Studies of Enzyme-mediated Reactions. Part VI.[1,2] Stereochemical Course of the Dehydrogenation of Stereospecifically Labelled Benzylamines by the Amine Oxidase from Pea Seedlings (E.C. 1.4.3.6.)

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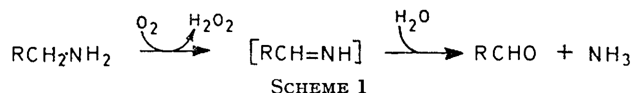
(*R*)-[*methylene*-$^2H_1$]Benzylamine has been synthesised and its absolute configuration established by degradation to (2*R*)-[2-$^2H_1$]glycine. By incubating the corresponding chiral tritiated benzylamines with the amine oxidase from pea seedlings, it has been shown that the *pro-S*-hydrogen atom is removed stereospecifically from the methylene group in the oxidative deamination of benzylamine to benzaldehyde. The enzyme from pea seedlings is readily isolated and is stable; it is now a valuable reagent for assaying the configurational purity of substances which are chiral owing to isotopic substitution at a methylene residue carrying a primary amino-group.

THE oxidation of a methylene group adjacent to a nitrogen atom is of vital importance in the metabolism of primary amines in a wide range of organisms;[3] the process is also a key step in the biosynthesis of many alkaloids.[4] In continuation of our studies of the stereochemistry of such transformations in a number of systems, this paper presents the results of work on the mode of action of the amine oxidase from pea seedlings.[5]

The amine oxidases form a widely distributed family of enzymes which utilise molecular oxygen to effect the oxidative deamination of a primary amine to an aldehyde, as in Scheme 1. The imine is shown speculatively as an



SCHEME 1

intermediate but it must be emphasised that the detailed mechanism is not known even for one oxidase. Moreover, since some amine oxidases employ pyridoxal phosphate as cofactor and others apparently do not,[5] it is possible that this class of enzymes makes use of more than one type of reaction.

The aim of current work is to throw light on the nature of such transformations by determining the stereochemistry of hydrogen removal from the methylene group. The approach involves incubating the enzyme with a suitable amine in which the prochiral methylene group adjacent to the nitrogen atom is labelled stereospecifically by replacement of one of the two enantiotopic hydrogen atoms by deuterium or tritium. The stereochemistry of hydrogen atom removal is then evident, depending on whether the isotope is lost or retained in the formation of the product aldehyde.

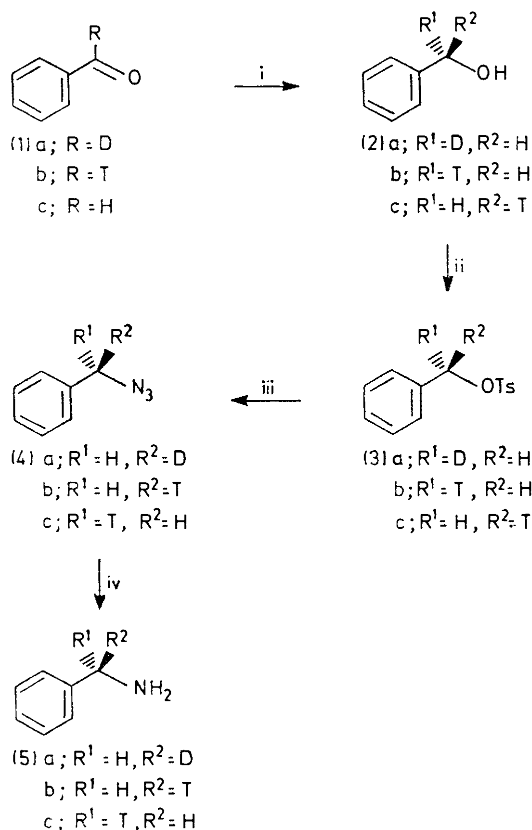
The amine oxidase from pea seedlings can metabolise an exceptionally wide range of substrates, including alkylamines, phenethylamines, tryptamines, benzylamines, and 1,4- or 1,5-diamines.[5] For reasons of convenience and ease of handling, we selected benzylamine as the substrate for this investigation.

