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^{2}H NMR SPECTROSCOPY AS A PROBE OF THE STEREOCHEMISTRY OF ENZYMIC REACTIONS AT PROCHIRAL CENTRES

DIAMINE OXIDASE

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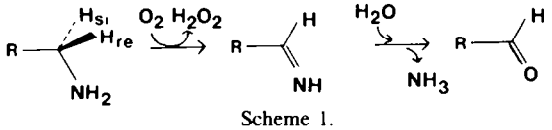
Abstract—In the conversion of cadaverine into Δ^1-piperideine, of putrescine into Δ^1-pyrroline, and of agmatine into 4-guanidinobutanal, catalyzed by hog kidney diamine oxidase (DAO) (E.C. 1.4.3.6 diamine:oxygen oxidoreductase (deaminating)), the *si*-H from C-1 of the substrate is removed while the *re*-H from C-1 of the substrate is maintained at the sp^2 C atom of each of the products.

DAO catalyzed oxidation of cadaverine takes place without detectable isotope effect, while an intramolecular primary isotope effect ($k_{H_{si}}/k_{D_{si}} = 4$) is observed in the DAO catalyzed oxidation of putrescine.

In conflict with earlier reports, incubation of cadaverine in deuterium oxide in the presence of bacterial L-lysine decarboxylase (E.C. 4.1.1.18) did not lead to entry of deuterium into the α-position of cadaverine. Likewise, L-ornithine decarboxylase (E.C. 4.1.1.17) did not catalyze exchange of the α-H of putrescine, nor did L-arginine decarboxylase (E.C. 4.1.1.19) catalyze such an exchange in agmatine.

Monoamine oxidase (E.C. 1.4.3.4 monoamine:oxygen oxidoreductase (deaminating)) and diamine oxidase (E.C. 1.4.3.6 diamine:oxygen oxidoreductase (deaminating)) catalyze the oxidation of primary amines to aldehydes (Scheme 1). The enzymes occur in mammalian tissues, in microorganisms and in plants. They are of low substrate specificity, each acting upon a wide range of primary amines.



Scheme 1.

The absolute stereochemistry of the abstraction of a H atom from the prochiral methylene group adjacent to N has been determined in several instances, by a variety of methods. A precondition for all these methods was the availability of substrates which were chirally labelled with deuterium or tritium at the methylene group, and whose absolute stereochemistry had been determined by correlation with compounds of known chirality.

Thus, mitochondrial monoamine oxidase of rat liver, acting upon tyramine, was shown, by a kinetic method, to catalyze removal of the *re*-H. The rate of oxidation of S-(α-^{2}H)tyramine was found to be 2.3 times faster than that of R-(α-^{2}H)tyramine, indicating a kinetic isotope effect $k_{H_{re}}/k_{D_{re}}$ of 2.3.[1,2] Abstraction of the *re*-H in the reaction catalyzed by mitochondrial monoamine oxidase of rat liver has recently been verified by radioactive tracer methods, employing S- and R-[α-^{3}H]tyramine[3] as well as S- and R-1-amino[1-^{3}H]heptane[4] as substrates.

Plasma amine oxidase, on the other hand, appears to catalyze the reaction of opposite chirality: radioactivity was retained in *p*-hydroxybenzaldehyde obtained in the oxidation of *p*-hydroxy-R-[α-^{3}H]benzylamine, catalyzed by bovine plasma amine oxidase.[5] Similarly, the ^{3}H/^{14}C ratio was maintained in the product when R-[α-^{3}H,α-^{14}C]benzylamine served as the substrate of this enzyme, whereas most of the tritium relative to ^{14}C was lost from S-[α-^{3}H, α-^{14}C]benzylamine.[6] Thus, the *si*-proton had been removed in each case. Attack on dopamine by the enzyme appears to be non-stereospecific, however.[7]

The stereospecificity of the reaction mediated by diamine oxidase has also been investigated. That the reaction catalyzed by hog kidney diamine oxidase, acting upon [α-^{3}H]histidine, proceeds with stereospecific release of tritium was first noted by T. Hesselbo (quoted in Ref. 8). It is now shown that hog kidney diamine oxidase, acting upon (1-^{2}H)cadaverine,[9] (1-^{2}H)putrescine and (1-^{2}H)agmatine, catalyzes removal of the *si*-proton of the substrate, in each case.

Evidence will be presented, however, that, even so, the rate limiting step in the reaction with cadaverine as the substrate must be different from that with putrescine as the substrate.

The stereochemistry of the reaction catalyzed by diamine oxidase from pea seedlings has also been determined. With S- and R-[α-^{3}H]benzylamine as substrates, it was shown[10] that the *si*-H is abstracted in the course of enzymic oxidation. More recently it was established that the *si*-H is lost also in the course of the oxidation, catalyzed by pea seedling diamine oxidase, of S-[α-^{3}H]- and R-[α-^{3}H]-3′-*O*-methyldopamine,[11] S-[1-^{3}H]- and R-(1-^{2}H)cadaverine,[12] R-[α-^{3}H]histamine[13] and S-1-amino[1-^{3}H] heptane.[14]

The stereochemical consistency with such a wide range of substrates would appear to justify the assumption that, in the reaction catalyzed by diamine oxidase, oxidation of every substrate is accompanied by loss of the *si*-H. Indeed, configurational assignments of the absolute stereochemistry of chiral samples of 4-amino[4-^{3}H]butan-1-ol,[14] of 5-amino[5-^{3}H] pentan-1-ol[15] and of 3-methylthio[1-^{3}H]propylamine[16] have been made on the basis of this assumption.

Similarly, the assumption that mitochondrial rat liver monoamine oxidase catalyzes removal of the *re*-H not only from tyramine[1-3] and heptylamine[4] but also from other substrates, has served as the basis of configurational assignment of chiral samples of [α-^{3}H]tryptamine.[17]

It had been our intention, in a study of the stereochemistry of the enzymic decarboxylation, catalyzed by the appropriate decarboxylase, of ornithine and arginine using ^{2}H as the chiral marker, to base the configurational assignments of the resulting chiral samples of (1-^{2}H)putrescine and (1-^{2}H)agmatine on a similar assumption.

We had earlier established the absolute configuration of the enantiomeric [1-^{3}H]cadaverines obtained by enzymic decarboxylation of L-[2-^{3}H]lysine in water, and of L-lysine in tritiated water, to be S-[1-^{3}H]- and R-[1-^{3}H]cadaverine, respectively, by correlation with [2-^{3}H]glycine of known chirality.[18] By carrying out the enzymic decarboxylation of L-(2-^{2}H)lysine in water and of L-lysine in deuterium oxide, we were thus able to obtain the enantiomeric (1-^{2}H)cadaverines of known chirality.[9]

These enantiomeric (1-^{2}H)cadaverines were then employed to determine the absolute stereochemistry of the reaction catalyzed by hog kidney diamine oxidase, using ^{2}H NMR spectroscopy to determine the labelling patterns of the resulting (^{2}H)aminoaldehydes by a method to be fully described in this paper.[9] On the assumption that the absolute stereochemistry of the reaction catalyzed by hog kidney diamine oxidase was the same, whatever the substrate, we then intended to employ similar ^{2}H NMR methods to determine the labelling patterns of the aminoaldehyde samples, obtained on incubation with hog kidney diamine oxidase, from the enantiomeric samples of (1-^{2}H)putrescine and of (1-^{2}H)agmatine. These, in turn, were to be obtained by enzymic decarboxylation of L-(2-^{2}H)ornithine and L-(2-^{2}H)arginine in water and of L-ornithine and L-arginine in ^2H_2O. These results would, in turn, lead to the absolute stereochemistry of the enzymic reactions catalyzed by L-ornithine decarboxylase and of L-arginine decarboxylase.

As will be seen in the sequel, the results that were obtained with cadaverine and with putrescine appeared to be qualitatively consistent. But since a kinetic isotope effect was observed in the diamine oxidase catalyzed reaction with putrescine as the substrate, but none with cadaverine as the substrate, it was evident that the rate-limiting step in the reaction with each of the two substrates was not the same.

To make the assumption that the stereochemistry of the reaction with the two substrates was the same, even though the rate-limiting step, and therefore possibly also the mechanism, was different, and to use this assumption as a basis for assignment of absolute chirality, would clearly have been unsound.

The absolute stereochemistry of the enantiomeric samples of (1-^{2}H)putrescine and of (1-^{2}H)agmatine was therefore determined by an independent method.[19]

With chiral samples, of known stereochemistry, of (1-^{2}H)cadaverine, (1-^{2}H)putrescine, and (1-^{2}H)agmatine in hand, the stereochemistry of the reaction of hog kidney diamine oxidase, acting upon each of the three substrates, was determined by ^{2}H NMR spectroscopy. In each case a *si*-proton is dislodged in the course of the reaction.

METHODS AND RESULTS

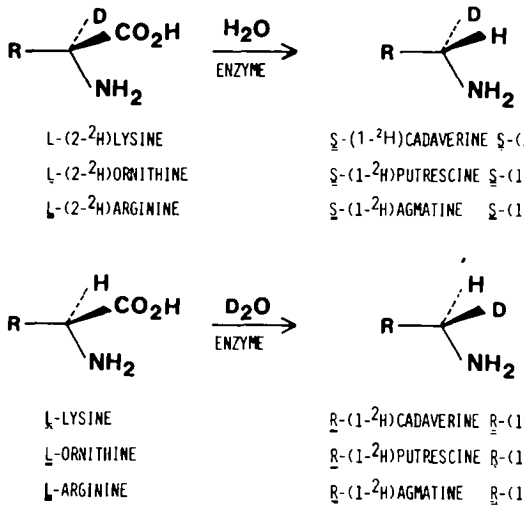
The enantiomers of (1-^{2}H)cadaverine, (1-^{2}H) putrescine, and (1-^{2}H)agmatine, required for this study were prepared, respectively, by the decarboxylation of L-lysine, catalyzed by L-lysine decarboxylase (E.C. 4.1.1.18 L-lysine carboxylyase) from *Bacillus cadaveris*, of L-ornithine, catalyzed by L-ornithine decarboxylase (E.C. 4.1.1.17 L-ornithine carboxylyase) from *Escherichia coli*, and of L-arginine, catalyzed by L-arginine decarboxylase (E.C. 4.1.1.19 L-arginine carboxylyase) of *E. coli* (Scheme 2). Each of these three enzymic reactions is known to proceed with retention of configuration.[18,19] S-(+)-(1-^{2}H)Cadaverine dihydrochloride (Expt. 2), S-(+)-(1-^{2}H)putrescine dihydrochloride (Expt. 5), (each > 95% deuteriated at the *si*-position of one of the terminal carbon atoms), and S-(+)-(1-^{2}H)agmatine sulfate (Expt. 8) (> 85% deuteriated at the *si*-position of the primary amino carbon atom) were obtained from the L-component of DL-(2-^{2}H)lysine, the L-component of DL-(2-^{2}H)ornithine and from L-(2-^{2}H)arginine, respectively. R-(−)-(1-^{2}H)Cadaverine dihydrochloride (Expt. 1), R-(−)-(1-^{2}H)putrescine dihydrochloride (Expt. 4), (each > 95% deuteriated at the *re*-position of one of the terminal carbon atoms) and R-(−)-(1-^{2}H)agmatine sulfate (Expt. 7) (> 98% deuteriated at the *re*-position of the primary amino carbon atom) were obtained by decarboxylation of the L-components of DL-lysine[9] and of DL-ornithine, and of L-arginine, respectively, in ^2H_2O (> 98 at. % ^{2}H). Incubation, in ^2H_2O solution, of cadaverine with L- lysine decarboxylase, of putrescine with L-ornithine decarboxylase, and of agmatine with L-arginine decarboxylase, under the conditions which yielded the deuteriated samples of the bases from the amino acids, did not lead to exchange of deuterium into the unlabelled amines. No decrease in the signal area due to the α-protons was detectable by ^{1}H NMR spectroscopy; and no signal due to ^{2}H was observable (by ^{2}H NMR) on examination of one of the amines, which was reisolated.

Samples of (1,1-$^2H_2$)cadaverine dihydrochloride (Expt. 3) and of (1,1-$^2H_2$)putrescine dihydrochloride (Expt. 6) (each > 90% perdeuteriated at one of the terminal C atoms) were prepared by enzymic decarboxylation of DL-(2-^{2}H)lysine and of DL-(2-^{2}H)ornithine, respectively, in ^2H_2O.

In separate experiments each of the three deuteriated samples of cadaverine (Expts. 1–3), the three deuteriated samples of putrescine (Expts. 4–6) and the two deuteriated samples of agmatine (Expts. 7, 8) were incubated with hog kidney diamine oxidase (E.C. 1.4.3.6 diamine:oxygen oxido-reductase (deaminating)). The details of these experiments are presented in Table 1.

The incubation in Experiments 1–6 was carried out in the presence[20,21] of *o*-aminobenzaldehyde which serves to trap[21,22] the oxidation products which, themselves, are difficult to isolate,[23,24] since they tend to trimerize at physiological pH.[23,25,26]

The product of the enzymic oxidation of cadaverine



Scheme 2.

