Studies of Enzyme-mediated Reactions. Part 15.[1] Stereochemical Course of the Formation of γ-Aminobutyric Acid (GABA) by Decarboxylation of (2*S*)-Glutamic Acid with Glutamate Decarboxylase from *Escherichia coli*

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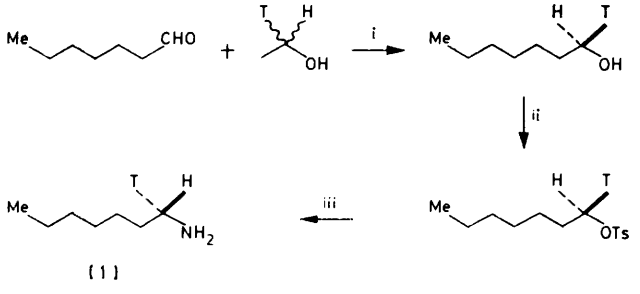
Incubation of (1*S*)-1-amino[1-$^3H_1$]heptane (1) with the amine oxidase from pea seedlings (E.C. 1.4.3.6) results in formation of heptaldehyde with complete removal of tritium showing that the *Si*-stereospecificity which had already been demonstrated for benzylamine also applies to this aliphatic amine. (2*RS*)-[2-^{3}H]Glutamic acid is prepared and then decarboxylated with an enzyme from *E. coli* (E.C. 4.1.1.15) to give γ-amino[4-$^3H_1$]butyric acid (GABA). Hydride reduction of this product gives 4-amino[4-$^3H_1$]butan-1-ol which is shown to have the (4*S*)-configuration by assay with the pea seedling amine oxidase; this completely removes the tritium from C-4. The decarboxylation of glutamic acid therefore proceeds with retention of configuration.

γ-AMINOBUTYRIC ACID (GABA) is an important inhibitory neurotransmitter in both the invertebrate nervous system and the vertebrate central nervous system.[2,3] As early as 1954, Mandeles *et al.*[4a] gave support for the view that the glutamate decarboxylases responsible for its formation acted stereospecifically. More recently two groups[4b,c] have provided evidence that the decarboxylation reaction proceeds with retention of configuration though their approach was completely different from that described here.

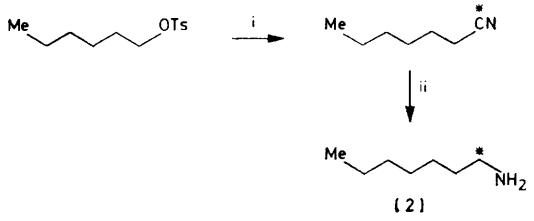
Recent work in this Laboratory and elsewhere has demonstrated that the enzymatic decarboxylation of tyrosine,[5] histidine,[6] and lysine[1,7] proceeds with retention of configuration. In the Cambridge work, the new centre formed by decarboxylation, which was chiral because of isotopic substitution, was assayed with the amine oxidase from pea seedlings, DAO (E.C. 1.4.3.6). This enzyme was shown to remove the *Si*-proton stereospecifically in the oxidation of both benzylamine[8] and tyramine.[5b] In order to extend the use of the DAO assay to aliphatic amines, (1*S*)-1-amino[1-$^3H_1$]heptane (1) was prepared and mixed with 1-amino[1-^{14}C]heptane (2) which acted as an internal standard. The preparation of these compounds is outlined in Schemes 1 and 2.



SCHEME 1 *Reagents*: i, Liver alcohol dehydrogenase, NAD^+; ii, TsCl, pyridine; iii, NaN_3; $LiAlH_4$

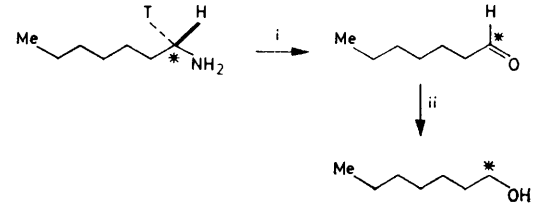
For the preparation of chiral [^{3}H]heptylamine, Scheme 1, the initial asymmetric introduction of tritium was achieved by transfer of tritium from (1*RS*)-[1-$^3H_1$]-ethanol using liver alcohol dehydrogenase[9] (LADH) (E.C. 1.1.1.1.) This is known to transfer hydrogen from the reduced co-factor NADH specifically to the *Re*-face of the aldehyde.[10]

