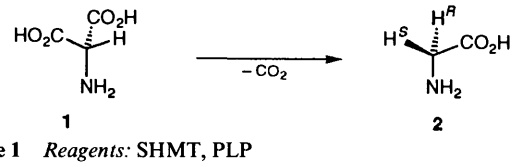
Decarboxylation of 2-Aminomalonic Acid by Serine Hydroxymethyltransferase is, in fact, a Stereospecific Process[1]

Neil R. Thomas, Janet E. Rose and David Gani*
School of Chemistry, The Purdie Building, The University, St. Andrews, Fife, KY16 9ST, UK

Contrary to the results of an earlier study, 2-aminomalonic acid is decarboxylated stereospecifically by serine hydroxymethyltransferase. The newly introduced hydrogen occupies the 2-*pro*-*S* position of the glycine product and, by analogy to studies using 2-amino-2-methylmalonic acid as the substrate (preceding paper in this issue), it is expected that the *pro*-*R* carboxy group is lost during the reaction. A study of the rates of C^{α}–H hydrogen exchange for 2-aminomalonic acid with solvent hydrogen from the aqueous buffer indicates that hydrogen exchange and racemisation would have complicated the analysis of the results in the earlier study.

Serine hydroxymethyltransferase (SHMT) is a ubiquitous pyridoxal 5'-phosphate (PLP)-dependent enzyme which catalyses the retro-aldol cleavage of L-serine to give glycine and formaldehyde.[2] The enzyme exhibits a low regard for reaction-type specificity with α-amino acid substrates and is able to catalyse retro-aldol cleavage/aldol condensation, transamination, racemisation and decarboxylation reactions with the appropriate substrates.[2,3]

Of relevance to our interest in the mechanism of PLP-dependent decarboxylase reactions, it had been reported that the SHMT-catalysed decarboxylation of aminomalonic acid **1** (Scheme 1), when conducted in tritiated water, apparently gave equal quantities of (2*R*)- and (2*S*)-tritiated glycine (**2**, $H^R = {}^3H$, $H^S = H$, and $H^R = H$, $H^S = {}^3H$).[4]



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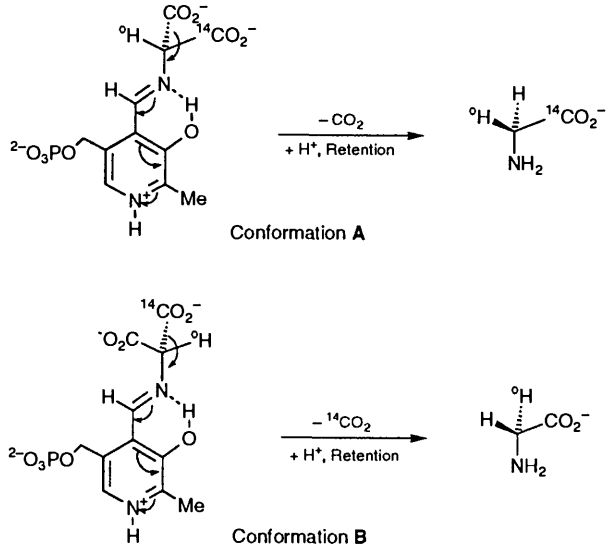
Scheme 1 *Reagents:* SHMT, PLP

Verification of the non-stereospecific nature of the reaction was obtained by using (2*R*)-amino[1-^{14}C]malonic acid as the substrate. In these experiments half of the radiolabel was lost as $^{14}CO_2$ and half was retained in the glycine product. The conclusion that SHMT catalysed a completely non-stereoselective decarboxylation reaction, therefore, seemed to be irrefutable and was of particular interest because it was the only reported example of a non-stereospecific decarboxylation catalysed by a PLP-dependent enzyme.[4] Furthermore, the result appeared to contradict Dunathan's proposals regarding stereoelectronic control in PLP-dependent enzymes.[5]