Table 1. Incubation of labelled substrates with diamine oxidase

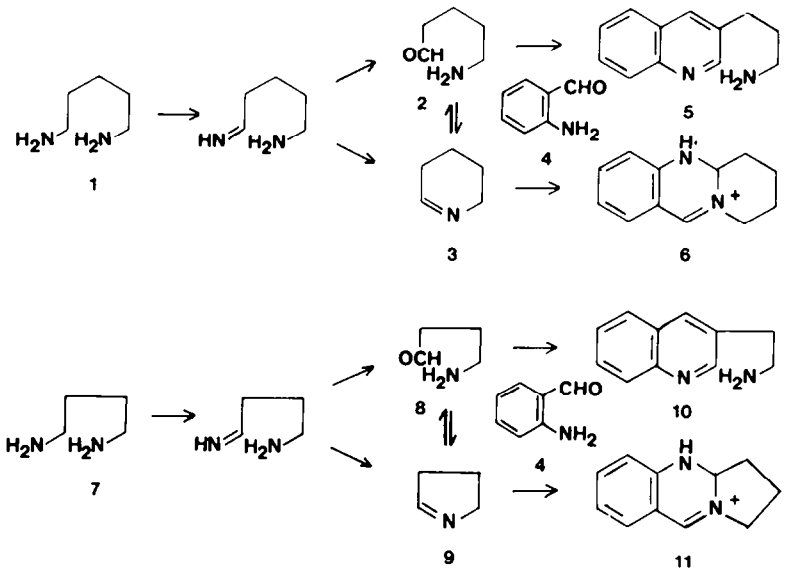
Expt. No.	Substrate	^{2}H content (atom %)	weight (mg)	conc. (mM)	Diamine Oxidase concn. (mg ml^{-1})	o-aminobenz aldehyde concn. (mM)	Incubation Time (h)
1	R-(-)-(1-^{2}H)cadaverine dihydrochloride	> 95 ($^2H_1$)	40	7.6	7.3	8.4	40
2	S-(+)-(1-^{2}H)cadaverine dihydrochloride	> 95 ($^2H_1$)	40	7.4	7.2	8.8	39
3 (a)	(1,1-$^2H_2$)cadaverine dihydrochloride	> 93 ($^2H_2$)	40	7.6	7.4	9.1	39
(b)	(1,1-$^2H_2$)cadaverine dihydrochloride	> 93 ($^2H_2$)	30	9.3	10.1	9.8	6
4 (a)	R-(-)-(1-^{2}H)putrescine dihydrochloride	99.7 ($^2H_1$)	30	9.3	10.4	8.4	6
(b)	R-(-)-(1-^{2}H)putrescine dihydrochloride	98.7 ($^2H_1$)	22	13.4	9.6	10.0	6
5	S-(+)-(1-^{2}H)putrescine dihydrochloride	97.2 ($^2H_1$)	31	9.6	9.6	8.9	6
6	(1,1-$^2H_2$)putrescine dihydrochloride	91 ($^2H_2$); 9 ($^2H_1$)	39	12.1	10.8	9.8	6
7	R-(-)-(1-^{2}H)agmatine sulfate	> 98 ($^2H_1$)	33	12.1	6.7	-	25
8	S-(+)-(1-^{2}H)agmatine sulfate	> 85 ($^2H_1$)	41	12.0	6.6	-	26

(**1**), 5-aminopentanal (**2**), in equilibrium with Δ^1-piperideine (**3**), is trapped by *o*-aminobenzaldehyde (**4**) to yield, as the major product, 3-(3′-aminopropyl)quinoline (**5**) which is isolated as the dipicrate or the dihydrochloride.[23] A second product, 2,3-tetramethylene-1,2-dihydroquinazolinium ion (**6**), isolated as the picrate,[22,23] is formed in lower yield (Scheme 3). The oxidation product of putrescine (**7**), 4-aminopropanal (**8**), in equilibrium with Δ^1-pyrroline (**9**), is trapped as 2,3-trimethylene-1,2-dihydroquinazolinium ion (**11**), isolated as the picrate,[22,24,27] while 3-(2′-aminoethyl)quinoline (**10**) is formed as a minor product (Scheme 3).

The samples of 3-(3′-aminopropyl)quinoline dihydrochloride, obtained from the incubation, with diamine oxidase, of the three deuteriated cadaverines (Expts 1–3) (Table 2) and the samples of 2,3-trimethylene-1,2-dihydroquinazolinium picrate, obtained similarly from the three deuteriated putrescines (Expts 4–6) (Table 3) each contained ^{2}H. The location of the ^{2}H in these samples was determined by ^{2}H NMR spectroscopy.

Assignment of the NMR signals was facilitated by the chemical synthesis of (2-^{2}H)-3-(3′-aminopropyl) quinoline dihydrochloride (δ 8.8 ppm) and of (2-^{2}H)-2,3-trimethylene-1,2-dihydroquinazolinium picrate (δ 5.3 ppm), by oxidation of DL-(2-^{2}H)lysine (*cf*, Ref. 28) and DL-(2-^{2}H)ornithine (*cf*, Ref. 24), respectively, with *N*-bromosuccinimide, to yield (1-^{2}H)-5-aminopentanal and (1-^{2}H)-4-aminobutanal, respectively, which were trapped, as before, as their *o*-aminobenzaldehyde adducts.

The products obtained from the incubation with diamine oxidase of R-(−)-(1-^{2}H)cadaverine (Expt. 1),



Scheme 3.

Table 2. Products of the incubation of cadaverine (1) with diamine oxidase

Expt. No.	Substrate	Product (5) 3-(3'-Aminopropyl)quinoline Dihydrochloride		
		Yield (mg)	^{2}H-NMR Signals (ppm; rel. area if applicable)	Retention of Deuterium (%) (determined by ^{1}H NMR)
1	R-(-)-(1-^{2}H)cadaverine dihydrochloride	2	2.9, 8.8 [(1.0 ± 0.2)/1]	103 ± 19
2	S-(+)-(1-^{2}H)cadaverine dihydrochloride	5	2.9	50 ± 19
3 (a)	(1,1-$^2H_2$)cadaverine dihydrochloride	7	2.9, 8.8 [(2.2 ± 0.4)/1]	74 ± 11
(b)	(1,1-$^2H_2$)cadaverine dihydrochloride	7		75 ± 11

Table 3. Products of the incubation of putrescine (7) with diamine oxidase

Expt. No.	Substrate	Product (11) 2,3-Trimethylene-1,2-dihydroquinazolinium Picrate		
		Yield (mg)	^{2}H-NMR Signals (ppm; rel. area if applicable)	Retention of Deuterium (%) (determined by ^{1}H NMR)
4(a)	R-(-)-(1-^{2}H)putrescine dihydrochloride	15	4.1, 5.2 [(1.2 ± 0.1)/1]	100 ± 9
(b)	R-(-)-(1-^{2}H)putrescine dihydrochloride	7	4.1, 5.2 [(1.0 ± 0.3)/1]	
5	S-(+)-(1-^{2}H)putrescine dihydrochloride	10	4.1	82 ± 4
6	(1,1-$^2H_2$)putrescine dihydrochloride	12	4.1, 5.2 [(7.0 ± 0.5)/1]	90 ± 8

(1,1-$^2H_2$)cadaverine (Expt. 3a), R-(−)-(1-^{2}H)putrescine (Expt. 4) and (1,1-$^2H_2$)putrescine (Expt. 6) each showed two signals in their ^{2}H NMR spectra. The products from S-(+)-(1-^{2}H)cadaverine (Expt. 2) and S-(+)-(1-^{2}H)putrescine (Expt. 5) each showed a single signal in their ^{2}H NMR spectra (Tables 2 and 3, Figs. 1 and 2). This shows that in the oxidation of cadaverine to Δ^1-piperideine, and of putrescine to Δ^1-pyrroline, catalyzed by diamine oxidase, a *si*-proton is lost.

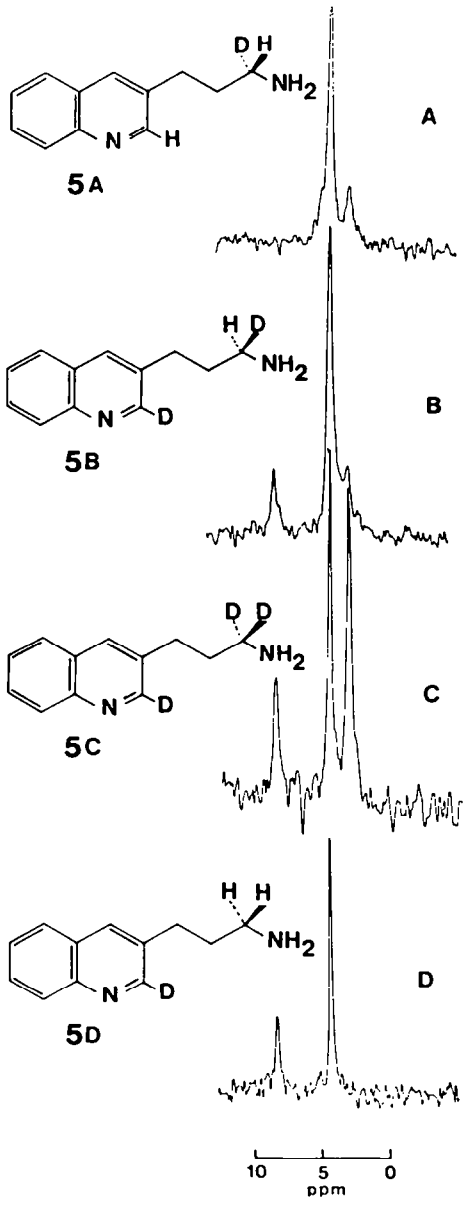


Fig. 1. Proton decoupled ^{2}H NMR spectra of deuterium labelled samples of 3-(3′-aminopropyl)quinoline dihydrochloride (**5**): A (**5A**), derived from s-(1-^{2}H)cadaverine (by enzymic oxidative deamination) (5 mM, 56628 transients); B (**5B**), derived similarly from R-(1-^{2}H)cadaverine (5 mM, 57000 transients); C (**5C**), derived similarly from (1,1-$^2H_2$)cadaverine (13 mM, 3475 transients); D (**5D**), obtained from DL-(2-^{2}H)lysine by chemical oxidation (8 mM, 4808 transients).

Correspondingly, in the oxidation of agmatine (**12**) to 4-guanidinobutanal (**13**)[21] which was isolated as the phosphate salt of its 2,4-dinitrophenylhydrazone (**15**), ^{2}H is preserved in the oxidation product of R-(−)-(1-^{2}H)agmatine sulfate (Expt. 7) but is lost in the formation of the product from s-(+)-(1-^{2}H)agmatine sulfate (Expt. 8) (Table 4, Scheme 4).

Hog kidney diamine oxidase thus mediates stereospecific removal of the *si*-H from C-1 of cadaverine, putrescine and agmatine. The products of the enzymic oxidation, Δ^1-piperideine (**3**), in equilibrium with 5-aminopentanal (**2**), Δ^1-pyrroline (**9**), in equilibrium with 4-aminobutanal (**8**), and 4-guanidinobutanal (**13**), respectively, each retains, at their sp^2 C atom, the *re*-H from C-1 of the starting material.

While the enzymic reaction with the three substrates thus takes a qualitatively similar stereochemical course, a quantitative difference in the reactions of cadaverine on the one hand and of putrescine on the other is revealed by the ^{2}H NMR spectra of the products of these reactions: Whereas the deuterium enrichment at the two deuteriated positions in the product from (1,1-$^2H_2$)cadaverine gives rise to relative areas 2:1 for the signals at δ 2.9 and 8.8 ppm, with a deuterium retention of 75% (Expt. 3a, Table 2; Fig. 1c), the corresponding product from (1,1-$^2H_2$)putrescine showed a deuterium enrichment at

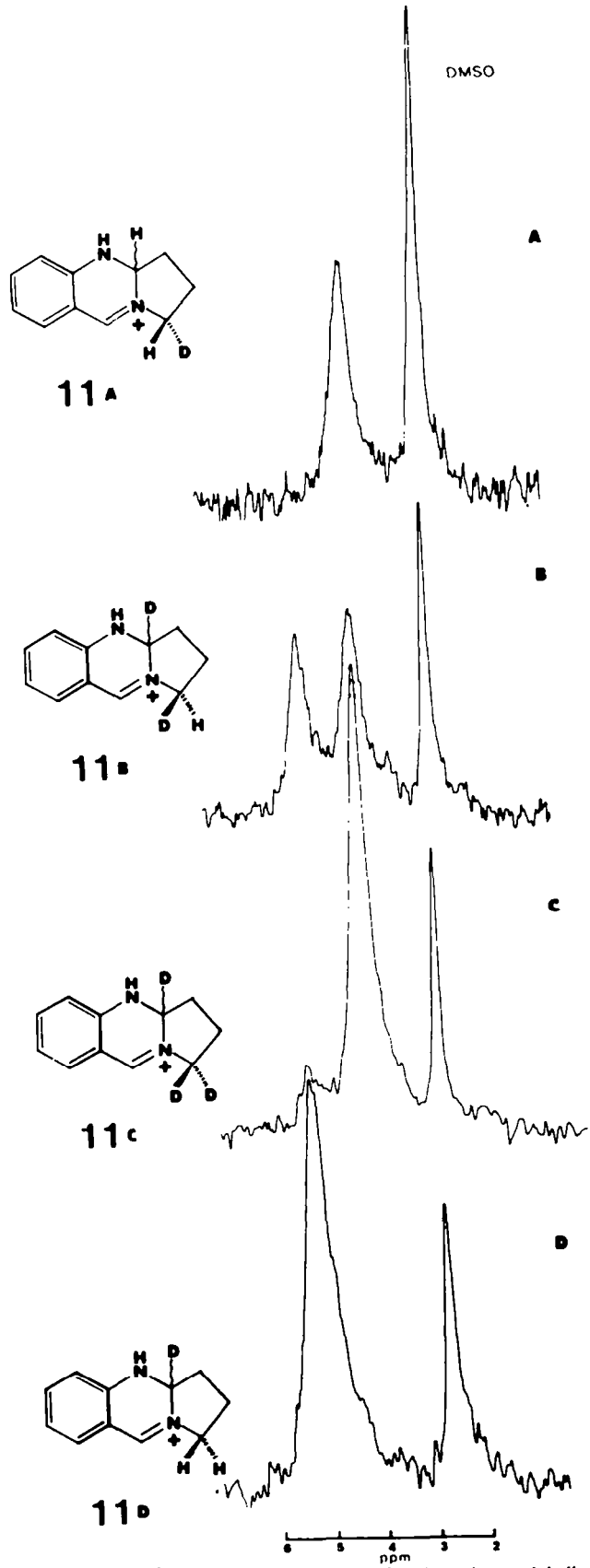
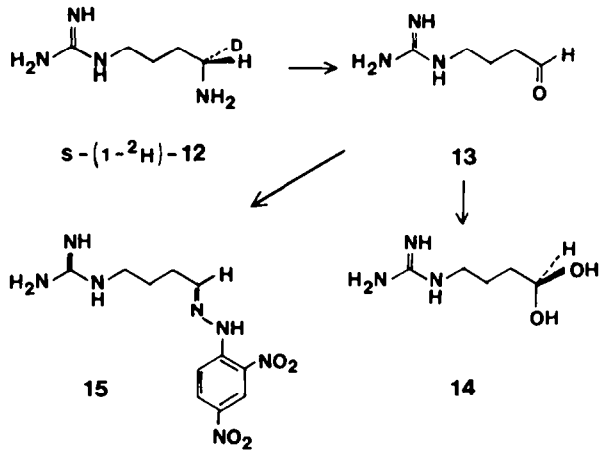


