Stereochemistry of Cysteinesulphinic Acid Decarboxylase

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An h.p.l.c.-based assay for cysteinesulphinic acid decarboxylase has been developed. The assay simultaneously measures glutamate decarboxylation and/or taurine formation. Using the cysteinesulphinic acid decarboxylase in rat liver homogenate, L-[2-$^2H_1$]cysteinesulphinic acid was converted into [2-$^2H_1$]hypotaurine. Similarly, unlabelled L-cysteinesulphinic acid was transformed by rat liver homogenate in a D_2O-containing medium into the enantiomeric [2-$^2H_1$]hypotaurine. The products were desulphurized by treatment with Raney nickel, and the resulting enantiomeric [1-$^2H_1$]ethylamines converted into the corresponding (—)-camphanamides. Deuterium n.m.r. spectra of the latter revealed that the decarboxylations proceeded stereospecifically with retention of configuration.

The decarboxylation of L-cysteinesulphinic acid by cysteinesulphinic acid decarboxylase [L-cysteinesulphinate carboxylyase, E.C. 4.1.1.29] (CSA * decarboxylase) constitutes a step in the biosynthesis of the putative inhibitory neurotransmitter and essential nutrient, taurine.[1–3] Various isomeric forms of CSA decarboxylase have been detected in brain and liver tissues (as well as others), some of which also show glutamic acid decarboxylating activity.[4–9] The enzyme uses pyridoxal-5'-phosphate as coenzyme.[1] We now report the results of an investigation concerning the stereochemistry of CSA decarboxylase in rat liver, which show that replacement of carboxyl by solvent hydrogen proceeds with *retention* of configuration.

Results

Our approach to the determination of the steric course of CSA decarboxylase was analogous to that used in our recently published studies on the steric course of 4'-phosphopantothenoyl-L-cysteine decarboxylase.[10] Thus, unlabelled L-cysteinesulphinic acid was incubated with the enzyme in a D_2O-containing medium. Similarly, L-[2-$^2H_1$]cysteinesulphinic acid was incubated in H_2O with the enzyme. The resultant enantiomers of [2-$^2H_1$]hypotaurine were reduced to the corresponding [1-$^2H_1$]ethylamines, the configurations of which were determined by ^2H n.m.r. on the (−)-camphanamide derivatives.[11]

Seeking a source of enzyme for these studies, in preliminary work we examined the activity of CSA decarboxylase in homogenates of rat brain and rat liver. Previously published assays of CSA decarboxylase employed the measurement of $^{14}CO_2$ formed from (±)-[1-^{14}C]CSA. As this compound was not readily available, an alternative new h.p.l.c.-based assay was developed. A convenient isocratic technique for the rapid separation of the OPA derivatives of CSA and HT by reversed phase h.p.l.c. was adapted from a previously published method.[12] Although CSA eluted too quickly for accurate quantitation, the appearance of HT in incubation mixtures could be readily followed. Also, since HT was well separated from GABA, the presence of contaminating glutamate decarboxylase activity could be measured by including L-glutamate in the assay mixture. This was of importance since there is evidence suggesting that glutamate decarboxylase (particularly in the brain) can also decarboxylate CSA,[13] or, alternatively, that some form(s) of CSA decarboxylase have glutamate decarboxylating activity.[3,5,6] Furthermore, since HT was also well separated from taurine, over-oxidation of the decarboxylation product (HT) could be detected in a single assay procedure. For typical assays of CSA/glu decarboxylase in rat brain and liver homogenates, see Figure 1a, b. The h.p.l.c. results show that rat brain homogenate exhibited very substantial glutamate decarboxylating activity relative to CSA decarboxylase activity. This would have necessitated extensive enzyme purification work in order to obtain a CSA decarboxylase preparation free from glutamate decarboxylating activity, as desired. In contrast, with rat liver homogenate the decarboxylation of glutamate was negligible relative to the decarboxylation of CSA, and over-oxidation to taurine was also essentially negligible. These results strongly suggested the