In order to explain the apparent lack of stereospecificity Palekar *et al.*[4] suggested that the substrate, aminomalonic acid, might be able to bind in two equally populated conformations (A and B in Scheme 2) at the active site of the enzyme, such that each of the two carboxy groups was positioned correctly for decarboxylation at 90° to the plane of the coenzyme. If the decarboxylation and subsequent protonation steps occurred stereospecifically for each form, then the observed results would have been obtained (see Scheme 2). Support for this proposal is provided by the fact that both D- and L-antipodes of several amino acid substrates bind to and are processed by the enzyme.[3]

On the other hand, if racemisation of the substrate occurred prior to decarboxylation, the same observations might have been expected. Prior racemisation it appeared, however, could be ruled out in view of Palekar's earlier



Scheme 2 Proposed explanation for non-stereospecific decarboxylation

finding; that another PLP-dependent enzyme, aspartate β-decarboxylase, catalysed the stereospecific decarboxylation of aminomalonate.[6]

Our recent work with mammalian cystosolic and *E. coli* SHMT,[7] using the chiral ^{13}C-isotopomers of the very slow decarboxylation substrate 2-amino-2-methylmalonic acid,[8] had shown that cleavage of the pro-R carboxy group of the substrate occurred with retention of configuration to give (2*R*)-alanine. Thus, in the context of the earlier work, either the bulkier substrate was only able to bind to the enzyme in one of the two conformations available to 2-aminomalonic acid (Scheme 2, conformation **B**), or Palekar's explanation[4] for non-stereospecificity with 2-aminomalonic acid as the substrate was incorrect.[7]

In order to test these possibilities, Palekar's experiments[4] with 2-aminomalonic acid were repeated, except that chemical assays for the chirality of the glycine product were used in which the isolated glycine was converted into its *N*-(1*S*,4*R*)-camphanamide derivative **3**.[9]

2-Aminomalonic acid **1** (30 mmol dm^{-3} at 3 times the Michaelis constant K_m) was incubated with rabbit liver cytosolic SHMT and PLP in deuteriated buffer under the exact conditions described by Palekar *et al.*[4] but on a 20-fold larger scale, and the decarboxylation product, glycine, was purified and isolated. This was treated with (1*S*,4*R*)-camphanoyl