Fig. 2. Proton decoupled ^2H NMR spectra of deuterium labelled samples of 2,3-trimethylene-1,2-dihydroquinazolinium (**11**) picrate: A (**11A**), derived from s-(1-^{2}H)putrescine (by enzymic oxidative deamination) (13 mM, 15140 transients); B (**11B**), derived similarly from R-(1-^{2}H)putrescine (13 mM, 10,000 transients); C (**11C**), derived similarly from (1,1-$^2H_2$)putrescine (26 mM, 4824 transients); D (**11D**), obtained from DL-(2-^{2}H)ornithine by chemical oxidation (20 mM, 5000 transients).



Scheme 4.

Table 4. Products of the incubation of agmatine (12) with diamine oxidase

Expt. No.	Substrate	Product (15) 4-Guanidinobutanal 2',4'-dinitrophenylhydrazone Phosphate		
		Yield (mg)	^{2}H-NMR Signals (ppm)	Retention of Deuterium (%) (determined by ^{1}H NMR)
7	R-(-)-(1-^{2}H)agmatine sulfate	25	7.6	100 ± 5
8	S-(+)-(1-^{2}H)agmatine sulfate	32	-	0 ± 5

the two deuteriated positions which gives rise to relative areas 7:1 for the signals at δ 4.1 and 5.2 ppm, with a ^{2}H retention of almost 90% (Expt. 6, Table 3; Fig. 2c). This observation will be referred to later in the discussion.

DISCUSSION

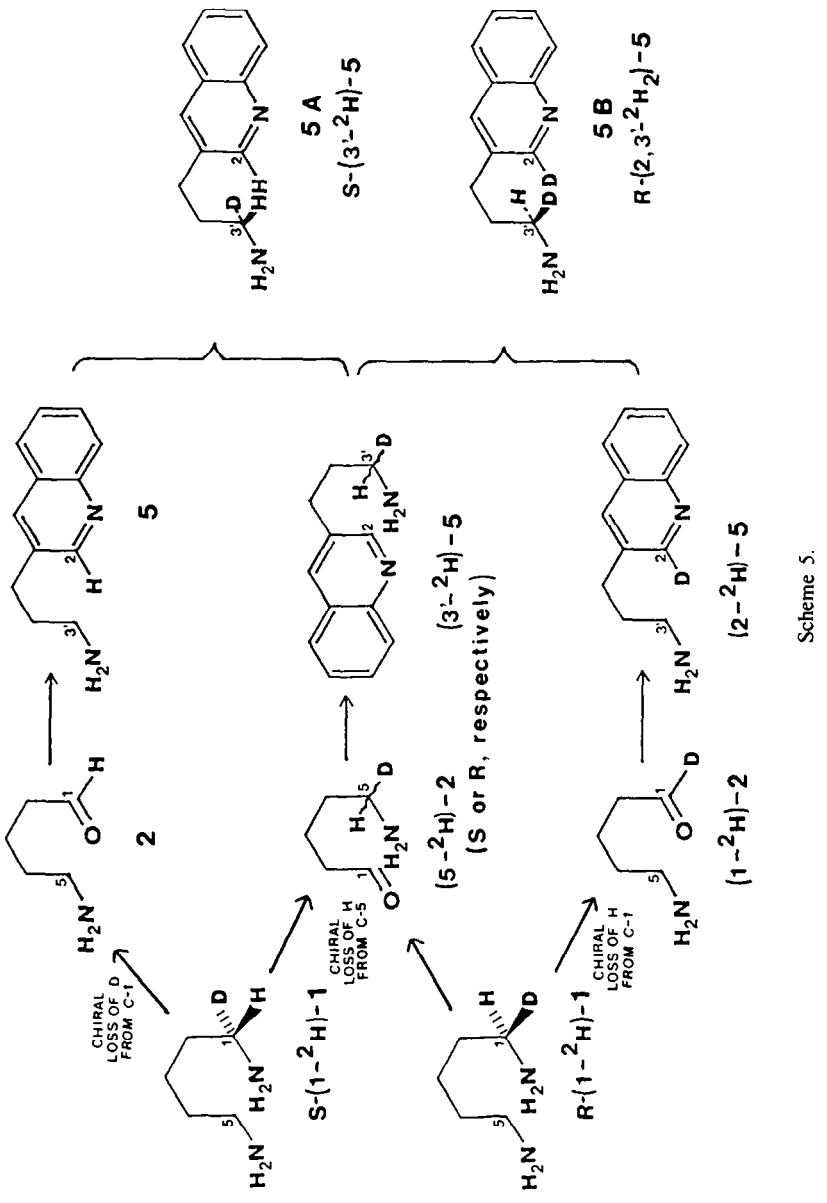
Hog kidney diamine oxidase catalyzes the oxidation of a wide range of primary amines to the corresponding aldehydes.[8,29-32] Putrescine (7) and cadaverine (1) are amongst substrates of the enzyme which have the lowest Michaelis constant (K_M)[30] and the highest oxidation rate (V_{max}).[32] Agmatine (12) is also readily attacked.[31]

The stereochemistry of the removal of an α-hydrogen atom in this reaction may be determined using as substrates chiral samples of known chirality, of each of the amines, in which one or the other of the two enantiotopic H atoms on the C atom adjacent to the reacting primary amino group is replaced by deuterium or tritium. Such chiral samples of putrescine, cadaverine and agmatine are readily available.[18,19] When ^{2}H serves as the marker, the ^{2}H content of the products of the enzyme catalyzed processes serves as a diagnostic indicator of the steric course of the reaction. Since starting materials with a ^{2}H enrichment approaching 100 at.% at the desired position can be used, the ^{2}H content of the products can be determined by ^{1}H or ^{2}H NMR spectroscopy.

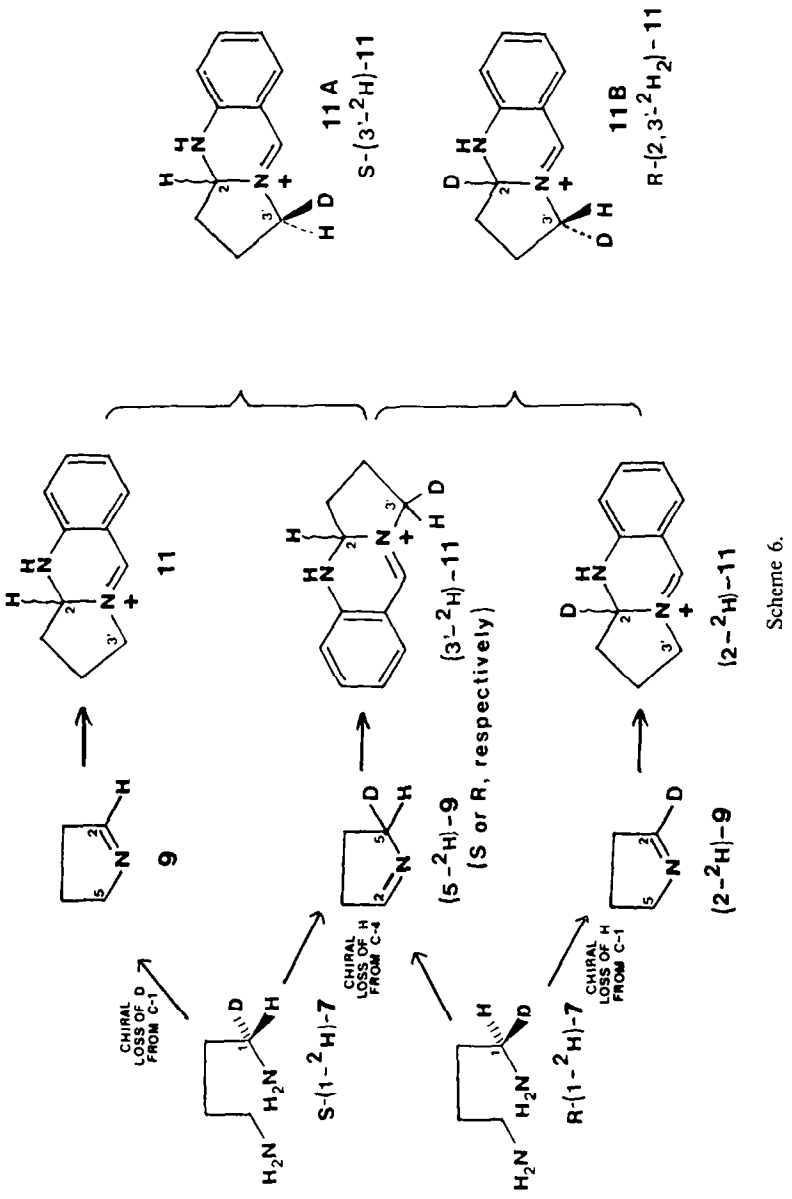
In the case of (1-^{2}H)agmatine, one enantiomer, by stereospecific loss of ^{2}H, yields a product which is devoid of label (Scheme 4), whereas the other enantiomer, by stereospecific loss of ^{1}H, yields a product which retains the label. Determination of presence or absence of ^{2}H in the product is sufficient to obtain a definitive answer to the question of the chirality of the process which leads to the products.

When chiral (1-^{2}H)putrescine or (1-^{2}H)cadaverine serves as substrate, however, the reaction will not lead to ^{2}H-free product with one enantiomer, and to deuteriated product with the other. Due to the C_{2v} symmetry of these amines (when unlabelled), both enantiomers yield products containing ^{2}H.

Oxidation of a sample of (1-^{2}H)cadaverine or (1-^{2}H)putrescine may occur either with removal of the amino group adjacent to the labelled methylene group or of that adjacent to the unlabelled methylene group. When oxidation takes place at the unlabelled end of the molecules, the products are (5-^{2}H)-5-aminopentanal and (4-^{2}H)-4-aminobutanal from either of the two enantiomers of (1-^{2}H)cadaverine and (1-^{2}H)putrescine, respectively, by loss of protium. When oxidation occurs at the labelled end of the substrates, one of the enantiomers will yield unlabelled 5-aminopentanal and 4-aminobutanal, respectively, by stereospecific loss of deuterium, whereas the other enantiomer will yield (1-^{2}H)-5-aminopentanal and (1-^{2}H)-4-aminobutanal, respectively, by stereospecific loss of protium. Concurrent oxidation at the labelled and the unlabelled ends will yield a mixture of products. One enantiomer of (1-^{2}H)substrate will yield a mixture of a monodeuteriated and an unlabelled species, the other enantiomer a mixture of two different monodeuteriated species (Schemes 5 and 6). Thus the presence or absence of ^{2}H within the product will not be a diagnostic criterion for the stereochemical course of the reaction. Quantitative determination of ^{2}H within the pro-


5 A
S-(3'-2H)-5
5 B
R-(2,3'-2H2)-5
5
2
S-(1-2H)-1
R-(1-2H)-1
(5-2H)-2
(S or R, respectively)
(3'-2H)-5
(1-2H)-2
(2-2H)-5
CHIRAL LOSS OF D FROM C-1
CHIRAL LOSS OF H FROM C-5
CHIRAL LOSS OF H FROM C-1

Scheme 5.


11A
S-(3'-2H)-11
11B
R-(2,3'-2H2)-11
11
9
(5-2H)-9
(S or R, respectively)
(3'-2H)-11
(2-2H)-9
(2-2H)-11
S-(1-2H)-7
R-(1-2H)-7
CHIRAL LOSS OF D FROM C-1
CHIRAL LOSS OF H FROM C-4
CHIRAL LOSS OF H FROM C-1

Scheme 6.

duct will be necessary in order to obtain the required information. A further problem arises if the reaction were accompanied by a substantial isotope effect. Measurement of the extent of retention of label within the product would, in that case, not yield information concerning the stereochemistry of H removal in the oxidation of (1-^{2}H)putrescine and (1-^{2}H)cadaverine. To solve the question of the stereochemical course of the reaction, determination of the distribution of ^{2}H within the products becomes mandatory.

