Studies of Enzyme-mediated Reactions. Part 14.[1] Stereochemical Course of the Formation of Cadaverine by Decarboxylation of (2*S*)-Lysine with Lysine Decarboxylase (E.C. 4.1.1.18) from *Bacillus cadaveris*

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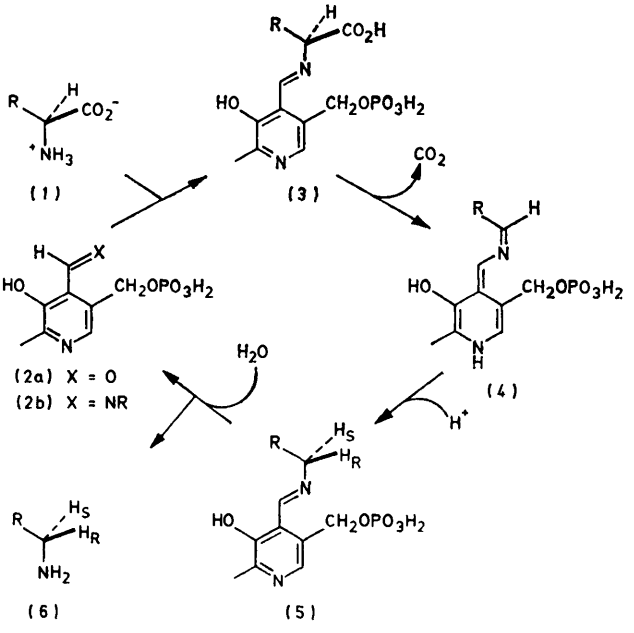
The decarboxylation of (2*S*)-lysine by lysine decarboxylase (E.C. 4.1.1.18) from *B. cadaveris* is shown to be stereospecific and to proceed with retention of configuration by incubation of [2-^{3}H,U-^{14}C]lysine in unlabelled water and [U-^{14}C]lysine in tritiated water to give labelled samples of [1-^{3}H,U-^{14}C]cadaverine (1,5-diaminopentane) which are chiral by isotopic substitution. The configuration of each diamine is assayed by deamination with the diamine oxidase from pea seedlings (E.C. 1.4.3.6) which stereospecifically removes the hydrogen from the *Si*-position of the tritiated methylene group.

THE decarboxylation of α-amino-acids is an important source of biologically active amines and such reactions form a key step in the biosynthesis of many alkaloids.[2] In a continuation of our studies of the stereochemistry of enzymic reactions, this paper presents the results of work on the mode of action of the enzyme (*S*)-lysine decarboxylase from *Bacillus cadaveris*.

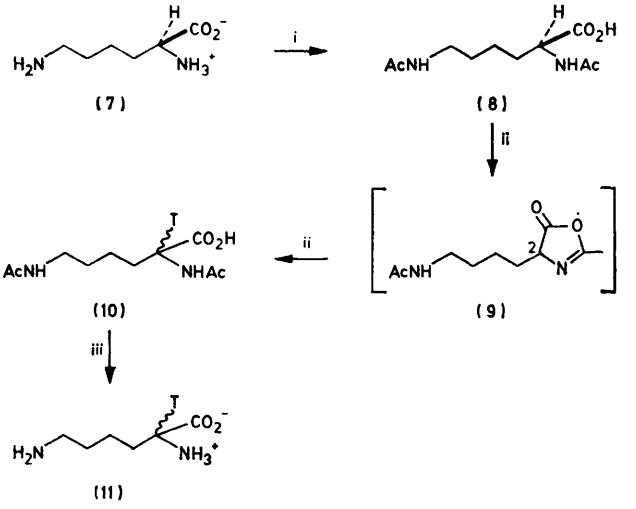
With very few exceptions, the amino-acid decarboxylases require pyridoxal phosphate (2a) as co-factor,[3] and the general view is that the prosthetic group (2b) reacts with the α-amino-group of the amino-acid substrate (1) to form an α-imino-acid[4] (3), as shown in Scheme 1.



SCHEME 1

Upon decarboxylation, this intermediate gives the azomethine (4), which protonates to form the Schiffs base (5) and importantly (see later) the proton is derived from water of the medium.[4] Hydrolysis of the base (5) then regenerates the co-factor with release of the amine product (6). The enzymic decarboxylation of (*S*)-lysine (7) was known to occur stereospecifically[4] but the stereochemistry of the reaction was not known. During the early stages of our work, the decarboxylation was reported[5] to occur with retention of configuration but the differing approach and assay used there made it worthwhile to continue the work described here.

