washed in phosphate-buffered saline and centrifuged for 5 min at 190*g*. The cell pellet obtained was resuspended in 300 μL of 210 mL/L glycerol containing thymidine (203 μmol/L), and stored at −80 °C until analysis. Cell count was determined with a Coulter Counter Model ZM (Coulter Electronics, Luton, UK).

Preparation of Rat Liver and Cell Homogenates

A 4-month-old male rat (*Rattus norvegicus*, BD9) was stunned and bled to death, and the liver (5 g) was removed without delay and chilled on ice. The liver was chopped into small pieces and homogenized in 4.5 volumes of distilled water with three strokes at 720 rpm with a Potter-Elvehjem homogenizer (B. Braun) equipped with a loose-fitting Teflon pestle. The homogenate was centrifuged at 750*g* at 4 °C for 10 min, and the supernate stored at −80 °C. Before analysis, frozen rat liver samples were thawed and diluted 1:15 (by vol) in 210 mL/L glycerol containing 203 μmol/L thymidine.

Total homogenates of cultured cells and liver in 200 mL/L glycerol were sonicated at 30W for 4 s in a minimum volume of 300 μL in conical polystyrene tubes (110 × 17 mm; Nunc). The procedure was carried out on ice and in red dim light.

Instrumentation

The programmable sample processor Gilson ASPEC (Gilson Medical Electronics) was equipped with an injector valve (Model 7010; Rheodyne, Cotati, CA) and a 20-μL sample loop, and coupled to a Model SP 8800 ternary solvent delivery system (Spectra-Physics, San Jose, CA) equipped with a solvent mixer (Model SP 8500; Spectra-Physics). The ODS column was mounted in a column heater (Model SP 8792; Spectra-Physics). The absorbance was recorded at 254 nm with an absorbance detector (Spectroflow 773; Kratos Analytical Instruments, Westwood, NJ) coupled to an integrator (Model SP 4290; Spectra-Physics).

Storage of Samples and Reagents During Unattended Analysis

Samples (100 μL) of sonicated cell or rat liver homogenates (crude enzyme) were stored in dark-colored conical plastic vials (1.5 mL; Sarstedt, Oslo, Norway) placed in a cooled (4 °C) code-31 rack (rack 1). This rack also contained a solution of AdoCbl (1 mmol/L) for the determination of total enzyme activity, and water for the determination of holoenzyme activity. AdoCbl was protected against light inactivation in a 1.5-mL colored polypropylene vial (Treff, Degersheim, Switzerland). The substrate, DL-methylmalonyl CoA, in 300 mmol/L Tris-phosphate buffer, pH 7.5, was placed in a conical plastic vial (50 mL; Nunc) in the neighboring code-33 rack thermostated at 37 °C (rack 2). Rack 2 also held three plastic tubes containing TCA (100 g/L), mineral oil (M-5904), and distilled water, respectively. The racks were covered with sheets of wiping paper (Gilson Medical Electronics).

Automated Methylmalonyl CoA Mutase Assay

The enzyme reaction was performed in the dark in 1.5-mL polypropylene tubes (reaction vials) in a third code-31 rack (rack 3) thermostated at 37 °C. The procedure was divided into five steps, which were carried out automatically by the Gilson ASPEC sample processor.

In step 1 (preincubation), liver or cell homogenate was first thoroughly mixed, and an aliquot of 50 μL transferred to and mixed with 20 μL of AdoCbl (1 mmol/L) or water in a reaction vial placed in rack 3. The enzyme was preincubated with AdoCbl (total en-

zyme activity) or water (holoenzyme activity) for 5 min at 37 °C.

In step 2 (enzyme incubation), the enzyme reaction was initiated by adding 120 μL of DL-methylmalonyl CoA (600 μmol/L) solution to the reaction vial. The resulting solution was mixed and, after 5 min of incubation at 37 °C, the reaction was terminated by adding 70 μL of TCA (100 g/L).

In step 3, included to prevent evaporation of the acid-treated mixtures, the samples were covered with 90 μL of mineral oil. Afterwards, the oil was removed from the inner side of the needle by repeated aspiration and forceful expulsion of 150 μL of water, and from the outer side by piercing the wiping paper that covered rack 2.

