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Comparison of two methods for the measurement of rat liver methylmalonyl-coenzyme A mutase activity: HPLC and radioisotopic assays

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Methylmalonyl-coenzyme A mutase (MCM) is a 5′-deoxyadenosylcobalamin-linked mitochondrial enzyme that catalyzes the isomerization of L-methylmalonyl-coenzyme A to succinyl-coenzyme A. In vitro assays of total and holo-MCM activities are important tools for investigating the cobalamin pathway. Several methods have been described for measuring MCM activity. The most commonly-used method is a radioassay based on the permanganate oxidation of DL[CH_3-^{14}C]methylmalonyl-coenzyme A, but radiometric methods are insensitive, laborious, and time-consuming. Therefore, we have compared this method with a nonradiometric assay, potentially most sensitive, based on the separation of methylmalonyl-coenzyme A and succinyl-coenzyme A by high performance liquid chromatography (HPLC). We determined the optimal assay conditions and the reproducibility and sensitivity of each technique. The results obtained by the two techniques were very different: the specific activities obtained by the permanganate oxidation method (0.039 ± 0.013 nmol/min/mg protein for the holo-MCM activity and 1.90 ± 0.69 nmol/min/mg protein for the total-MCM activity) were threefold lower than those obtained with the HPLC method (0.124 ± 0.011 nmol/min/mg protein for the holo-MCM activity and 6.15 ± 0.76 nmol/min/mg protein for the total-MCM activity). The coefficients of variation for the radiometric method (18.4–40.6%) were three to five times greater than those for the HPLC assay (3.5–12.2%). This demonstrates the lack of sensibility and reproducibility of the permanganate radioassay. Thus, the radiometric method is not suitable for measuring low mutase activities such as the holo activities in tissues. The intrinsic inconvenience of the radiometric assay indicates that the HPLC method is a method of choice for measuring MCM activity. (J. Nutr. Biochem. 10:56–62, 1999)

Keywords: methylmalonyl-coenzyme A mutase; high-performance liquid chromatography; radio-assay; methodology; rat; liver

Introduction

Methylmalonyl-coenzyme A mutase (MCM; EC 5.4.99.2) is one of the two known cobalamin-dependent enzymes present in mammalian tissues.[1] It is a mitochondrial 5′-deoxyadenosylcobalamin (AdoCbl)-linked enzyme that catalyzes the isomerization of L-methylmalonyl-CoA to succinyl-CoA.[2] A lack of MCM activity leads to the accumulation of unmetabolized methylmalonic acid and other organic acids in body fluids.[3–6] The severity of this disorder, termed methylmalonic acidemia (MMA), may range from mild[7] to fatal.[8]

A subnormal MCM activity and the resulting MMA may be due to a defect in the enzyme methylmalonyl-CoA racemase;[9] a cobalamin deficiency caused by a nutritional lack of or a genetic defect in the enzymes responsible for AdoCbl synthesis, called *cbl* mutations[10]; or a defect in the structural gene for the apoenzyme MCM, termed a *mut* mutation, which leads to residual (mut^-) or no (mut^0) activity.[11–16] The various forms of MMA cannot be distinguished from one another clinically. MMA may be diagnosed by measuring MCM activity, with or without the