Incubation of R-(−)-(1-^{2}H)agmatine sulfate with diamine oxidase (Expt 7) yielded a sample of the corresponding aldehyde, 4-guanidinobutanal (**13**) (isolated as the phosphate salt of the 2′,4′-dinitrophenylhydrazone (**15**)) which contained ^{2}H at the aldehyde *C* atom. The product derived from the enantiomeric S-(+)-(1-^{2}H)agmatine sulfate (Expt. 8) was free of ^{2}H (Table 4; Scheme 4). Thus, complete retention of ^{2}H in the product (**15B**) from R-(1-^{2}H)agmatine, and complete loss of ^{2}H in the course of formation of the product (**15A**) from S-(1-^{2}H)agmatine, provides clear evidence that in the course of the diamine oxidase catalyzed dehydrogenation of agmatine the *si*-proton is removed from the α-C atom and the *re*-proton is preserved in the product.

The ^{1}H NMR spectra of the two dinitrophenylhydrazones confirm the inference that ^{2}H is maintained in one of the products but not in the other (Fig. 3). The downfield region of the spectrum of the aldehyde dinitrophenylhydrazone from S-(+)-(1-^{2}H)agmatine sulfate (Fig. 3, spectrum A) shows a proton triplet, centred at δ 7.74 ppm ($J_{1,2}$ 5.1 Hz), due to the proton at the imino sp^2-carbon, C-1. In this spectrum, the central line of the triplet, δ 7.74 ppm, coincides with the downfield line of a doublet, δ 7.68 ppm ($J_{5',6'}$ 9.8 Hz), due to H-6′ of the aromatic nucleus. This triplet is missing entirely in the spectrum of the sample derived from R-(−)-(1-^{2}H)agmatine (Fig. 3, spectrum B). Thus, this sample is completely deuteriated at C-1.

In order to determine the stereochemistry of the reaction, with putrescine and cadaverine as substrates, it was of the utmost importance to ensure that the chiral samples of the substrate were deuteriated only at one of the terminal methylene groups and not at both. In the case of the S-enantiomers of the two (1-^{2}H)amines, this single deuteriation is a consequence of the mode of preparation, enzyme catalyzed decarboxylation in water, of L-(2-^{2}H)ornithine[19] and of L-(2-^{2}H)lysine,[18] respectively (Scheme 1). In the case of the R-enantiomers, on the

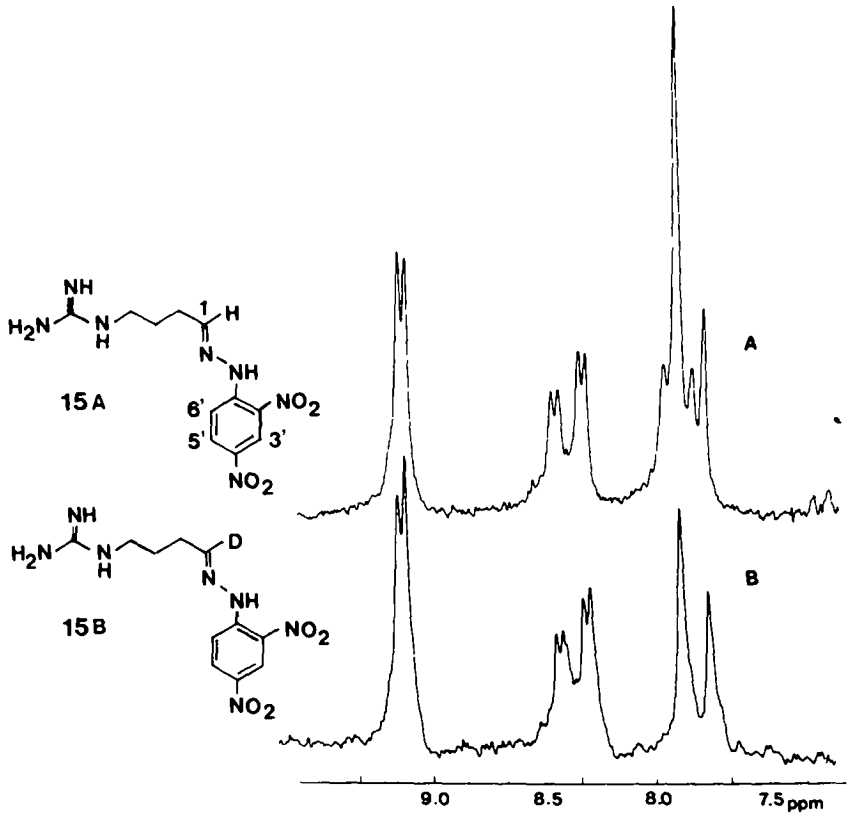
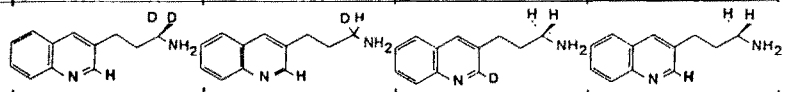
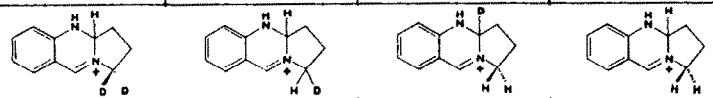


Fig. 3. Downfield region of the ^{1}H NMR spectra of samples of 4-guanidinobutanal dinitrophenylhydrazone (**15**), derived from deuterium labelled samples of agmatine (**12**) by enzymic oxidative deamination. A (**15A**), derived from S-(1-^{2}H)agmatine. B (**15B**), derived from R-(1-^{2}H)agmatine. The signals assigned to the protons at C-3′, C-5′ and C-6′ of the aromatic nucleus of the 2′,4′-dinitrophenylhydrazone appear at δ 9.03 (d, $J_{3',5'}$ 2.7 Hz, H-3′), 8.23 (d of d, $J_{3',5'}$ 2.7 Hz, $J_{5',6'}$ 9.8 Hz, H-5′) and 7.68 (d, $J_{5',6'}$ 9.8 Hz, H-6′) ppm, respectively. The triplet due to the presence of protium at C-1 (Spectrum A) is centred at δ 7.74 ($J_{1,2}$ 5.1 Hz) ppm.

Table 5. Analysis of the ^{1}H NMR spectra of the ^{2}H-labelled samples of 3-(3′-aminopropyl)quinoline and 2,3-trimethylene-1,2-dihydroquinazolinium picrate obtained in Expts 1–6.

			Relative area [a] of signals due to: H-2	Relative area [a] of signals due to: H-3′	Relative contribution to the product of each of the following species [f] (%)			
	3-(3′-aminopropyl)-quinoline							
					(3′,3′-$^2H_2$)-(5)	(3′-^{2}H)-(5)	(2-^{2}H)-(5)	(5)
(5B)	(R)	Expt. 1[b]	0.51 ± 0.04	1.51 ± 0.18[d]		49(± 18)	49(± 4)	2(± 18)
(5A)	(S)	Exp. 2[b]	1.00 ± 0.05	1.53 ± 0.18[d]		47(± 18)		53(± 18)
(5C)	($^2H_2$)	Expt. 3a[b]	0.49 ± 0.05	1.10 ± 0.17[d]	41(± 18)	8(± 20)	51(± 5)	- [g]
(5C)	($^2H_2$)	Expt. 3b[c]	0.50 ± 0.04	1.07 ± 0.20[d]	43(± 20)	7(± 22)	50(± 4)	- [g]
	2,3-trimethylene-1,2-dihydroquinazolinium picrate							
					(3′,3′-$^2H_2$)-(11)	(3′-^{2}H)-(11)	(2-^{2}H)-(11)	(11)
(11B)	(R)	Expt. 4a[e]	0.50 ± 0.03	1.50 ± 0.08		50(± 8)	50(± 3)	0(± 9)
(11A)	(S)	Expt. 5[e]	1.00 ± 0.03	1.20 ± 0.04		80(± 4)		20(± 4)
(11C)	($^2H_2$)	Expt. 6[e]	0.76 ± 0.04	0.50 ± 0.03	74(± 5)	2(± 9)	24(± 4)	- [h]

a Area of the signals assigned to the protons at C-2 (H-2) and C-3′ (H-3′) in the products obtained from each experiment are measured relative to the area of the signal assigned to the proton at C-4 (H-4′).

b The products were isolated as the 3-(3′-aminopropyl) quinoline dihydrochlorides. The signals assigned to H-2 and H-3′ appeared at 8.68 (s) and 2.95 (t, $J_{3',2'}$ 7.2 Hz) ppm, respectively. The signal due to H-4 occurred at 8.32 (s) ppm.

c The products were isolated as the 3-(3′-aminopropyl) quinoline dipicrates. The signals assigned to H-2 and H-3′ appeared at 9.30 (d, $J_{2,4}$ 1.2 Hz) and 3.16 (t, $J_{3',2'}$ 7.2 Hz) ppm, respectively. The signal due to H-4 occurred at 9.04 (s (br)) ppm.

d Since the signals assigned to the protons at C-3′ (H-3′) and C-1′ (H-1′) appeared as two overlapping triplets, only the combined relative area of these signals could be measured. The relative area of the signal due to H-3′ was determined by subtraction of the contribution due to H-1′ (taken as 2.0 hydrogen atoms).

e The signals assigned to H-2 and H-3′ appeared at 5.20 (t, $J_{2,1'}$, 6.0 Hz) and 4.12 (t, $J_{3',2'}$ 6.8 Hz) ppm, respectively. The signal due to H-4 occurred at 8.97 (s) ppm.

f Determined from the relative area of the ^{1}H NMR signals due to H-2 and H-3′.

g The contribution of the unlabelled species (5) was taken to be zero.

h The contribution of the unlabelled species (11), found to be < 1%, was taken to be zero.

other hand, the mode of preparation did not necessarily exclude the possibility that amines were formed which were chirally deuteriated at both α-methylene groups. The R-enantiomers of the amines were prepared by enzymic decarboxylation of unlabelled L-ornithine[19] and L-lysine,[18] respectively, in ^2H_2O solution. Even though this reaction leads, in the first instance, to R-(1-^{2}H)amines,[18,19] prolonged incubation might have led to significant further conversion to RR-(1,4-$^2H_2$)putrescine and RR-(1,5-$^2H_2$)cadaverine, respectively, if enzyme mediated stereospecific hydrogen/deuterium exchange had taken place. Such exchange has been reported to take place in the course of incubation of each of several biogenic amines with the decarboxylase that acts upon the corresponding amino acid (Table 6).

One of these reports[33] was subsequently shown[34] to be in error. It was crucial, in the present context, to gain independent evidence on possible exchange. Prolonged incubation, in ^2H_2O (99.8 at.% ^{2}H) solution, of cadaverine with L-lysine decarboxylase, of putrescine with L-ornithine decarboxylase, and also of agmatine with L-arginine decarboxylase (Experimental), followed by reisolation, yielded samples whose ^{1}H NMR and mass spectra were indistinguishable from those of the starting materials. Thus, exchange of hydrogen by deuterium was not detectable under the conditions used to prepare the R-(1-^{2}H)amines. Within the limits of detection, the chiral samples of (1-^{2}H)cadaverine and (1-^{2}H)putrescine were indeed monodeuteriated. The ^{1}H NMR spectra of these samples showed a triplet due to H-1,4 of putrescine and H-1,5 of cadaverine, respectively, at δ 3.06 ppm, whose integration corresponded to *ca* 3 protons, relative to the 4-proton multiplet centered at δ 1.77, due to H-2,3 of putrescine and the 6-proton multiplet at δ 1.3–1.9, due to H-2,3,4 of cadaverine, respectively. The mass spectra of the samples (cadaverine as the dibenzoyl derivative, putrescine by C.I. MS) showed monodeuteriation only.

Incubation of the enantiomeric samples of (1-^{2}H)cadaverine (**1**) with diamine oxidase yielded deuteriated samples of 5-aminopentanal (**2**) which were trapped with *o*-aminobenzaldehyde (**4**) to yield deuteriated 3-(3′-aminopropyl)quinoline (**5**) (Scheme 5). Similarly, the samples of (1-^{2}H)putrescine (**7**) gave deuteriated samples of 4-aminobutanal (**8**) which were trapped as deuteriated 2,3-trimethylene-1,2-dihydroquinazolinium ion (**11**) (Scheme 6). The location of deuterium in the samples of the quinoline and the quinazolinium derivatives was determined by ^{2}H NMR spectroscopy (Tables 2 and 3; Figs. 1 and 2).

The quinoline derivative of the sample of 5-aminopentanal derived from S-(1-^{2}H)cadaverine contained deuterium only at C-3′ of the sidechain (δ 2.9 ppm, Fig. 1, spectrum A), that derived from R-(1-^{2}H)cadaverine contained deuterium at C-3′ of the side chain as well as at C-2 of the quinoline ring (δ 2.9 and 8.8 ppm, Fig. 1, spectrum B). Thus, the 5-aminopentanal, derived from S-(1-^{2}H)cadaverine, was a mixture of (5-^{2}H)-5-aminopentanal and non-deuteriated 5-aminopentanal (**2**), whereas the product from R-(1-^{2}H)cadaverine was a mixture of (5-^{2}H)-5-aminopentanal and (1-^{2}H)-5-aminopentanal. Deuterium had been stereospecifically lost from S-(1-^{2}H)cadaverine, protium from R-(1-^{2}H)cadaverine (Scheme 5). The diamine oxidase catalyzed oxidation of cadaverine proceeds with loss of the *si*-proton from the methylene group adjacent to N.