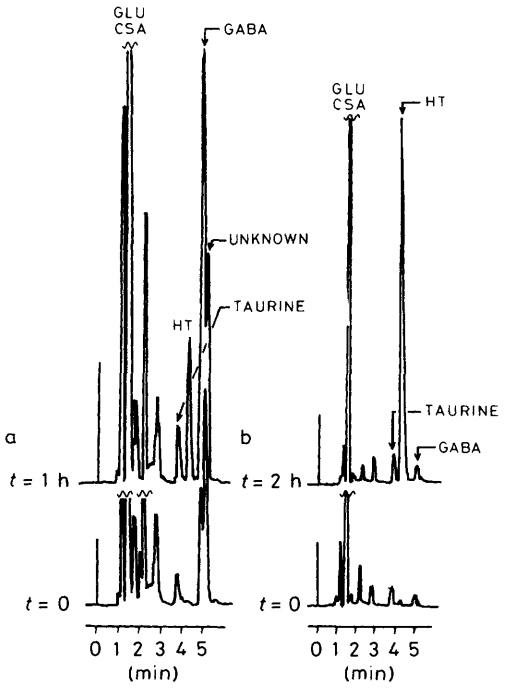


Figure 1. H.p.l.c.-based assays of CSA and glutamate decarboxylase activity in rat brain and liver homogenates (300 μl of each homogenate used in each assay; assays were performed as described in Methods). The incubation with brain homogenate was continued for 1 h, that with liver homogenate for 2 h. Lower traces show composition *before* incubation (t = 0); upper traces show composition *after* incubation. Detector range: Figure 1a, 0.1; Figure 1b, 1.0

* Abbreviations: AET, *S*-(2-Aminoethyl)isothiouronium bromide; CSA, cysteinesulphinic acid; GABA, γ-aminobutyric acid; Glu, L-glutamic acid; HT, hypotaurine; LB, line broadening; OPA, *o*-phthalaldehyde; PLP, pyridoxal 5'-phosphate; PW, pulse width; RD, relaxation delay.

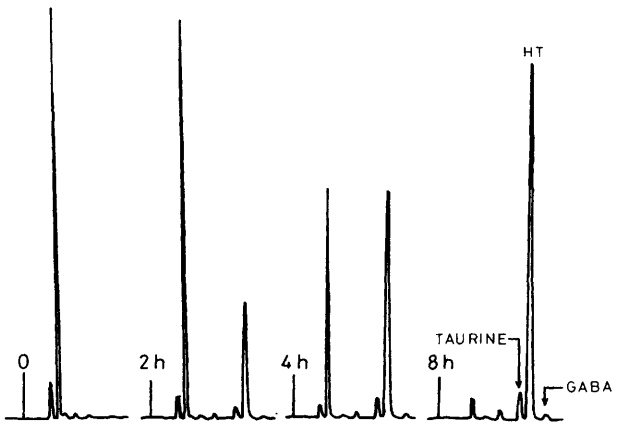


Figure 2. H.p.l.c.-based assay of the time course of decarboxylation of CSA (high concentration run) by rat liver homogenate (an analysis after 20 h was virtually identical to that at 8 h)

preferential use of rat liver homogenate rather than rat brain as a source of CSA decarboxylase for the stereochemical study. The CSA decarboxylase in rat liver has been reported to be identical, or very similar, to one (or more) of the isomers of CSA decarboxylase (CSA decarboxylase I) in rat brain which does not show glutamate decarboxylase activity.[3,5] The decarboxylation of CSA by rat liver homogenate was so clean that it was apparent that purification of the enzyme would be unnecessary. Using *ca.* 20 ml of rat liver homogenate, it was possible to transform over 1 g of unlabelled CSA into HT in high yield in a *ca.* 8 h incubation, with a small degree of oxidation to taurine being the only apparent side-reaction, see Figure 2. Since the activity of CSA decarboxylase was essentially the same in frozen liver as in fresh liver, commercially available frozen liver was used for these studies.