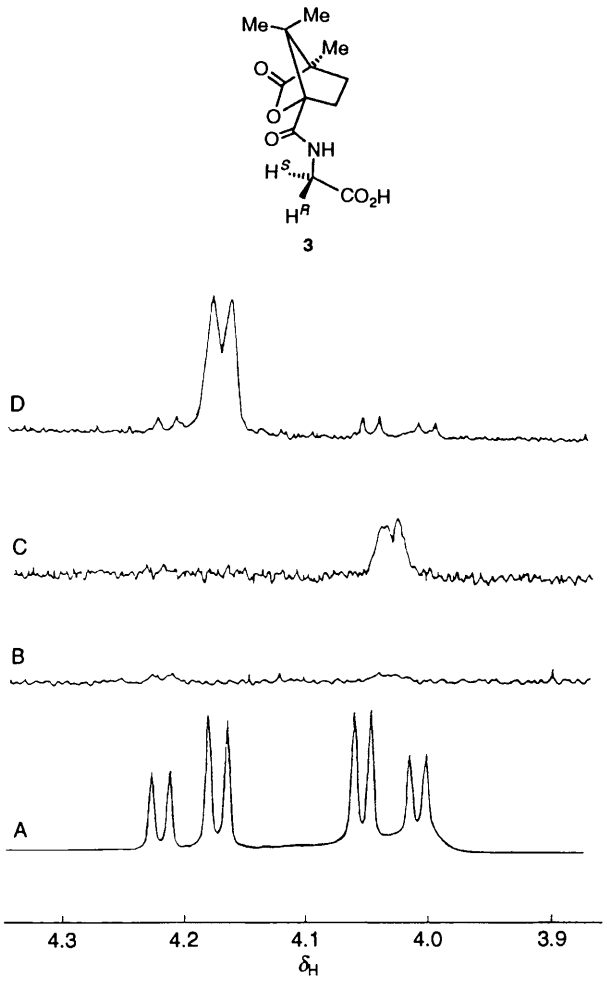


Fig. 1 Partial 400 MHz ^1H NMR spectra of *N*-camphanoylglycines **3** showing the signals due to the diastereotopic C-2 methylene protons of the glycine moiety; A, unlabelled *N*-camphanoylglycine (**3**; $H^R = H^S = H$); B, deuterium-labelled *N*-camphanoylglycine (**3**; $H^R = H^S = {}^2H$) from glycine isolated from the incubation of unlabelled 2-aminomalonic acid with cytosolic SHMT in dideuterium oxide under Palekar's conditions (small amounts of SHMT activity); C, *N*-camphanoylglycine (**3**, $H^R = H$, $H^S = {}^2H$) from glycine isolated from the incubation of unlabelled 2-aminomalonic acid with large amounts of cytosolic SHMT in dideuterium oxide; D, *N*-camphanoylglycine (**3**; $H^R = {}^2H$, $H^S = H$) from glycine isolated from the incubation of 2-amino[2-^2H]malonic acid with large amounts of cytosolic SHMT in water. See Experimental section for details.

chloride to give the camphanamide derivative, which was examined by NMR spectroscopy. The unlabelled derivative and the 2-dideuterio isotopomer were known to display well separated AB-type signals for the diastereotopic C-2 hydrogens of the glycine moiety in the ^1H and ^2H NMR spectra.[9] H^R and H^S resonances occur at δ 4.05 and 4.16, respectively, in the 360 MHz ^1H NMR spectrum. Hence, on the basis of Palekar's experiments, the spectra of the decarboxylation products were expected to show the incorporation of ~50 atom% solvent-derived hydrogen in each of the C-2 positions of the product. In actuality the incorporation of solvent-derived deuterium was close to 100 atom% for each of the C-2 positions as judged by the NMR spectrum (Fig. 1, spectrum B) and the mass spectrum of the camphanamide derivative.

Thus, it was evident from the high level of deuterium incorporation that either α-hydrogen exchange had occurred prior to the enzyme-catalysed decarboxylation or that the product, glycine, had racemised.

Whatever the cause, the high level of incorporation of solvent hydrogen would have elicited severe problems for Palekar *et al.*[4]

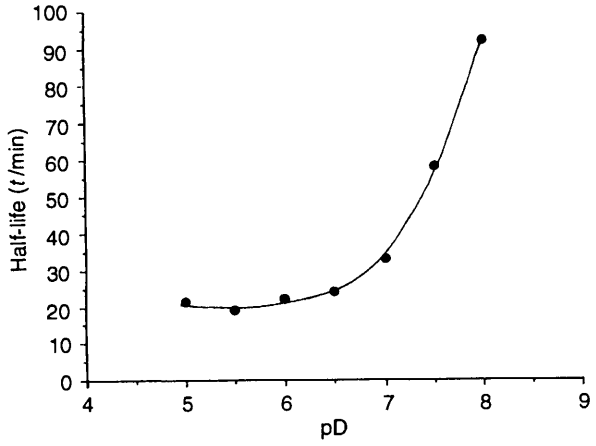


Fig. 2 Graph showing the pD-dependence of the half-life for the exchange of the α-hydrogen of unlabelled 2-aminomalonic acid with solvent hydrogen in dideuterium oxide at 20 °C. Exchange rates were measured as described in the Experimental section.

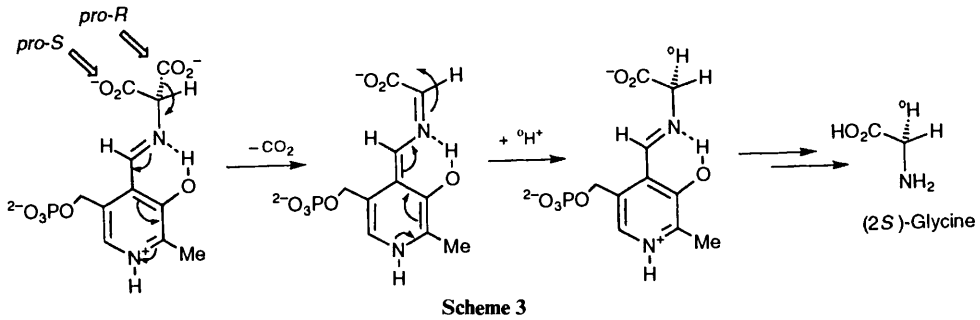
who had used tritium in their experiments and had analysed the chirality of the tritiated glycine product using D-amino acid oxidase, which is known to transfer the 2-*pro-S* methylene hydrogen to the solvent. Clearly, for any given specific amount of label, an assay based on D-amino acid oxidase cannot differentiate between a racemic mixture of labelled glycines and a mixture of unlabelled and doubly labelled glycines. Their apparent results were, therefore, identical with our own and an explanation as to why Palekar *et al.* believed that the decarboxylation occurred non-stereospecifically emerges. Nevertheless, our result had shown that too much solvent-derived hydrogen had been incorporated into the glycine and we now wished to identify the cause.