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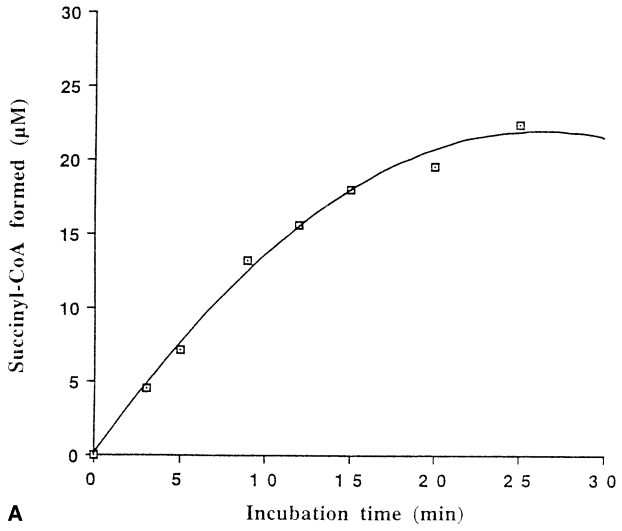
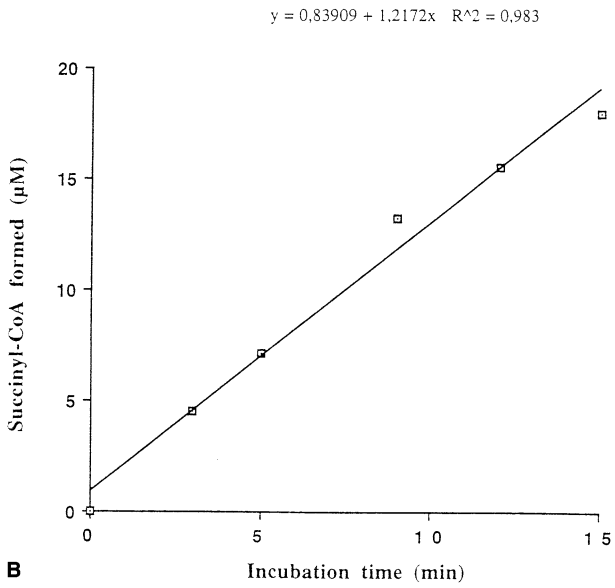




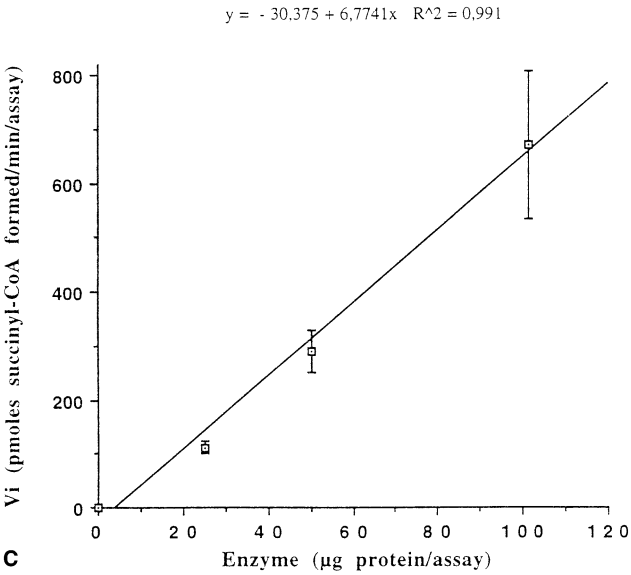


Figure 1 High performance liquid chromatography assay of rat liver total MCM activity. *Figure 1A:* Amount of succinyl-CoA formed at 37°C as a function of incubation time. The amount of enzyme was constant (40 µg protein/assay). *Figure 1B:* Detail of the above curve showing incubation times of 0 to 15 minutes. *Figure 1C:* Rate of formation of succinyl-CoA per min (Vi) as a function of protein concentration (0–100 µg protein/assay). The assay was performed with four different incubation time values (3, 6, 9, and 12 minutes) per protein concentration.

addition of AdoCbl. This can be used to distinguish between two variants of MMA (Cbl-responsive and Cbl-unresponsive) and to detect the heterozygotes within the Cbl unresponsive class. Thus, the in vitro measurement of total (with AdoCbl) and holo activities (without cofactor) of MCM activity is useful to investigate the Cbl pathway, diagnose MMA, identify *mut* and *cbl* mutations, and gain insight into the biochemical changes accompanying vitamin B12 deficiency.