Following the same procedure, unlabelled L-CSA (**1**), was transformed in a D_2O-containing medium (*ca.* 80—90% D) into [2-$^2H_1$]HT (**2**) which was recovered in crude form by a cation exchange procedure. The decarboxylation reaction appeared to proceed at essentially the same rate as in H_2O, but this has not been accurately measured. The substrate for the parallel run, L-[2-$^2H_1$]CSA (**3**) was prepared in two steps from *S*-benzyl[2-$^2H_1$]-L-cysteine[10] following a published method.[14] The product retained a small amount of protium at 2-H (10—20%) as shown by ^1H n.m.r., but was adequately deuteriated for the purposes of this study. This product was then incubated with rat liver homogenate (in H_2O), and the resultant [2-$^2H_1$]HT (**4**) recovered in crude form by cation exchange. The decarboxylation appeared to proceed substantially more slowly than in the case of unlabelled CSA, but the relative rates have not been accurately measured. Also, in this run, more taurine (20—30%) was formed than in runs with unlabelled CSA in H_2O or D_2O. Fortunately, the taurine did not interfere with subsequent steps.

The recovered hypotaurines from the two runs were then subjected to reduction with Raney nickel. The reductions were carried out using the lowest temperature (45 °C) at which the reaction (followed by h.p.l.c.) proceeded at a useful rate (*ca.* 1.5—2 h required for completion) in an attempt to avoid hydrogen–deuterium scrambling. Fortuitously, preliminary development studies on this reaction revealed that, while HT was readily reducible by Raney nickel under mild conditions, taurine did not react, even under much more vigorous conditions (prolonged treatment at 70 °C or 100 °C). This fact made it unnecessary to remove taurine from the crude HT before reduction (although both would have given the same desulphurization product in any case).