The synthetic route to the various chirally labelled benzylamines is shown in Scheme 2. In the preparation of (*R*)-[*methylene*-$^2H_1$]benzylamine (5a), for example, [*formyl*-^{2}H]benzaldehyde (1a) was reduced to (*S*)-[*methylene*-$^2H_1$]benzyl alcohol (2a) (*cf.* refs 6 and 7) by hydrogen transfer from ethanol mediated by a catalytic quantity of NAD^+ in the presence of liver alcohol dehydrogenase. The derived toluene-*p*-sulphonate ester (3a) [6a] was converted under conditions designed to promote an S_N2 displacement into (*R*)-[*methylene*-$^2H_1$]benzyl azide (4a). Reduction of the latter with lithium aluminium hydride gave the chirally labelled benzylamine (5a), isolated as its hydrochloride.[8]

A crucial step in this sequence is the conversion of the ester (3a) into the azide (4a). The change in the n.m.r. signal from the protons of the methylene group [D_2O–$(CD_3)_2CO$] as this reaction occurred showed it to be extremely rapid; it was complete in less than 10 s when a four-molar excess of sodium azide was used. If the

[1] Part V, A. R. Battersby, J. Staunton, H. R. Wiltshire, B. J. Bircher, and C. Fuganti, *J.C.S. Perkin I*, 1975, 1162.
[2] Preliminary report, A. R. Battersby, J. Staunton, and M. C. Summers, *J.C.S. Chem. Comm.*, 1974, 548.
[3] (*a*) H. Blaschko and E. A. Zeller in ' The Enzymes,' ed. P. D. Boyer, H. Lardy, and K. Myrbäck, 2nd edn., Academic Press, New York, 1963, vol. 8, p. 377; (*b*) A. N. Davison, *Physiol. Rev.*, 1958, **38**, 729; (*c*) H. Blaschko, *Pharmacol. Rev.*, 1952, **4**, 415; (*d*) K. F. Tipton, *Brit. Med. Bull.*, 1973, **29**, (2), 116; (*e*) Monoamine Oxidases: New Vistas, in 'Advances in Biochemical Psychopharmacology,' ed. E. Costa and M. Sandler, Raven Press, New York, vol. 5, 1972.
[4] *Chem. Soc. Specialist Periodical Reports*, ' The Alkaloids,' 1974, vol. 4, p. 1, and earlier volumes of this series.
[5] Reviewed by R. Kapeller-Adler in 'Amine Oxidases and Methods for their Study,' Wiley–Interscience, New York, 1970.
[6] *Cf.* (*a*) A. Streitweiser, J. R. Wolfe, and W. D. Schaefler, *Tetrahedron*, 1959, **6**, 338; (*b*) V. E. Althouse, D. M. Feigl, W. A. Sanderson, and H. S. Mosher, *J. Amer. Chem. Soc.*, 1966, **88**, 3595; (*c*) A. Horeau and H. Nouaille, *Tetrahedron Letters*, 1966, 3953; (*d*) H. Gerlach, *Helv. Chim. Acta*, 1966, **49**, 2481.
[7] R. H. Wightman, J. Staunton, A. R. Battersby, and K. R. Hanson, *J.C.S. Perkin I*, 1972, 2355.
[8] A. Streitweiser and J. R. Wolfe, *J. Org. Chem.*, 1963, **28**, 3263.

displacement proceeds according to precedent [7-9] with inversion of configuration, then the amine will have the



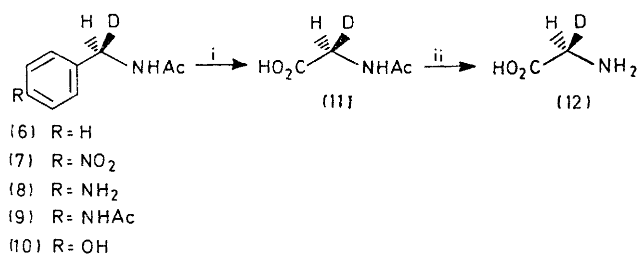
SCHEME 2 i, liver alcohol dehydrogenase, NAD+, and $MeCH_2$·OH or MeCHT·OH; ii, TsCl–pyridine; iii, NaN_3; iv, $LiAlH_4$