In step 4, the protein precipitated by TCA was removed by transferring 200 μL of the mixture to filtration columns mounted in two mobile disposable extraction column racks. The filtration process was enforced by air pressurization, and the effluent drained into 3.5-mL collection tubes (Gilson).

During the final step (5), the filtered samples (100 μL) were subjected to HPLC for quantification of succinyl CoA and methylmalonyl CoA.

With the present version of the program, groups of three samples were carried through steps 1–3 over 18 min. When all samples had been processed up to step 4, the temperature of rack 3 was reset from 37 °C to 22 °C.

Chromatography

Samples of 20 μL were injected immediately after each filtration into the reversed-phase C_{18} column. The column was equilibrated with 740 mL/L of solvent A (75 mmol/L acetic acid in 100 mmol/L phosphate buffer, pH 4.6) and 260 mL/L of solvent B (300 mL/L methanol in solvent A). The flow rate was 2 mL/min and the temperature 39.7 °C. The column was eluted with a methanol gradient obtained by increasing the volume percentage of solvent B from 26% to 45% during the first 6 min, and from 45% to 60% in the interval of 6–9 min. Then the column was reequilibrated for 5 min. Total run time was 14 min. The mobile phases were degassed with helium, which stabilized the retention times of methylmalonyl CoA (3.0 min) and succinyl CoA (4.4 min). Thymidine, added to the crude enzyme before the enzyme assay, eluted after 2 min. Methylmalonyl CoA and succinyl CoA were quantified by estimating peak area. External standards were run after every 14 samples.

Linearity of the Enzyme Reaction

We studied the linearity of the enzyme reaction vs time for at least 5 min, analyzing total enzyme and holoenzyme activity in rat liver homogenate (0.8 g/L protein in assay mixture). The assay was also tested for linearity vs enzyme concentration with different dilutions of homogenates from both rat liver and lung fibroblasts.

Comparison of Methods and Precision

The automated method was compared with the same manual procedure for analyzing triplicates of five dilutions of rat liver homogenate.

The precision of the method was evaluated by measuring both holoactivity and total activity in homogenates of glioma cells, lung fibroblasts, and rat liver.

To determine the within-run imprecision of the automated assay, homogenates of cells or rat liver were prepared in one batch. Twenty replicates of these homogenates were assayed.

To test the within-run imprecision of the total procedure, including cell harvesting, sonication, and activity determination, we separately cultured, harvested, sonicated, and assayed 20 cell-culture dishes.

Protein Determination

Protein content was determined according to the method of Bradford (*14*), with bovine gamma globulin as calibrator.

Results and Discussion

Inexpensive programmable sample processors carrying out complex handling and mixing of liquids combined with sample injection into an HPLC column have been marketed for the last 7 years. These sample processors have been used for automated precolumn derivatization of samples before on-line HPLC (*15–20*). We previously developed an automated method for plasma homocysteine based on a sample processor from Gilson (Model 232) that confers both reliability and high precision to the assay (*21*, *22*).

Principles, Preconditions, and Assay Construction

Automated enzyme assays coupled to on-line determination of product formation by HPLC have hitherto not been adapted to programmable sample processors. This approach requires two features, i.e., sample racks thermostated at temperatures required for the enzyme reaction (usually 37 °C) and for storage of thermolabile enzyme or reagents (0–4 °C), and a device for removing precipitated protein before injection into the HPLC column.

The instrument used here was equipped with one cooled rack (rack 1) storing the enzyme samples and the cofactor, AdoCbl, at 4 °C, and two racks thermostated at 37 °C holding the substrate (DL-methylmalonyl CoA) in assay buffer (rack 2) and the reaction vials (rack 3). A unique option of the Gilson ASPEC is the mobile disposable extraction column racks (racks 4 and 5), which in the present assay were adapted for on-line filtration of acid-treated samples through glass fiber filters immediately before injection of the sample into the HPLC column.

In addition to the product succinyl CoA, the amounts of the substrate methylmalonyl CoA and of thymidine in the injection mixture were routinely recorded. These variables and their relation served to identify occasional errors in volumes delivered by the autosampler,

due to clogging of the needle or incomplete filtration of sample before injection. Thymidine was added to the crude enzyme, and a low ratio between thymidine relative to the sum of methylmalonyl CoA and succinyl CoA pointed to low volume of enzyme preparation in the assay mixture. On the other hand, low values but a normal ratio between these variables suggested impaired filtration or injection.