The resultant crude [1-$^2H_1$]ethylamines from the two

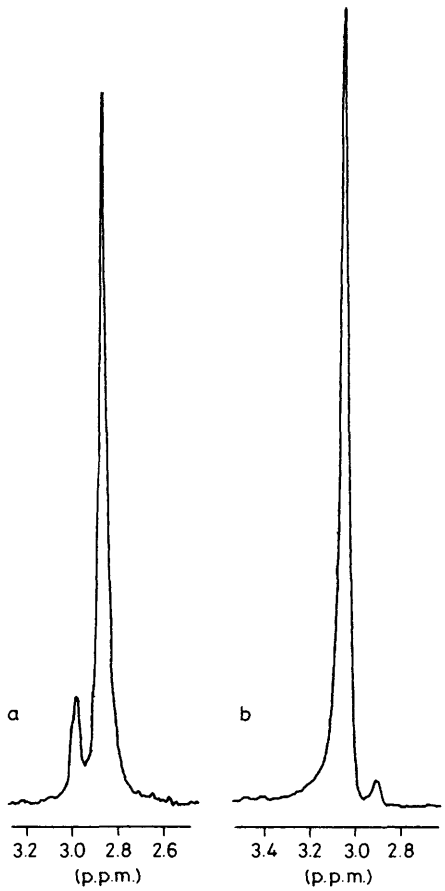


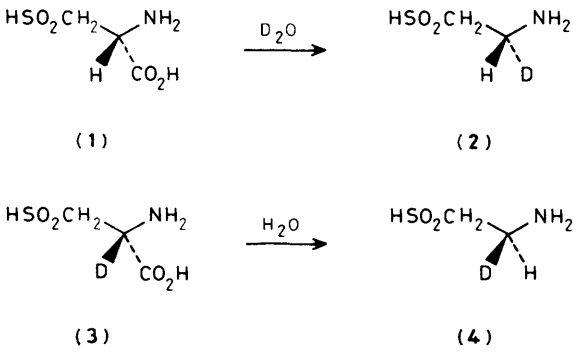
Figure 3. ^1H-Decoupled 76.77 MHz ^2H n.m.r. spectra of [1-$^2H_1$]ethylamine (−)-camphanamide derivatives, taken in 5 mm tubes in C_6H_6 solution with added C_6D_6 (δ 7.15) as reference (peak not shown). a = (1*R*)-[1-$^2H_1$]-enantiomer (**2**) (−)-camphanamide derivative, obtained *via* incubation of unlabelled CSA in D_2O: SW, 1 000 Hz; 0.488 Hz/point; PW, 25 μs; RD, zero; LB, 0.5 Hz; 156 scans; repetition rate, 2.048 s. b = (1*S*)-[1-$^2H_1$]-enantiomer (**4**) (−)-camphanamide derivative, obtained *via* incubation of L-[2-$^2H_1$]-CSA, (**3**) in H_2O; SW, 1 000 Hz; 0.488 Hz/point; PW, 40 μs; RD, zero; LB, 0.3 Hz; 234 scans; repetition rate, 2.048 s

incubation runs were then converted into the (−)-camphanamide derivatives, which were examined by ^2H n.m.r. spectroscopy.* The product obtained from the sequence in which unlabelled CSA was incubated in a D_2O-containing medium showed (Figure 3a) a strong deuterium signal (87%) at δ 2.87, with a much weaker signal (13%) at δ 2.98. (In a second run, not shown, these peaks were in a slightly different proportion: δ 2.87, 79%; δ 2.98, 21%.) The fact that a single peak was not obtained from the D_2O runs is attributed to a small degree of scrambling in the Raney nickel reductions, rather than to partial nonstereospecificity in the decarboxylation reaction. That the decarboxylation has, in fact, a much higher stereospecificity was shown by the ^2H n.m.r. spectrum of the ethylamine (−)-camphanamide derivative obtained in the complementary sequence starting with L-[$^2H_1$]CSA (Figure 3 b). Although again two peaks were observed, the relative areas:

* ^2H N.m.r., rather than ^1H n.m.r., was used, as in our previous work (Aberhart *et al.*, 1985) to avoid complications in interpretation due to the presence of unlabelled ethylamine (−)-camphanamide resulting from incomplete deuteriation of the solvent in the D_2O run, or incomplete deuteriation of [2-$^2H_1$]CSA. Also, it should be noted that AET, used in small amounts in all incubations as a stabilizer of CSA decarboxylase[4] will yield unlabelled ethylamine upon desulphurization.

δ 2.90 (3%), δ 3.04 (97%) show that the decarboxylation must be at least 95% stereospecific. (In an earlier run with L-[2-$^2H_1$]CSA, where the resultant HT was reduced with Raney nickel at reflux as in our previous studies on 4′-phosphopantothenoyl-L-cysteine decarboxylase,[10] the resultant [1-$^2H_1$]ethylamine (−)-camphanamide was essentially completely racemic.) It is more likely that the decarboxylation is completely stereospecific, and that the varying amounts of racemization observed in the several runs carried out is a consequence of our use of different batches of Raney nickel in each case. In the previous work,[10] where a $HSCH_2$-group was being reduced, such racemization was not observed.

Since Parker[11] has shown that the 1-pro-*R* and 1-pro-*S* proton (deuterium) resonances of ethyl (−)-camphanamide appear in the *upfield* and *downfield* positions, respectively, it follows from the complementary spectra obtained from the two enzymatic runs (Figure 3 a, b) that the decarboxylation takes place in a *retention* mode, as shown in (**1**) ⟶ (**2**) and (**3**) ⟶ (**4**).