The present study required lysine (7) labelled with tritium at C-2. This was prepared following the route in Scheme 2.[6] Pilot studies of deuterium incorporation by

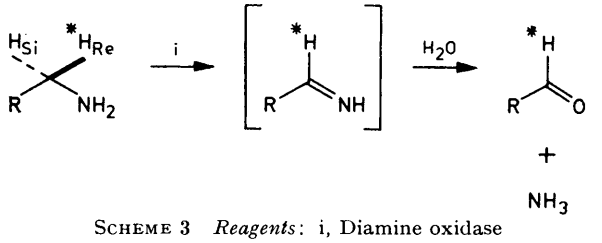


SCHEME 2 *Reagents*: i, Ac_2O; ii, Ac_2O, HTO; iii, 6M-HCl

this route gave a good yield of [2-^{2}H]lysine (80% deuteriated). The reaction is thought to involve the oxazole (9), formed transiently by dehydration of *N*,*N*′-diacetyllysine (8). Rapid interconversion of (9) with its enol form at C-2 results in racemisation and exchange of hydrogen with the medium. Use of tritiated water gave *N*,*N*′-diacetyl[2-^{3}H]lysine (10) as a racemate which was hydrolysed to (2*RS*)-[2-^{3}H]lysine (11). This was not resolved prior to incubation with the decarboxylase because the enzyme is specific for the (*S*)-isomer.[7] A suitable quantity of (2*S*)-[U-^{14}C]lysine was added to provide an internal standard against which tritium retention levels could be measured in the subsequent assay procedure.

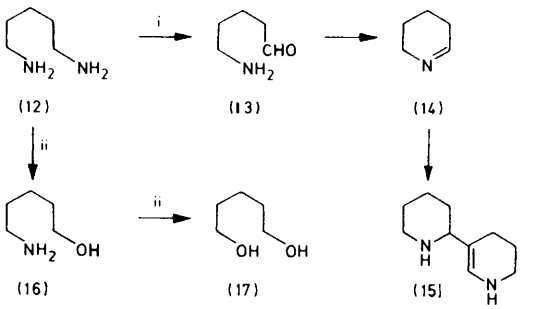
The product of the decarboxylation is cadaverine (1,5-

diaminopentane) (12). The configuration of the labelled methylene of (12) was determined by incubation with diamine oxidase (DAO) isolated from pea seedlings which converts primary amines into aldehydes by oxidatively deaminating a methylene residue carrying a primary amino-group. Earlier work had established that when DAO acts on benzylamine, hydrogen is removed from the *Si*-position of the methylene group.[8] Experiments described in Part 15 (following paper) extend this knowledge to the aliphatic series by proving that DAO works with the same *Si*-stereospecificity (Scheme 3) as it deaminates heptylamine; this extension is important for the present assay on cadaverine (12).