The maximal sample number to be assayed in a single run was limited by the capacity of the rack (rack 3) containing the reaction vials, and was 56 samples. With the present version of the program, groups of three samples were successively preincubated (for 5 min, step 1), incubated (for 5 min, step 2), inactivated by adding acid, and then finally covered with mineral oil (step 3). One cycle (steps 1–3) lasted 18 min. The time interval between initiation of step 1 for the first and last enzyme sample in a series of 56 samples was 5.6 h, and stability of the enzyme at 4 °C for this time period is a prerequisite for automation.

After all samples had been assayed, acid-treated, and overlaid with mineral oil, they were successively filtered (step 4) and subjected to HPLC (step 5) every 14th min. With a maximal sample load (56 enzyme samples and four calibrator samples), the time interval between completion of step 3 and start of steps 4 and 5 was 5.3 h for the first sample and 14 h for the last sample. Thus, another necessity is stability of the product succinyl CoA at 37 °C for 5.3 h and at 22 °C for ~14 h.

The program can be modified to carry out the separate steps (1–5) in a sequential mode. This is required in case the strategy is adapted to the determination of enzyme activities where unstable products are formed.

Stability of Enzyme, Substrate, and Product

We investigated the stability of methylmalonyl CoA mutase in crude homogenates placed in the sample rack (rack 1) thermostated at 4 °C. The enzyme activity declined at a rate of ~0.08 h^{-1} (Fig. 1A). We then tested the ability of several agents to stabilize the enzyme, and among these (sodium disulfite, mercaptoethanol, dithioerythritol, glutathione), glycerol prevented the enzyme inactivation in a concentration-dependent manner (Fig. 1B). In the presence of 200 mL/L glycerol, essentially no inactivation occurred after 20 h (Fig. 1A). This time is sufficient for the unattended enzyme assay of >56 samples.

The time course of the methylmalonyl CoA mutase reaction measured in the absence (holoenzyme activity) and presence of AdoCbl (total enzyme activity) was linear for up to 5 min, and was not affected by glycerol (final concentration of 52.6 mL/L) in the assay mixture (data not shown). This corresponds to the concentration of glycerol carried over from the crude enzyme preparation containing 200 mL/L glycerol.

The enzyme reaction was terminated by the addition of 100 g/L TCA (final concentration, 27 g/L) to the assay mixture. The acid-treated samples were then left in the reaction vials (rack 3) thermostated to 37 °C until