The stereochemistry (and, presumably, the mechanism) thus parallels that found for a wide variety of other pyridoxal-5′-phosphate-dependent L-α-amino acid decarboxylases.[15,16]

Experimental

General.—^1H N.m.r. spectra were run on a Varian EM-390 instrument. ^2H N.m.r. spectra were run on a Bruker WM-500 instrument. H.p.l.c. was performed by using an instrument consisting of a Waters Associates U6K injector and M-6000A pump, and a Kratos Model FS950 fluorimeter equipped with a 365 nm excitation filter and a 418 nm emission filter. The column was a Waters Associates Resolve C_{18} 3.9 mm × 15 cm column. M.p.s were taken on a hot stage apparatus and are corrected. T.l.c. was performed by using Analtech silica gel GF Uniplates. Preparative t.l.c. was performed by using homemade plates (20 cm × 20 cm × 1 mm thick) prepared with E. Merck Kieselgel HF254 + 366. Rats (female, Sprague-Dawley, *ca.* 250 g) were obtained from Charles River Breeding Laboratories. Frozen rat livers were obtained from Pel-Freeze Biologicals, Rogers, AR, and were stored at −70 °C. (−)-Camphanyl chloride was obtained from Fluka.

Protein assays were performed by using the Bio-Rad protein assay kit, with bovine serum albumin as standard.

OPA Reagent was prepared fresh daily by mixing 100 μl of a solution of *o*-phthalaldehyde (350 mg) in absolute EtOH (5 ml) (stored at −20 °C in the dark) with 10 ml of 0.1M borate buffer, pH 10.4 (1:10 diluted Pierce Fluoropa Reagent Diluent). Finally, 2-mercaptoethanol (20 μl) was added. The OPA concentration was *ca.* 5 mM.

Synthesis of L-[2-^2H]*Cysteinesulphinic Acid* (**3**).—Ammonium molybdate (50 mg) in H_2O (3 ml) was treated with 60—65% $HClO_4$ (0.40 ml), and the mixture was gently boiled over an open flame for 5 min, giving a white precipitate. After cooling to room temperature, H_2O (1 ml) was added, and the mixture was filtered through a Gelman Acro LC13 filter (0.45 μM nylon). The filtrate was added to *S*-benzyl[2-$^2H_1$]-L-cysteine[10] (2.0 g) and 30—35% H_2O_2 (3.8 ml) was added dropwise over *ca.* 10 min, keeping the temperature below 10 °C. The mixture was kept in ice–H_2O for 2 h, then left at room temperature for 18 h. The resultant thick paste was mixed with cold H_2O (10 ml) and vacuum filtered. The solid was dissolved in *ca.* 20 ml of boiling H_2O, and the solution was allowed to cool to room temperature, then to 4 °C. The white crystalline product was collected by suction filtration and washed with a little cold H_2O to yield *S*-benzyl[2-$^2H_1$]-L-cysteine sulphone (2.24 g), m.p. 176—178 °C (corr.) (lit.,[14] m.p. 174.5 °C); δ($CDCl_3$) 3.93 (A) and 4.19 (B) (3-H, 2 H, AB, J_{AB} 14 Hz; shows small amount of coupling with residual 2-H), 4.82 (2 H, s, $PhCH_2$), 4.85 (2-H, *ca.* 0.1—0.2 H, m-overlapped by δ 4.82 singlet), and 7.61 (5 H, s, Ph). Unlabelled *S*-benzyl-L-cysteine sulphone had δ 3.93 (A) and 4.19 (B) (2 H, AB, J_{AB} 14, J_{AX} 7, and J_{BX} 4 Hz), 4.82 (2 H, s), 4.85 (X) (1 H, m), and 7.61 (5 H, s).