SCHEME 3 *Reagents*: i, Diamine oxidase

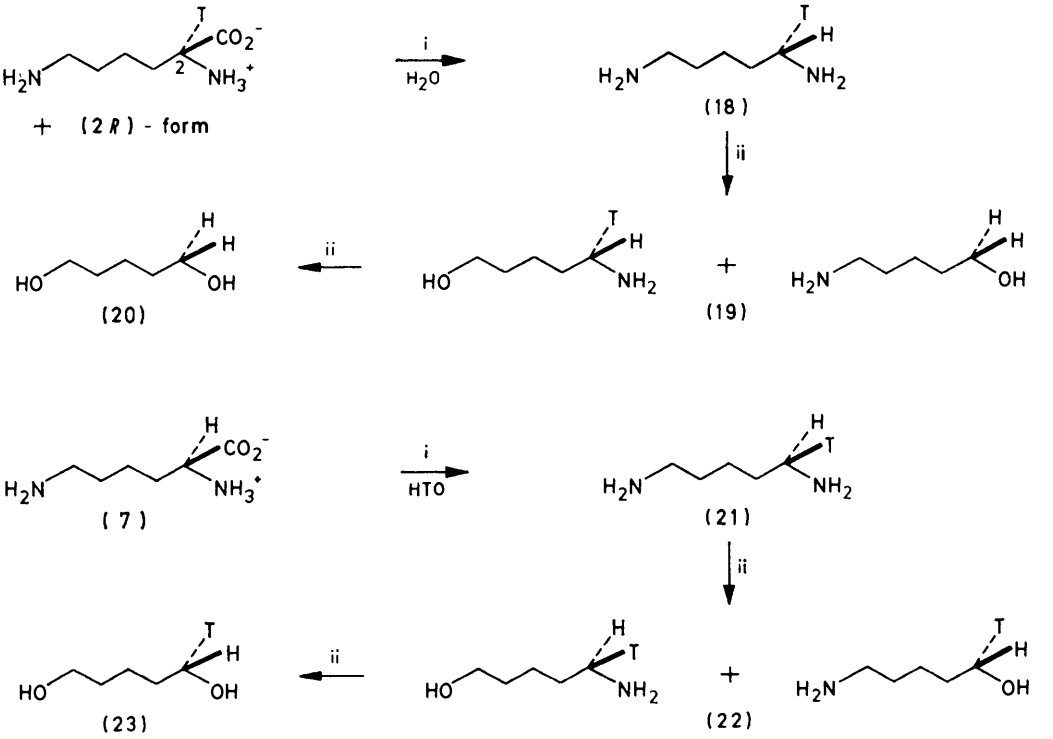
The existence of two primary amino-groups in cadaverine complicates the assay procedure in two ways. First, the initial product of deamination, 5-aminopentanal (13), can cyclise spontaneously under the conditions of the incubation to give tetrahydropyridine (14) and this, in turn, can undergo self-condensation to give tetrahydroanabasine (15) (Scheme 4). To avoid these side reactions, the enzymic deamination was carried out in the presence of an alcohol dehydrogenase and ethanol (as hydride donor) so that the product (13) was rapidly reduced to the chemically stable alcohol 5-aminopentan-1-ol (16). Yeast alcohol dehydrogenase (YADH) was used in the present work in preference to the more usual



SCHEME 4 *Reagents*: i, Diamine oxidase (DAO); ii, DAO–YADH couple

choice of equine liver alcohol dehydrogenase (LADH) (*e.g.* refs. 1 and 8) because it proved to be a more effective catalyst and therefore less protein needed to be added to the incubation mixture.

The second difficulty with cadaverine arises from the symmetry of the molecule which means that either of the two primary amino-groups can be attacked initially by the DAO enzyme. The competing oxidation at the unlabelled methylene means that measurements of tritium retention in the amino-alcohol (16) are inconclusive as will be seen later. Fortunately the amino-alcohol (16) is also a substrate for the DAO and is oxidatively deaminated under the conditions of the incubation (though at a much slower rate); the final product



SCHEME 5 i, *S*-Lysine decarboxylase; ii, DAO–YADH couple

was pentane-1,5-diol (17). In practice a mixture of compounds (16) and (17) was produced in the enzymic assay. The corresponding crystalline bis-*p*-nitrobenzoyl derivatives of (16) and (17) are readily separated by t.l.c.; both were isolated and monitored for tritium retention in the assay procedure.

Two decarboxylation experiments were carried out involving, respectively, incubation of the tritiated lysine (11), prepared as described earlier, in unlabelled water and a complementary run in which [U-^{14}C]lysine was enzymically decarboxylated in tritiated water (Scheme 5). The two resulting [1-$^{3}H_1$,U-^{14}C]cadaverine samples (18) and (21) were then subjected to the foregoing stereochemical assay by incubation with DAO.

Incubation of [1-$^{3}H_1$,U-^{14}C]cadaverine with the DAD-YADH enzyme couple

Expt. No.	Origin of cadaverine	Cadaverine [a] ^{3}H : ^{14}C ratio	5-Aminopentan-1-ol [a] ^{3}H : ^{14}C ratio (retention ^{3}H) [b]	Pentane-1,5-diol [a] ^{3}H : ^{14}C ratio (retention ^{3}H) [b]
1	[2-^{3}H,U-^{14}C]Lysine in H_2O	13.5	12.8 (95 ± 5%)	0.3 (2 ± 1%)
2	[U-^{14}C]Lysine in HTO	4.4	4.3 (98 ± 5%)	4.2 (95 ± 5%)

[a] Counted as bis-*p*-nitrobenzoyl derivative. [b] Calculated relative to cadaverine (100%).