Incubation of the (1*S*)-1-amino[1-$^3H_1$]heptane with



SCHEME 2 *Reagents*: i, $K^{14}CN$; ii, H_2–Pd

the DAO and reduction *in situ* of the product heptaldehyde with liver alcohol dehydrogenase resulted in heptan-1-ol containing only 7% of the tritium present in the amine (Scheme 3). This result establishes that the



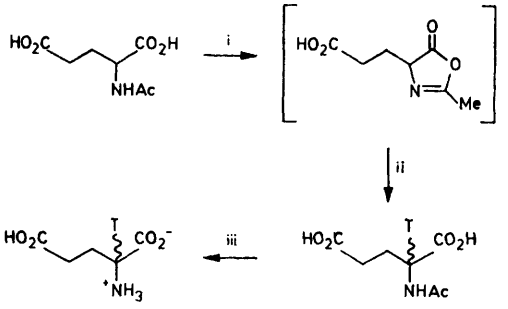
SCHEME 3 *Reagents*: i, Diamine oxidase; ii, Liver alcohol dehydrogenase, ethanol, NADH

dehydrogenation step involves loss of the *Si*-proton. The small degree of retention of tritium can be attributed to slight loss of stereochemical integrity in the conversion of (1*R*)-[1-$^3H_1$]heptan-1-ol into (1*S*)-[1-$^3H_1$]heptylamine (Scheme 1).

With the stereochemical assay now established, (2*RS*)-[2-^{3}H]glutamic acid (3) was prepared (Scheme 4; *cf.* ref. 1). This racemic acid could be safely used for the decarboxylation studies as the (2*S*)-glutamate decarboxylase is known[11] to be specific for the (2*S*)-isomer (8). The (2*RS*)-[2-^{3}H]glutamic acid was mixed with uniformly carbon labelled (2*S*)-glutamic acid which acted as

the internal standard; the $^3H : ^{14}C$ ratio was set at 19.0.

Decarboxylation of glutamic acid gave γ-aminobutyric acid (4). The change in isotopic ratio ($^3H : ^{14}C$) from 19.0 to 11.7 (Table) is consistent with complete retention (98.5%) of tritium if allowance is made for the

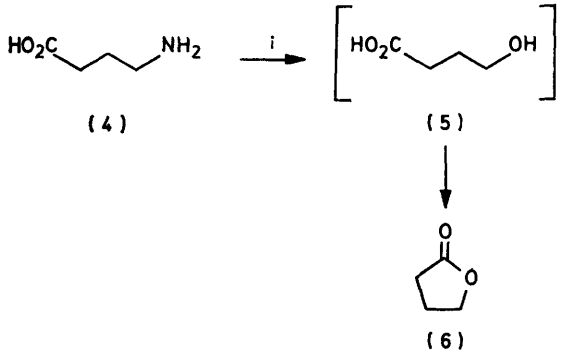


(3)

SCHEME 4 *Reagents*: i, Ac_2O; ii, $MeCO_2T$ then H_2O; iii, Hot 3M-HCl, NaOH to pH 3

racemic nature of the tritiated material and the loss of one labelled carbon. γ-Aminobutyric acid itself was not considered suitable for stereochemical assay with the coupled system of amine oxidase and alcohol dehydrogenase used above for heptylamine, as the product 4-hydroxybutyric acid (5) would be expected to cyclise to produce butyrolactone (6) (unlabelled series, Scheme 5). This would be difficult to handle and purify on a small scale or to form derivatives for radiochemical assay.