R-configuration as illustrated. For our purpose, however, it was imperative to have an independent proof of the absolute configurations of the amine and also a measure of its stereochemical purity. Accordingly the amine was chemically correlated with a suitable reference standard, [2-$^2H_1$]glycine.[10] Direct ozonolysis of *N*-acetylbenzylamine (6) did not give a significant yield * of *N*-acetylglycine (11) so an electron-donating group was introduced to activate the aryl nucleus of *N*-acetylbenzylamine towards attack by ozone. To this end, the 4-nitro-derivative (7), prepared in good yield by nitration [11] of the amide (6), was reduced to the amine (8) by catalytic hydrogenation over palladium. Surprisingly neither the amine (8) nor its *N*-acetyl derivative (9) could be ozonised satisfactorily to give *N*-acetylglycine (11). However, the corresponding phenol (10), prepared from the amine (8) by diazotisation, did react readily with ozone to give the desired fragment (11) in good yield (Scheme 3). This product was hydrolysed to the free α-amino-acid (12) by treatment with hog-kidney acylase-I under conditions known not to affect the chiral centre.[12] O.r.d. measurements [10] on the resultant sample of [2-$^2H_1$]-glycine (12) proved it to be the (+)-(*R*)-isomer and therefore the starting benzylamine was as expected, the (*R*)-isomer (5a). Moreover the high specific rotation of the [2-$^2H_1$]glycine, $[\alpha]^{20}_{328}$ +38.8°, showed that the synthesis had yielded amine of very high configurational purity.

The synthesis was then adapted to produce the various tritiated benzylamines required for the enzymic experiments. First, starting from [*formyl*-^{3}H]benzaldehyde,[7] (*R*)-[*methylene*-$^3H_1$]benzylamine (5b) was prepared by essentially the same sequence of reactions used for the deuteriated analogue, *i.e.* (1b) ⟶ (2b) ⟶ (3b) ⟶ (4b) ⟶ (5b). To prepare the enantiomer (5c), (1*RS*)-[1-$^3H_1$]ethanol † (prepared by reduction of acetaldehyde with borotritiide) was used for reduction of benzaldehyde (1c) to yield (*R*)-[*methylene*-$^3H_1$]benzyl alcohol (2c). Subsequent steps, (2c) ⟶ (3c) ⟶ (4c) ⟶ (5c), paralleled exactly the preparation of the (*R*)-amine (5b). This use of essentially equivalent syntheses to prepare the two enantiomers is an important strength of this approach because it ensures that the two benzylamines not only have opposite configurations but also have the same degree of configurational purity; they should thus produce strictly complementary results in the enzymic experiments.

The final required compound, (*RS*)-[*methylene*-$^3H_1$]-benzylamine (5b and c), was prepared by reduction of benzaldehyde with sodium borotritiide to produce (*RS*)-[*methylene*-$^3H_1$]benzyl alcohol (2b and c), which was converted into the amine by the now standard route.

The amine oxidase from pea seedlings has been extensively studied by Hill, Mann, and Kenten.[14] Significantly, the enzyme requires Cu^{II} for activity but it has



SCHEME 3 i, O_3 followed by HCO_3H; ii, Acylase-I

not been shown to require pyridoxal phosphate as a cofactor.[15] When highly purified, the enzyme rapidly

* We later learned that Arigoni and Besmer [10a] had successfully achieved this conversion under conditions different from those we tested; we are grateful to Professor Arigoni (Züruch) for this information.

† For an alternative method, which allows tritium to be transferred in high yield, see ref. 13.

[9] (*a*) A. Streitweiser and J. R. Wolfe, *J. Amer. Chem. Soc.*, 1959, **81**, 4912; (*b*) A. Streitweiser and W. D. Schaeffer, *ibid.*, 1956, **78**, 5597.

[10] (*a*) P. Besmer, Diss. No. 4435, E.T.H. Zürich, 1970; (*b*) P. Besmer and D. Arigoni, *Chimia* (*Switz.*), 1968, **22**, 494; (*c*) D. Arigoni and E. L. Eliel, *Topics Stereochem.*, 1969, **4**, 127.

[11] H. H. Fox, *J. Org. Chem.*, 1948, **13**, 438.

[12] C. Fuganti, *J.C.S. Perkin I*, 1973, 954.

[13] For discussion of this point, see A. R. Battersby, J. Staunton, and H. R. Wiltshire, *J.C.S. Perkin I*, 1975, 1156.

[14] P. J. G. Mann and R. H. Kenten, *Biochem. J.*, 1952, **50**, 360; P. J. G. Mann, *ibid.*, 1955, **59**, 609; 1961, **79**, 623; J. M. Hill and P. J. G. Mann, *ibid.*, 1962, **85**, 198; 1964, **91**, 171.