The deuteriated sulphone (2.0 g) was dissolved in liquid ammonia (100 ml) at −33 °C and treated with sodium spheres (*ca.* 0.1 g each) until a blue colour persisted for 15 min. The ammonia was allowed to evaporate and the dry residue dissolved in H_2O (50 ml). The solution was extracted once with ether, and the aqueous extract was added to a 2 × 20 cm column of Dowex 50 W-X8, 50—100 mesh H^+ form. The column was eluted with H_2O until all acidic material had been collected. The acidic effluent was then freeze-dried to yield L-[2-$^2H_1$]cysteinesulphinic acid (**3**) as a powder (470 mg), δ(D_2O; external TMS/$CDCl_3$ as reference) 2.96 (A) and 3.15 (B) [2 H, AB, J_{AB} 15 Hz shows small amount of additional coupling (10—20%) with residual 2-H], *ca.* 4.6 (*ca.* 0.1—0.2 H, m, partially overlapped by HOD sideband). Unlabelled cysteinesulphinic acid had δ 2.96 (A) and 3.15 (B) (2 H, AB, J_{AB} 15, J_{AX} 7, and J_{BX} 4 Hz), and 4.66 (X) (1 H, dd, J_1 4 and J_2 7 Hz).

Simultaneous Assay of CSA *Decarboxylase and Glutamate Decarboxylase.*—The procedure is based on published assays for CSA decarboxylase using [1-^{14}C]CSA.[4,5,17] Assay mixtures were prepared consisting of: sodium phosphate buffer (1M; 200 μl), pH 7.2; L-CSA (0.1M; 100 μl) (adjusted to pH 7.2 with NaOH); L-glutamic acid (0.1M; 100 μl) (adjusted to pH 7.2 with NaOH); PLP (1 mM; 100 μl); AET (10 mM; 100 μl); Triton X-100 (1%; 100 μl); and enzyme + H_2O (300 μl). The mixture was incubated at 37 °C for 1 h (or more), and then 10% sodium tungstate solution (50 μl) and 6M HCl (50 μl) were added. The mixture was centrifuged (2 000 r.p.m., 10 min) and the protein-free supernatant withdrawn. A portion of the latter (20 μl) was treated with OPA reagent (200 μl)* for 1 min, and then a 20 μl aliquot of the mixture was subjected to h.p.l.c. using as solvent 20% MeCN–80% 0.1M potassium phosphate buffer, pH 7.5 (filtered through an 0.45 μ Nylon filter and degassed by vacuum pumping). The flow rate was 1.0 ml/min^{-1}. The detector settings were: sensitivity, 3.5; range, 1.0 (or in some cases 0.1). Under these conditions, the retention times were: CSA and glu, 1.5 min; taurine, 3.9 min; HT, 4.4 min; GABA, 5.0 min. Peak areas were determined by triangulation and compared with peak areas from reference solutions having known concentrations of HT, GABA, or taurine.

Typically, rat liver homogenate (prepared as described below) had CSA decarboxylase activity of *ca.* 0.4 units mg^{-1}

* If the amino compound (*i.e.*, CSA, glutamate, taurine, etc.) concentration is greater than *ca.* 20 mM in the test solution, it should be diluted before assay. Otherwise this amount of OPA will be insufficient for complete reaction.

protein (*ca.* 100 mg protein ml^{-1}). A unit of CSA decarboxylase is defined as the amount of enzyme required to produce one μmol of HT in one hour under the assay conditions.*[7,8]

Conversion of L-CSA *into* HT *by Rat Liver Homogenate.*—Frozen rat liver (20 g) was blended for 1—2 min in 40 ml of a cold (4 °C) solution of sodium phosphate (50 mM), AET (1mM), and PLP (0.1 mM) prepared in D_2O (99.5% D) and adjusted to pH 7.2 (meter reading) with 40% NaOD–D_2O. The mixture was then homogenized using a chilled teflon Potter-Elvejhem homogenizer, and the homogenate was centrifuged at 20 000 r.p.m. for 30 min at 0 °C. The supernatant was decanted after aspiration of a fatty layer.