In order to determine whether non-enzymic α-hydrogen exchange between the substrate and the solvent could occur during the course of the enzymic decarboxylation, the rates of α-deuterium atom incorporation into the unlabelled substrate were measured in a vast excess of deuterium oxide under pseudo-first-order conditions over the pD range 5.0–8.0 at 20 °C. The exchange reaction was followed by ^1H NMR spectroscopy using an internal 1,4-dioxane concentration reference and 19 spectra were accumulated at 5 min intervals for a period of 100 min at each pD.

For the experiments conducted at pD 5.0–6.5, the half-lives for exchange, at 20 °C, were ~20 min whilst those for the experiments conducted at pD >7.0 were 35 min or more (Fig. 2). Thus, under Palekar's conditions, pH 6.0 at 37 °C, the half-life for exchange would have been ~5 min, a substantially shorter period than the duration of the experiments upon which Palekar *et al.* performed the original stereochemical analyses (*e.g.*, 30 min at 37 °C for the experiment performed in tritiated water).[4] Therefore, it is now evident that, in these experiments, the concentration of the unexchanged (unlabelled) substrate that was available to the enzyme would have been crucially dependent on the amount of enzyme used in the experiments and, of course, the initial concentration of the substrate and how long it was pre-exposed to the isotopically labelled buffer medium.

It should be noted that arguments pertaining to non-enzyme-catalysed α-hydrogen exchange in the substrate also apply to pre-racemisation. This is because the enol (*aci*-carboxylic acid) is an intermediate in both reactions and possesses homotopic faces. Thus, in Palekar's experiments in which chiral ^{14}C-labelled substrate was used, the substrate would have racemised before significant enzymic decarboxylation had occurred.

Since it was now evident that a substantial amount of the



Scheme 3

2-aminomalonic acid substrate had undergone α-hydrogen exchange or had been racemised prior to the actual enzyme catalysed decarboxylation reaction in the original experiments, it was absolutely clear that SHMT, in fact, had *not* been shown to catalyse decarboxylation non-stereospecifically. From our kinetic analysis of the competition for the unlabelled substrate between the exchange/racemisation reaction, a pseudo-first-order process, and the enzyme-catalysed decarboxylation, an effectively zeroth-order process with respect to substrate at [S] > K_m (the standard conditions at the start of the reactions), it seemed expedient to reassess the stereochemical course in incubations containing much larger amounts of enzyme.

Accordingly, 2-aminomalonic acid was decarboxylated under the conditions of Palekar *et al.*, as before, in deuteriated buffer but in the presence of much more enzyme and for a shorter period of time, 10 min. To prevent undue opportunity for the substrate to exchange its α-hydrogen atom with the solvent, the substrate was added to the incubation solution containing the enzyme in two portions. The interval between additions was timed so that virtually all of the substrate had been consumed from the first addition before the second portion of substrate was added. After a further 10 min the glycine products were isolated and were then converted into their camphanamide derivatives. Examination of the ^1H and ^2H NMR and mass spectra of the derivatives indicated that very little dideuterioglycine had been formed and that the monodeuteriated glycine was of the (2*S*)-configuration only (Fig. 1, spectrum C). When a similar experiment was performed in diprotium oxide with 2-amino[2-^{2}H]malonic acid as the substrate, the spectral analyses of the derivatised glycine indicated that only (2*R*)-glycine had been formed (Fig. 1, spectrum D). Thus, SHMT, in fact, catalyses the stereospecific decarboxylation of 2-aminomalonic acid.

