Mechanisms and Stereochemistry of the Activation of (2*S*)- and (2*R*)-Serine *O*-Sulfate as Suicide Inhibitors for *Escherichia coli* Glutamic Acid Decarboxylase[1]

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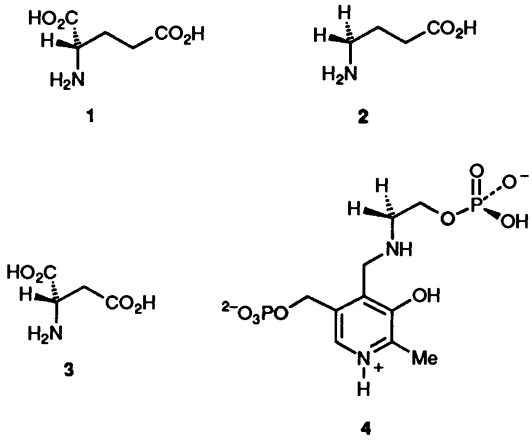
E. coli glutamic acid decarboxylase is inactivated by both enantiomers of the suicide inhibitor serine *O*-sulfate; inactivation by the (2*S*)-enantiomer involves C^{α}–H bond cleavage while inactivation by the (2*R*)-isomer involves C^{α}-decarboxylation. Both processes occur on the 4′-*Re*-face of the coenzyme, the opposite face to that utilised in physiological decarboxylation.

Pyridoxal 5′-phosphate (PLP) dependent glutamic acid decarboxylase (GAD) catalyses the decarboxylation of (2*S*)-glutamic acid **1** to give γ-aminobutyric acid **2** (GABA) and carbon dioxide. The enzyme is completely specific for the (2*S*)-enantiomer of the substrate and the reaction occurs with retention of configuration at C^{α}.[2] Recent work in our own laboratory has shown that (2*S*)-aspartic acid **3** is also decarboxylated with retention of configuration and that reactivation mediated by $N^{4'}$-(2″-phosphoethyl)pyridoxamine 5′-phosphate **4** involves the removal of the 1″-*pro*-*R* hydrogen



atom.[3] This work also demonstrated that during an abortive transamination the C-4′ protonation of the quinonoid intermediate derived from the natural substrate **1** occurs on the 4′-*Si*-face of the coenzyme.[3] These results, which are entirely analogous to those obtained for the methionine decarboxylase system,[4] strongly suggest that C^{α} and C-4′ protonations and deprotonations occur from the same 4′-*Si*-face of the coenzyme. There is also much evidence to suggest that the imidazolium side chain of a His residue and the ε-ammonium group of the active-site Lys residue serve as conjugate acids and bases for C^{α} and C-4′, respectively.[3,5] In the light of these findings it is entirely reasonable to expect that the distal anionic ω-carboxylate group of the natural substrate (Scheme 1, X = C) and the anionic phosphate group of the 2″-phosphoethyl moiety of $N^{4'}$-(2″-phosphoethyl)pyridoxamine 5′-phosphate **4** (Fig. 1) would reside on the 3′-OH side of the coenzyme.

Replacing the ω-carboxylate group in glutamic acid by a sulfonate group gives homocysteic acid **5**. In accord with the expectations, we show here that only the (2*S*)-enantiomer serves as a substrate for the enzyme (Scheme 1, X = SO). However, in contrast to expectations, Metzler and co-workers had shown that the isosteric glutamate analogue, (2*S*)-serine *O*-sulfate **6**

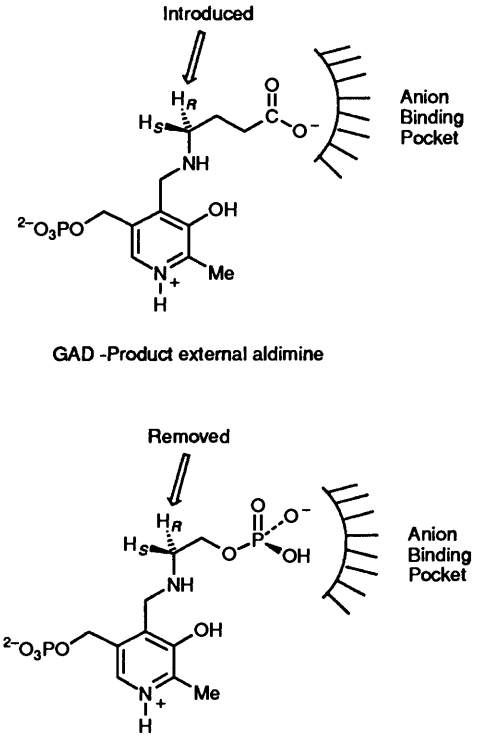
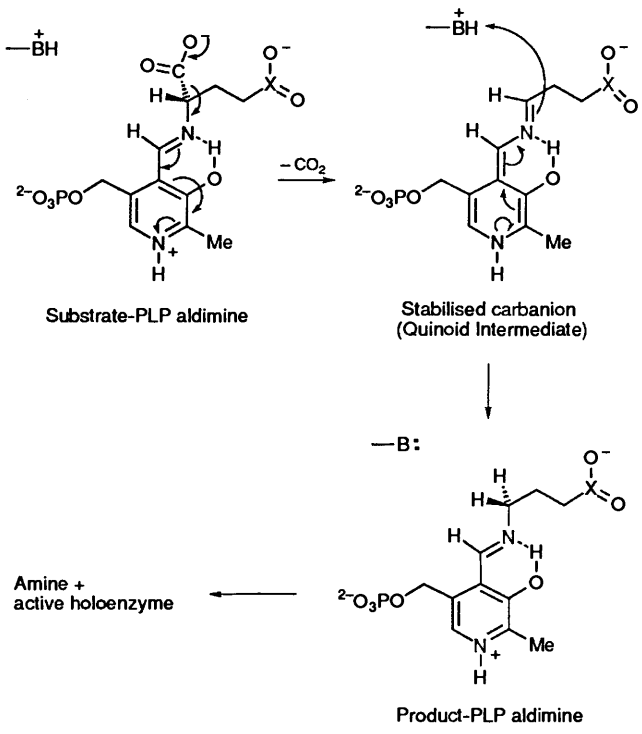