[15] J. M. Hill, *Biochem. J.*, 1967, **104**, 1048.

loses activity, whereas partially purified preparations are stable at −10 °C for months. In the present study, therefore, a partially purified preparation [5] was used; importantly, assay [16] of the gel from polyacrylamide electrophoresis of our preparation showed only one band with amine oxidase activity. The activity was measured spectrophotometrically with benzylamine as substrate by following the formation of benzaldehyde, as shown by the rise in absorption at 250 nm. The preparation used in this investigation oxidised 0.026 μmol of benzylamine per min per mg of protein at 25 °C.

Extensive studies [17] of the effect of substrate and product concentration and temperature on the amine oxidase acitivity gave the optimum conditions for preparative incubations; under these conditions, the rate of aldehyde production was proportional to the amount of enzyme added. In designing the enzymic experiments with the labelled benzylamines, it was necessary to take into account the susceptibility of the product benzaldehyde to further oxidation, particularly since oxygen is required by the amine oxidase and hydrogen peroxide is a by-product (Scheme 1). If serious oxidation of the benzaldehyde occurred, the ^{3}H-specific activity of the residual aldehyde would be increased, owing to the kinetic isotope effect. Therefore it was decided not to allow the aldehyde to accumulate but to reduce it *in situ* to benzyl alcohol. A reducing system compatible with the amine oxidase was required and a coupled redox reaction based on liver alcohol dehydrogenase was the logical choice. Less obvious was the choice of alcohol to serve as hydrogen donor. Ethanol, the usual candidate, is far from ideal because of the very large excess which would be required to maintain a suitably low equilibrium concentration of benzaldehyde,[13] leading to the difficult problem of isolating a small amount of benzyl alcohol from a large excess of ethanol. This problem was avoided by employing cyclopentanol as a more potent hydrogen donor; the benzyl alcohol was then readily separated from the relatively small excess of the reductant by chromatography of the corresponding 2,4-dinitrobenzoates.

Each of the three tritiated benzylamines (5b), (5c), and (5b and c) was mixed prior to incubation with a suitable quantity of [*methylene*-^{14}C]benzylamine † so as to provide an independent internal standard against which to measure the degree of tritium retention. The results (Table 1) confirm the high configurational purity of the two enantiomers of tritiated benzylamine. More importantly, they establish that the oxidative deamination is a stereospecific process involving removal of the *pro-S*-hydrogen atom from the methylene group.

The amine oxidase preparation from pea seedlings is readily available and is reasonably stable. As a result of the foregoing work, a convenient method is now available for determining the configurational purity of chirally labelled *N*-methylene groups in suitable amines. Several examples of its use in this way will be described in subsequent papers.

† Prepared from commercially available [*methylene*-^{14}C]benzyl alcohol as in Scheme 2.

[16] *E.g.* B. H. M. Youdim, in ref. 3*d*, p. 120, and ref. 3*e*, p. 67; M. D. Houslay and K. F. Tipton, *Biochem. J.*, 1973, **135**, 173; G. G. S. Collins, M. Sandler, E. D. Williams, and B. H. M. Youdim, *Nature*, 1970, **225**, 817; K. F. Tipton, M. D. Houslay, and N. J. Garrett, *ibid.*, 1973, **246**, 213; H. Yamada and K. T. Yasunobu, *J. Biol. Chem.*, 1962, **237**, 1511.

TABLE 1

Incubation of labelled benzylamines with the amine oxidase

Benzylamine [a]		Benzyl alcohol [b]		
	^{3}H/^{14}C		^{3}H/^{14}C	% ^{3}H retention
(*R*)-[*methylene*-^{3}H$_1$, *methylene*-^{14}C] (5b)	11.0 ± 0.3	Expt. 1	11.1 ± 0.3	100 ± 3
		Expt. 2	11.0 ± 0.3	
(*S*)-[*methylene*-^{3}H$_1$, *methylene*-^{14}C] (5c)	9.6 ± 0.3	Expt. 3	0.2 ± 0.05	2 ± 0.5
		Expt. 4	0.2 ± 0.05	
(*RS*)-[*methylene*-^{3}H$_1$,*methylene*-^{14}C] (5b and c)	16.3 ± 0.3	Expt. 5	8.5 ± 0.2	52 ± 2
		Expt. 6	8.3 ± 0.2	

[a] Radioassay as hydrochloride or phenylthiourea. [b] Radioassay as 2,4-dinitrobenzoate ester.