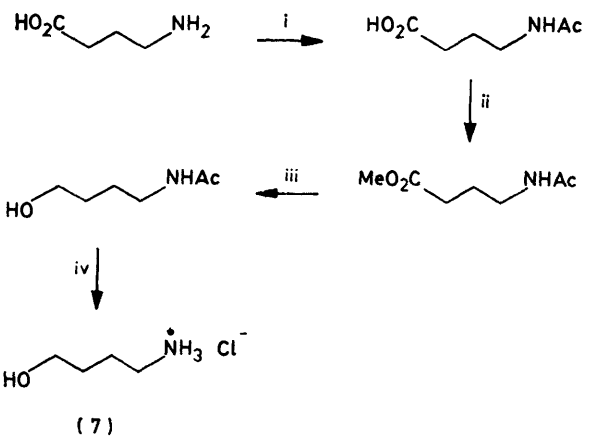
Consequently, γ-aminobutyric acid from enzymic decarboxylation of doubly-labelled glutamic acid was reduced to 4-aminobutan-1-ol (7), unlabelled series Scheme 6, which was assayed by enzymic conversion into



SCHEME 5 *Reagents*: i, Diamine oxidase, alcohol dehydrogenase, ethanol

butane-1,4-diol with the coupled system of DAO yeast alcohol dehydrogenase.[1] The results are summarised in the Table and the conversions in the labelled series are outlined in Scheme 7.

The loss of 91% of tritium observed in the transformation of 4-aminobutan-1-ol (10) into butane-1,4-diol (11) indicates that 91% of the tritium originally present in the amino-alcohol (10) occupied the *Si*-position at



(7)

SCHEME 6 *Reagents*: i, Ac_2O; ii, CH_2N_2; iii, $LiAlH_4$, 0 °C; iv, Hot 2M-HCl

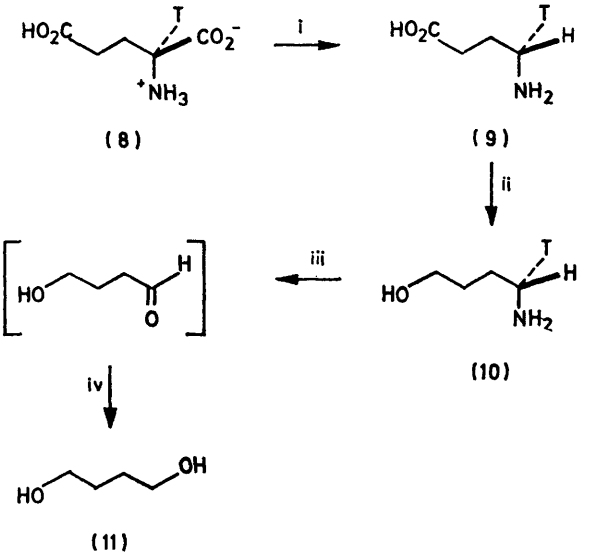
carbon-4, Scheme 7. This corresponds to overall retention of configuration for the decarboxylation step.

Stereochemical assay of doubly labelled γ-aminobutyric acid

$^3H : ^{14}C$ ratio (% retention of ^3H)		
γ-Aminobutyric Acid (9)	4-Aminobutan-1-ol (4) [a]	Butane-1,4-diol (10) [a]
11.7 (100)	11.4 (97)	1.03 (8.8)

[a] Assayed as bis-*p*-nitrobenzoyl derivative

The foregoing conclusion is rigorous but retention of



(11)

SCHEME 7 *Reagents*: i, glutamate decarboxylase; ii, as Scheme 6; iii, diamine oxidase; iv, yeast alcohol dehydrogenase, ethanol, NADH.

8.8% of tritium in the butane-1,4-diol (11) (Table) required examination since it was greater than the experimental error. Lack of enzymic stereospecificity seemed highly unlikely whereas the presence of some tritium at C-4 of the labelled glutamic acid (8) (Scheme 7) was a clear possibility. This tritium could have been introduced during the exchange reaction, Scheme 4, used to

generate the (2*RS*)-[2-^{3}H]glutamic acid by formation, for example, of a mixed anhydride. Tritium present at C-4 of glutamic acid (8) would not be removed in any of the subsequent enzymic or chemical steps leading to butane-1,4-diol (11).

A method for tritium assay at C-4 of glutamic acid (8) was, therefore, necessary and this was achieved by oxidising a sample of the derived diol (11) to succinic acid. None of the tritium present at C-2 of the initial glutamic acid (8) can remain in the succinic acid because C-2 becomes a terminal carbon of the diol (11). Thus, any tritium in the succinic acid must have originated from C-4 (or far less likely, C-3) of the glutamic acid (8). In fact, permanganate oxidation of the labelled butane-1,4-diol (11) produced succinic acid with an isotopic ratio (^{3}H : ^{14}C) of 0.96, which corresponds to 8.4% of the total tritium activity of 4-aminobutan-1-ol (8).