Similarly, the quinazolinium derivative (**11**) of the sample of Δ^1-pyrroline (**9**), in equilibrium with 4-aminobutanal (**8**), derived from S-(1-^{2}H)putrescine contained deuterium only at C-3′ of the trimethylene chain (δ 4.1 ppm, Fig. 2, spectrum A), while that derived from R-(1-^{2}H)putrescine contained ^{2}H at C-3′, as well as at C-2 of the dihydroquinazoline ring system (δ 4.1 and 5.2 ppm, Fig. 2, spectrum B). Thus, the 4-aminobutanal derived from S-(1-^{2}H)putrescine was a mixture of (4-^{2}H)-4-aminobutanal and non-deuteriated 4-aminobutanal, while the product from R-(1-^{2}H)putrescine was a mixture of (4-^{2}H)-4-aminobutanal and (1-^{2}H)-4-aminobutanal. Deuterium had been stereospecifically lost from S-(1-^{2}H)putrescine, protium from R-(1-^{2}H)putrescine (Scheme 6). The dehydrogenation of putrescine thus

Table 6. Reported hydrogen/deuterium (or tritium) exchange at the α-methylene group of biogenic amines, catalyzed by the appropriate amino acid decarboxylase

Enzyme (source)	Substrate Amine	Reported Exchange	No exchange
Lysine decarboxylase[a] (*B. cadaveris*)	Cadaverine	33	-
Glutamate decarboxylase[a] (*E. coli*)	γ-Aminobutyrate	33	34
Histidine decarboxylase[b] (*Lactobacillus*)	Histamine	13	35
(*C. welchii*)		13	-
Tyrosine decarboxylase[a] (*S. faecalis*)	Tyramine	3	-
S-Adenosylmethionine[b] decarboxylase (*E. coli*)	S-Adenosyl-S-methyl-3-thiopropylamine	-	16

[a] cofactor: pyridoxal phosphate

[b] cofactor: pyruvate

proceeds with loss of the *si*-proton from the α-methylene group.

The stereospecificity of the reaction catalyzed by hog kidney diamine oxidase with cadaverine, putrescine and agmatine as substrates, removal of the *si*-proton in the course of the conversion of the primary amino group to an aldehyde group, is identical with the stereospecificity of the corresponding reactions, catalyzed by diamine oxidase from pea seedlings.[10-14]

The ^{2}H NMR spectra of the products obtained by the action of hog kidney diamine oxidase on the chiral samples of (1-^{2}H)cadaverine (Fig. 1, spectra A and B) and of (1-^{2}H)putrescine (Fig. 2, spectra A and B) show that the stereochemical course of the reaction is the same with both substrates. Even so, comparison of the ^{2}H NMR spectra of the corresponding products obtained from (1,1-$^2H_2$)cadaverine (Fig. 1, spectrum C) and from (1,1-$^2H_2$)putrescine (Fig. 2, spectrum C) reveals that the extent of reaction at the deuterium labelled site, relative to that at the unlabelled site, is significantly different with the two substrates.

The product (**5C**) from (1,1-$^2H_2$)cadaverine showed deuterium enrichment at C-3′ (δ 2.9 ppm) which was approximately twice that at C-2 (δ 8.8 ppm) (Expt. 3a, Table 2; Fig. 1, spectrum C), whereas the product (**11C**) from (1,1-$^2H_2$)putrescine, which bears ^{2}H at the corresponding positions, showed ^{2}H enrichment at C-3′ (δ 4.1 ppm) which was seven times greater than that at C-2 (δ 5.2 ppm) (Expt. 6, Table 3; Fig. 2, spectrum C).

Since enzymic oxidative deamination of the (1,1-$^2H_2$)diamines can take place at one of two sites within a given molecule, either at the labelled site (C-1), with release of deuterium, or at the unlabelled site (C-4 or C-5, respectively), with release of protium, two isotopically distinct species are formed. The relative contribution of each of these two species to the product, which is trapped *in situ* with *o*-aminobenzaldehyde, can be determined from the ^{2}H NMR spectrum of the quinoline or the quinazolinium derivative which is isolated.

The ^{2}H NMR spectrum of the sample of 3-(3′-aminopropyl)quinoline dihydrochloride (**5C**) derived from (1,1-$^2H_2$)cadaverine (Expt. 3a, Table 2; Fig. 1, spectrum C) indicates the presence of a mixture of the dideuteriated species, (3′,3′-$^2H_2$)-(**5**) (δ 2.9 ppm, relative area 2.2 (± 0.4)) and the monodeuteriated species, (2-^{2}H)-(**5**) (δ 8.8 ppm, relative area 1) in the ratio 1:1. It follows that the enzyme mediates release of hydrogen from C-5 and deuterium from C-1 of (1,1-$^2H_2$)cadaverine to an equal extent.

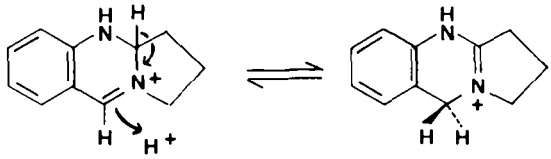
By contrast, the ^{2}H NMR spectrum of the sample of 2,3-trimethylene-1,2-dihydroquinazolinium picrate, (**11C**) derived from (1,1-$^2H_2$)putrescine (Expt. 6, Table 3; Fig. 2, spectrum C) indicates preferential formation of the dideuteriated species (3′,3′-$^2H_2$)-(**11**) (δ 4.1 ppm, relative area 7.0 (± 0.5)) over the monodeuteriated species (2-^{2}H)-(**11**) (δ 5.2 ppm, relative area 1), by a factor of *ca* 3.5.

Two obvious possibilities must be considered for the predominance of the dideuteriated product, (3′,3′-$^2H_2$)-(**11**), over the monodeuteriated product, (2-^{2}H)-(**11**) in the product mixture.

The first possibility is that, as in the case of cadaverine, enzymic oxidation leads to a 1:1 mixture of mono- and dideuteriated products, but that deuterium is subsequently lost from C-2 of the monodeuteriated product, (2-^{2}H)-(**11**) by solvent mediated exchange, e.g. via tautomerization (Scheme 7). This would lead to unlabelled (**11**) from the monodeuteriated species, (2-^{2}H)-(**11**), but would not affect deuterium enrichment in the dideuteriated species, (3′,3′-$^2H_2$)-(**11**). The net effect of such a process would thus be to change the ratio of dideuteriated to monodeuteriated (**11**) within the product from 1:1 to the observed high value.

The second possibility for the preponderance of the dideuteriated species in the reaction product is that the enzymic reaction is accompanied by a large primary intramolecular hydrogen/deuterium isotope effect, protium being released more readily (from C-5) than deuterium is released (from C-1) in the course of oxidation of (1,1-$^2H_2$)putrescine.

Solvent mediated exchange as the cause of the high ratio of dideuteriated to monodeuteriated species within the product may be excluded on the basis of the ^{1}H NMR spectrum of the reaction product (**11C**). The relative areas of the signals assigned to the protons at C-2 (δ 5.20 ppm) and C-3′ (δ 4.12 ppm) correspond to 0.76 and 0.50 H atoms, respectively. This indicates that the product consists of 24% (2-^{2}H)-(**11**) and 74% (3′,3′-$^2H_2$)-(**11**) (Table 5, Expt. 6). Since these two species thus account for approx. 98% of the product, and since non-deuteriated (**11**) contributes less than 1%, loss of ^{2}H by tautomerization of the dihydroquinazolinium ion cannot be significant under the conditions of the experiment.



11 Scheme 7. **16**

This inference is further supported by the results of Expts 4a and 4b. The sample of dihydroquinazolinium salt (**11B**), derived from R-(1-^{2}H)putrescine, consisted of approximately equal amounts of (3′-^{2}H)-(**11**) and (2-^{2}H)-(**11**), as determined from the ^{2}H NMR spectrum (Table 3, Expts. 4a and 4b). The ^{1}H NMR spectrum of (**11B**) (Table 5, Expt. 4a) reveals that 100% of the deuterium of the putrescine is retained in the components of the product. Thus, deuterium at C-2 of (2-^{2}H)-(**11**), derived from R-(1-^{2}H)putrescine, is maintained without loss in the course of the enzymic reaction. It can be concluded that the integrity of deuterium at C-2 is also maintained in the (2-^{2}H)-(**11**), which is derived, under similar conditions (Table 1, Expts. 6 and 4), from (1,1-$^2H_2$)putrescine.

Since exchange is excluded as the cause of the high ratio of dideuteriated to monodeuteriated species in the product of the diamine oxidase catalyzed oxidation of (1,1-$^2H_2$)putrescine, the conclusion is inescapable that it is the result of isotope discrimination between the two symmetry-equivalent, i.e. homotopic, but isotopically distinct sites of the substrate, in the rate limiting step of the oxidation of

(1,1-$^2H_2$)putrescine. The mole ratio of the two components of the product, (3′,3′-$^2H_2$)-(**11**)/(2-^2H)-(**11**), derived from (1,1-$^2H_2$)putrescine (91% $^2H_2$)† indicates an intramolecular isotope effect, $k_H/k_D \sim 4$ (Table 7, Expt. 6). An intramolecular isotope effect of *ca* 4 in the oxidation of (1,1-$^2H_2$)putrescine catalyzed by hog kidney diamine oxidase was recently reported.[36] The contribution of mono- and dideuteriated species to the product was determined by mass spectrometry. By contrast, oxidation of (1,1-$^2H_2$)cadaverine (*ca* 93% $^2H_2$) leads to a product containing (3′,3′-$^2H_2$)-(**5**) and (2-^2H)-(**5**) in the ratio‡ 1:1, indicating a negligible intramolecular isotope effect (Table 7, Expt. 3a).

Correspondingly, an intramolecular isotope effect is observed in the oxidation of s-(1-^2H)putrescine, but none in the oxidation of s-(1-^2H)cadaverine.

The ^1H NMR spectrum of the product (**11A**) of the oxidation of s-(1-^2H)putrescine indicates the presence of two components, (3′-^2H)-(**11**) and unlabelled (**11**), in the approximate ratio 4:1 (Table 5, Expt. 5). Thus, the *si*-proton at C-4 of s-(1-^2H)putrescine is abstracted in preference to the *si*-deuteron at C-1. On the other hand, the composition of the product from s-(1-^2H)cadaverine (Table 5, Expt. 2) indicates the absence of an isotope effect (Table 7, Expt. 2).

Since the magnitude of hydrogen/deuterium isotope effects is sensitive to variation of experimental conditions,[37,38] the reaction with (1,1-$^2H_2$)cadaverine was repeated (Table 1, Expt. 3b) employing conditions similar to those used in the oxidation of (1,1-$^2H_2$)putrescine (Table 1, Expt. 6). The product, 5-aminopentanal (**2**), was trapped with *o*-aminobenzaldehyde, and the quinoline derivative (**5**), as well as the dihydroquinazolinium ion (**6**) were isolated. In agreement with the earlier results these products again contained mono- and dideuteriated species in the ratio 1:1 (Table 7, Expt. 3b), confirming the absence of an intramolecular isotope effect.

Thus, an intramolecular isotope effect is not observed with cadaverine as the substrate of hog kidney diamine oxidase. Similarly, oxidation of cadaverine, catalyzed by pea seedling diamine oxidase also takes place without significant primary isotope effect, as shown by the result of the oxidative deamination of s-[1-^3H]cadaverine in the presence of [1-^{14}C]cadaverine.[12] The resulting Δ^1-piperideine, which was trapped as pelletierine, retained 55 (± ?)§ % ^3H, relative to ^{14}C, indicating little, if any, preference for removal of the *si*-hydrogen from C-5 over that of the *si*-tritium from C-1 of cadaverine.

The oxidation of s-[1-^3H]cadaverine, together with [1-^{14}C]cadaverine, catalyzed by pea seedling diamine oxidase, was recently reinvestigated.[15] The reaction was carried out in the presence of a second enzyme, yeast alcohol dehydrogenase, together with ethanol as a hydride donor. Under these conditions the oxidation product of cadaverine, 5-aminopentanal, is reduced *in situ* to yield 5-aminopentan-1-ol. Further diamine oxidase mediated reaction of this compound yields pentan-1,5-diol (via 5-hydroxypentanal). 5-Aminopentan-1-ol as well as pentan-1,5-diol were isolated. When s-[1-^3H]cadaverine served as the substrate, the pentan-1,5-diol was essentially free of tritium, relative to ^{14}C. The 5-Aminopentan-1-ol, on the other hand, retained almost all the tritium (95 ± 5%) relative to ^{14}C. This was interpreted[15] to indicate a large isotope effect in the oxidation of the s-[1-^3H]cadaverine. In view of the result reported by Gerdes and Leistner[12] it is much more likely that the retention of tritium, relative to ^{14}C, in the sample of s-5-amino[5-^3H,1,5-$^{14}C_2$]pentan-1-ol was the result of a substantial intermolecular isotope effect in its further oxidation to [1-^{14}C]pentan-1,5-diol, rather than the consequence of an intramolecular isotope effect in its generation from s-[1-^3H, 1-^{14}C]cadaverine.