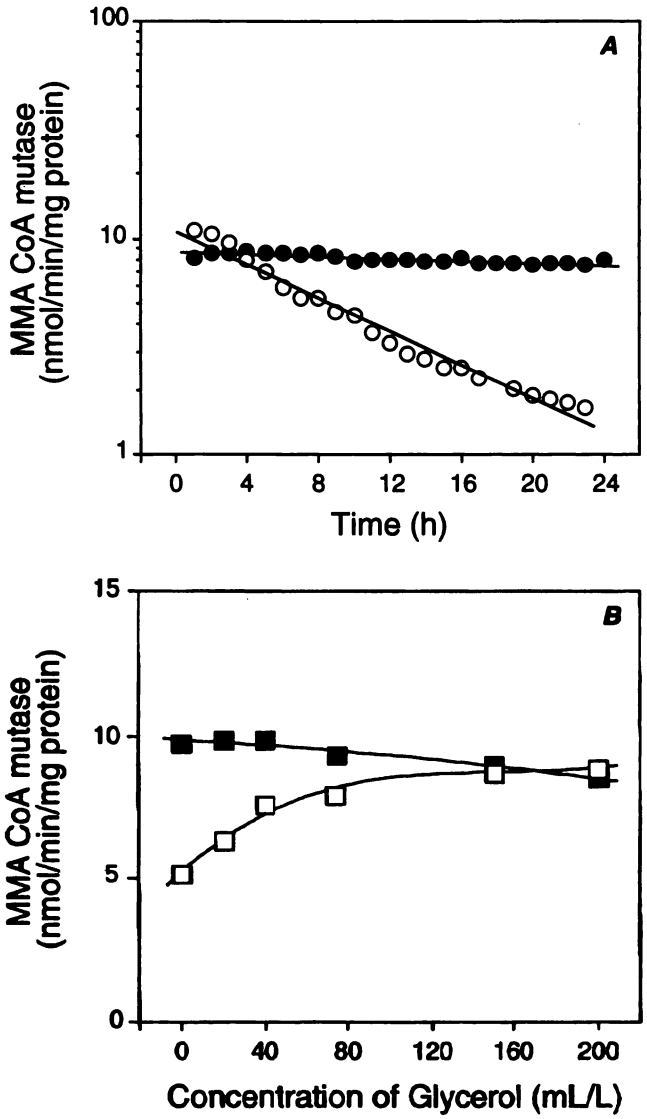

Fig. 1. Stabilization of methylmalonyl (MMA) CoA mutase by glycerol.

(*A*) Rate of inactivation of methylmalonyl CoA mutase at 4 °C in absence (○) and presence (●) of glycerol. The enzyme in liver homogenate (3 g/L protein) was stored with and without 200 mL/L glycerol (52.6 mL/L final concentration during enzyme assay) for an increasing period of time. The enzyme reaction was started by addition of methylmalonyl CoA, as described in the text. (*B*) Protection of the enzyme by adding increasing amounts of glycerol to the liver homogenate. Samples were assayed immediately (■) or after 6 h (□). The experimental conditions were as described under *A*. Each point is the mean of duplicate determinations.

HPLC was started, i.e., after the last enzyme reaction was completed. Evaporation of the injection mixtures was prevented by placing a layer of mineral oil on the top.

Depending on the number of samples assayed in one series, this design required stability of the substrate, methylmalonyl CoA, at 37 °C for 5.6 h in assay buffer and of the product, succinyl CoA, in 2.7% TCA for up to 14 h (5.3 h at 37 °C and 14 h at 22 °C).

We observed no degradation of methylmalonyl CoA at 37 °C in assay buffer (data not shown). We also investigated the stability of the methylmalonyl CoA and succinyl CoA at 37 °C in the TCA-treated samples for up to 20 h (Fig. 2). Methylmalonyl CoA was stable

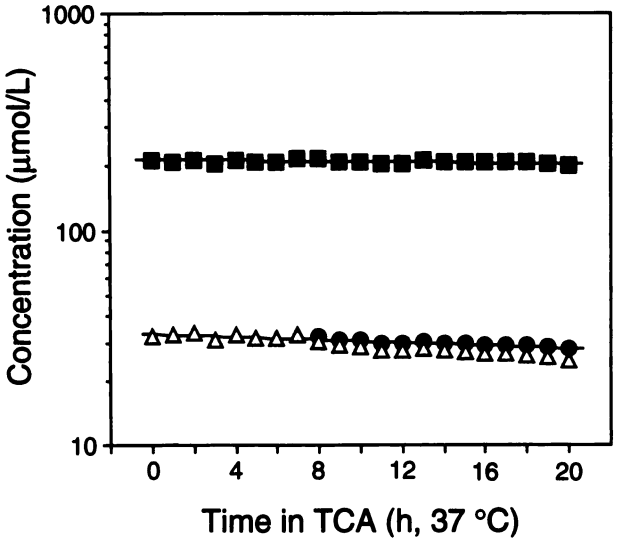

Fig. 2. Stability of methylmalonyl CoA and succinyl CoA at 37 °C in TCA-treated samples.

Rat liver homogenate (3 g/L protein) was assayed for methylmalonyl CoA mutase. The incubation time was 5 min, and the reaction was terminated by addition of TCA (27 g/L final concentration), as described in the text. The figure shows the concentration of methylmalonyl CoA (■) and succinyl CoA (△) as a function of the time interval between addition of acid and injection of the sample into the HPLC column. Succinyl CoA (4.4 min) was slowly decomposed and converted to a compound with a retention time of 4.68 min. The amount of succinyl CoA plus the decomposition product, given as succinyl CoA equivalents, is also shown (●).

under these conditions, whereas succinyl CoA (retention time 4.4 min) slowly decomposed at a rate of 0.012 h^{-1}, but was recovered as a compound with a retention time of 4.7 min (Fig. 2) (which coeluted with the degradation product formed during storage of authentic succinyl CoA in phosphoric acid at −18 °C). Minimal (rate <0.004 h^{-1}) decomposition of succinyl CoA was observed at 22 °C (data not shown). We corrected for the slight decomposition of succinyl CoA, which was <10% in series of 56 samples.