In the light of our earlier work with the methyl homologue of the substrate[7] and our findings with the enzymes L-methionine[10] and L-glutamate decarboxylase,[11] we suggest that cleavage of the *pro-R* carboxy group of the substrate occurs with retention of configuration, such that protonation introduces the 2-*pro*-S hydrogen of glycine, and that this chemistry occurs on the 4′-*si*-face of the coenzyme (see Scheme 3). The stereochemical course of the decarboxylation of 2-aminomalonic acid would thus appear to be completely analogous to that for the retro-aldol cleavage of (2*S*)-serine in which the *pro-R* carboxy group of 2-aminomalonic acid binds to the same site as does the hydroxymethyl group of serine. Presumably the carboxy group of serine and the *pro-S* carboxy group of 2-aminomalonic acid bind to a common site on the enzyme.

Given that SHMT does not catalyse the non-stereospecific decarboxylation of 2-aminomalonic acid, but that the substrate rapidly exchanges its α-hydrogen with the solvent, it is useful to consider why Palekar *et al.*[6] were able to observe the stereospecific decarboxylation of (2*R*)-2-amino[1-^{14}C]malonate by aspartate β-decarboxylase. A probable explanation is that the enzyme was rapidly inactivated *via* a transamination reaction with the substrate in which all of the PLP in the solution was converted into pyridoxamine phosphate. This is in keeping with the fact that only 7% of the substrate was converted into glycine. The limited number of decarboxylation events that did take place would, therefore, have occurred immediately after addition of the substrate to the incubation solution and before it had an opportunity to racemise.

Experimental

Elemental analyses were carried out in the departmental microanalytical laboratory. Specific rotations were determined on an Optical Activity Ltd AA-1000 polarimeter using 10 or 20 cm pathlength cells at 21 °C, and are given in units of 10^{-1} deg cm^2 g^{-1}. ^1H NMR spectra were recorded at 200 MHz on a Varian Gemini-200 and at 300 MHz on a Bruker AM-300 spectrometer. 400 MHz ^1H NMR spectra were obtained from the SERC service at Warwick. Aqueous solutions were referenced using either the H ^{2}HO signal (at δ_H 4.61), the signal for 1,4-dioxane (at δ_H 3.66), or the sodium salt of 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionic acid [TMS(Na)] signal (at δ_H 0.0). *J* Values are given in Hz. ^2H NMR spectra were recorded at 61.44 MHz and are quoted in ppm relative to C^2HCl_3 (at δ_D 7.27). $\{^1H\}$ ^{13}C NMR spectra were recorded 50.3 Hz on a Varian Gemini-200 and 75.47 MHz on a Bruker AM-300 spectrometer, and chloroform (δ_C 77.20), 1,4-dioxane (δ_C 67.40) or methanol (δ_C 47.00) were used as chemical-shift references, as indicated.

Mass spectra and accurate mass measurements were recorded on a Kratos MS 50 spectrometer locally, or on the VG ZAB E (SERC service) instrument at Swansea. Major fragments are given as percentages of the base peak density (100%). Fast-atom bombardment (FAB) spectra were recorded with glycerol as matrix. pH Measurements were performed using a Jenway PNA2 pH meter.

Yeast alcohol dehydrogenase was obtained from Sigma Chemical Co., Poole, UK and serine hydroxymethyltransferases were kindly provided by Professor Verne Schirch of the Virginia Commonwealth University.

Ammonium Hydrogen Aminomalonate **1**·NH_3.—Diethyl aminomalonate hydrochloride (11.11 g, 5.25 mmol) was dissolved in 2 mol dm^{-3} aq. KOH (110 cm^3) and the resulting solution was heated to 100 °C for 15 min. The solution was cooled in ice and the pH was adjusted to 6.0 using 6 mol dm^{-3} acetic acid. Ethanol (300 cm^3) was added, and the resulting precipitate was filtered off, and washed with ethanol. The precipitate was then dissolved in a minimum amount of water and was applied to a column of Dowex 1-X8 acetate resin (30 × 2 cm). The column was washed with water (300 cm^3) and then with 2.0 mol dm^{-3} ammonium acetate. The ninhydrin-positive fractions were combined, and concentrated under reduced pressure (< 40 °C) to 100 cm^3. The aminomalonic acid monoammonium salt was precipitated by addition of ethanol, and was recrystallised twice from 3 mol dm^{-3} ammonium