Intramolecular isotope effects in enzyme catalyzed H abstraction in substrates with C_{2v} symmetry have been investigated in a number of instances.[39–41] and values for k_H/k_D as high as 11 have been observed. A kinetic model for the interpretation of intramolecular isotope effects between two *N*-methyl groups in substrates with C_s symmetry has recently been advanced.[42] This model is adaptable to the C_{2v} case. If H abstraction were irreversible, the intramolecular isotope effect is a function of the relative rate of this H abstraction compared to the rate of the exchange, at the active site of the enzyme, of the two symmetry equivalent but isotopically distinct regions of the substrate. Thus, if the labelled and unlabelled homotopic regions of the substrate rapidly equilibrate at the active site, a large intramolecular isotope effect results, as was observed in the diamine oxidase catalyzed oxidation of s-(1-^2H)putrescine. If equilibration of labelled and unlabelled regions is slow, relative to C–H (or C–^2H) bond cleavage, i.e. when there is little rotational mobility once substrate is bound at the active site, the intramolecular isotope effect is small. This is the case when cadaverine serves as the substrate of diamine oxidase. Since intramolecular isotope effects are regarded as insufficient evidence for a rate-limiting C–H bond cleaving step in an enzyme reaction,[42] mechanistic inferences can-

†The sample of (1,1-$^2H_2$)putrescine was found to contain 91.0 ± 1.0 at.% $^2H_2$ and 9.0 ± 1.0 at.% ^2H, by mass spectrometry. Enzymic decarboxylation of the L-component of DL-(2-^2H)ornithine (*ca* 92 at.% ^2H at C-2, as determined by ^1H NMR) in ^2H_2O thus led to incorporation of *ca* 98.9 at.% ^2H from the medium into the 1-*re*-position[19] of putrescine. It follows that this sample consisted of 91% (1,1-$^2H_2$)-, 7.9% R-(1-^2H)-, 1.0% s-(1-^2H)-, and 0.1% non-deuteriated putrescine. The contribution to the product of the two species derived solely from the (1,1-$^2H_2$)-species is 73.3 ± 6.8% (3′,3′-$^2H_2$)-(**11**) and 17.7 ± 1.0% (2-^2H)-(**11**), as determined from the relative areas of the ^2H NMR signals corresponding to ^2H enrichment at C-3′ and C-2 (Expt. 6, Table 3); and 72.4 ± 7.2% (3′,3′-$^2H_2$)-(**11**) and 18.6 ± 0.5% (2-^2H)-(**11**) as determined from the relative areas of the ^1H NMR signals assigned to the protons at C-3′ and C-2.

‡Corrected for 5% R-(1-^2H)- and 2% s-(1-^2H)cadaverine, present in the sample of (1,1-$^2H_2$)cadaverine. The relative amounts of the chiral monodeuteriated species were calculated on the basis that enzymic decarboxylation of the nondeuteriated fraction of the L-component of DL-(2-^2H)lysine (*ca* 95 at.% ^2H at C-2 as determined by ^1H NMR) in ^2H_2O (Experimental)yields R-(1-^2H)cadaverine[18] which is assumed to contain 98 at.% ^2H at the 1-*re* position. This assumption is based on the finding that the corresponding products, obtained by enzymic decarboxylation of ornithine and arginine in ^2H_2O, contained >98 at.% ^2H, as determined by MS.[19]

§The confidence limits of the counting data were not given; the result[12] was taken to indicate a loss of 50% of the tritium, relative to ^{14}C, i.e. a reaction not accompanied by an isotope effect.

Table 7. Intramolecular isotope effect in the oxidation of (1-^{2}H)- and (1,1-$^2H_2$)cadaverine and putrescine, catalyzed by diamine oxidase

Expt. No.	Substrate	^{2}H content (Atom %)	Intramolecular Isotope effect
1	R-(1-^{2}H)cadaverine	ca. 95	1.0 ± 0.2 [a] 1.0 ± 0.4 [b,c]
2	S-(1-^{2}H)cadaverine	ca. 95	1.0 ± 0.5 [b,c]
3a	(1,1-$^2H_2$)cadaverine	ca. 93	1.1 ± 0.2 [a,d] 0.9 ± 0.2 [b,d]
3b	(1,1-$^2H_2$)cadaverine	ca. 93	1.0 ± 0.2 [b,d]
4a	R-(1-^{2}H)putrescine	99.7	1.2 ± 0.1 [a] 1.0 ± 0.2 [b,c]
4b	R-(1-^{2}H)putrescine	98.7	1.0 ± 0.3 [a]
5	S-(1-^{2}H)putrescine	97.2	4.7 ± 1.1 [b,c]
6	(1,1-$^2H_2$)putrescine	91	4.2 ± 0.3 [a,e] 3.9 ± 0.3 [a,e]

[a] determined from the relative areas of the ^{2}H NMR signals corresponding to deuterium enrichment at C-2 and C-3' (from Tables 2 and 3).

[b] determined from the relative area of the ^{1}H NMR signals corresponding to H-2 and H-3' (from Table 5).

[c] corrected for the presence of unlabelled species in the (1-^{2}H)-substrate.

[d] corrected for 5% R-(1-^{2}H)- and 2% S-(1-^{2}H)-species present in the sample of (1,1-$^2H_2$)cadaverine.

[e] corrected for 7.9% R-(1-^{2}H)-, 1.0% S-(1-^{2}H)- and 0.1% unlabelled species present in the sample of (1,1-$^2H_2$)putrescine.

not be based on the observed differences in the behaviour of putrescine and cadaverine as substrates of diamine oxidase.

It is noteworthy in this context that the values of intermolecular isotope effects in reactions catalyzed by hog kidney diamine oxidase are smaller than those of the intramolecular isotope effects. Thus, in the oxidation of putrescine, k_H/k_D (intermolecular)[36] was found to be *ca* 1.3 (compared to k_H/k_D (intramolecular) = 4). With *p*-dimethylaminomethylbenzylamine and the corresponding (α,α-$^2H_2$)-derivative as substrates,[43] the hydrogen/deuterium isotope effect on the kinetic parameters V_{max}/K_M varied from 2.1 at pH 7.0 and 40° to 5.2 at pH 6 and 20°. The isotope effect on V_{max} was less sensitive to changes in pH and temperature, varying from 1.6 to 2.8. Values for k_H/k_D ranged from 4.8 to 1.8 for pH values from pH 6 to pH 8, at 20°. Similar values for intermolecular isotope effects have been observed in reactions catalyzed by rat liver monoamine oxidase.[1,2,44] ($k_H/k_D = 2.3$), by rabbit liver[45] ($k_H/k_D \sim 2$) and by hog plasma monoamine oxidase[46] ($(V_{max}/K_M)_H/(V_{max}/K_M)_D \sim 3$).

EXPERIMENTAL

(2-^{2}H)*Amino acids*

DL-(2-^{2}H)*Ornithine and* L-(2-^{2}H)*Arginine.*

These compounds were prepared as described in an earlier publication.[19]

†Abs EtOH purified by the Lund-Bjerrum method[47] was used throughout.

DL-(2-^{2}H)*Lysine.*

Diethyl 2-acetamido-2-(4-phthalimidobutyl)malonate. Diethyl acetamidomalonate (Aldrich Chemical Co.) (2.17 g) was dissolved in hot dry EtOH† (10 ml) in a 2-necked round bottom flask, fitted with a reflux condenser, $CaSO_4$ drying tube, and a pressure equalizing dropping funnel. A soln of NaOEt (250 mg Na in 14 ml EtOH) was added and the resulting soln was heated at reflux while N-(4-bromobutyl)phthalimide (Aldrich Chemical Co.) (2.83 g) in hot EtOH (14 ml) was added dropwise over 10 min. The mixture was refluxed 20 hr, and was then cooled to 0°. Water (35 ml) was added to precipitate the product (2.77 g) which was used without further purification in the preparation of (2-^{2}H)lysine (see below). Concentration of the filtrate *in vacuo* to one half the original volume yielded additional product (1 g), yield 90% from diethyl acetamidomalonate; m.p. 110–111° (from 95% EtOH); ^{1}H NMR (C^2HCl_3), δ 1.0–1.3 (2H, unresolved), 1.23 (6H, t, J 7.2 Hz), 1.67 (2H, quin., J 7.2 Hz), 2.04 (3H, s), 2.34 (2H, m), 3.60 (2H, t, J 7.5 Hz), 4.27 (4H, q, J 7.2 Hz), 6.74 (1H, s), 7.80 (4H, m); MS, *m/e* 418 (M, 2%), 345 (100), 303 (89), 299 (80), 229 (97), 160 (42), 148 (23), 130 (44). (Found: C, 59.65; H, 6.25; N, 6.41. Anal. Calc for $C_{21}H_{26}N_2O_7 \cdot \frac{1}{2}C_2H_5OH$: C, 59.85; H, 6.62; N, 6.35%.)

DL-(2-^{2}H)*Lysine monohydrochloride* (*cf Ref.* 48). Diethyl 2-acetamido-2-(4-phthalimidobutyl)malonate (2.77 g) was suspended in a solution of ^{2}HCl (38% w/w, in ^2H_2O, 25 ml) (Merck, Sharp, and Dohme, 99.7 at.% ^{2}H) in a small flask fitted with a reflux condenser and $CaSO_4$ drying tube. The mixture was heated at reflux for 18 hr, cooled to 0°, and diluted with water (10 ml). Phthalic acid, which precipitated, was filtered off and washed with water (2 × 1 ml). The combined filtrate and washings were concentrated to dryness *in vacuo*, and the residue was repeatedly dissolved in water and evaporated to dryness to remove exchangeable ^{2}H. The residue, crude lysine dihydrochloride, was dissolved in hot 95% EtOH (8 ml), the soln was cooled

to room temp, filtered and pyridine (*ca* 600 mg) was added. DL-(2-^{2}H)Lysine monohydrochloride which precipitated was recrystallized from aqueous EtOH, yield 1.05 g; m.p. 259–260° (dec.) (lit. (nondeuteriated) m.p. 259–262°,[48] 260–261°,[49] 263–264°[50]); ^{1}H NMR (^2H_2O), δ 1.2–2.1 (6H, m, H-3,4,5), 3.05 (2H, t, J 7.2 Hz, H-6), 3.77 (< 0.05H, H-2); the ^{2}H NMR spectrum (H_2O 22 mM, 8600 transients) showed one signal at 3.5 ppm relative to that due to natural abundance ^{2}H in water (4.5 ppm).

Decarboxylation of (2-^{2}H)*amino acids to* (1-^{2}H)*amines*

Lysine

Decarboxylation of lysine, catalyzed by L-*lysine decarboxylase* (*E.C.* 4.1.1.18) *from* B. cadaveris. The enzyme reaction was carried out either in ^1H_2O or ^2H_2O solns, buffered with potassium phosphate. The phosphate–^2H_2O buffer solution was prepared in the following way. A mixture of dipotassium hydrogen phosphate (120 mg, Fisher) and potassium dihydrogen phosphate (600 mg, Analar) was repeatedly dissolved in ^2H_2O and evaporated to dryness (3 × 5 ml) to effect removal of exchangeable protons. The residue was then dissolved in 25 ml ^2H_2O (99.8 at.% ^{2}H, Stohler Isotopes) yielding a buffer soln, *ca* 0.2 M, p^{2}H 6.

(i) R-(−)-(1-^{2}H)*Cadaverine dihydrochloride by decarboxylation of the* L-*component of unlabelled* DL-*lysine in deuterium oxide solution* (*Expt* 1). DL-Lysine monohydrochloride (Sigma) (152 mg) was twice dissolved in ^2H_2O (3 ml) and the soln evaporated to dryness. After being dried *in vacuo* overnight, the residue was dissolved in phosphate–^2H_2O buffer soln (0.2 M, p^{2}H 6, 24 ml) (see above) and transferred into a flask containing L-lysine decarboxylase (Sigma, "Type VI", 2.6 U/mg†) (24 mg). The flask, fitted with a $CaSO_4$ drying tube, was warmed at 36° in a shaking constant temperature bath. After 48 hr, this enzymic mixture was acidified with 4M hydrochloric acid, heated on a steam bath for 30 min and cooled to room temp. The precipitated enzyme was removed by centrifugation, followed by filtration of the supernatant solns through Celite. The filtrate was then lyophilyzed, the residue dissolved in 10% NaOHaq (3 ml), and the alkaline soln saturated with NaCl. (^{2}H)Cadaverine was extracted into 1-BuOH (4 × 5 ml). The extract was acidified by addition of a soln of hydrogen chloride in BuOH and the soln was then concentrated *in vacuo* (5 ml). R-(1-^{2}H)Cadaverine dihydrochloride which precipitated was crystallized from 95% EtOH, yield 61 mg; ^{1}H NMR (^2H_2O) δ 1.3–1.9 (6H, m, H-2,3,4), 3.06 (*ca* 3H, t, J 7.2 Hz, H-1,5); ORD, $[\alpha]_{365} - 0.33 \pm 0.02°$, $[\alpha]_{407} - 0.23 \pm 0.03°$, $[\alpha]_{546} - 0.14 \pm 0.01°$, $[\alpha]_{579} - 0.10 \pm 0.02°$ (*c*, 4.92% in 0.1 N HCl, 26°, uncorrected for incomplete deuteriation).

A sample of the dihydrochloride was converted into N,N′-dibenzoyl-R-(1-^{2}H)cadaverine: m.p. 132–133° (from aqueous EtOH) (lit. (nondeuteriated) m.p. 129–131°,[51] 135°[52]); MS *m/e* 312 (M + 1, 1.7%), 311 (M, 7.7), 310 (M-1, 1.0), 206 (9.0), 189 (19.4), 188 (3.4), 105 (100), 77 (75).

An authentic sample of unlabelled N,N′-dibenzoylcadaverine, m.p. 132–133°, showed MS *m/e* 311 (M + 1, 0.3%), 310 (M, 1.6), 205 (2.5), 189 (3.6), 188 (10.2), 105 (100), 77 (75).

(ii) S-(+)-(1-^{2}H)*Cadaverine dihydrochloride by decarboxylation of the* L-*component of* DL-(2-^{2}H)*lysine* (*Expt* 2). DL-(2-^{2}H)Lysine monohydrochloride (> 95% 2-^{2}H, 123 mg) and L-lysine decarboxylase (2.6 U/mg†) (16 mg) were dissolved in phosphate buffer (0.2 M, pH 6, 20 ml). After incubation at 36° for 40 hr, the enzymic reaction mixture was worked up as described above to give S-(1-^{2}H)cadaverine dihydrochloride (47 mg); ORD, $[\alpha]_{407} + 0.25 \pm 0.03°$, $[\alpha]_{546} + 0.15 \pm 0.03°$, $[\alpha]_{579} + 0.10 \pm 0.03°$, (*c*, 3.17% in 0.1 N HCl, 26°, uncorrected for incomplete deuteriation). The proton NMR spectrum (^2H_2O) was similar to that of R-(1-^{2}H)cadaverine dihydrochloride. N,N′-Dibenzoyl-S-(1-^{2}H)-cadaverine: m.p. 132–133°; MS, *m/e* 312 (M + 1, 1.6%), 311 (M, 6.8), 310 (M-1, 3.6), 206 (7.9), 189 (20.9), 188 (11.9), 105 (100), 77 (75).