Chromatography

The filtered injection mixture was subjected to on-line reversed-phase liquid chromatography with a C_{18} column. Fig. 3 shows the chromatograms of a standard assay mixture containing rat liver homogenate or cell homogenate incubated with methylmalonyl CoA for 5 min before the addition of TCA (upper traces). The corresponding blank samples were obtained by adding TCA before incubating the enzyme with methylmalonyl CoA (lower traces). In this chromatographic system thymidine eluted after 2.00 min, methylmalonyl CoA after ~3 min, and succinyl CoA after 4.4 min (Fig. 3).

In the liver blank, but not in blanks obtained with cultured cells, there was a small peak (with a retention of 4.22 min) eluting ahead of succinyl CoA (lower left trace, Fig. 3). This material caused no interference with succinyl CoA.

The identity of methylmalonyl CoA and succinyl CoA was verified by chromatography of authentic compounds added to both cell and liver homogenates (data not shown).

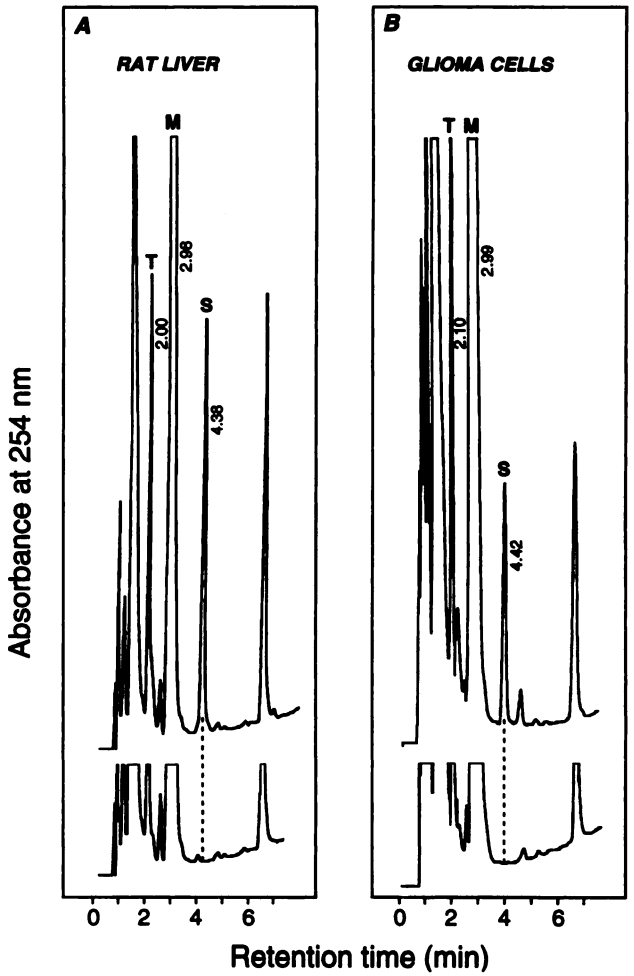

Fig. 3. Chromatographic resolution of thymidine, methylmalonyl CoA, and succinyl CoA in samples of (*A*) rat liver and (*B*) human glioma cells

Homogenates of rat liver (3 g/L protein) and human glioma cells (13 g/L protein) were assayed for methylmalonyl CoA mutase activity. *Lower traces* show the chromatographic profiles of the assay blank; *upper traces* show the profiles obtained after 5 min of incubation with methylmalonyl CoA. *T*, thymidine; *M*, methylmalonyl CoA; *S*, succinyl CoA.

Performance of the Assay

Using rat liver homogenate (0.8 g/L protein in assay mixture), we verified that the reaction was linear vs time for at least 5 min for both total enzyme and holoenzyme activities, and linear vs enzyme concentration up to 13.3 g/L protein (5 min incubation) (data not shown). Linearity vs enzyme concentration (up to 14.5 g/L protein) was also demonstrated for the cell homogenate (data not shown).