hydroxide–ethanol to give a solid (4.23 g, 59%), m.p. 208 °C (decomp.) (Found: C, 26.65; H, 5.95; N, 20.45. $C_3H_8N_2O_4$ requires C, 26.45; H, 5.90; N, 20.60%); $\delta_H(^2H_2O$; ref. $H^2HO)$ 4.17 (1 H, s, 2-H); *m/z* (EI) 136 (M^+, 0.1%), 91 ($[M - CO_2H]^+$, 1.3), 75 ($[M + H - CO_2NH_4]^+$, 15.12) and 30 (100).

Monoammonium Salt of Perdeuteriated Aminomalonic Acid.—Ammonium hydrogen aminomalonate (60 mg, 29.4 mmol) was dissolved in dideuterium oxide (1 cm^3) in an NMR tube and 1,4-dioxane (1 g) was added as an internal concentration reference standard. After 19.5 h at 37 °C, the aminomalonate was 90% deuteriated at C-2 as judged by ^1H NMR spectroscopy. The sample was sealed and kept frozen until required.

(1'S,4'R)-(−)-N-*Camphanoylglycine* **3**.—Glycine (150 mg, 2.0 mmol) was dissolved in 1.0 mol dm^{-3} aq. NaOH (3 cm^3) and the solution was then shaken with a solution of freshly prepared (1*S*,4*R*)-camphanoyl chloride (435 mg, 2.01 mmol) in toluene (2 cm^3). After 15 min the mixture was vigorously stirred for 2 h and was then diluted with both water (5 cm^3) and dichloromethane (5 cm^3). The organic phase was separated and discarded, and the aqueous phase was adjusted to pH 2 with 5 mol dm^{-3} HCl. The solution was then extracted with dichloromethane (3 × 10 cm^3), and the combined organic layers were *then dried* ($MgSO_4$), and concentrated under reduced pressure to yield a thick oil which crystallised on scratching. Camphanic acid was removed by flash column chromatography on silica with CH_2Cl_2–EtOAc (9:1) as eluent. The (1'*S*,4'*R*)-camphanoylglycine **3** was then obtained as crystals after recrystallisation from diethyl ether–light petroleum (40–60 °C) (389 mg, 78%), m.p. 74 °C (lit.,[9] 73.5–75 °C) (Found: C, 49.5; H, 7.1; N, 4.9. Calc. for $C_{12}H_{17}NO_5 \cdot 2H_2O$: C, 49.47; H, 7.27; N, 4.81%); *m/z* (CI–NH_3) (Found: $[M + H]^+$, 256.1168. Calc. for $C_{12}H_{18}NO_5$: *m/z* 256.1185); $[\alpha]_D$ −18.9 (*c* 1.5, MeOH) [lit.,[8] −19.7 (*c* 1.55, MeOH)]; $\nu_{max}(CHCl_3)/cm^{-1}$ 3570 (OH), 3470 (NH), 2990 (CH), 1775 (C=O, lactone), 1705 (C=O, acid), 1665 and 1557 (C=O, amide); δ_H(400 MHz; C^2HCl_3; ref. TMS) 0.95 (3 H, s, 4'-Me), 1.10 (6 H, s, 7'-Me_2), 1.60–2.54 (4 H, m, 5'- and 6'-H_2), 4.14 (2 H, ABX, J_{AB} 18, J_{AX} = J_{BX} = 7.2, *J* 6, 2-H_2), 7.39 (1 H, t, *J* 7, 2-NH) and 10.35 (1 H, br s, 1-OH); δ_C(50.3 MHz; C^2HCl_3) 9.94, 16.41, 16.64, 28.92, 30.10, 40.73, 54.31, 55.39, 92.52, 168.07, 172.57 and 178.79; *m/z* (EI) 255 (M^+, 12.9%), 237 ($[M - H_2O]^+$, 3.6), 227 ($[M - CO]^+$, 5.7) and 209 ($[M - CO_2H - H]^+$, 100).