EXPERIMENTAL

Solutions were dried over anhydrous magnesium sulphate monohydrate and evaporated under reduced pressure and below 40 °C. M.p.s were determined with a Reichert–Kofler hot-stage apparatus. All solvents were dried and distilled before use. Column chromatography was carried out on silica gel (Silicar CC7 Special) or alumina (Woelm neutral, grade II or III). Preparative t.l.c. was carried out on glass plates coated with Merck Kieselgel GF$_{254}$. Radioactive compounds were checked by t.l.c. and scanned with a Panax RTLS-1A radiochromatogram scanner. Radioactive samples were counted in 7 ml of organic scintillator or aqueous scintillator on a Packard Tri-Carb 3385 instrument and standardised with radiolabelled n-hexadecane as internal standard. Unless otherwise stated, u.v. spectra were recorded with a Unicam SP 800 spectrometer for solutions in 95% ethanol, i.r. spectra for solutions in chloroform with a Unicam SP 200 or SP 1000 spectrometer, and n.m.r. spectra for solutions in deuteriochloroform with a Perkin-Elmer R12B or Varian HA-100 spectrometer (tetramethylsilane standard). Mass spectra were determined with an A.E.I. MS9, MS12, or MS902 spectrometer by direct insertion at appropriate temperatures. Spectra of the relevant labelled and unlabelled materials were run consecutively and in duplicate; the deuterium content was calculated according to Biemann.[18] Assays of enzymic activity were carried out with a Unicam SP 500 or SP 550 or a Zeiss RPQ 20A recording spectrometer coupled to an accurate temperature control system. Protein was estimated by the method described by Lowry and Layne.[19] Buffer solutions were prepared from KH_2PO_4 and KOH.

[*formyl*-^{2}H]Benzaldehyde (1a).—This was prepared by the dithian route; [7,20] the deuteriated dithian showed no n.m r. signal at τ 4.93 and contained 99% deuteriated species (M^+ 197). The recovered aldehyde,[20] b.p. 87—90° at 35 mmHg,

[17] M. C. Summers, Ph.D. Thesis, Cambridge, 1974.

[18] K. Biemann, 'Mass Spectrometry,' McGraw-Hill, New York, 1962, p. 223.

[19] (*a*) O. M. Lowry, N. J. Rosenbrough, A. L. Farr, and R. S. Randall, *J. Biol. Chem.*, 1951, **193**, 265; (*b*) E. Layne, *Methods Enzymol.*, 1957, **3**, 448.

[20] D. Seebach, B. W. Erickson, and G. Singh, *J. Org. Chem.*, 1966, **31**, 4303.

showed no n.m.r. signal at τ 0.05. The dithian could be cleaved more conveniently and in better yield (83%) as follows.[21] Cerium(IV) ammonium nitrate (100 g) was added to a solution of the dithian (9 g) in 1 : 4 water–acetonitrile (500 ml). After 3 min, the mixture was poured on to ice and water (500 g) and the benzaldehyde was extracted with ether (2 × 300 ml) and purified as before; it showed no formyl signal at τ 0.05.

(R)-[methylene-$^2H_1$]*Benzylamine Hydrochloride* [*as* (5a)].—To aqueous 0.01M-phosphate buffer (400 ml; pH 7.0) containing dioxan (*ca.* 5% by vol) were added ethanol (50 ml), NAD^+ (20 mg), a suspension of liver alcohol dehydrogenase (1 ml; 10 mg per ml; Boehringer), and [*formyl*-^{2}H]-benzaldehyde (1 g). After incubation at 37 °C for 12 h, the solution was saturated with solid sodium chloride and extracted with ether (3 × 200 ml). The liquid remaining after removal of the ether was chromatographed on silica gel with dichloromethane and ether as eluants to yield unchanged benzaldehyde (90 mg) and (+)-(*S*)-[*methylene*-$^2H_1$]-benzyl alcohol (2a) (850 mg, 85%); $\nu_{max.}$ 3 350, 2 160, and 2 110 cm^{-1}; τ 2.78 (5 H, s, ArH) and 5.5 (1 H, d, J_{HD} 3 Hz, CHD). Repetition on a larger scale yielded more alcohol (2.72 g, 87%).