Several methods have been described and employed to measure MCM activity. These include radiometric methods in which [^{14}C]succinyl-CoA is produced and separated from the substrate DL[CH_3-^{14}C]methylmalonyl-CoA by paper chromatography,[17,18] thin layer chromatography,[12,19] electrophoresis,[20] potassium permanganate oxidation,[21,22] extraction into ethyl acetate,[22,23] and high performance liquid chromatography (HPLC)[24] or gas chromatography.[25] There are also nonradioactive assays based on the separation of methylmalonyl-CoA and succinyl-CoA by reverse-phase HPLC[26,27] or on the direct spectrophotometric assay of succinyl-CoA.[28–30]

The first five methods are reputed to be laborious and time-consuming, and they have been criticized for their lack of sensitivity.[24,25] The permanganate oxidation method is also open to criticism because the optimal conditions for oxidation vary depending on the permanganate concentration and heating time.[18] The gas chromatographic radiometric assay method appears to be sensitive,[25] but is also time-consuming.[24] On the contrary, the nonradioactive HPLC assay seems to be satisfactory for measuring the conversion of small fractions of methylmalonyl-CoA to succinyl-CoA, and is said to be simple, rapid, and highly reproducible.[26,27]

Otherwise, authors often have not provided any data (details of the optimal assay conditions or coefficients of variation) to support their conclusions and not all authors

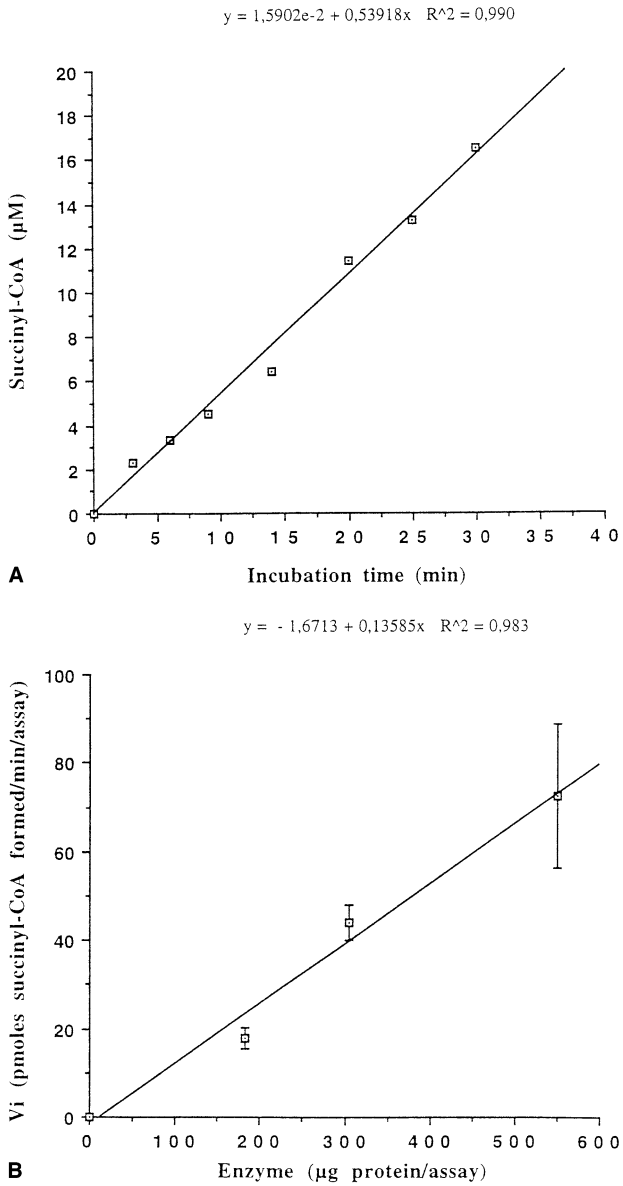


Figure 2 High performance liquid chromatography assay of rat liver holo-MCM activity. *Figure 2A:* Amount of succinyl-CoA formed at 37°C as a function of incubation time. The enzyme concentration was constant (360 µg protein/assay). *Figure 2B:* Rate of formation of succinyl-CoA per min (Vi) as a function of protein concentration (0–550 µg protein/assay). The assay was performed with three incubation time values (8, 15, and 20 minutes) per protein concentration.