(1'S,4'R)-N-*Camphanoyl*[2-$^2H_2$]*glycine*(H^R = H^S = ^2H).—This was prepared in an identical manner to *N*-camphanoylglycine, starting from [2-$^2H_2$]glycine (15.4 mg, 0.2 mmol); δ_H(400 MHz; C^2HCl_3) 0.95 (3 H, s, 4'-Me), 1.10 (6 H, s, 7'-Me_2), 1.60–2.54 (4 H, m, 5'- and 6'-H_2), 7.39 (1 H, t, *J* 7, 2-NH) and 10.35 (1 H, br s, 1-OH); δ_D(60 MHz; $CHCl_3$) 4.15 and 4.00 (C^2H_2 of dideuteriated glycine moiety); *m/z* (EI) 257 (M^+).

Serine Hydroxymethyltransferase.—SHMT from rabbit liver cystosol was obtained as a lyophilised powder from Professor V. Schirch. The enzyme was dissolved in potassium phosphate buffer (20 mmol dm^{-3}, pH 7.3) containing (2*S*)-serine (20 mmol dm^{-3}), dithiothreitol (1 mmol dm^{-3}) and ethylenediaminetetraacetic acid (EDTA) (0.1 mmol dm^{-3}) and was treated to remove unbound PLP and then assayed for activity with [L-*allo*-](2*S*,3*S*)-threonine, in the presence of yeast alcohol dehydrogenase and nicotinamide adenine dinucleotide (reduced form NADH), as described in the preceding paper. One unit of enzyme activity is the amount of enzyme required to convert one micromole of L-*allo*-threonine into acetaldehyde and glycine per minute.

Decarboxylation of 2-Aminomalonic Acid under Palekar's Conditions.—The incubation contained 2-aminomalonic acid (75 mmol dm^{-3}), dithiothreitol (1 mmol dm^{-3}), PLP (0.1 mmol dm^{-3}) and SHMT (1 unit) in deuteriated sodium phosphate buffer (60 mmol dm^{-3}, pD 6.0) in a total volume of 1.0 cm^3. The reaction was maintained at 37 °C for 1 h, when all of the substrate had been consumed, and was terminated by adjusting the pH to 10.5 with 2 mol dm^{-3} aq. KOH. The protein was precipitated by addition of absolute ethanol and was removed by centrifugation (3000 rpm for 20 min). The supernatant solution was concentrated under reduced pressure (< 40 °C) to give a residue containing glycine and buffer salts. This solid was dissolved in water (1 cm^3) and the pH of the solution was adjusted to 11.0 by using 2 mol dm^{-3} aq. NaOH. A solution of freshly prepared (1*S*,4*R*)-camphanoyl chloride (21.6 mg, 0.1 mmol) in toluene (1.5 cm^3) was added, and the mixture was shaken vigorously for 15 min and then stirred for 2 h. The *N*-camphanoylglycine was isolated as described above and the extent of label incorporation and the chirality of the glycine moiety were deduced. The sample contained greater than 90 atom% deuterium in each of the diastereotopic methylene positions of glycine, indicating that solvent-hydrogen exchange in the substrate was occurring before decarboxylation; δ_H(400 MHz; C^2HCl_3; ref. TMS) 0.95 (3 H, s, 4'-Me), 1.10 (6 H, s, 7'-Me_2), 1.60–2.54 (4 H, m, 5'- and 6'-H_2), 7.39 (1 H, t, *J* 7, 2-NH) and 10.35 (1 H, br s, 1-OH) (see Fig. 1, spectrum B); δ_D(60 MHz; $CHCl_3$; ref. C^2HCl_3 at δ_D 7.25) 4.14 (1 ^2H, s, 2-$^2H^S$) and 3.99 (1 ^2H, s 2-$^2H^R$); *m/z* (EI) 257 ($[^2H_2]M^+$, 17%), 256 ($[^2H]M^+$, 4), 255 ($[^2H_0]M^+$, 2) and 211 (base peak for $[^2H_2]$-compound).