This product was converted [6a] into its *p*-tolylsulphonyl derivative (57% yield), m.p. 56° (lit.,[6a] 58°). Part (2 g) was added to a solution of sodium azide (1 g) in 9 : 1 acetone–water (75 ml), and after 1 h the acetone was evaporated off and the azide extracted with ether (2 × 20 ml). The oil was distilled (b.p. 110° at 24 mmHg) to give (*R*)-[*methylene*-$^2H_1$]-benzyl azide (980 mg, 97%); τ 2.6 (5 H, s, ArH) and 5.7 (1 H, d, J_{HD} 1 Hz, CHD).

The azide (980 mg) in anhydrous ether (20 ml) was added dropwise to a stirred suspension of lithium aluminium hydride (500 mg) in the same solvent (30 ml). The mixture was then heated under reflux for 1 h; this was followed by a basic work-up [22] into ether, and addition of ethereal hydrogen chloride then gave a solid which was crystallised from ethanol–water to give (*R*)-[*methylene*-$^2H_1$]benzylamine hydrochloride [as (5a)] (750 mg, 70%), m.p. 254—256°. The corresponding base showed τ 2.7 (5 H, s, ArH), 6.18 (1 H, s, CHD), and 8.32 (2 H, s, NH_2).

Correlation of the Foregoing Product (5a) *with* (+)-(2R)-[2-$^2H_1$]*Glycine* (12).—The foregoing labelled benzylamine (5a) (2.0 g), was treated with acetic anhydride (20 ml) in pyridine (50 ml) at 0 °C overnight, and the product was recrystallised from ethyl acetate–light petroleum (b.p. 60—80°) to give the amide (1.61 g), m.p. 58—60° (lit.,[11] 60° for non-deuteriated material); $\nu_{max.}$ 3 440, 3 290, 1 670, and 1 640 cm^{-1}; τ 2.88 (5 H, s, ArH), 5.2 (1 H, s, CHD), 5.7 (1 H, d, *J* 6 Hz, NHCO), and 8.2 (3 H, s, Ac); *m/e* 150 (M^+, 79%), 149 (5), and 107 ($M^+ - CH_3CO$, 100). Mass spectrometric analysis showed 0.97 ± 0.02 atom equiv. of deuterium.

(a) *Nitration and reduction*. The foregoing amide (6) (1.6 g) in glacial acetic acid (2 ml) was treated with fuming nitric acid (6.5 ml) at 0°.[11] After 2 h, the solution was carefully neutralised with concentrated ammonia solution and the resultant nitro-derivative was recrystallised from ethanol–water (920 mg, 45%); m.p. 129—131° (lit.,[11] 130—131° for non-deuteriated material); $\lambda_{max.}$ ($CHCl_3$) 272 nm; $\nu_{max.}$ 1 670, 1 515, and 1 350 cm^{-1}; τ 1.8 and 2.6 (2 H each, d, *J* 8.5 Hz, ArH), 3.7 (1 H, s, NH), 5.5 (1 H, s, CHD), and 7.9 (3 H, s, Ac); *m/e* 195 (M^+, 100%). This material (900 mg) in 95% ethanol (20 ml) was shaken with hydrogen and 10% palladised charcoal (150 mg) at 1 atm. and 20 °C until uptake was complete. The residue was chromatographed on alumina (100 g; activity III) with 3 : 17 methanol–ether. The product (8) (700 mg, 92%) (from ethyl acetate–cyclohexane) had m.p. 89—91° (lit.,[11] 93—95° for non-deuteriated material); $\lambda_{max.}$ 288 nm; $\nu_{max.}$ 3 440, 1 665, 1 620, and 1 514 cm^{-1}; τ 3.0 and 3.4 (2 H each, d, *J* 8.5 Hz, ArH), 4.1 (1 H, s, NH), 5.8 (1 H, s, CHD), 6.5 (2 H, s, NH_2), and 8.05 (3 H, s, Ac); *m/e* 165 (M^+, 90%) and 122 ($M^+ - CH_3CO$, 100).