With this point settled, it follows that well within the limits of experimental error, all the tritium present in the $RCHTNH_2$ group of 4-aminobutan-1-ol (10) was eliminated during deamination with DAO and that this group therefore had the *S*-configuration which corresponds to complete retention overall for the enzymic decarboxylation (Scheme 7).

EXPERIMENTAL

Materials.—(2*S*)-Glutamate decarboxylase, liver alcohol dehydrogenase (LADH), yeast alcohol dehydrogenase (YADH), nictotinamide adenine dinucleotide (NAD^+) and catalase were obtained from the Sigma Chemical Company; reduced β-nicotinamide adenine dinucleotide (NADH) was obtained from Boehringer Mannheim GmbH; tritiated water, potassium [^{14}C]cyanide, sodium [^{3}H]borohydride and (*S*)-[U-^{14}C]glutamic acid were obtained from the Radiochemical Centre, Amersham.

General.—For general experimental details see ref. 1.

1-*Amino*[1-^{14}C]*heptane.*—This compound was prepared as earlier [12] using potassium [^{14}C]cyanide in place of sodium [^{14}C]cyanide and a reaction time of 48 h, 40 °C. Specific activity of picrate 1.2×10^6 d.p.m./mg.

(1R)-[1-$^3H_1$]*Heptan*-1-*ol.*—Albumin (40 mg) and NAD^+ (20 mg) were dissolved in a solution containing potassium phosphate buffer (pH 7, 25 ml, 0.2M,) and distilled water (80 ml) and (1*RS*)-[1-^{3}H]ethanol [8] (*ca.* 1 mmol, 100 mCi) in water (7 ml) were added. LADH (5 mg) was then added and the system flushed with nitrogen and warmed to 30 °C; after this heptanal (253 mg, 2.2 mmol) was added and the reaction kept at 30 °C in the dark for 24 h. LADH (10 mg) and NAD^+ (10 mg) were then added and after 31 h, unlabelled heptan-1-ol (400 mg) was added and the solution saturated with potassium carbonate and extracted with methylene chloride (3 × 50 ml). The combined organic extracts were dried (K_2CO_3) and the product after evaporation of solvent was distilled (bulb-to-bulb, 30 mmHg) to give (1*R*)-[1-$^3H_1$]heptan-1-ol (451 mg, 1.3×10^8 d.p.m./mg).

(1R)-[1-$^3H_1$]*Heptyl Tosylate.*—Toluene-*p*-sulphonyl chloride (1.6 g) was added to a solution of the foregoing alcohol (450 mg) in anhydrous pyridine (7 ml) cooled in an ice–salt bath. The solution was kept overnight at 4 °C, poured onto ice–water (50 ml), and extracted with ether (3 × 25 ml). The combined ethereal extracts were washed with 5M-hydrochloric acid (2 × 30 ml) and water (30 ml), dried ($CaCl_2$), and evaporated to give (1*R*)-[1-$^3H_1$]heptyl tosylate (1.1 g), δ($CDCl_3$) 0.9 (3 H, t, CH_3), 1.1—1.8 (10 H, m, CH_2), 2.4 (3 H, s, CH_3-Ar), 4.0 (2 H, t, *J* 6Hz, CH_2O), 7.35 (2 H, d, *J* 8 Hz, ArH), and 7.85 (2 H, d, *J* 8 Hz, ArH).