SHMT–Aminomalonic Acid α-Proton Exchange Experiments.—Potassium dihydrogen phosphate monohydrate (0.68 g, 5.0 mmol) was dissolved in the minimum amount of dideuterium oxide and the solution was then concentrated to dryness under reduced pressure. The residue was redissolved in dideuterium oxide and the pD of the solution was adjusted to pD 5.0 [pD = (pH meter reading) + 0.4][12] by using both 5 mol dm^{-3} NaOD in dideuterium oxide and dideuterium oxide such that the final volume was 25 cm^3. To a portion (1 cm^3) of this solution were added ammonium hydrogen aminomalonate (4.0 mg, 30 μmol) and 1,4-dioxane (1.0 mm^3). The exchange of the α-proton of the aminomalonate with solvent deuterium was monitored by 270 MHz ^1H NMR spectroscopy at 20 °C and the rate of exchange was determined by integrating and comparing the integral for the signals for the α-proton (δ ~4.2) and those for an internal dioxane reference standard.

Without removing the sample from the NMR probe, one spectrum was recorded every 5 min for 90 min. The data were fitted to the first-order rate equation ([protiated substrate] = [protiated substrate]$_0\{\exp(-k_{5.0}t)\}$, where [protiated substrate]$_0$ is the initial concentration of unlabelled aminomalonate and *k* is the first-order rate constant at pD 5.0) and the half-life for the reaction was deduced. This experiment was repeated at pD 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 to give a pH profile for the exchange reaction (see Fig. 2).

Rapid Decarboxylation Experiments.—1. *In dideuterium oxide.* Cytosolic SHMT (25 units) was dissolved in dideuterium oxide (5 cm^3) and the pD of the solution was adjusted to 7.4 as described above. Ammonium hydrogen aminomalonate (6 mg, 40 μmol) was added in two equal portions at $t = 0$ and $t = 5$ min and the solution was incubated at 37 °C for 15 min. The protein was denatured by the addition of ethanol (5 cm^3), and removed by centrifugation. The solution was concentrated to dryness under reduced pressure and rewashed with water–ethanol and then centrifuged until no further precipitation of protein occurred. The final solution was concentrated once more to yield a solid (6.95 mg) containing glycine but no aminomalonate as judged by TLC and ^1H NMR spectroscopy.

The solid was dissolved in water and was purified by preparative TLC on cellulose with Pr^iOH–conc. NH_3–water (26:6:5) as developer. The band containing the glycine was removed from the plate and was stirred with ethanol–water (3:2; 25 cm^3). The cellulose was removed by filtration through Celite and the filtrate was concentrated under reduced pressure. The residual solid was dissolved in water (1 cm^3), and the solution was adjusted to pH 11.0 by using 2 mol dm^{-3} NaOH and then treated with a solution of freshly prepared camphanoyl chloride (25 mg, 0.118 mmol) in toluene (1.5 cm^3). The reaction flask was shaken for 15 min, the mixture was stirred vigorously for 2 h, and the *N*-camphanoylglycine was isolated as described above; δ_H(400 MHz; C^2HCl_3): this spectrum was similar to that for the unlabelled material except that the glycyl signal for H^S at δ 4.20 was missing and that for H^R at δ 4.03 occurred as a doublet (see Fig. 1, spectrum C); δ_D(60 MHz; $CHCl_3$; ref. C^2HCl_3 at δ_D 7.25) 4.18 (1 ^2H, s, 2-$^2H^S$); *m/z* (EI) 256 (M^+).

2. *In diprotium oxide.* Cytosolic SHMT (25 units) was dissolved in water (5 cm^3) and the pH of the solution was adjusted to 7.0. Freshly prepared deuteriated ammonium hydrogen aminomalonate (6.2 mg, 40 μmol deuterium oxide, 100 mm^3) was added in two equal aliquots at $t = 0$ and $t = 5$ min, and the solution was incubated at 37 °C. After 15 min the protein was denatured and the glycine product was purified and then converted into its camphanamide derivative, as described above; δ_H(400 MHz; C^2HCl_3): this spectrum was similar to that for the unlabelled material except that the glycyl signal for H^R at δ 4.03 was missing and that for H^S at δ 4.18 occurred as a doublet (see Fig. 1, spectrum D); δ_D(60 MHz; $CHCl_3$; ref. C^2HCl_3 at δ_D 7.25) 3.99 (1 ^2H, s, 2-$^2H^R$).

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