Fig. 1

5 H_A = H, Y = CH_2
6 H_A = H, Y = O
7 H_A = ^{2}H, Y = O

8 H_A = H, Y = CH_2
9 H_A = H, Y = O
10 H_A = ^{2}H, Y = O

did not undergo decarboxylation during a suicide inhibition process but, instead, underwent α,β-hydrogen sulfate elimination.[6] On the basis of the arguments presented above, the carboxylate groups of the external aldimines of both compounds, homocysteic acid and serine *O*-sulfate, should be optimally disposed for C^{α}–CO_2 bond cleavage on the 4′-*Si*-face of the coenzyme (Scheme 1). This is a curious result and, hitherto, could not be explained. Moreover, our recent finding that (2*R*)-serine *O*-sulfate **9** acts as an irreversible inhibitor for GAD[1] could not be rationalised within the context of the known chemistry of pyridoxal phosphate. Clearly this suicide



Scheme 1

substrate would be expected to eliminate hydrogen sulfate without decarboxylation, given that its α-proton should be correctly disposed for removal by the conjugate base of the active site acid which serves to protonate the stabilised carbanion of GABA following the decarboxylation step in the physiological reaction.[4] This conjugate base is very probably the imidazole side chain of a His residue, *vide supra.*

In order to verify and further probe Metzler's mechanism for suicide inactivation by (2*S*)-serine *O*-sulfate[6] and to gain insight into the mechanism of inactivation by the (2*R*)-enantiomer, α-deuteriated (2*S*)- and (2*R*)-serine were prepared from deuteriated 3-isopropyl-2,5-dimethoxy-3,6-dihydropyrazines as described recently.[7] Samples of the unlabelled and deuteriated serines were then converted into the corresponding *O*-sulfates (**6**, **7**, **9** and **10**), using literature methods,[8] and the rates of GAD inactivation were measured for each of the isotopomers at a range of concentrations. Using this data, the deuterium isotope effects were determined for each enantiomer.

For the (2*S*)-enantiomer, the observed isotope effects were normal, ^{D}V [that is k_H/k_D] was unity, within experimental error (see Table 1), and $^{D}(V/K)$ [that is $(k_H/k_D)/(K_H/K_D)$] was 2.3, indicating that C^{α}–H bond cleavage does, indeed, occur during the inactivation of GAD. The large size of $^{D}(V/K)$ compared to ^{D}V indicates that the reaction commitments to C–H bond cleavage are not large and that C–H bond cleavage is not the most significant transition state in the inactivation process (see Cleland[9] for a review on the interpretation of isotope effects). Thus, the inactivation of GAD by (2*S*)-serine *O*-sulfate involves the removal of an electrofuge from the 4′-*Re*-face of the coenzyme. The slightly inverse value of ^{D}V for the (2*S*)-antipode could be due to the expression of secondary deuterium isotope effects in the formation of the external aldimine complex prior to inactivation. However, within experimental error its difference from unity is not significant.

For (2*R*)-serine *O*-sulfate, the observed isotope effects for inactivation were inverse at all measured concentrations of the suicide substrate. The averaged apparent values of k_H/k_D ranged from 0.9 at 2 mmol dm^{-3} to 0.34 at 7 mmol dm^{-3} and, while clearly inverse, ^{D}V could not be determined accurately from the narrow range of inhibitor concentrations amenable to kinetic analysis. Maximum and minimum values of ^{D}V were calculated to be 0.27 and 0.07, respectively, see Table 1. The result indicates that C^{α}–H bond cleavage does not occur during the deactivation process and suggests that C^{α}–CO_2^- bond cleavage occurs. The observed values of the inverse isotope effects are too large to represent only the expression of secondary α-isotope effects, where α-H is more stiffly bonded in the transition state than in the external aldimine.[10] However, they could represent the suppression of alternative pathways for suicide substrate processing (upon the introduction of deuterium) which do not cause enzyme inactivation (an induced isotope effect), or a combination of both secondary α-isotope and induced isotope effects.