measured the holo-MCM activity.[24,25] Lastly, the most popular method for measuring MCM activity, despite its inconveniences, is the one based on the permanganate oxidation of DL[CH_3-^{14}C]methylmalonyl-CoA.[31–33]

Therefore, we conducted this study to compare the most commonly used method (radioassay based on the separation of the substrate and product by permanganate oxidation)[22] with the reputedly more sensitive nonradioactive HPLC assay.[26,27] All assays were performed on rat liver homogenates. Several criteria were taken in account. Within-run reproducibility was determined by assaying ten replicate samples from the same batch and by assaying samples from ten batches. We also monitored assay sensitivity. We paid special attention to the determination of the best conditions for measuring holo activities.

Materials and methods

Reagents

Methylmalonyl-coenzyme A (lithium salt), succinyl-coenzyme A (sodium salt), AdoCbl, perchloric acid ($HClO_4$), propionic acid, and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich S.A.R.L. (St. Quentin Fallavier, France). HPLC-gradient grade methanol was obtained from Merck (Nogent-sur-Marne, France). DL[CH_3-^{14}C]methylmalonyl-coenzyme A was obtained from NEN (NEN Life Science Products, Les Ulis, France). Picofluor 40 scintillation cocktail was provided from Packard Instrument (Rungis, France). Millex-HV13 syringe operated filter unit 0.45 µm was from Millipore S.A. (St. Quentin Fallavier, France).

For the HPLC assay, a stock solution of 600 µM methylmalonyl-coenzyme A in 300 mM Tris-phosphate, pH 7.5, was prepared and stored at −30°C. Calibration solutions of 100 µM methylmalonyl-coenzyme A in 100 mM phosphate buffer, pH 4.0, and 100 µM succinyl-coenzyme A in 200 mM phosphoric acid, pH 1.8, were kept at −30°C. For the radiometric assay, a solution of 3 mM methylmalonyl-coenzyme A in 3 mM HCl was stored at −30°C. A solution of 56.4 mCi/mmol DL[CH_3-^{14}C]methylmalonyl-coenzyme A was stored at −80°C in 6 µL aliquots.

Preparation of the enzyme extract

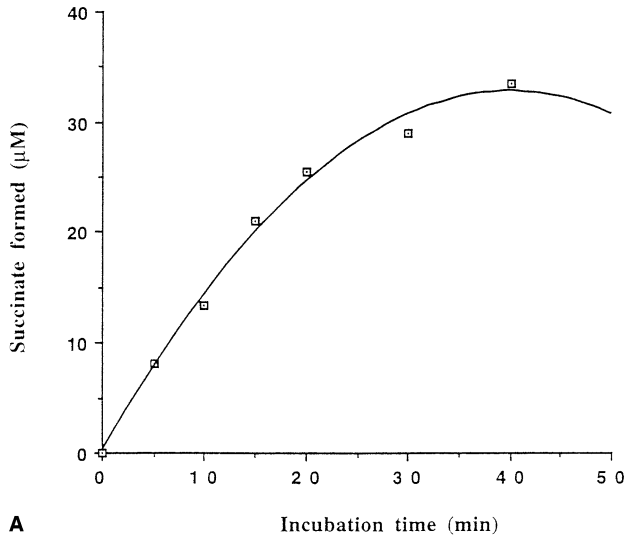
Ten rats (*Rattus norvegicus*, Wistar) were decapitated and the livers immediately removed, chopped into small pieces on ice, frozen in liquid nitrogen, and stored at −80°C. Before enzymatic assay, the pieces of tissue were homogenized on ice in 4.5 volumes (w/v) cold distilled water (Ultra-Turrax T25, Janke and Kunkel, IKA-Werk, Staufen, Germany) at 20,000 rpm for 3 minutes. The homogenates were centrifuged at 750 g for 10 minutes at 4°C. The supernatants were then aliquoted (1 mL) and stored in black microcentrifuge tubes at −80°C. Just before analysis, the aliquots were diluted with distilled water to obtain the optimal protein concentration for the assay. One part was used to measure the protein concentration (Bio-Rad protein assay kit, Bio-Rad Laboratories S.A., Ivry sur Seine, France) by the method of Bradford[34] with bovine serum albumin as standard.