A mixture was prepared of $NaH_2PO_4 \cdot H_2O$ (0.73 g), L-CSA (1.22 g), AET (22.4 mg), PLP (2.1 mg), and Triton X-100 (80 mg) in D_2O (56 ml) and was adjusted to pH (reading) 7.2 with 40% NaOD–D_2O. Rat liver homogenate in D_2O (24 ml) was added and the mixture was incubated *stationary* in a water bath at 37 °C.

A sample (0.25 ml) of the mixture was immediately taken for assay. This was mixed with 10% Na_2WO_4 (25 μl) and 6M HCl (25 μl), followed by centrifugation (2 000 r.p.m., 10 min). A portion (25 μl) of the supernatant was diluted to 250 μl with H_2O. This solution was then assayed, as described above, for CSA and HT. Samples of the incubating mixture were taken at intervals and similarly assayed. The conversion of CSA into HT was essentially complete in 8 h, but the incubation was continued for a total of 23 h.

6M HCl (3 ml) Was added to adjust the pH to 2.0. The mixture was centrifuged (20 000 r.p.m., 30 min) and the supernatant decanted. This was added to a 2.0 × 20 cm column of Dowex 50W-X8, 50—100 mesh, H^+ form. The column was eluted with H_2O until neutral and then eluted with 2M NH_4OH (300 ml). The alkaline effluent was partially evaporated under reduced pressure on a rotary evaporator over hot water (70 °C) to remove most of the ammonia, and was then lyophilized, yielding crude [2-$^2H_1$]hypotaurine as a brown residue (*ca* 1.1 g).

A similar procedure (on a smaller scale) was used to transform L-[2-$^2H_1$]CSA (**3**) into [2-$^2H_1$]hypotaurine, except that all reagents were dissolved in H_2O.

Raney Nickel Reductions.—A portion (*ca.* 100—200 mg) of the crude recovered L-[2-$^2H_1$]hypotaurine was dissolved in H_2O to give a *ca.* 1 mg ml^{-1} solution (estimated by h.p.l.c. using the methods described above). This was treated with freshly prepared Raney nickel[18] (*ca.* 8 ml of a *ca.* 1:1 suspension in H_2O) with magnetic stirring under N_2 at 45 °C. The reduction was followed at intervals by filtering aliquots (0.3 ml) through syringe filters (0.45 μM) and estimating the residual concentration of HT by h.p.l.c. Additional Raney nickel suspension (3—5 ml) was added after 1 and 1.5 h. The HT concentration was reduced to less than 5% of the original within 2 h. The mixture was suction filtered through two layers of glass fibre filter, and the filtrate was acidified to pH 2.5 with HCl. The yield of ethylamine hydrochloride (*ca.* 80—90%) in the resulting solution was estimated by h.p.l.c., using the same conditions as for HT measurement, except that the solvent was 60% MeOH–40% H_2O pumped at 1.0 ml min^{-1}. Ethylamine peak areas were compared with those from a solution of known concentration. The acidified filtrate was then lyophilized and the residue (*ca.* 25—50 mg) used for derivatization.

[1-$^2H_1$]*Ethylamine* (−)-*Camphanamide Derivatives.*—These were prepared from the crude freeze-dried [1-$^2H_1$]ethylamine hydrochloride samples, essentially as described by Parker.[11] The products were purified by preparative t.l.c. (30% EtOAc–hexane), yield *ca.* 20—40 mg; δ($CDCl_3$) 0.89 (3 H, s), 1.10 (6 H, s), 1.1—2.8 (7 H, m), 3.35 (1 H, m), and 6.5 (1 H, br s, $W_{\frac{1}{2}}$ 30 Hz).

For ^2H n.m.r. spectra (Figure 3a, b), the products were dissolved in C_6H_6 containing a small amount (*ca.* 0.3 μl/0.5 ml sample) of C_6D_6 as reference.

* Other investigators[1,3,5] have used a wide variety of definitions of a unit of CSA decarboxylate activity.

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