The results given in the Table show that the decarboxylation of lysine proceeds with retention of configuration at C-2. This conclusion is based on comparison of the tritium retention values for the pentanediols with those of the corresponding cadaverine samples. The essentially complete loss of tritium in Experiment 1 means that in the cadaverine (18) the isotope must have occupied the *Si*-position (DAO stereospecifically removes the *Si*-hydrogen) which proves in turn that the configuration at C-2 is retained in the overall decarboxylation process. The complementary result from Experiment 2 with almost complete retention of tritium through all the steps confirms this conclusion.

The nature of the isotopically labelled species in these two experiments is shown in Scheme 5; Experiment 1, (11)→(18)→(19)→(20); Experiment 2, (7)→(21)→(22)→(23). One result which deserves comment is the high retention of tritium in the intermediate (19). In the absence of an isotope effect, a tritium retention value of 50% would be expected. The much higher value can be attributed to a relatively large isotope effect which favours oxidation at the unlabelled end of the diamine molecule (18) in the first deamination step.

The final conclusion that enzymic decarboxylation of lysine is a stereospecific process which proceeds with retention of configuration at C-2 matches the results for all α-amino-acids studied so far; a further example is covered in the following paper. Lastly, work published after completion of the above studies, showed [9] that a hog kidney DAO carries out the first deamination step on cadaverine (12), it too stereospecifically removes the *Si*-hydrogen atom.

EXPERIMENTAL

General Directions.—All buffers were made up in glass distilled water using NaH_2PO_4 and Na_2HPO_4, or tris-(hydroxymethyl)methylamine and hydrochloric acid. Paper chromatography was with Whatman No. 1 chromatography paper; radioactive samples were counted in 10 ml of Instagel scintillation fluid (Hewlett-Packard) on a Packard Tri-Carb 3385 instrument and standardised internally with radiolabelled n-hexadecane. I.r. spectra were recorded as Nujol mulls with a Perkin-Elmer 257 spectrometer, and n.m.r. spectra with solutions in deuteriomethanol or deuteriomethyl sulphoxide on a Varian HA-100 instrument. Mass spectra were determined with an A.E.I. MS-30 spectrometer by direct insertion at appropriate temperatures. Assays of enzymatic activity were carried out using a Unicam SP1800 or a Unicam SP8-100 recording spectrophotometer coupled to an accurate temperature-control system. Protein concentration was estimated by the Coomassie Brilliant Blue [10] method against bovine serum albumin as standard. L-Lysine decarboxylase and yeast alcohol dehydrogenase (YADH) were obtained from the Sigma Chemical Company.

(2RS)-[2-^{2}H]-α,ε-N,N′-*Diacetyl-lysine* (cf. *Ref.* 11).—This compound was prepared by the oxazole route; [6] (2*RS*)-α,ε-*N*,*N*′-diacetyl-lysine [11,12] (230 mg, 1 mmol) was dissolved in acetic anhydride (0.94 ml, 10 mmol) containing deuterium oxide (0.2 ml, 10 mmol), and the solution was heated under reflux for 1 h. The residue from evaporation was twice evaporated to dryness from an aqueous solution (*ca.* 5 ml) and the product was recrystallised from aqueous acetone to give (2*RS*)-[2-^{2}H]-α,ε-*N*,*N*′-diacetyl-lysine (197.8 mg, 86%), m.p. 141—142 °C.[11] Integration of the n.m.r. signal at δ 4.38, due to the proton at the 2-position of the product indicated 80% incorporation of deuterium specifically at that site.

(2RS)-[2-^{2}H]*Lysine Monohydrochloride.*—The foregoing product in hydrochloric acid (5 ml; 6M) was heated under reflux for 2 h. The solution was then evaporated, and the residue thrice evaporated from an aqueous solution. A solution of the final residue in water (1 ml) was adjusted to pH 6 with aqueous sodium hydroxide (1M) and then diluted with nine parts of ethanol. Crystallisation overnight in the cold gave (2*RS*)-[2-^{2}H]lysine monohydrochloride (42.2 mg, 53%), m.p. 265—267 °C (decomp).[13] Integration of the n.m.r. signal at δ 3.73, due to the α-proton, indicated complete retention of deuterium.