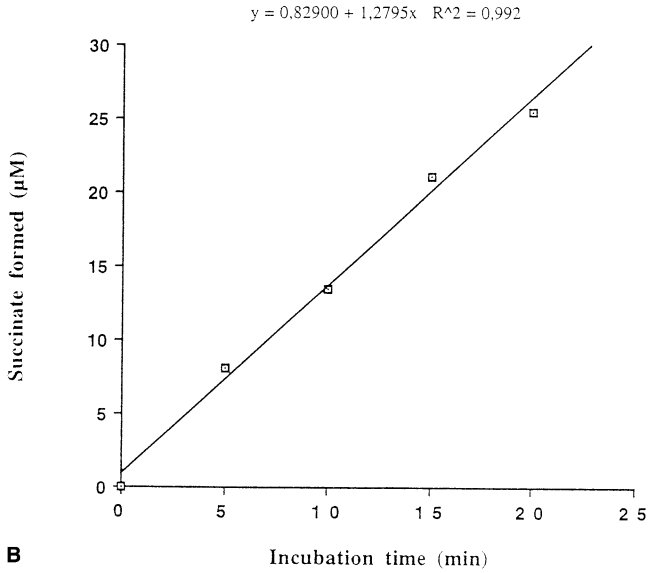
MCM assay

All assays were performed using saturating concentrations of substrate (120 µM methylmalonyl-CoA for the HPLC assay and 300 µM for the radioassay) and cofactor (100 µM AdoCbl for the radioassay and 130 µM for the HPLC assay).

HPLC method

Material. HPLC was carried out on a modular system (Thermo-Quest, Les Ulis, France) consisting of an SCM 1000 vacuum membrane degasser, a P1000 XR HPLC pump module, and an AS 100 autosampler. Compounds were detected by their absorbance at 254 nm (UV 2000 detector, Thermo-Quest, Les Ulis, France) and analyzed with a microcomputer equipped with a chromatographic software (Borwin, JMBS Developpements, Le Fontanil, France). The HPLC column (Hypersil 3 µm C18 100 × 0.3) was from Interchim (Paris, France).





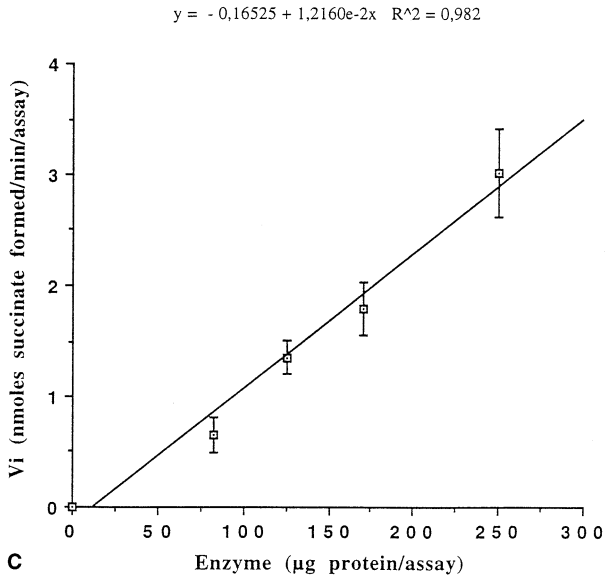


Figure 3 Assay of the rat total liver MCM activity by the $KMnO_4$/radioassay. *Figure 3A:* Amount of succinate formed at 37°C as a function of incubation time. The amount of enzyme was constant (80 μg protein/assay). *Figure 3B:* Detail of the above curve showing incubation time of 0 to 20 minutes. *Figure 3C:* Succinyl-CoA formed per minute (Vi) as a function of protein concentration (0–250 μg protein/assay). The assay was performed with two incubation time values (4 and 9 minutes) per protein concentration.