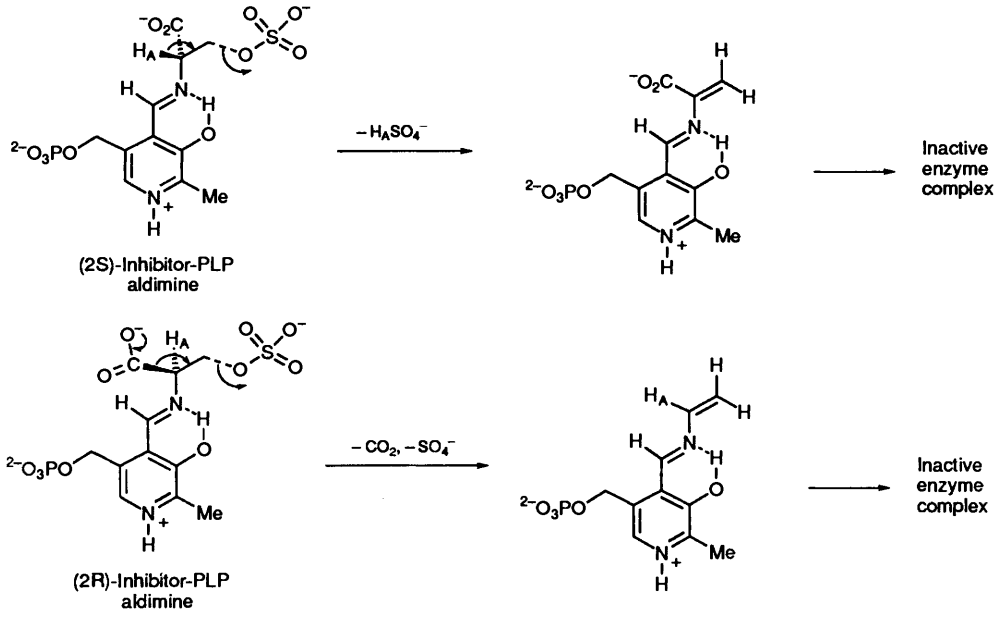
Interestingly, the observed large inverse isotope effect is not easily accommodated by a step-wise mechanism where the loss of carbon dioxide gives a pyridoxal-stabilised carbanion which slowly eliminates sulfate. This is because any such second slow elimination step would dominate the reaction coordinate profile and mask the isotope effect. Note that the determined value of $^{D}(V/K)$ for the inactivation of GAD by (2*R*)-serine *O*-sulfate was 1.0 indicating that there is a large forward reaction commitment to the first isotopically sensitive step. This result shows that the suicide inhibitor is extremely sticky[9] (*i.e.* does not freely dissociate from the Michaelis complex) in contrast to the (2*S*)-enantiomer, where $^{D}(V/K) = 2.3$.

In order to verify the unexpected conclusions that (2*S*)- and (2*R*)-serine *O*-sulfate lose a proton and a carboxyl group, respectively, in their inactivations of GAD, samples of (2*S*)-[U-^{14}C]- and (2*RS*)-[U-^{14}C]-serine *O*-sulfate were prepared from the appropriately labelled serines. The uniformity of the label in the (2*S*)-[U-^{14}C]serine was checked and verified by chemical decarboxylation[11] and the racemic material was prepared from this sample *via* the racemisation of the azlactone derived from the *N*-acetylserine.

Each of the [^{14}C]serine *O*-sulfates were incubated with GAD and any liberated CO_2 was collected in aqueous barium hydroxide. The radioactivities of the incubation solutions and the aqueous barium hydroxide were determined by scintillation counting before the start of the reactions and after the inactivations were complete. No $^{14}CO_2$ whatsoever was released during the inactivation of the enzyme by the (2*S*)-isomer but, the sample of (2*RS*)-[U-^{14}C]serine *O*-sulfate gave some $^{14}CO_2$. Confirmations of the results were obtained by comparing the residual radioactivities in the incubation solutions. Only the solution containing the racemic sample of [U-^{14}C]serine *O*-sulfate showed a decrease in radioactivity and this corresponded to several times the theoretical maximum.

Table 1 Kinetic parameters for GAD inactivation by isotopomers of (2*S*)-and (2*R*)-serine *O*-sulfate

Inhibitor	$10^3 k_{cat}$/min^{-1}	K_i/mmol dm^{-3}	k_{cat}/K_m	^{D}V	^{D}(V/K)
(2*S*)-Serine *O*-sulfate	10.8 ± 1.0	2.6 ± 0.5	4.20	0.79	2.3
(2*S*)-[2-^{2}H]serine *O*-sulfate	13.7 ± 2.7	7.6 ± 2.2	1.80		
(2*R*)-Serine *O*-sulfate	3.0 ± 0.7	4.3 ± 2.1	0.70	0.12	1.0
(2*R*)-[2-^{2}H]serine *O*-sulfate	24.1 ± 10.2	35.9 ± 12.6	0.67		



Scheme 2

That is, on average, each of the six active sites of the enzyme processed several molecules of the (2*R*)-enantiomer of the inhibitor *via* decarboxylation before the inactivation was complete.

Unfortunately, these results cannot be quantified for several reasons. First, it is not known how many of the six active sites per hexamer need to react to give inactive enzyme. Second, the supposedly racemic sample contained, in actual fact, a 16:9 mixture of the (2*S*):(2*R*)-enantiomers of [U-^{14}C]serine *O*-sulfate as determined by measuring the optical rotation of the sample. Third, the rate of inactivation of the enzyme by the (2*S*)-enantiomer is faster than that of the (2*R*)-enantiomer and the ratio of $(V/K)_S/(V/K)_R$, which determines how the substrates compete for the enzyme, is 6.0. Thus, under the conditions of the experiment, only 6% of the enzyme would have been inactivated by the (2*R*)-enantiomer. Fourth, from our earlier work it is apparent that some of the (2*R*)-enantiomer can be processed without giving rise to inactivation. Presumably, this reaction involves the removal of the α-H since the inactivation reaction shows an apparent inverse primary deuterium isotope effect, *vide supra*. Nevertheless, the results are clear and confirm the conclusions of the isotope effect studies. The (2*R*)-enantiomer releases $^{14}CO_2$ on exposure to the enzyme, whereas the (2*S*)-enantiomer does not. The large amount of CO_2 released during the inactivation of the enzyme by the racemic sample indicates that for both enantiomers, several turn-over events can occur before inactivation irreversibly inhibits the enzyme. This is apparent because if each processed molecule of (2*S*)-serine *O*-sulfate caused irreversible inhibition, very little active enzyme would be available to process the (2*R*)-enantiomer *via* C–CO_2 bond cleavage, *vide supra*.

In view of the strong evidence that the distal SO_3^- binding group in each of the external aldimines of the two suicide substrates should occupy the same position,[3] both inactivation events must involve the loss of electrofuges from the 4′-*Re*-face of the coenzyme (Scheme 2). In the light of the fact that the enzyme has evolved to decarboxylate and reprotonate substrates on the 4′-*Si*-face, these are intriguing results.