(iii) (1,1-$^2H_2$)*Cadaverine dihydrochloride by decarboxylation of the* L-*component of* DL-(2-^{2}H)*lysine in deuterium oxide solution* (*Expt* 3). Decarboxylation of the L-component of DL-(2-^{2}H)lysine monohydrochloride (> 95% 2-^{2}H; 110 mg), catalyzed by L-lysine decarboxylase (18 mg) in phosphate–^2H_2O buffer solution (0.2 M, p^{2}H 6, 20 ml) was carried out as described under (i), yielding (1,1-$^2H_2$)cadaverine dihydrochloride (50 mg). ^{1}H NMR (^2H_2O), δ 1.66 (6H, m, H-2,3,4), 3.05 (*ca* 2.1 H, t, J 7.2 Hz, H-1,5). N,N′-Dibenzoyl-(1,1-$^2H_2$)cadaverine: m.p. 133–134°; MS, *m/e* 313 (M + 1, 0.9%), 312 (M, 4.6), 311 (M-1, 2.3), 310 (M-2, 0.5) 207 (2.3), 206 (0.9), 190 (8.9), 189 (6.9), 105 (100), 77 (75).

Incubation of cadaverine with L-*lysine decarboxylase in deuterium oxide solution.* Cadaverine dihydrochloride was prepared by passing a stream of dry hydrogen chloride through a soln of cadaverine free base (Aldrich) in ethanol at room temp. The dihydrochloride precipitated on cooling; m.p. 255–257° (lit.[52] m.p. 255°).

A soln of cadaverine dihydrochloride (23 mg) and L-lysine decarboxylase (Sigma, "Type II") (9 mg) in phosphate-^2H_2O buffer soln (0.2 M p^{2}H 6, 10 ml) was incubated at 36° under anhyd conditions (i.e. exclusion of ^1H_2O). After 28 hr the soln was acidified with conc hydrochloric acid, heated on a steam bath for 1 hr, and the precipitated enzyme removed by centrifugation. The supernatant soln was lyophylized and exchangeable ^{2}H was removed by repeated soln of the residue in water followed by evaporation to dryness. The residue was then dissolved in water (2 ml). Part of the aqueous soln (1 ml) containing cadaverine was converted into the dibenzoyl derivative, yield 23 mg; m.p. 129–131° (from aqueous EtOH); ^{1}H NMR (C^2HCl_3), δ 1.3–1.8 (6H, m, H-2,3,4), 3.48 (4H, q, J ~ 6 Hz, H-1,5), 6.44 (2H, s (br), 2NH), 7.3–7.5 (6H, m, ArH), 7.7–7.9 (4H, m, ArH); MS, *m/e* 311 (M + 1, 0.3%), 310 (M, 1.6), 309 (M-1, 0.1), 205 (2.5), 188 (10.2), 105 (100), 77 (75). The ^{1}H NMR spectrum and the mass spectrum were identical with those of unlabelled material.

Ornithine

Decarboxylation of ornithine, catalyzed by L-*ornithine decarboxylase* (*E.C.* 4.1.1.17) *from* E. coli.

(i) R-(−)-(1-^{2}H)*Putrescine dihydrochloride by decarboxylation of the* L-*component of unlabelled* DL-*ornithine in deuterium oxide solution* (*Expt* 4). Anhyd Na_2CO_3 (Analar) (111 mg) was added to a stirred soln of perdeuterioacetic acid (Merck, Sharp, and Dohme, 99.7 at.% ^{2}H) in ^2H_2O (Stohler Isotopes, 99.8 at.% ^{2}H) (0.2 M, 15 ml) under dry N_2. After stirring for 1 hr, DL-ornithine monohydrochloride (Aldrich) (98 mg), which had been stripped of exchangeable protons by repeated soln in ^2H_2O and evaporation to dryness (3 times), and L-ornithine decarboxylase (Sigma, 0.06 U/mg‡) (45 mg) were dissolved in the buffered soln (*ca* p^{2}H 5). The enzymic mixture, in a flask fitted with a $CaSO_4$ drying tube, was agitated at 36° for 40 hr, and worked up as described for the decarboxylation of lysine, to give R-(−)-(1-^{2}H)putrescine dihydrochloride (46 mg) which was recrystallized from 95% EtOH; ^{1}H NMR (^2H_2O), δ 1.77 (4H, m, H-2,3), 3.06 (*ca* 3H, m, H-1,4); C.I. MS 90 (M + H, 5%), 74 (5), 73 ($M-NH_3$, 100), 72 (2), (C.I. MS of authentic unlabelled material, 89 (M + H, 5%), 73 (5), 72 ($M-NH_3$, 100), 71 (2)); 99.7 ± 0.3% $^2H_1$ determined by C.I. MS. The sample showed a plane negative ORD curve. Another sample of (−)-(1-^{2}H)putrescine dihydrochloride obtained as above from L-ornithine monohydrochloride (rather than from DL-) was 98.7 ± 1.2% mono-^{2}H labelled at C-1, determined by C.I. MS, and gave ORD, $[\alpha]_{302} - 1.12 \pm 0.11°$, $[\alpha]_{313} - 1.03 \pm 0.08°$, $[\alpha]_{334} - 0.78 \pm 0.09°$, $[\alpha]_{365} - 0.66 \pm 0.05°$, $[\alpha]_{405} - 0.50 \pm 0.04°$, $[\alpha]_{436} - 0.43 \pm 0.04°$, $[\alpha]_{546} - 0.24 \pm 0.05°$, $[\alpha]_{577} - 0.20 \pm 0.06°$ (*c*, 2.26% in 0.1 N HCl, 26°, uncorrected for incomplete deuteriation).

†1 U will release 1.0 μmole carbon dioxide from L-lysine per min at pH 6.0 at 37°.

‡1 U will release 1.0 μmole carbon dioxide from L-ornithine per min at pH 5.0 at 37°.

(ii) S-(+)-(1-^{2}H)*Putrescine dihydrochloride by decarboxylation of the* L-*component of* DL-(2-^{2}H)*ornithine* (*Expt* 5. A soln of DL-(2-^{2}H)ornithine monohydrochloride (*ca* 98% ^{2}H) (100 mg) and L-ornithine decarboxylase (40 mg) in acetate buffer (0.2 M, pH 5.0, 15 ml) was incubated at 36° for 20 hr. After workup, S-(+)-(1-^{2}H)putrescine dihydrochloride was obtained and recrystallized from 95% EtOH (yield 45 mg). The ^{1}H NMR spectrum (^2H_2O) was identical with that of R-(−)-(1-^{2}H)putrescine. ORD, $[\alpha]_{313} - 0.94 \pm 0.08°$, $[\alpha]_{334} + 0.81 \pm 0.06°$, $[\alpha]_{365} + 0.68 \pm 0.04°$, $[\alpha]_{405} + 0.57 \pm 0.04°$, $[\alpha]_{436} + 0.39 \pm 0.04°$, $[\alpha]_{546} + 0.28 \pm 0.04°$, $[\alpha]_{577} + 0.20 \pm 0.04°$ (*c*, 3.84% in 0.1 N HCl, 26°, uncorrected for incomplete deuteriation). C.I. MS 90 (M + H, 3%), 74 (5), 73 (M−NH_3, 100), 72 (5); 97.2 ± 0.3% $^2H_1$ determined by C.I. MS.

(iii) (1,1-$^2H_2$)*Putrescine dihydrochloride by decarboxylation of the* L-*component of* DL-(2-^{2}H)*ornithine in deuterium oxide solution* (*Expt* 6). Decarboxylation of the L-component of DL-(2-^{2}H)ornithine monohydrochloride (*ca* 92% ^{2}H) (117 mg), catalyzed by L-ornithine decarboxylase (40 mg) in perdeuterioacetate-^2H_2O buffer soln (0.2 M, *ca* p^{2}H 5, 20 ml) to give (1,1-$^2H_2$)putrescine dihydrochloride (52 mg) was carried out as described under (i): ^{1}H NMR (^2H_2O), δ 1.78 (4H, m, H-2,3), 3.08 (*ca* 2H, m, H-1,4); C.I. MS 91 (M + H, 5%), 75 (5), 74 (M−NH_3, 100), 73 (10), 72 (5); *ca* 91 ± 1% $^2H_2$, 9 ± 1% $^2H_1$ determined by C.I. MS.

Incubation of putrescine with ornithine decarboxylase in deuterium oxide solution. A soln of putrescine dihydrochloride (25 mg) and L-ornithine decarboxylase (9 mg) in perdeuterioacetate-^2H_2O buffer (0.2 M, *ca* p^{2}H 5, 3 ml) was incubated at 36° under N_2. After 42 hr a ^{1}H NMR spectrum of the mixture indicated no apparent change in the signals due to putrescine: δ 1.77 (4H, m), 3.05 (*ca* 4H, t (br)).

Arginine

Decarboxylation of arginine catalyzed by L-*arginine decarboxylase* (*E.C.* 4.1.1.19) *from* E. coli.

(i) R-(−)-(1-^{2}H)*Agmatine sulfate by decarboxylation of* L-*arginine in deuterium oxide solution* (*Expt* 7). L-Arginine monohydrochloride (Eastman) (90 mg) was twice dissolved in ^2H_2O and evaporated to dryness to effect replacement of exchangeable protons with ^{2}H. The amino acid was then dissolved in perdeuterioacetate-^2H_2O buffer (*ca* p^{2}H 5.2, 20 ml) to yield a 0.02 M soln. The buffer had been prepared from perdeuterioacetic acid (0.2 M in ^2H_2O) and anhyd Na_2CO_3 (8.36 mg/ml) (see under Decarboxylation of ornithine). L-Arginine decarboxylase (Sigma; 2.9 U/mg)† (11 mg) was added and the resulting soln incubated at 36° in a flask equipped with a $CaSO_4$ drying tube. After 30–40 hr the incubation mixture was acidified (*ca* pH 2) with conc hydrochloric acid, treated with Norite, heated on a steam bath for 30 min, and filtered through Celite. The filtrate was evaporated, the residue dried *in vacuo* over NaOH and then dissolved in 5% NaOHaq (1–2 ml). The alkaline soln was saturated with NaCl and extracted with 1-BuOH (4 × 3 ml). Evaporation of the BuOH gave an oil which was redissolved in H_2SO_4aq (0.1 M, *ca* 1 ml). After addition of MeOH and cooling at 0° overnight the crystalline sulfate of R-(−)-(1-^{2}H)agmatine separated and was recrystallized from water: methanol, yield 72 mg; m.p. 238–240° (lit.[53] (nondeuteriated) m.p. 236–239°); ^{1}H NMR (^2H_2O), δ 1.66 (4H, m, H-2,3), 2.95 (*ca* 1H, t (br), $J \sim 6.8$ Hz, H-1), 3.18 (2H, m, H-4); ORD, $[\alpha]_{302} - 1.37 \pm 0.08°$, $[\alpha]_{313} - 1.17 \pm 0.07°$, $[\alpha]_{334} - 1.01 \pm 0.06°$, $[\alpha]_{365} - 0.76 \pm 0.04°$, $[\alpha]_{405} - 0.57 \pm 0.03°$, $[\alpha]_{436} - 0.50 \pm 0.03°$, $[\alpha]_{546} - 0.19 \pm 0.02°$, $[\alpha]_{579} - 0.16 \pm 0.03°$ (*c*,6.52% in water, 25°, uncorrected for incomplete deuteriation); ^{2}H NMR (H_2O, 180 mM, 100 transients) showed one signal at 2.9 ppm apart from that due to natural abundance ^{2}H in water (4.5 ppm).

†1 U will release 1.0 μmole carbon dioxide from L-arginine per min at pH 5.2 at 37°.

(ii) S-(+)-(1-^{2}H)*Agmatine sulfate by decarboxylation of* L-(2-^{2}H)*arginine*. (*Expt* 8). A soln of L-(2-^{2}H)arginine monohydrochloride (*ca* 85% ^{2}H) (0.02 M) and L-arginine decarboxylase (2.9 U/mg)† (19 mg) in acetate buffer (0.2 M, pH 5.2, 35 ml) was incubated at 36° for 40 hr. The mixture was worked up, as described under (i), to give S-(+)-(1-^{2}H)agmatine sulfate (100 mg). The m.p. and NMR spectra (^{1}H and ^{2}H) were similar to those obtained for R-(−)-(1-^{2}H)agmatine sulfate. The sample showed a plane positive ORD curve; $[\alpha]_{297} + 1.42 \pm 0.11°$, $[\alpha]_{302} + 1.21 \pm 0.08°$, $[\alpha]_{313} + 1.07 \pm 0.07°$, $[\alpha]_{334} + 0.84 \pm 0.06°$, $[\alpha]_{365} + 0.70 \pm 0.04°$, $[\alpha]_{405} + 0.53 \pm 0.03°$, $[\alpha]_{436} + 0.42 \pm 0.03°$, $[\alpha]_{546} + 0.27 \pm 0.03°$, $[\alpha]_{577} + 0.23 \pm 0.04°$ (*c*, 4.18% in water, 25°, uncorrected for incomplete deuteriation).