Method. The nonradioactive assay of MCM activity was based on the separation of unlabeled methylmalonyl-CoA and succinyl-CoA by HPLC,[26] as described by Riedel et al.,[27] with some personal modifications.

All manipulations involving AdoCbl were done in black microcentrifuge tubes. The assay reaction mixture for total MCM activity contained, in a total volume of 150 μL, 25 to 550 μg of liver homogenate protein (100 μL), 20 μL 1 mM AdoCbl, and 30 μL 600 μM methylmalonyl-CoA. AdoCbl was replaced by the same volume of distilled water for measuring holoactivity. The liver homogenate was first incubated with AdoCbl for 5 minutes at 37°C, and the enzyme reaction was initiated by adding the substrate methylmalonyl-CoA. Incubation was at 37°C for 0 to 30 minutes and was stopped by adding 50 μL 100 g/L TCA. The proteins precipitated by the TCA were removed by filtering the reaction mixture through a 0.45-μm syringe-operated filter unit. Aliquots (20 μL) of each sample were taken for HPLC. Blanks were included in every run, and prepared by adding TCA just after methylmalonyl-CoA (incubation time = 0 minutes). Specific activity is defined as nanomoles of succinyl-CoA formed per minute per mg protein.

Methylmalonyl-CoA and succinyl-CoA were separated at 39.7°C on a Hypersil 3 μm C18 100 × 0.3 reverse phase column (Interchim, Paris, France) equilibrated with 56% solvent A (75 mM acetic acid in 100 mM phosphate buffer, pH 4.6) and 44% solvent B [18% (v/v) methanol in solvent A]. Elution was with a linear methanol gradient: 0 to 3 minutes (44% solvent B); 3 to 9 minutes (44–75%); 9 to 12 minutes (75–100%); 12 to 17 minutes (100–44%); and 17 to 26 minutes (44%). The flow rate was 0.2 mL/min. Solvent A was filtered through a 0.22-μm membrane and then degassed with a vacuum–pump.

The amount (y) of succinyl-CoA formed (μM) was calculated from the standard curve ($y = 1.432852\ 10^{-5}x + 0.36522936$; $r^2 = 0.992$), where peak area is x. The exact concentration of the succinyl-CoA stock solution was determined spectrophotometrically using the molecular extinction coefficient $\epsilon_{259\ nm}$ of 15.4 $mmol^{-1}cm^{-1}$ in phosphate buffer, pH 7.0.

MCM activity was measured by the conversion of [^{14}C]methylmalonyl-CoA to [^{14}C]succinyl-CoA using the $KMnO_4$–$HClO_4$ procedure, described previously[22] with modifications, as follows.

The assay reaction mixture for total MCM activity (250 μL) contained 40 to 250 μg liver homogenate protein (100 μL), 25 μL

Table 1 Rat liver methylmalonyl-coenzyme A mutase (MCM) activity measured by $KMnO_4$-radioassay and by HPLC: Assay precision and specific activities of MCM*

	Total-MCM activity		Holo-MCM activity	
	Specific activity (mean ± SD)	CV (%) (n = 10)	Specific activity (mean ± SD)	CV (%) (n = 10)
HPLC method				
Within-run†	6.61 ± 0.52	7.92	0.126 ± 0.004	3.53
Within-run‡	5.68 ± 0.69	12.16	0.121 ± 0.015	12.19
Mean SA§	6.15 ± 0.76		0.124 ± 0.011	
$KMnO_4$ radioassay				
Within-run†	1.94 ± 0.65	33.38	0.044 ± 0.008	18.43
Within-run‡	1.86 ± 0.76	40.58	0.033 ± 0.014	43.75
Mean SA§	1.90 ± 0.69		0.039 ± 0.013	

*MCM specific activities are expressed as nmoles succinyl-CoA/min/mg protein. Total activity was assayed with excess AdoCbl [100 μM for the radioassay and 130 μM for the high performance liquid chromatography (HPLC) assay].
†Mean specific activity measured by assaying ten replicate samples from the same batch.
‡Mean specific activity measured in ten batches.
§Mean specific activity of 2 and 3.