There was a good correlation between the enzyme activities obtained with the automated assay and the same assay carried out manually (Fig. 4): $y = 1.0109x - 0.012752$, $r = 0.999$.

The automated assay was sufficiently sensitive to determine holomutase activity in human lung fibroblasts and glioma cells as well as in rat liver. Measured total methylmalonyl CoA mutase activity in fibroblasts (~1.3 U/g) and rat liver (~7 U/g) (Table 1) were comparable with published values (*5, 6, 9, 12, 23, 24*).

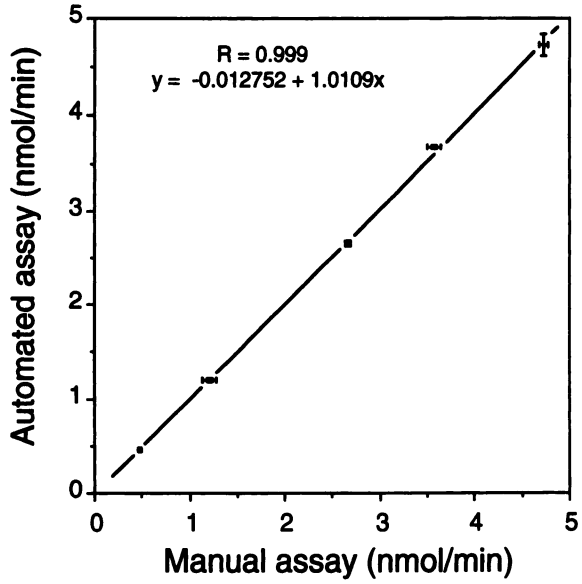

Fig. 4. Comparison of the automated method with the manual procedure.

Homogenates of rat liver (1.3–13.3 g/L protein) were assayed for methylmalonyl CoA mutase by the automated method and by a procedure in which each step is carried out manually. Results of triplicate samples are given as mean ± SD. The *straight line* shows the linear regression of the mean values.

The imprecision of the assay for total mutase and holomutase activity in cultured cells and in rat liver was determined by using cell and liver homogenates prepared in one single batch. The within-run CVs were in the range of 2–5% and 6–9% for total and holoactivity, respectively (Table 1), and mainly reflect the precision of the sample and liquid handling and the injection carried out by the instrument.

We also determined the imprecision of the mutase assay when the enzyme activity was measured in cell homogenates obtained from separate dishes. The within-run CVs were in the range of 7–10% and 9–12% for total activity and holoactivity, respectively (Table 1). The higher imprecision is probably related to the variability in cell culture and processing between the separate samples.

In conclusion, we have demonstrated the utility of an inexpensive programmable autosampler equipped with thermostated sample racks and a sample filtering device for unattended enzyme assay based on determination of the product by HPLC. The method is precise and completely automated. During a period of 24 h, 56 samples can be analyzed. A prerequisite is the construction of a timetable compatible with the stability of the enzyme, reagents in the assay mixture, and product formed. This strategy may be adapted to measure other enzyme activities. The methylmalonyl CoA mutase assay developed here should be evaluated as a tool to monitor cobalamin status in experimental and clinical studies.

Table 1. Assay precision and specific activities.

	CV, % (n = 20)		Specific activity, U/g protein (mean ± SD)	
Enzyme preparation	Total activity	Holo-activity	Total activity	Holoactivity
Automated assay[a]				
Fibroblasts	3.7	5.9	1.3 ± 0.05	0.25 ± 0.01
Glioma cells	5.2	9.3	2.8 ± 0.14	0.45 ± 0.04
Rat liver	1.9	7.5	8.4 ± 0.15	0.39 ± 0.03
Total procedure[b]				
Fibroblasts	7.7	12.0	1.5 ± 0.12	0.40 ± 0.05
Glioma cells	9.6	8.8	4.4 ± 0.40	0.24 ± 0.02
Rat liver	6.7	8.6	8.2 ± 0.16	0.35 ± 0.02

[a] Enzyme determination of replicate samples prepared in one batch.
[b] Enzyme determination in samples prepared separately.

This work was supported by grants from the Norwegian Cancer Society. B. R. is a fellow of the Norwegian Cancer Society. The technical assistance of Eli Gundersen, Gry Kvalheim, and Halvard Bergesen is highly appreciated.

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