(2RS)-[2-^{3}H]*Lysine Monohydrochloride.*—A solution of (2*RS*)-α,ε-*N*,*N*′-Diacetyl-lysine (230 mg, 1 mmol) in acetic anhydride (0.94 ml, 10 mmol) containing tritiated water (0.15 ml, 750 mCi; specific activity 5.0 Ci mol^{-1}), was heated under reflux for 1 h. The solvent was then removed by vacuum transfer and the residue was heated under reflux for 2 h with aqueous hydrochloric acid (5 ml; 6M). The resultant solution was evaporated to dryness, and the residue was thrice evaporated to dryness from an aqueous solution. A solution of the residue in water (1.0 ml) was treated exactly as for the ^{2}H-case above to give (2*RS*)-[2-^{3}H]lysine monohydrochloride (71.2 mg, 39%), m.p.

265—267 °C (decomp.),[13] specific activity = 7.78 mCi/mmol.

The product and radioinactive (2*RS*)-lysine monohydrochloride (180 mg) were dissolved in water (2 ml) and the solution was diluted with ethanol (18 ml). The product which crystallized at 0 °C during 16 h was recrystallised to constant activity from aqueous ethanol; final specific activity = 2.02 mCi/mmol.

(2RS)-[U-^{14}C,2-^{3}H]*Lysine Monohydrochloride*.—(2*RS*)-[2-$^{3}H_1$]Lysine monohydrochloride (5.82 mg, 64.3 μCi) and (2*RS*)-lysine monohydrochloride (16.94 mg) were dissolved in water (0.5 ml) and an aqueous solution of (2*S*)-[U-^{14}C]-lysine (0.1 ml, 2.69 μCi; 2.9 μg of lysine) was added. The solution was diluted with ethanol (4.5 ml) and cooled at 0° C for 16 h; the doubly labelled lysine was recrystallised from aqueous ethanol to constant activity and ratio; final specific activity [^{3}H] = 790 (±5%) μCi/mmol, [^{14}C] = 33 (±5%) μCi/mmol; final ratio ^{3}H : ^{14}C = 23.9 ± 1.0.

N,N′-*Bis*-p-*Nitrobenzoylcadaverine*.—Cadaverine dihydrochloride (175.1 mg, 1 mmol) in aqueous sodium hydroxide (20 ml; 2M), was rapidly stirred for 3 h with a solution of *p*-nitrobenzoyl chloride (1.855 g, 10 mmol) in benzene (5 ml). The product was then collected and recrystallized from aqueous acetone to give the *nitrobenzoyl derivative* (288.1 mg, 72%), m.p. 185—185.5 °C (Found: C, 56.9; H, 5.05; N, 13.8. $C_{19}H_{20}N_4O_6$ requires C, 57.0; H, 5.05; N, 14.0%); $\nu_{max.}$ 3 310 and 1 635 cm^{-1}; δ 1.60 (6 H, m, CH_2), 3.37 (4 H, m, NCH_2), 8.16 (8 H, AB q, ArH), and 8.70 (2 H, m, NH); *m/e* 400 (M^+, 1%).

5-(p-*Nitrobenzamido*)*pentyl* p-*Nitrobenzoate*.—5-Aminopentan-1-ol (103.2 mg, 1 mmol) and *p*-nitrobenzoyl chloride (1.855 g, 10 mmol) were heated under reflux for 3 h in anhydrous pyridine (10 ml) and the solution was then evaporated. A solution of the residue in hot acetone was filtered and diluted with water to give the product which was recrystallized from aqueous acetone. 5-(p-*Nitrobenzamido*)*pentyl* p-*nitrobenzoate* (294 mg, 73%) had m.p. 163.5—164.5 °C (decomp.) (Found: C, 57.0; H, 4.25; N, 10.3. $C_{19}H_{19}N_3O_7$ requires C, 56.85; H, 4.8; N, 10.5%); $\nu_{max.}$ 1 720, 1 695, and 1 660 cm^{-1}; δ 1.5—1.9 (6 H, m, CH_2), 3.37 (2 H, t, *J* 7 Hz, NCH_2), 4.42 (2 H, t, *J* 7 Hz, OCH_2) 8.0 (8 H, m, ArH), and 8.3 (1 H, bs, NH); *m/e* 401 (M^+, 7%).