1 M Tris/HCl buffer, pH 8.0, 9 μL 5 M NaCl, 66 μL distilled water, and 25 μL 1 mM AdoCbl. The same procedure was used to measure holomutase activity, except that AdoCbl was replaced by 25 μL distilled water. Sample tubes were first incubated for 8 minutes at 37°C and the enzyme reaction was initiated by adding 25 μL 3 mM DL-methylmalonyl-CoA (containing 1,200 μCi of DL[CH_3-^{14}C]methylmalonyl-CoA per millimole) and continued at 37°C for 0 to 30 minutes. The reaction was stopped by adding 50 μL 2 M $HClO_4$, followed by incubation at 100°C and 4°C for 3 minutes each. After cooling, the tubes were centrifuged at 1,500 g for 15 minutes at 4°C. Supernatant (125 μL) was combined with 25 μL 2 M $HClO_4$ and 250 μL 4% $KMnO_4$ (w/v), incubated at 100°C for 10 minutes, and centrifuged at 1,500 g for 15 minutes at 4°C. This supernatant was centrifuged at 1,500 g for 5 minutes at 4°C. An aliquot (200 μL) of supernatant was placed in a glass scintillation vial containing 125 μL propionic acid and evaporated to dryness at 80°C overnight. Residues were dissolved in 500 μL distilled water, and 2 mL of Picofluor 40 scintillation cocktail were added. Radioactivity was counted using a Minaxi Tri-Carb 4000 Packard model (Packard Instrument SA, Rungis, France). Assays were carried out in the dark until the addition of perchloric acid. Blanks were run in parallel with each series of assays by adding $HClO_4$ just after the methylmalonyl-CoA (incubation time was 0 minutes). A total count tube was also prepared, without adding $HClO_4$ and without drying.

Enzyme activity was calculated from the percentage of [^{14}C]methylmalonyl-CoA converted to [^{14}C]succinyl-CoA and specific activity was defined as nanomoles of succinic acid formed per minute per milligram of protein.

Statistical analysis

All data are presented as means with their standard errors (± SD). In figures, values are means, with their standard errors represented by vertical bars.

Results

Optimal assay conditions

Preliminary experiments established the conditions under which the conversion of methylmalonyl-CoA to succinyl-CoA was linear with respect to time and the amount of liver crude extract.

HPLC method

The conversion of methylmalonyl-CoA to succinyl-CoA was linear with time for up to 15 minutes in excess AdoCbl (100 μM) (*Figures 1A and 1B*) and was linear with respect to protein concentration up to 100 μg liver crude extract protein per assay (*Figure 1C*).

The time course of the holomutase assay was linear for up to 30 minutes (*Figure 2A*) and linear with respect to protein concentration over the range 0 to 550 μg protein per assay (*Figure 2B*).

Radiometric method

Total MCM activity was linear with time up to 20 minutes (*Figure 3A and 3B*) and with protein concentration up to 250 μg liver crude extract protein per assay (*Figure 3C*).

The percentage conversion of methylmalonyl-CoA to succinyl-CoA was low but significantly higher than the radioactive blank ($P = 0.005$ according to Wilcoxon test) in experiments without added AdoCbl, with a protein concentration of 400 μg per assay and incubation time of 20 minutes.

Assay precision and specific activities

All subsequent measurements were performed using saturating concentrations of substrate and AdoCbl (for total activity) and under conditions described above giving linearity with time and protein concentration (i.e., under Vmax conditions).

The within-batch precision of the HPLC assay from ten replicate analyses of the same liver crude extract gave a coefficient of variation (CV) of 3.5% for the holomutase activity and 7.9% for the total mutase activity. The radiometric method gave CVs of 18.4% for the holomutase activity and 33.4% for the total MCM activity (*Table 1*). The enzyme activity measured by HPLC in ten different homogenates gave CVs of 12.2% for both holo- and total-MCM activity. The radiometric assay gave CVs of 43.8% for the holomutase activity and 40.6% for the total

MCM activity (*Table 1*). The CVs for the radiometric method were three to five times greater than those for HPLC. These results reveal the lack of reproducibility of the $KMnO_4$/radioassay for measuring MCM activity.