(1S)-1-*Amino*[1-$^3H_1$]*heptane.*—A stirred solution of (1*R*)-[1-$^3H_1$]heptyl tosylate (1.1 g) in methanol (7 ml) and water (1.4 ml) at 70 °C was treated with sodium azide (0.45 g). After 24 h, the reaction was cooled, ether (12 ml) and water (8 ml) were added followed by calcium chloride solution [13 ml, from $CaCl_2$ (8 g) and water (20 ml)]. The equilibrated layers were separated and the aqueous phase was extracted with ether (3 × 10 ml). The combined organic extracts were dried ($CaCl_2$) and evaporated to give (1*S*)-[1-$^3H_1$]heptyl azide (0.45 g).[13]

This, in anhydrous ether (12 ml, was added dropwise to a stirred suspension of lithium aluminium hydride (0.25 g) in ether (5 ml) during 30 min. The mixture was then heated under reflux for 1 h, cooled, and treated with water (0.25 ml), 3M-sodium hydroxide (0.25 ml), and water (0.75 ml). The filtered ethereal solution was extracted with 3M-hydrochloric acid (2 × 25 ml) and the combined aqueous extracts were brought to pH 11 with 3M-sodium hydroxide. This aqueous solution was extracted with methylene chloride (3 × 30 ml), and the combined organic extracts were washed with saturated brine (30 ml), dried (K_2CO_3), and evaporated to give the crude (1*S*)-1-amino[1-$^3H_1$]heptane. Unlabelled 1-aminoheptane (0.2 g) was added and the product distilled (bulb-to-bulb, 30 mmHg) to give (1*S*)-1-amino[1-$^3H_1$]heptane (0.362 g); specific activity of picrate 1.17 mCi mmol^{-1}.

Stereochemical Assay of (1S)-1-*Amino*[1-$^3H_1$,1-^{14}C]*heptane with Pea Seedling Diamine Oxidase.*—Pea seedling diamine oxidase (E.C. 1.4.3.6) was isolated by the method of Hill and Mann.[14, 15]

The (1*S*)-1-amino[1-$^3H_1$,1-^{14}C]heptane (*ca.* 3 mg, ^{3}H : ^{14}C = 7.4) was dissolved in 0.05M-Tris-HCl buffer (5 ml, pH 8.5) and treated at 37 °C for 22 h with ethanol (0.1 ml), LADH (5 mg), NADH (5 mg), and the pea seedling diamine oxidase (0.02 units). The solution was cooled, acidified to pH 1 with concentrated hydrochloric acid, diluted with unlabelled heptan-1-ol (50 μl) and extracted with ether (4 × 15 ml). The combined ethereal extracts were dried (K_2CO_3) and the product from evaporation was treated with 1-naphthyl isocyanate (70 μl) at 100 °C for 10 min; the resulting urethane was recrystallised from light petroleum (b.p. 100—120 °C) to constant specific activity and ^{3}H : ^{14}C ratio of 0.52; m.p. 58—59 °C, lit.,[16] 62 °C. Further fractionation by t.l.c. on silica (3 : 7 ether–light petroleum) and sublimation (80 °C/0.1 mmHg) left the specific activity and ratio unchanged.

(R,S)-[2-^{3}H]*Glutamic Acid.*—Acetic anhydride (5 ml, 53 mmol) was added dropwise at 0 °C to a stirred solution of (*RS*)-glutamic acid (2.0 g, 13.6 mmol) in 2.5M-sodium hydroxide (11 ml, 27.5 mmol). After 15 h at 0 °C, the solution was neutralised with Amberlite IR 120 (H^+) and filtered. Evaporation afforded the (*RS*)-*N*-acetylglutamic acid, m.p. 182—183 °C from methanol–benzene (lit.,[17] m.p. 185 °C).

A mixture of (*RS*)-*N*-acetylglutamic acid (150 mg, 0.79 mmol), acetic anhydride (0.63 ml, 6.7 mmol), and tritiated water (0.084 ml, 4.7 mmol, 420 mCi) was heated in a sealed tube at 120 °C for 2.5 h. The mixture was cooled and the solvents removed by vacuum transfer (0.5 mmHg); the crude product was then dissolved in water (2 ml) before the vacuum transfer was repeated. The residue in 3M-hydrochloric acid (4 ml) was heated under reflux for 1.5 h after

which the solvent was again removed by vacuum transfer and a solution of the product in water was adjusted to pH 3 with 1.5M-sodium hydroxide. Concentration of the solution to *ca.* 1.5 ml and cooling to 2 °C afforded the (*RS*)-[2-^{3}H]-glutamic acid (0.095 g), specific activity 19.1 mC mmol^{-1}.