5-(p-*Nitrobenzamido*)*pentan*-1-*ol*.—5-Aminopentan-1-ol (103.2 mg, 1 mmol) in aqueous sodium hydroxide (10 ml; 2M) was rapidly stirred for 3 h with a solution of *p*-nitrobenzoyl chloride (927.5 mg, 5 mmol) in benzene (5 ml). The product was collected and recrystallized from aqueous acetone to give 5-(*p-nitrobenzamido*)*pentan*-1-*ol* (158.9 mg, 63%), m.p. 102.5—103.5 °C (Found: C, 56.8; H, 6.3; N, 11.0. $C_{12}H_{16}N_2O_4$ requires C, 57.1; H, 6.4; N, 11.1%); $\nu_{max.}$ 3 360 and 1 655 cm^{-1}; δ 1.58 (6 H, m, CH_2), 3.37 (2 H, t, *J* 7 Hz, NCH_2), 3.6 (2 H, t, *J* 7 Hz, OCH_2), 8.16 (4 H, ABq, ArH), and 8.6 (1 H, bs, NH); *m/e* 252 (M^+, 21%).

Pentane-1,5-*diyl Bis*-(p-*nitrobenzoate*).—Pentane-1,5-diol (104 mg, 1 mmol) and *p*-nitrobenzoyl chloride (1.855 g, 10 mmol) were kept in anhydrous pyridine (10 ml) for 16 h at 20 °C and then poured into ice–water; the product was recrystallized from aqueous acetone to give *pentane*-1,5-*diyl bis*-(p-*nitrobenzoate*) (342 mg, 85%), m.p. 104—105 °C (Found: C, 56.5; H, 4.55; N, 6.7. $C_{19}H_{18}N_2O_8$ requires C, 56.7; H, 4.5; N, 7.0%); $\nu_{max.}$ 1 720 cm^{-1}; δ 1.6—2.0 (6 H, m, CH_2), 4.42 (4 H, t, *J* 6 Hz, OCH_2), and 8.20 (8 H, s, ArH); *m/e* 402 (M^+, 12%).

(1S)-[U-^{14}C,1-$^{3}H_1$]*Cadaverine*.—(2*RS*)-[U-^{14}C,2-^{3}H]Lysine monohydrochloride (4 mg; total activity [^{3}H] 17.2 μCi, [^{14}C] 0.73 μCi) and *S*-lysine decarboxylase (4 mg, 4 units) were incubated in phosphate buffer (5.0 ml, 0.2M, pH 6.0) at 37 °C for 6 h. Hydrochloric acid (2 ml; 6N) was then added and the solution evaporated to dryness. The residue in methanolic potassium hydroxide (5 ml; 2% w/v) was applied to Whatman No. 1 chromatography paper and developed for 12 h with propan-2-ol–ammonia solution (*d* 0.88)–water (8 : 1 : 1); the band corresponding to cadaverine (R_F 0.5) was eluted with methanol. Ethanolic hydrogen chloride (10 ml; 1M) was added and the solution was evaporated to dryness to give (1*S*)-[U-^{14}C,1-$^{3}H_1$]-cadaverine dihydrochloride (1.30 mg, 34%). This in water (9 ml) was mixed with radioinactive cadaverine dihydrochloride (30 mg) and a portion (3.0 ml) of this solution was added to a solution of radioinactive cadaverine dihydrochloride (10 mg) in aqueous sodium hydroxide (10 ml; 2M). The amine was converted as described above into the bis-*p*-nitrobenzoyl derivative of (1*S*)-[U-^{14}C,1-$^{3}H_1$]cadaverine (35.7 mg, 71%), m.p. 185—185.5 °C, specific activity [^{3}H] = 4.2 (±5%) μCi/mmol^{-1}, [^{14}C] = 0.310 (±5%) μCi/mmol^{-1}; final ratio ^{3}H : ^{14}C = 13.5 ± 0.6.

The remainder of the solution (6 ml) of cadaverine dihydrochloride was assayed with pea seedling diamine oxidase as described below.

Stereochemical Assay of (1S)-[U-^{14}C,1-$^{3}H_1$]*Cadaverine 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] Pea seeds (2 kg) were grown in boxes of damp sand at 18 °C for 14 days. The seedlings (10—14 cm high) were washed free of sand and the roots removed to leave the aerial parts, which yielded the enzyme used for the stereochemical assay.