Interestingly, the stereochemical and kinetic data reported here is most consistent with concerted processes, an *E*2 type elimination in the case of (2*S*)-serine *O*-sulfate and an early C–OSO_3^- bond ionisation in the case of (2*R*)-serine *O*-sulfate. The binding geometry of the distal OSO_3^- group which leaves during each reaction should then be *anti*- to the 4′-*Re*-face electrofuge, where the activation energies for such reactions are lower than those for the corresponding stepwise (stabilised carbanion) 4′-*Si*-face processes which are expected for pyridoxal systems[4] but, are not observed here (Scheme 2). Upon prolonged exposure to the enzyme neither (2*R*)-glutamic acid nor (2*R*)-aspartic acid exchanged hydrogen at C^{α} with the solvent.

Experimental

Elemental microanalyses were performed in the departmental microanalytical laboratory. NMR spectra were recorded on a Bruker AM-300 (300 MHz; f.t. ^{1}H NMR, and 74.76 MHz; ^{13}C NMR), a Varian Gemini 200 (200 MHz; f.t. ^{1}H NMR and 50.31 MHz; ^{13}C NMR), or a JEOL FX90Q (90 MHz; f.t. ^{1}H NMR, and 22.5 MHz; ^{13}C NMR) spectrometers. ^{1}H NMR recorded at 400 MHz were obtained from the SERC NMR service at Warwick University. ^{1}H NMR spectra were referenced internally on ^{2}HOH (4.68 ppm), $CHCl_3$ (7.27 ppm) or DMSO (2.47 ppm). ^{13}C NMR spectra were referenced on CH_3OH (49.9 ppm), C^2HCl_3 (77.5 ppm), or DMSO (39.70 ppm). *J* Values are given in Hz. IR spectra were recorded on a Perkin-Elmer 1710 f.t. IR spectrometer. The samples were prepared as Nujol mulls, solutions in chloroform or thin films between sodium chloride discs. Mass spectra and accurate mass measurements were recorded on a VG 70-250 SE, a Kratos MS-50 or by the SERC service at Swansea using a VG AZB-E. Fast atom bombardment (FAB) spectra were recorded using glycerol as a matrix. Major peaks are given as percentages of the base peak intensity (100%). UV spectra were recorded on Pye-Unicam SP8-500 or SP8-100 spectrophotometers. Melting points were taken on an electrothermal melting point apparatus and are uncorrected. Optical rotations were measured at 23 °C on a Optical Activity AA-100 polarimeter using 10 or 20 cm path length cells and are given in units of 10^{-1} deg cm^2 g^{-1}. Flash chromatography was performed according to the method of Still *et al.*[12] using Sorbsil C 60 (40–60 μm mesh) silica gel. Analytical thin layer chromatography was carried out on 0.25 mm precoated silica gel plates (Macherey-Nagel SIL g/UV_{254}) and compounds were visualised using UV fluorescence, iodine vapour, ethanolic phosphomolybdic acid, or ninhydrin.

Amino acid substrates, PLP, PMP, buffers, salts, deuterium

oxide and glutamic acid decarboxylase were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Amberlite IR 120 (H^+) and Amberlite IRA 400 (acetate) ion exchange resins were obtained from British Drug Houses (Poole, Dorset, UK) and [1-^{14}C]-L-amino acid substrates were obtained from Amersham International (Amersham, Bucks, UK). Water-miscible scintillant (ES-199) was obtained from Canberra Packard (Pangbourne, Berks, UK). All other chemicals were of analytical grade or were recrystallised or redistilled before use.

Potassium (2S)-*Serine* O-*Sulfate*.—(2*S*)-Serine (1 g, 9.6 mmol) was added at room temperature to conc. sulfuric acid (*d* 1.84; 2 cm^3) according to the method of Tudball.[13] The mixture was stirred *in vacuo* until the serine had fully dissolved (*ca.* 2 h). Ice-cold water (10 cm^3) was added to the solution, the pH of which was then adjusted to 7.25 with aqueous barium hydroxide (saturated). The precipitated barium sulfate was removed by centrifugation and the clear supernatant was concentrated under reduced pressure to a small volume (*ca.* 2 cm^3) at 38 °C. This solution was passed through an Amberlite IR-120(H) ion exchange column and eluted with water. Fractions (10 cm^3) were collected and checked by TLC [using Pr^iOH–NH_3–H_2O (26:6:5) and visualised with ninhydrin] for the presence of the product. The product-containing fractions were combined, adjusted to pH 7.8 with 5% aq. KOH and concentrated to dryness under reduced pressure at 38 °C. The white residue was dissolved in the minimum amount of cold water and precipitated by the dropwise addition of ice-cold ethanol. The (2*S*)-serine *O*-sulfate was filtered off and dried overnight (550 mg, 30%), m.p. 150 °C (decomp.) (Found: C, 16.15; H, 2.55; N, 6.15%; M^+, 223.0981. Calc. for $C_3H_6KNO_6S$: C, 16.15; H, 2.7; N, 6.25%; *M*$^+$, 223.0936); $[\alpha]_D^{23}$ −10.25 (*c* 1.0 in H_2O) {lit.,[13] $[\alpha]_D^{16}$ −9.5 (*c* 5 in H_2O)}; ν_{max}(Nujol)/cm^{-1} 3400–3100 (OH) and 1650 (amino acid C=O); δ_H(90 MHz; ^2H_2O/NaO^2H) 3.53 (1 H, t, *J* 5, 2-CH) and 4.18 (2 H, m, 3-CH_2); δ_C(67.5 MHz; ^2H_2O/NaO^2H) 47.7 (3-CH_2), 71.5 (2-CH) and 178.4 (CO_2H).