γ-Amino[4-$^3H_1$,U-^{14}C]*butyric Acid.*—A solution of (*S*)-[U-^{14}C]glutamic acid (50 μCi, 68 μCi/mg) and the foregoing (*RS*)-[2-^{3}H]glutamic acid (6.7 mg) in 0.2M-acetate buffer (5 ml, pH 5) was prepared. A small sample was diluted with unlabelled glutamic acid and crystallised from water–ethanol to constant isotopic ratio (^{3}H : ^{14}C 19.0). The remainder was incubated at 37 °C for 2 h with (*S*)-glutamate decarboxylase (2 mg) and then concentrated to *ca.* 0.5 ml under nitrogen. The residue was applied to Whatman No. 1 paper and developed for 19 h using a mixture of ethanol, water, and ammonia (*d* 0.88) (20 : 4 : 1). The band corresponding to γ-aminobutyric acid (detected by spraying strips with Ninhydrin solution) was eluted with the same solvent mixture (200 ml). Unlabelled γ-aminobutyric acid (100 mg) was added and the solution evaporated to dryness. An aliquot of the resultant γ-amino[4-$^3H_1$,U-^{14}C]butyric acid was diluted with unlabelled γ-aminobutyric acid and recrystallised from ethanol–water to constant isotopic ratio (^{3}H : ^{14}C 11.7). The remainder was converted into 4-amino[4-$^3H_1$,U-^{14}C]butan-1-ol as described below.

4-*Amino*[4-$^3H_1$,U-^{14}C]*butan-1-ol.*—The foregoing γ-amino-[4-$^3H_1$,U-^{14}C]butyric acid in 3M-sodium hydroxide solution (0.53 ml, 2 mmol) was stirred at 0 °C while acetic anhydride (0.38 ml, 4 mmol) was added dropwise. After further stirring for 2 h at 0 °C, water (10 ml) was added and the solution neutralised with an excess of Amberlite IR120 (H^+). The solution was then filtered and the solvent evaporated. Methanol (20 ml) was added and evaporated to give 4-acetamido[4-$^3H_1$,U-^{14}C]butyric acid which in anhydrous methanol (10 ml) was treated at 0 °C with an excess of ethereal diazomethane. Removal of solvent gave the doubly labelled methyl 4-acetamidobutyrate (0.14 g), δ ($CDCl_3$) 1.7—2.1 (2 H, m, CH_2), 2.0 (3 H, s, CH_3CO), 2.2—2.5 (2 H, m, CH_2CO), 3.3 (2 H, q, CH_2N), and 3.7 (3 H, s, CH_3CO) (Found: M^+, 159.0899. $C_7H_{13}NO_3$ requires 159.0895).

The amide ester in anhydrous ether (15 ml) was added dropwise during 30 min at 0 °C to a stirred suspension of lithium aluminium hydride (0.6 g) in anhydrous ether (15 ml). Stirring was continued at 0 °C for 20 min more and then water (0.6 ml), 3M-sodium hydroxide, (0.6 ml) and water (1.8 ml) were cautiously added. The precipitate was filtered off and extracted with boiling ethanol (3 × 20 ml). The combined organic solutions were evaporated and the residue extracted with boiling chloroform (30 ml), the evaporation of which afforded the 4-acetamido[4-$^3H_1$,U-^{14}C]-butan-1-ol, identical by n.m.r. with an authentic sample; [16] δ (CD_3OD) 1.5 (4 H, quint, CH_2), 1.9 (3 H, s, CH_3), 2.15 (2 H, t, CH_2N), and 3.5 (2 H, t, CH_2O) (Found: M^+ 131.0950. $C_6H_{13}NO_2$ requires 131.0946).

A solution of the foregoing amide in 2M-hydrochloric acid (10 ml) was heated under reflux for 2 h. Removal of the solvent gave 4-amino[4-$^3H_1$,U-^{14}C]butan-1-ol hydrochloride (92 mg) identical with an authentic sample by n.m.r. and t.l.c. on silica with n-butanol–acetic acid–water (12 : 3 : 5) as eluant; δ(CD_3OD) 1.5—1.9 (4 H, m, CH_2), 3.0 (2 H, t, *J* 7 Hz, CH_2N), and 3.6 (2 H, t, *J* 6 Hz, CH_2O).