The solution of (1*S*)-[U-^{14}C,1-$^{3}H_1$]cadaverine dihydrochloride from above was evaporated to dryness, and the residue dissolved in phosphate buffer (18 ml; 0.05M, pH 8.5). To this solution were added ethanol (1.0 ml), NADH (5 mg), YADH (5 mg), pea seedling diamine oxidase solution (0.5 ml, 0.055 units) and aqueous ethylenediaminetetraacetic acid (EDTA) (1.0 ml; 1.0M), and the solution was incubated at 26 °C for 24 h. The incubation mixture was diluted with phosphate buffer (20 ml; 0.5M, pH 8.6) and stirred rapidly for 3 h with a solution of *p*-nitrobenzoyl chloride (500 mg) in benzene (5 ml). The crude products were collected and separated by preparative t.l.c. (developed in 5% ethanol in ether) into two bands of R_F 0.8 and 0.3, the products from which being recrystallized from aqueous acetone.

The product (R_F 0.3) was identified as 5-(*p*-nitrobenzamido)[U-^{14}C,5-$^{3}H_1$]pentyl *p*-nitrobenzoate (11.47 mg, 24%), m.p. 163—164 °C (decomp.); specific activity [^{3}H] = 4.1 (±5%) μCi mmol^{-1}, [^{14}C] = 0.32 (±5%) μCi/mmol^{-1}; ratio ^{3}H : ^{14}C = 12.8 ± 0.6.

The product (R_F 0.8) was identified as [U-^{14}C]pentane-1,5-diyl bis-(*p*-nitrobenzoate) (13.31 mg, 28%), m.p. 104—105 °C; specific activity [^{3}H] = 0.007 (±5%) μCi mmol^{-1}, [^{14}C] = 0.30 (±5%) μCi mmol^{-1}; ratio ^{3}H : ^{14}C = 0.3 ± 0.15.

(1R)-[U-^{14}C,1-$^{3}H_1$]*Cadaverine*.—(2*S*)-Lysine (2.5 mg) and *S*-lysine decarboxylase (2.5 mg) were dissolved in phosphate buffer (0.5 ml; 0.2M, pH 6.0) containing aqueous (2*S*)-[U-^{14}C]lysine monohydrochloride (0.05 ml; 2.5 μCi; specific activity 50 μCi ml^{-1}) and tritiated water (0.04 ml, 200 mCi). The solution was incubated at 37 °C for 6 h, and then worked up as above to give (1*R*)-[U-^{14}C,1-$^{3}H_1$]cadaverine (0.89 mg, 37%). The crude product in water (9.0 ml) was diluted

with radioinactive cadaverine dihydrochloride (30 mg). A portion (3.0 ml) of this solution was added to a solution of radioinactive cadaverine dihydrochloride (10 mg) in aqueous sodium hydroxide (10 ml; 2M), and it was converted into the bis-*p*-nitrobenzoyl derivative of (1*R*)-[U-^{14}C,1-^{3}H$_1$]-cadaverine (32.9 mg, 71%), m.p. 185—185.5 °C; specific activity [^{3}H] = 10.6 (±5%) μCi mmol^{-1}, [^{14}C] = 2.4 (±5%) μCi mmol^{-1}; final ratio ^{3}H : ^{14}C = 4.4 ± 0.3.

The remainder of the solution of cadaverine dihydrochloride was assayed with pea seedling diamine oxidase as described below.

Stereochemical Assay of (1R)-[U-^{14}C,1-^{3}H$_1$]*Cadaverine with Pea Seedling Diamine Oxidase.*—The remaining solution (6 ml) containing (1*R*)-[U-^{14}C,1-^{3}H$_1$]cadaverine dihydrochloride was incubated with pea seedling diamine oxidase, as described previously, and the products acylated as above to yield the same two derivatives.

One was 5-(*p*-nitrobenzamido)[U-^{14}C,1,5-^{3}H$_1$]pentyl *p*-nitrobenzoate (16.48 mg, 35%), m.p. 164—165 °C (decomp.); specific activity [^{3}H] = 10.7 (±5% μCi mmol^{-1}, [^{14}C] = 2.5 (±5%) μCi mmol^{-1}; ratio ^{3}H : ^{14}C = 4.3 ± 0.2.

The second compound was [U-^{14}C,1,5-^{3}H$_1$]pentane-1,5-diyl bis-(*p*-nitrobenzoate) (4.71 mg, 10%), m.p. 104—105 °C; specific activity [^{3}H] = 10.50 (±5%) μCi mmol^{-1}, [^{14}C] = 2.5 (±5%) μCi mmol^{-1}; ratio ^{3}H : ^{14}C = 4.2 ± 0.2.

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