(2S)-*Serine* O-*Sulfate* **6**.—Chlorosulfonic acid (1 cm^3, 15 mmol) was added dropwise to a stirred solution of (2*S*)-serine (1.05 g, 10 mmol) in trifluoroacetic acid (10 cm^3) according to the method of Previero[8] to give a white solid precipitate. When the addition was complete, the mixture was left at room temperature for 20 min. The excess of chlorosulfonic acid was destroyed by addition to the mixture of ethanol (1 cm^3), followed by four volumes of diethyl ether (40 cm^3) to complete the precipitation. The solid was filtered off, dried and recrystallised from aqueous ethanol to give the product (1.38 g, 62%), m.p. 228–230 °C (Found: C, 19.7; H, 3.65; N, 7.55. Calc. for $C_3H_7NO_6S$: C, 19.45; H, 3.8; N, 7.55%); *m*/*z* (Found: $[M + H]^+$, 186.0072. $C_3H_8NO_6S$ requires 186.0072); $[\alpha]_D^{23}$ +10.03 (*c* 3.4 in 1 mol dm^{-3} HCl) {lit.,[8] $[\alpha]_D$ +9.8 (*c* 3.4 in 1 mol dm^{-3} HCl)}; $[\alpha]_D^{23}$ −2.96 (*c* 1.145 in 5% KOH); ν_{max}(Nujol)/cm^{-1} 3200 (NH/OH str), 2800–3000 (CH str and Nujol) and 1755 (acid CO_2H monomer); δ_H(200 MHz; ^2H_2O/^2HCl) 4.1–4.35 (m, 2-CH and 3-CH_2); δ_C(50 MHz; $2H_2O$/^2HCl) 54.81 (3-CH_2), 68.95 (2-CH) and 171.01 (CO_2H).

Potassium (2R)-*serine* O-*sulfate*. This compound, prepared in a manner identical with that used for the potassium salt of the (2*S*)-antipode, starting from the (2*R*)-serine, was obtained in 30% yield; m.p. 164–166 °C (Found: C, 16.3; H, 2.65; N, 6.2; M^+, 223.0954. Calc. for $C_3H_6KNO_6S$: C, 16.15; H, 2.7; N, 6.25%; *M*$^+$, 223.0936); $[\alpha]_D^{23}$ 6.3 (*c* 1 in H_2O); ν_{max}-(Nujol)/cm^{-1} 3400–3100 (OH), 1650 (amino acid C=O), 1609 (NH_2 bend), 1210 (S–O) and 794 (C–O–S); δ_H(90 MHz; ^2H_2O/NaO^2H) 3.55 (1 H, t, *J* 4.3, 2-CH) and 4.2 (2 H, m, 3-CH_2); δ_C(22.5 MHz; ^2H_2O) 52.06 (3-CH_2), 64.52 (2-C) and 168.63 (CO_2H).

(2R)-*Serine* O-*sulfate* **9**. This compound, prepared in a manner identical with that used for (2*S*)-serine *O*-sulfate **6**, starting from (2*R*)-serine and following the method of Previero,[8] was obtained in 70% yield; m.p. 188–190 °C (Found: C, 19.35; H, 3.6; N, 7.5. Calc. for $C_3H_7NO_6S$: C, 19.45; H, 3.8; N, 7.55%); $[\alpha]_D^{23}$ −9.0 (*c* 3.4 in 1 mol dm^{-3} HCl), $[\alpha]_D^{23}$ +3.5 (*c* 1.085 in 5% KOH) {lit.,[14] $[\alpha]_D^{20}$ +9.8 (*c* 3.4 in 1 mol dm^{-3} HCl) for (2*S*)-isomer}; ν_{max}(Nujol)/cm^{-1} 3200 (NH/OH str), 2800–3000 (CH str) and 1760 (acid CO_2H monomer); δ_H(200 MHz; ^2H_2O/NaO^2H) 4.29 (1 H, m, 2-CH) and 4.39 (2 H, m, 2-CH_2); δ_C(50 MHz; ^2H_2O/NaO^2H) 54.94 (3-CH_2), 68.52 (2-CH) and 171.35 (CO_2H).

(2S)-[2-^{2}H]*Serine* O-*sulfate* **7**. This compound, prepared in a manner identical with that used for (2*S*)-serine *O*-sulfate **6**, starting from (2*S*)-[2-^{2}H]serine[7] and following the method of Previero,[8] was obtained in 55% yield; m.p. 220 °C (Found: C, 19.35; H, 3.2; N, 7.45. Calc. for $C_3H_6NO_6{}^2HS$: C, 19.35; H, 3.25; N, 7.5%); $[\alpha]_D^{23}$ −0.92 (*c* 1.035 in 5% KOH); ν_{max}(Nujol)/cm^{-1} 2800–3000 (CH str), 1766 (acid CO_2H monomer) and 1216 and 724 (C–O–S); δ_H(200 MHz; ^2H_2O/NaO^2H) 3.35 (0.05 H, t, *J* 4.5, 2 CH undeuteriated), 4.00 (1 H, d, *J* 10, 3-CH_A) and 3.97 (1 H, d, *J* 10, 3-CH_B); δ_C(50 MHz; ^2H_2O/NaO^2H) 55.4 (t, 2-C^2H), 72.34 (3-CH_2) and 180.0 (CO_2H); δ_D(H_2O/NaOH) 3.47 (2-C^2H).