(b) *Diazotisation and ozonolysis*. The foregoing amine (8) (700 mg) in aqueous 5% acetic acid (140 ml) at 5 °C was treated with sodium nitrite (340 mg) in water (10 ml). The solution was kept at 5 °C for 1 h, then heated at 60—70° for 30 min before cooling and extracting with ethyl acetate (3 × 100 ml). The product was chromatographed on silica (80 g) with 5% methanol–ether and recrystallised from ethyl acetate–cyclohexane to give (R)-N-*acetyl-4-hydroxy*-[methylene-$^2H_1$]*benzylamine* (420 mg), m.p. 131—133° (m.p. 133—135° for non-deuteriated material) (Found for non-deuteriated material: C, 65.5; H, 6.7; N, 8.3. $C_9H_{11}NO_2$ requires C, 65.5; H, 6.7; N, 8.5%); $\lambda_{max.}$ (MeOH) 276 nm, $\lambda_{max.}$ (MeOH–NaOH) 287 nm; $\nu_{max.}$ 3 300, 1 660, and 1 600 cm^{-1}; τ (CD_3OD) 2.9 and 3.3 (2 H each, d, *J* 9 Hz, ArH), 5.78 (1 H, s, CHD), and 8.05 (3 H, s, Ac); *m/e* 166 (M^+, 80%), 165 (5), and 123 ($M - COCH_3$, 100).

The non-deuteriated phenol was *O*-methylated by treatment with an excess of ethereal diazomethane in methanol at 0 °C for 1 day, and the product was recrystallised from ethanol–water; m.p. 95—96° (lit.,[23] 96°); τ 2.8 and 3.2 (2 H each, d, *J* 8.5 Hz, ArH), 4.0 (1 H, s, NH), 5.7 (2 H, d, CH_2), 6.23 (3 H, s, OMe), and 8.02 (3 H, s, NAc); *m/e* 179 (M^+), 136 ($M^+ -$ OMe), and 121 (136 − Me).

Ozonised oxygen (*ca.* 5%; 20 l h^{-1}) was bubbled through a solution of the deuteriated phenol (10) (400 mg) in methanol (30 ml) for 16 h at room temperature. The excess of ozone was then removed in a stream of nitrogen, the solution was evaporated, and the residue was heated under reflux for 1 h with water (10 ml), formic acid (1 ml), and hydrogen peroxide (100 vol.; 1 ml). Palladised charcoal (10%; *ca.* 20 mg) was added, and when effervescence ceased the solution was filtered. The residue left by evaporation of the filtrate was dissolved in warm methanol (*ca.* 5 ml), and ethyl acetate (15 ml) was added. Partial evaporation of the solution at 0 °C caused crystallisation of *N*-acetylglycine (95 mg, 33%), m.p. 204—206°; τ (CD_3OD) 6.1 (1 H, s, CHD) and 8.0 (3 H, s, NAc); *m/e* 118 (M^+, 36%), 73 (90), and 43 (100).

The foregoing deuteriated *N*-acetylglycine (80 mg) in 0.1M-phosphate buffer (pH 7.0; 15 ml) was treated with hog-kidney acylase-I (15 mg; Sigma) at 30 °C for 16 h. After the solution had been heated at 80 °C for 5 min it was filtered through Celite. (+)-(2*R*)-[2-$^2H_1$]Glycine (12) (22 mg) was isolated by chromatography on Dowex-50 resin (H^+) (1 × 10 cm) (elution with 0.5N-ammonia and recrystallisation five times from water–methanol). The deuteriated material showed negligible u.v. absorption above 230 nm. Part of this material was converted into its *N*-acetyl derivative and the deuterium content was determined by mass spectrometric analysis: 0.99 ± 0.02 atom equiv. deuterium.

(R)-[methylene-$^3H_1$]*Benzylamine Hydrochloride* [*as* (5b)] *and* (S)-[methylene-$^3H_1$]*Benzylamine Hydrochloride* [*as* (5c)].—[2-^{3}H]-2-Phenyl-1,3-dithian (860 mg; 25 mCi) was pre-

[21] Ho. Tse-Lok, Ho. C. Honor, and C. M. Wong, *J.C.S. Chem. Comm.*, 1972, 791.

[22] L. F. Fieser and M. Fieser, ' Reagents for Organic Synthesis,' vol. I, Wiley, New York, 1967.

[23] H. Goldshmidt and H. Polanowska, *Ber.*, 1887, **20**, 2409.

pared as earlier [7] and the [*formyl*-^{3}H]benzaldehyde derived from it was converted exactly as for the deuterium sequence into (*R*)-[*methylene*-$^3H_1$]benzylamine hydrochloride, m.p. 257—258°.