The two techniques for assaying the MCM activity gave very different specific activities. The HPLC assay gave a mean holomutase activity of 0.124 ± 0.011 nmol succinyl-CoA/min/mg protein and a total mutase activity of 6.15 ± 0.76 nmol succinyl-CoA/min/mg protein, whereas the radiometric method gave activities of 0.039 ± 0.013 nmol succinyl-CoA/min/mg protein for the holomutase activity and 1.90 ± 0.69 nmol succinate/min/mg protein for the total activity (*Table 1*). The specific activities measured by HPLC were three times greater than those obtained with the radiometric method described by Kolhouse et al.[22]

Discussion

The in vitro measurement of total- and holo-MCM activities is important for studies on the Cbl-pathway. Several approaches have been used to measure MCM activity. The main current method is a radioassay based on the permanganate oxidation of DL[CH_3-^{14}C]methylmalonyl-CoA,[22] although radiometric methods for mutase activity have been criticized for their cumbersome nature and their lack of sensitivity.[24,25] This study compares this method with the nonradiometric assay based on the separation of methylmalonyl-CoA and succinyl-CoA by HPLC.[26] The reproducibility and the sensitivity of each technique were evaluated to determine which method was the most suitable for measuring total- and holo-MCM activity.

We found many differences in the results obtained by the two techniques. The specific mutase activities (SA) were very different. The permanganate oxidation method gave SAs three times lower than the HPLC method. The HPLC results agreed fairly well with those obtained by Riedel et al.[27] using a comparable technique. The mutase activity has not been measured by others using the radiometric method under the same strict conditions as ours (with rat liver extract and the permanganate radioassay). Other authors using the same experimental conditions but liver extracts from other species obtained specific activities varying greatly from one another (total SA from 2.83 nmol succinate/min/mg protein[32] to 24 nmol succinate/min/mg protein[35] using crude human liver extract).

There were also considerable differences in the CV for the two methods. The CVs for the radiometric method were three to five times higher than those for the HPLC assay. The HPLC CVs are in agreement with those of Riedel et al.[27] using a comparable technique. No CVs seem to have been published for the permanganate oxidation radioassay.

These results demonstrate the lack of sensibility and reproducibility of the permanganate radioassay. The HPLC method is known for its sensitivity. The HPLC method for measuring MCM activity assays succinyl-CoA, the product of the enzymatic reaction directly. The radiometric method uses permanganate to oxidize the succinyl-CoA and then measures the amount of succinate. This could explain why the radiometric method is less sensitive and less reproducible than the HPLC method.

Many authors[18,24,25] have criticized the radioassay (without giving their position concerning HPLC). Our findings confirm that the radiometric method is not suited for measuring low mutase activities. This confirms the results of Goodey and Gompertz,[25] which show that the permanganate oxidation radioassay for the leukocyte enzyme was satisfactory only for measuring high percentage conversions of methylmalonyl-CoA to succinate. Consequently, the radiometric method for assaying the MCM activity based on the permanganate oxidation is not adequate for measuring holo-MCM activities in tissues and cells or for assaying the total activity in some cells, such as leukocytes.[25]

The radiometric assay, in contrast to the HPLC assay, has several other inconvenient features: it is time consuming because of its numerous incubation times and its overnight drying and it is laborious because it requires many manipulations. DL[CH_3-^{14}C]methylmalonyl-CoA is not always available commercially[36] and its synthese is laborious and dangerous.

The lack of sensitivity and reproducibility, and the inconvenience of the radiometric assay for MCM activity using permanganate oxidation makes the HPLC method the method of choice. This method provides rapid, reliable, and reproducible results and is sufficiently sensitive to measure low MCM activity such as the holo-MCM activity in tissue extracts or the total-MCM activity of cells. We therefore believe that this method is suitable for detecting abnormal MCM apoenzyme, whether to diagnose MMA or to detect errors of cobalamin metabolism.

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