(2R)-[2-^{2}H]*Serine* O-*sulfate* **10**. This compound, prepared in a manner identical with that used for (2*S*)-serine *O*-sulfate **6**, starting from (2*R*)-[2-^{2}H]serine[7] and following the method of Previero,[8] was obtained in 17% yield; m.p. 228 °C (Found: C, 19.25; H, 3.2; N, 7.53. Calc. for $C_3H_6NO_6{}^2HS$: C, 19.35; H, 3.25; N, 7.5%); $[\alpha]_D^{23}$ +0.9 (*c* 0.995 in 5% KOH); ν_{max}(Nujol)/cm^{-1} 2800–3000 (CH str), 1766 (acid CO_2H monomer) and 1216 and 724 (C–O–S); δ_H(400 MHz; ^2H_2O/NaO^2H) 3.32 (0.05 H, t, *J* 4.7, 2-CH undeuteriated), 3.94 (1 H, d, *J* 10, 3-CH_A) and 3.97 (1 H, d, *J* 10, 3-CH_B); δ_C(100 MHz; ^2H_2O/NaO^2H) 72.03 (3-CH_2); δ_D(H_2O/NaOH) 3.46 (2-C^2H).

Potassium (2S,3R)-*threonine* O-*sulfate*. This compound, prepared in a manner identical with that used for potassium (2*S*)-serine *O*-sulfate, starting from (2*S*,3*R*)-threonine, was obtained in 51% yield; m.p. 150 °C (decomp.) (Found: C, 18.6; H, 3.75; N, 5.35. Calc. for $C_4H_8KNO_6S{\cdot}H_2O$: C, 18.8; H, 3.95; N, 5.5%); $[\alpha]_D^{23}$ −20 (*c* 9.7 in H_2O) {lit.,[13] $[\alpha]_D^{18}$ −20.6 (*c* 9.7 in H_2O}; ν_{max}(Nujol)/cm^{-1} 2500–2800 (OH) and 1640 (acid C=O); δ_H(90 MHz; ^2H_2O/NaO^2H) 1.38 (3 H, d, *J* 5, 4-CH_3), 3.22 (1 H, d, *J* 6, 2-CH) and 4.55 (1 H, d of q, 3-CH); δ_C(67.5 MHz; ^2H_2O/NaO^2H) 18.2 (4-CH_3), 61.7 (3-CH), 79.4 (2-C) and 178.6 (CO_2H); *m*/*z* (FAB) 238 ($[M + H]^+$).

Potassium (2S,3S)-*threonine* O-*sulfate*. This compound, prepared in a manner identical with that used for potassium (2*S*)-serine *O*-sulfate, starting from (2*S*,3*S*)-threonine, was obtained in 30% yield; m.p. 224–226 °C (decomp.); $[\alpha]_D^{23}$ +0.8 (*c* 0.56 in H_2O); ν_{max}(Nujol)/cm^{-1} 3000–2800 (NH and OH) and 1650 (acid C=O); δ_H(90 MHz; ^2H_2O/NaO^2H) 1.10 (3 H, d, *J* 6.4, 4-CH_3), 3.32 (1 H, d, *J* 5.5, 2-CH) and 4.05 (1 H, d of q, 3-CH); δ_C(75 MHz; ^2H_2O/NaO^2H) 15.45 (4-CH_3), 59.58 (3-CH), 77.15 (2-C) and 175.53 (CO_2H); *m*/*z* (FAB) 238 ($[M + H]^+$).

N-*Acetyl*-(2RS)-*serine*. According to the method of Akabori,[15] acetic anhydride (6 cm^3) was added dropwise to a solution of (2*S*)-serine (2 g, 19 mmol) dissolved in water (20 cm^3) and pyridine (1.56 cm^3, 19 mmol). The resulting mixture was stirred at room temperature overnight after which it was poured onto an Amberlite IR-120(H) column. The column was eluted with water and fractions (25 cm^3) collected until the effluent was pH 6. The acidic fractions were pooled and concentrated to dryness under reduced pressure to yield an oil (2.1 g, 90%); ν_{max}(neat)/cm^{-1} 3348 (br, NH and OH str), 2977 (CH str), 1732 (acetyl C=O) and 1652 (CO_2^-); δ_H(200 MHz; ^2H_2O/NaO^2H) 1.9 (3 H, s, acetyl CH_3), 3.75 (2 H, m, 3-CH_2) and 4.35 (1 H, m, 2-CH); δ_C(50 MHz; ^2H_2O/NaO^2H) 22.7

(acetyl CH_3), 55.84 (3-CH_2), 62.07 (2-CH) and 174.52, 175.39 (acid and acetyl CO_2H); *m/z* (Cl) 148 ($[M + H]^+$, 100%), 130 (50, $[M - OH]^+$), 102 (20, $[M - CO_2H]^+$) and 60 (20, $[M - CO_2H - CH_3CO + H]$). The *N*-acetyl-(2*RS*)-serine was taken on to the next step without further purification.

(2RS)-*Serine*. *N*-Acetyl-(2*RS*)-serine (2 g, 13.6 mmol) was dissolved in 2 mol dm^{-3} HCl (80 cm^3) and the solution refluxed under nitrogen for 2 h.[15] The solution was cooled, concentrated to dryness under reduced pressure, and the residue dried over phosphorus pentoxide. The serine hydrochloride salt was dissolved in dry ethanol (20 cm^3) and propylene oxide (20 cm^3) and the mixture was refluxed for 15 min. The solid was filtered off and recrystallised from aqueous ethanol to yield a white crystalline solid (0.86 g, 60%), m.p. 235–237 °C (decomp.); $[\alpha]_D^{23}$ +0.15 (*c* 2 in 1 mol dm^{-3} HCl); ν_{max}(Nujol)/cm^{-1} 3200–2400 (NH_2, OH and CH str) and 1661 (amino acid CO_2H); δ_H(200 MHz; $^2H_2O/NaO^2H$) 3.49 (1 H, t, *J* 4.6, 2-CH) and 3.72 (2 H, m, 3-CH_2); δ_C(50 MHz; $^2H_2O/NaO^2H$) 57.09 (2-CH), 60.89 (3-CH_2) and 173.05 (CO_2H); *m/z* (Cl) 106 ($[M + H]^+$, 100%) and 60 (35, $[M - CO_2H]^+$).