For synthesis of the enantiomeric amine, (2*RS*)-[1-$^3H_1$]-ethanol was prepared by treating a solution of freshly distilled acetaldehyde (500 mg) in water (10 ml) with sodium borohydride (50 mg). After 1 h, sodium borotritiide (25 mCi) was added and the solution was kept at 20 °C for 16 h.

TABLE 2

O.r.d. spectrum of (2*R*)-[2-$^2H_1$]glycine [a]

λ/nm	[α] (°)	λ/nm	[α] (°)
303	2.73 (3.6)	263	10.43 (10.7)
294	3.58 (4.57)	256	13.83 (13.8)
286	4.67 (5.36)	250	19.65 (18.6)
278	6.16 (6.70)	244	27.31 (25.7)
270	8.10 (8.25)	238	38.83 (36.7)

[a] Values in parentheses from ref. 10.

An excess of sodium borohydride was then added, and after 1 h the solution was adjusted to pH 1 with sulphuric acid. The aqueous solution of tritiated ethanol was separated from the inorganic materials by vacuum transfer and the whole of it was mixed at 37 °C with 0.01M-phosphate buffer (100 ml; pH 7), ethanol-free liver alcohol dehydrogenase (5 mg; Sigma), NAD$^+$ (10 mg), and benzaldehyde (50 mg). After 20 h, ethanol (2 g), liver alcohol dehydrogenase (5 mg), and NAD$^+$ (10 mg) were added, and after incubation at 37 °C for a further 24 h the solution was saturated with sodium chloride and extracted with ether (3 × 100 ml). The extract was purified as before to give (−)-(*R*)-[*methylene*-$^3H_1$]benzyl alcohol (960 mg, 92%), which was converted as above into (*S*)-[*methylene*-$^3H_1$]benzylamine hydrochloride.

(*RS*)-[*methylene*-$^3H_1$]Benzylamine and [*methylene*-^{14}C]-benzylamine were prepared analogously.

Isolation of the Amine Oxidase from Pea Seedlings.—This was carried out essentially as in the method of Hill and Mann.[24] Pea seeds (1.75 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 were removed to leave the aerial parts (*ca.* 5 kg), which yielded [24] the enzyme used for all the work described in this paper.

[24] Ref. 5, p. 268.

Dehydrogenation of Benzylamine by the Amine Oxidase from Pea Seedlings.—The doubly labelled benzylamine hydrochloride (30 mg) was dissolved in 0.01M-phosphate buffer (40 ml; pH 7.0) containing cyclopentanol (100 mg), NAD$^+$ (10 mg), ethanol-free liver alcohol dehydrogenase (5 mg), and the foregoing amine oxidase preparation (5 ml). The mixture was incubated for 20 h at 37 °C in air. Further additions of NAD$^+$ (10 mg each) and liver alcohol dehydrogenase (5 mg each) were made after 8 and 19 h.

After acidification to pH 1 with 2N-hydrochloric acid, the solution was extracted continuously with ether for 24 h. The organic layer was washed with water and aqueous sodium hydrogen carbonate, then dried, and the ether was removed by distillation through a short column of glass helices. The residue with anhydrous pyridine (10 ml) and 2,4-dinitrobenzoyl chloride (500 mg) was kept at 0 °C overnight and then poured into ice–water. Extraction with methylene chloride (3 × 20 ml) gave a mixture of the cyclopentyl and benzyl esters, which were separated by p.l.c. on silica (double development with 1 : 6 ether–hexane). The *benzyl ester* was recrystallised to constant activity (see Table 1) from 95% ethanol (typical yield 20 mg); m.p. 101—102° (Found for unlabelled material: C, 55.2; H, 3.5; N, 9.5. $C_{14}H_{10}N_2O_6$ requires C, 55.3; H, 3.4; N, 9.4%); $\lambda_{max.}$ 239 nm (ε 15 000); $\nu_{max.}$ 1 735, 1 660, and 1 540 cm^{-1}; τ 1.21 [1 H, d, *J* 1 Hz, $Ar(NO_2)_2$], 1.5 [1 H, dd, *J* 1 and 4.2 Hz, $Ar(NO_2)_2$], 2.1 [1 H, d, *J* 4.2 Hz, $Ar(NO_2)_2$], 2.61 (5 H, s, ArH), and 4.62 (2 H, s, CH_2); *m/e* 302 (M^+, 5%) and 195 (100).

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