A small sample of the amino-alcohol, diluted with unlabelled 4-aminobutan-1-ol (49 mg) was dissolved in anhydrous pyridine (10 ml). The solution was cooled to 0 °C and *p*-nitrobenzoyl chloride (0.45 g) was added, with stirring. After being kept for 16 h at 0 °C, the mixture was poured onto ice–water (40 ml) and the precipitate was collected, dried, and recrystallised from methanol to constant isotopic ratio (^{3}H : ^{14}C 11.4), m.p. 160—162 °C (Found: C, 55.6; H, 4.7; N, 10.6%; M^+, 387.1064. $C_{18}H_{17}N_3O_7$ requires C, 55.8; H, 4.4; N, 10.8%; M^+, 387.1066). δ (CD_3SOCD_3) 1.5—2.0 (4 H, m, CH_2), 3.1—3.6 (2 H, m, CH_2N), 4.4 (2 H, t, CH_2O), 7.8—8.4 (8 H, m, ArH), and 8.7br (1 H, exchangeable with D_2O, NH).

Radiochemical yield of 4-amino[4-$^3H_1$,U-^{14}C]butan-1-ol from γ-aminobutyric acid was 64%.

Stereochemical Assay of Labelled 4-*Aminobutan-1-ol with Pea Seedling Diamine Oxidase.*—4-Amino[4-$^3H_1$,U-^{14}C]-butan-1-ol (1.6 mg) was purified by chromatography on silica (15 g, 80—200 mesh), with n-butanol–acetic acid–water (12 : 3 : 5) as eluant. The pure amino-alcohol was dissolved in 0.1M-sodium phosphate buffer (7 ml; pH 8) and treated with ethanol (0.15 ml), YADH (3.5 mg), NADH (12 mg), catalase (1.5 mg) and diamine oxidase (0.15 units) at 28 °C. After 27 h, unlabelled butane-1,4-diol (39 mg) was added and the solution saturated with sodium chloride and continuously extracted with ethyl acetate for 17 h. The ethyl acetate was evaporated off and the product was dissolved in anhydrous pyridine (10 ml); the stirred solution was then treated at 0 °C with *p*-nitrobenzoyl chloride (0.9 g) and kept for 16 h at 0 °C. Ice–water (50 ml) was added to the mixture which was then extracted with chloroform (3 × 50 ml). The combined organic extracts were washed with water (40 ml), 5M-hydrochloric acid (2 × 3 ml), M-sodium hydroxide (30 ml), and saturated brine (30 ml), and then dried ($CaCl_2$) and evaporated. The product was purified by t.l.c. on silica (chloroform as eluant) and then recrystallised from chloroform to constant isotopic ratio (^{3}H : ^{14}C = 1.01; a repeat run gave 1.05), m.p. 172—173 °C, lit.,[16] 175 °C. δ (C_6D_5N at 70 °C) 1.93 (4 H, m, CH_2), 4.47 (4 H, m, CH_2O), and 8.2 (s, 8 H, ArH).

Oxidation of [$^3H_1$,U-^{14}C]*Butane-1,4-diol.*—A sample of butane-1,4-diol (*ca.* 35 mg), produced from a DAO-YADH coupled system as described above, was dissolved in water (1.5 ml) and added dropwise to a stirred solution of potassium permanganate (0.24 g) in water (2 ml) at 60 °C. After 2 h, the excess of permanganate was decomposed with methanol (0.1 ml) and the hot solution was filtered. The precipitate was washed with hot water (2 × 5 ml) and the filtrate was acidified to pH 1 with concentrated hydrochloric acid and then evaporated. The solid was extracted with methanol, the solution filtered and the solvent evaporated to give a residue which was extracted with boiling ethyl acetate; the extracts were filtered and evaporated. The crude succinic acid was purified by sublimation [18] (130 °C, 0.5 mmHg) and recrystallised from ethyl acetate to constant isotopic ratio (^{3}H : ^{14}C = 0.96). This product was identified by full comparison with an authentic sample (Found: *m/e* 101.0246, loss of OH. $C_4H_5O_3$ requires 101.0239; *m/e* 100.0165, loss of H_2O. $C_4H_4O_3$ requires 100.0160.)

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