(2RS)-[U-^{14}C]*Serine*. (2*S*)-[U-^{14}C]Serine (5 mCi) in aqueous ethanol was added to (2*S*)-serine (200 mg, 1.89 mmol) after which the solvent was removed under reduced pressure. The racemic material was prepared in a manner identical with that described above for unlabelled (2*RS*)-serine and was obtained in 77.5% overall yield; $[\alpha]_D^{23}$ +3.0 {36% (2*R*)-, 64% (2*S*)-[U-^{14}C]serine}.

(2RS)-[U-^{14}C]*Serine* O-*sulfate*. (2*RS*)-[U-^{14}C]Serine (0.155 g, 1.47 mmol) was converted into (2*RS*)-[U-^{14}C]serine *O*-sulfate following the method of Tudball[13] in 18% yield.

GAD Activity Assay (*Method 1*).—Enzyme activity was determined in 0.1 mol dm^{-3} pyridine/HCl buffer at pH 4.6 using [1-^{14}C]glutamic acid as the substrate as described in the previous article.[3]

Inactivation of GAD by (2S)-*serine* O-*sulfate*. Several solutions of (2*S*)-serine *O*-sulfate (3.7 –10 mmol dm^{-3}) in 0.1 mol dm^3 pyridine/HCl buffer at pH 4.6 (1.2 cm^3) were prepared. GAD (0.2 mg) was added to each solution and to a reference solution containing buffer (1.2 cm^3) only. The solutions were assayed for enzyme activity in duplicate using method 1 at *t* = 0.5, 4.5, 24 and 72 h. Time *vs.* enzyme activity plots showed a gradual decrease in enzyme activity. The data was used to design experiments to determine the kinetic parameters for the inactivation process, see below.

Inactivation of GAD by (2S,3R)-*threonine* O-*sulfate*. Several solutions of (2*S*,3*R*)-threonine *O*-sulfate (3.7–10 mmol dm^{-3}) in 0.1 mol dm^3 pyridine/HCl buffer at pH 4.6 (1.2 cm^3) were prepared and GAD (0.2 mg) was added to each solution and to a reference solution containing buffer (1.2 cm^3) only. The solutions were assayed for enzyme activity in duplicate using method 1 at *t* = 0.5, 4.5, 24 and 72 h. Time *vs.* enzyme activity plots showed no loss of enzyme activity relative to the reference sample.

Inactivation of GAD by (2S,3S)-*threonine* O-*sulfate*. Experiments similar to those described for (2*S*,3*R*)-threonine *O*-sulfate were performed using (2*S*,3*S*)-threonine *O*-sulfate (10, 25, 50 mmol dm^3) in pyridine/HCl buffer at pH 4.6. Time *vs.* enzyme activity plots showed no decrease in enzyme activity relative to the reference sample.

Inactivation of GAD by (2R)-*serine* O-*sulfate*. Experiments similar to those described for (2*S*)-serine *O*-sulfate were performed using (2*R*)-serine *O*-sulfate (10, 25, 50 mmol dm^{-3}) in 0.1 mol dm^3 pyridine/HCl buffer at pH 4.6. Time *vs.* activity plots showed a gradual decrease in activity, see below.

Kinetic isotope effects for GAD inactivation by (2S)-*serine* O-*sulfates*. Solutions of both (2*S*)-serine *O*-sulfate and (2*S*)-[2-^{2}H]serine *O*-sulfate at concentrations of 0, 1, 2, 3.5 and 5 mmol dm^{-3} in pyridine/HCl buffer (0.1 mol dm^{-3}, pH 4.6) were prepared. GAD (0.1 mg) was added to 0.6 cm^3 aliquots of each solution, in duplicate. The solutions were incubated at 37 °C and assayed for enzyme activity using method 2 (below) at *t* = 0, 0.75, 2, 4 and 7 h after the addition of the enzyme. The data was corrected for non-inhibitor specific enzyme activity loss and plots of $\log_e$ (enzyme activity) *vs.* time gave straight lines of gradient $-k_{app}$ for each concentration. Plots of k_{app} *vs.* inhibitor concentration, fitted by non-linear regression analysis[16] gave the kinetic parameters k_{cat} and K_i (see Table 1).

Kinetic isotope effects for GAD inactivation by (2R)-*serine* O-*sulfates*. Kinetic experiments for the inactivation of GAD by (2*R*)-serine *O*-sulfate and (2*R*)-[2-^{2}H]serine *O*-sulfate at concentrations of 0, 2, 3.5, 5 and 7 mmol dm^{-3} in pyridine/HCl buffer were performed and analysed exactly as described above (see Table 1).

Modified Assay for Kinetic Studies of GAD Inactivation (*Method 2*).—The assay was performed by diluting a portion of the inhibitor–enzyme solution (60 mm^3) at specified times directly into 1.5 cm^3 of substrate solution containing (2*S*)-[1-^{14}C]glutamic acid (16 mmol dm^{-3}, 0.5 μCi) in 0.1 mol dm^{-3} pyridine/HCl buffer solution, pH 4.6. At *t* = 5, 10, 15, 20 and 25 min after the addition of the enzyme solution to the substrate solution, aliquots (200 mm^3) were removed and were added to H_2SO_4 (6 mol dm^{-3}, 200 mm^3) in a scintillation vial to stop the conversion of (2*S*)-glutamic acid into GABA. After 30 min, when the evolution of $^{14}CO_2$ had ceased, scintillation fluid was added to the vial and the sample was scintillation counted to determine the residual activity of the enzyme.

(2S)-[U-^{14}C]*Serine* O-*Sulfate*.—To (2*S*)-serine (50 mg, 0.22 mmol) was added (2*S*)-[U-^{14}C]serine (24.5 μCi) in aqueous ethanol and the solution was lyophilised. The (2*S*)-[U-^{14}C]serine *O*-sulfate, prepared in a manner identical with that described for the unlabelled material, was purified by ion exchange chromatography on Amberlite IR-120(H) (see above). Fractions containing the required product as judged by TLC (visualising with ninhydrin) and by scintillation counting a small portion of each fraction, were combined. The pH of the pooled solution was adjusted to 7.8 by the addition of aqueous 5% potassium hydroxide and the precipitated crystalline solid was collected (4 μCi, 109 mg, 16%).

Inactivation of GAD by (2S)-[U-^{14}C]*Serine* O-*Sulfate*.—In the side-arm chamber of a 50 cm^3 Warburg apparatus, (2*S*)-[U-^{14}C]serine *O*-sulfate (400 mm^3 of a 5.3 mmol dm^{-3} solution; 2.2×10^5 dpm) in 0.1 mol dm^{-3} potassium acetate buffer at pH 4.6 was treated with GAD (1.2 mg, 24 nmol). The main chamber of the apparatus contained barium hydroxide solution (25 cm^3) and a tube from the neck at the top passed through another vessel containing barium hydroxide solution (25 cm^3). After 26 h at 37 °C, several aliquots of the enzyme solution, the internal barium hydroxide solution and the external barium hydroxide solution were removed and scintillation counted. No radiolabelled CO_2 was recovered from the barium hydroxide and no decrease in the radioactivity of the incubation solution was detected.

Incubation of (2RS)-[U-^{14}C]*Serine* O-*Sulfate with GAD*.—(2*RS*)-[U-^{14}C]Serine *O*-sulfate (400 mm^3 of a 10 mmol dm^{-3}; 4×10^{-6} mol dm^{-3}; 46.5×10^3 dpm) in 0.1 mol dm^{-3} pyridine/HCl buffer at pH 4.6 was treated with GAD (1.1 mg, 22×10^{-9} mol active sites) in the side-arm of a Warburg apparatus as described above. After 24 h at 37 °C aliquots of the inactivated enzyme solution, and the barium hydroxide solution were removed. The ^{13}C-label contents of the solutions were determined by scintillation counting. The enzyme solution

contained 39.2 × 10^3 dpm and the baryta solution contained 7.6 × 10^3 dpm corresponding to the amount of radioactivity lost from the enzyme solution. The experiment was repeated under similar conditions to confirm the result. No attempt was made to gain quantitative information on the partition ratio for non-inhibitory substrate processing *vs.* irreversible inhibition.

NMR Spectroscopic Experiments to Measure Hydrogen Exchange.—Incubation of (2R)-*serine* O-*sulfate with GAD.* (2*R*)-Serine *O*-sulfate (50 mg, 0.226 mmol) was dissolved to 0.1 mol dm^{-3} pyridine/^{2}HCl buffer (pD 4.2; 1 cm^3) and GAD (2.54 mg, ~50 units) was added to the solution. The solution was incubated at 37 °C and ^{1}H and ^{13}C NMR spectra were recorded at t = 0, 17, 32.5 and 113 h. No hydrogen exchange with the solvent occurred during the incubation period as determined from the integrals and multiplicities of the NMR signals.

Incubation of (2S)-*serine* O-*sulfate with GAD.* (2*S*)-Serine *O*-sulfate (5 mmol dm^{-3} in 0.1 mol dm^{-3} pyridine/^{2}HCl buffer, pD 4.2) was added to GAD (0.1 mg, 4 units) and the solution was incubated at 37 °C. ^{1}H and ^{13}C NMR spectra recorded at t = 0, 1, 2, 3, 4, 5, 6.17, 29.92 and 73.5 h indicated that no hydrogen exchange with the solvent occurred.

Incubation of (2R)-*aspartic acid with GAD.* (2*S*)-Aspartic acid (50 mg, 0.37 mmol) was dissolved in pyridine/^{2}HCl buffer (pD 4.25, 0.1 mol dm^{-3}, 4 cm^3). An aliquot (0.7 cm^3) was placed in an NMR tube and after the addition of GAD (2 mg, 50 units) the solution was incubated at 37 °C. ^{1}H and ^{13}C NMR spectra recorded at t = 0, 1, 2, 3 and 4 weeks indicated that no hydrogen exchange between the substrate and the solvent occurred.

Incubation of (2R)-*glutamic acid with GAD.* This incubation was performed in a manner identical with that used for (2*S*)-aspartic acid using (2*R*)-glutamic acid (50 mg, 0.34 mmol) in pyridine/^{2}HCl buffer (pD 4.39, 0.1 mol dm^{-3}, 4 cm^3). No hydrogen exchange with the solvent was detected.

Incubation of (2S)- *and* (2R)-*homocysteic acid with GAD.* To GAD (2.5 mg) in 0.1 mol dm^{-3} pyridine/HCl buffer pH 4.6 was added (2*S*)-homocysteic acid (54 mmol dm^{-3}, 100 mm^3). The solution was incubated at 37 °C overnight and then concentrated under reduced pressure to dryness. Examination of the residue by ^{1}H and ^{13}C NMR spectroscopy revealed that decarboxylation to the amine was complete [δ_H(200 MHz; ^2H_2O/pyridine buffer) 2.0 (2 H, m, 2-CH_2) and 2.8–3.2 (4 H, m, 1- and 3-CH_2's); δ_C(100 MHz; ^2H_2O/pyridine buffer) 23.23 (2-CH_2), 39.07 and 48.71 (1- and 3-CH_2's)]. (2*R*)-Homocysteic acid was not a substrate for GAD as judged by NMR spectroscopic analysis of the lyophilised incubation mixture.

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