Incubation of agmatine with L-*arginine decarboxylase in deuterium oxide solution.* A soln containing agmatine sulfate (Aldrich) (47 mg) and L-arginine decarboxylase (Sigma, 2.9 U/mg)† (8 mg) in perdeuterioacetate-^2H_2O buffer (0.2 M, *ca* p^{2}H 5.2, 14 ml) was incubated at 36° under a stream of dry N_2. After 92 hr, paper chromatography (Whatman 3 MM, developed with 1:BuOH–AcOH–pyridine–water (4:1:1:2)) showed only agmatine, R_f 0.36, as indicated by spraying separate chromatograms with ninhydrin and Sakaguchi reagent. Agmatine sulfate was then reisolated from the enzymic mixture as previously described. The ^{1}H NMR spectrum (^2H_2O) of the reisolated sample was identical with that of authentic unlabelled material; only one signal was observed in its ^{2}H NMR spectrum (H_2O, 83 mM, 5000 transients), due to natural abundance ^{2}H in water.

Oxidation of (1-^{2}H)*amines to* ω*-aminoaldehydes*

Chemical synthesis of solid derivatives of the ω*-aminoaldehydes*

5-Aminopentanal (**2**). 3-(3′-*Aminopropyl*)*quinoline* (**5**) (*cf* Ref. 23) α-Tripiperideine (404 mg) was dissolved in hot citrate buffer (0.1 M, pH 4.7, 10 ml) and added to a hot soln of *o*-aminobenzaldehyde (Fluka A.G.) (610 mg) in the same buffer (190 ml). The mixture was heated on a steam bath for 6 hr, cooled to 0°, and filtered. The filtrate was basified with 5% w/v NaOH, saturated with NaCl, and extracted with $CHCl_3$ (4 × 100 ml). The combined $CHCl_3$ extracts were dried over $MgSO_4$, filtered and evaporated. 3-(3′-Aminopropyl)quinoline was obtained as a yellow oil (686 mg). ^{1}H NMR (C^2HCl_3), δ 1.57 (2H, s (br), NH_2), 1.82 (2H, quin, J 7.2 Hz, H-2′), 2.73 (2H, t, J 7.2 Hz, H-1′), 2.80 (2H, t, J 7.2 Hz, H-3′), 7.40–8.13 (5H, m, ArH), 8.77 (1H, d, J 2.1 Hz, H-2); ^{13}C NMR (C^2HCl_3, 2.69 mM, 6000 transients), δ 30.5 (C-2′), 34.7 (C-1′), 41.5 (C-3′), 126.7 (C-5), 127.4 (C-6), 128.3 (C-10), 128.7 (C-7 or C-8), 129.3 (C-7 or C-8), 134.2 (C-4), 134.8 (C-3), 147.0 (C-9), 152.1 (C-2).

A soln of the quinoline derivative (113 mg) in EtOH (3 ml) was acidified with ethanolic hydrogen chloride. The dihydrochloride precipitated on addition of a small amount of ether, and was recrystallized from EtOH–ether, yield, 110 mg; m.p. 228–230° (lit.[23] m.p. 225–230°); ^{1}H NMR (perdeuterioacetate-^2H_2O buffer (0.2 M, *ca* p^{2}H 5)), δ 1.94 (2H, quin., J 7.2 Hz, H-2′), 2.85 (*ca* 2H, t, J 7.2 Hz, H-1′), 2.95 (*ca* 2H, t, J 7.2 Hz, H-3′), 7.42–8.02 (4H, m, ArH), 8.32 (1H, s, H-4), 8.68 (1H, s (br), H-2).

A sample of the dipicrate of 3-(3′-aminopropyl)quinoline was prepared from the dihydrochloride, m.p. 209–212° (from water) (lit. m.p. 208–210°,[20] 216 217°[23]); ^{1}H NMR (DMSO-$^2H_6$-^2H_2O, 10:1), δ 2.12 (2H, quin., J 7.2 Hz, H-2′), 3.16 (4H, two overlapping t, J 7.2 Hz, H-1′, H-3′), 7.86–8.30 (4H, m, ArH), 8.66 (4H, s, picrate-H), 9.04 (1H, d, J 1.2 Hz, H-4), 9.30 (1H, d, J 1.2 Hz, H-2).

3-(3′-*Aminopropyl*)-(2-^{2}H)*quinoline dihydrochloride* (*cf* Ref. 28). N-Bromosuccinimide (131 mg) was added to a small flask containing an aqueous soln of DL-(2-^{2}H)lysine monohydrochloride (*ca* 95% ^{2}H, 66 mg) in water (7 ml). The flask was immersed in a water bath at 40° and rotated by means of a rotary evaporator under reduced pressure. When

the soln had become colourless (45 min), the mixture was basified with 5% NaOHaq, and was extracted with $CHCl_3$ (4 × 5 ml). The $CHCl_3$ extracts were dried over $MgSO_4$, filtered and the filtrate evaporated to give a pale yellow oil. The oil, containing (2-^{2}H)-Δ^1-piperideine, the cyclized form of (1-^{2}H)-5-aminopentanal, was dissolved in hot citrate buffer (0.1 M, pH 4.7, 1 ml) and reacted with *o*-aminobenzaldehyde (44 mg in 19 ml of the same buffer) as described for the preparation of unlabelled material. 3-(3′-Aminopropyl)(2-^{2}H)quinoline was obtained as its dihydrochloride and recrystallized from EtOH–ether, yield, 40 mg, 41%; m.p. 228–230°. The ^{1}H NMR spectrum (perdeuterioacetate-^2H_2O (0.2 M, *ca* p^{2}H 5)) was identical with that of unlabelled material except that the signal at 8.68 ppm corresponded to *ca* 0.05 hydrogen atoms. The ^{2}H NMR spectrum (H_2O, 8 mM, 4808 transients) showed one signal at 8.8 ppm as well as that due to natural abundance ^{2}H in water (4.5 ppm).

In a second experiment (2-^{2}H)-Δ-^{1}-piperideine, which had been obtained from DL-(2-^{2}H)lysine monohydrochloride (> 95% ^{2}H, 23 mg) by oxidative decarboxylation with N-bromosuccinimide (46 mg), was trapped as the dipicrate[23] of 3-(3′-aminopropyl)quinoline, yield 26 mg (33%); m.p. 212–215° (lit.[23] m.p. 216–217°); ^{1}H NMR (DMSO-$^2H_6$–^2H_2O, 10:1), δ 2.12 (2H, quin., J 7.2 Hz, H-2′), 3.16 (4H, two overlapping t, J 7.2 Hz, H-1′, H-3′), 7.86–8.30 (4H, m, ArH), 8.67 (4H, s, picrate H), 8.95 (1H, s, H-4). The signal present at 9.30 ppm in the spectrum of the unlabelled material was absent, indicating that this sample was ⩾98% deuteriated at C-2. The ^{2}H NMR spectrum (DMSO, 25 mM, 866 transients) showed one signal, at 9.2 ppm, in addition to that due to natural abundance ^{2}H in DMSO (2.6 ppm).

4-Aminobutanal (**8**). *2,3-Trimethylylene-1,2-dihydroquinazolinium* (**11**) *picrate*. Oxidation of DL-ornithine monohydrochloride (333 mg) with N-bromosuccinimide (350 mg) in aqueous soln (20 ml) to give Δ^1-pyrroline, the cyclized form of 4-aminobutanal, was carried out by a published procedure.[24] The pH of the solution containing Δ^1-pyrroline (20 ml, pH 3.5) was adjusted to pH 4.5 with 5% NaOHaq. *o*-Aminobenzaldehyde (253 mg) in citrate buffer (0.1 M, pH 4.7, 40 ml) was added. The colour of the resulting soln changed from yellow to bright orange during stirring at room temp. After 20 hr the mixture was filtered, and a soln of picric acid in MeOH (10% w/v, 5 ml) was added to the filtrate. The dihydroquinazolinium picrate, which precipitated at 0°, was recrystallized from MeOH–water, yield, 400 mg, 50%; m.p. 160–162° (lit. m.p. 170–171°;[27] 169–170;[24] 166–168°[22]); ^{1}H NMR (DMSO-$^2H_6$), δ 2.03–2.70 (4H, m, H-1′,2′), 4.12 (2H, t, J 6.8 Hz, H-3′), 5.20 (1H, t, J 6.0 Hz, H-2), 6.85 (2H, m, ArH), 7.53 (2H, m, ArH), 8.07 (1H, s, NH), 8.57 (2H, s, picrate-H), 8.97 (1H, s, H-4); ^{13}C NMR (DMSO-$^2H_6$, 125 mM, 85000 transients), δ 22.5 (C-1′ or C-2′), 30.2 (C-1′ or C-2′), 52.9 (C-3′), 71.0 (C-2), 114.5, 115.4, 119.5, 125.2, 132.8, 139.2, 142.0, 148.0 (C-5 to C-10 and picrate C atoms), 159.2 (C-4).

2,3 - Trimethylene - (2-^{2}H) - 1,2 - dihydroquinazolinium picrate. The (2-^{2}H)dihydroquinazolinium picrate (72 mg) was obtained from DL-(2-^{2}H)ornithine monohydrochloride (*ca* 98% ^{2}H, 57 mg) as described for the preparation of unlabelled material, m.p. 154–155° (from MeOH–water); ^{1}H NMR (DMSO-$^2H_6$), δ 2.17 (*ca* 2H, t, J 6.8 Hz, H-1′), 2.03–2.70 (*ca* 2H, m (unresolved), H-2′), 4.12 (2H, t, J 6.8 Hz, H-3′), 6.87 (2H, m, ArH), 7.55 (2H, m, ArH), 8.07 (1H, s, NH), 8.58 (2H, s, picrate-H), 9.00 (1H, s, H-4). The signal at 5.20 ppm, present in the spectrum of the unlabelled compound, was absent, indicating that the sample was ⩾98% deuterium labelled at C-2. The ^{2}H NMR spectrum (DMSO, 20 mM, 5000 transients) showed one signal at δ 5.2 ppm as well as that due to natural abundance ^{2}H in DMSO (2.6 ppm).

Enzymic synthesis of ω-aminoaldehydes

5-Aminopentanal (**2**). *Incubation of cadaverine with hog kidney diamine oxidase* (E.C. 1.4.3.6) *and isolation of the product as 3-(3′-aminopropyl)quinoline* (**5**) *dihydrochloride*. (i) 40 hr Incubation. A soln of cadaverine dihydrochloride (40 mg), *o*-aminobenzaldehyde (32 mg), diamine oxidase (Sigma, Grade II, 0.12 U/mg)† (200 mg), and beef liver catalase (E.C. 1.11.1.6) (Sigma, 137 U/mg)‡ (500 μg) in phosphate buffer (0.1 M, pH 7.2, 30 ml) was incubated at 36° for 40 hr. The mixture was then acidified (pH 4.5) with 4 M hydrochloric acid, heated on a steam bath for 30 min and centrifuged. *o*-Aminobenzaldehyde (20 mg) was added, and the supernatant soln was refluxed for 6 hr, cooled to room temp and filtered through Celite. The pH of the filtrate was adjusted to 11–12 with 5% NaOHaq, and the soln was extracted with diethyl ether (4 × 20 ml). The combined ether extracts were dried over Na_2SO_4, filtered, and the solvent was evaporated to dryness. The residue was purified by preparative TLC (silica gel plates, 0.5 mm, 20 × 20 cm; developed with 1-BuOH–AcOH–water (5:1:1)). The band corresponding to that of 3-(3′-aminopropyl)quinoline, R_f 0.3, was eluted with EtOH. After removal of the solvent *in vacuo*, the residue was suspended in NaOHaq (2% w/v, 1 ml) and the product extracted into ether (4 × 2 ml). The quinoline dihydrochloride was obtained by acidification with ethanolic hydrogen chloride, evaporation to dryness, and crystallization of the residue from EtOH–ether, yield 5 mg; m.p. 229–231°. The ^{1}H NMR spectrum (perdeuterioacetate-^2H_2O (0.2 M, p^{2}H 5)) was identical with that of 3-(3′-aminopropyl)quinoline dihydrochloride prepared from α-tripiperideine. (ii) 6 hr Incubation. Cadaverine dihydrochloride (38 mg) was incubated with diamine oxidase (200 mg) in the presence of *o*-aminobenzaldehyde (26 mg) in phosphate buffer (0.1 M, pH 7.2, 20 ml) for 6 hr. When picric acid in MeOH was added, the products precipitated. The solid was extracted with boiling MeOH (1 ml).

The yellow residue was recrystallized from water to give 3-(3′-aminopropyl)quinoline dipicrate (8 mg), m.p. 216–218°. The ^{1}H NMR spectrum (DMSO-$^2H_6$) was identical with that of an authentic sample of the quinoline dipicrate prepared from α-tripiperideine (see above).

When water (0.2 ml) was added to the MeOH extract the second product, 2,3-tetramethylene-1,2-dihydroquinazolinium picrate (2 mg) precipitated, m.p. 165–167° (from MeOH–water) (lit.[23] m.p. 166–172°); ^{1}H NMR (DMSO-$^2H_6$), δ 2.09 (6H, m, H-1′,2′,3′), 4.02 (*ca* 2H, t (br), J ~ 10 Hz, H-4′), 5.75 (1H, d of d (br), J_1 ~ 9 Hz, J_2 ~ 3 Hz, H-2), 6.80 (2H, m, ArH), 7.45 (2H, m, ArH), 7.73 (*ca* 1H, s (br), NH), 8.63 (2H, s, picrate-H), 8.80 (1H, s, H-1).

Enzymic conversion of (*^{2}H)cadaverine into* (*^{2}H)-3-(3′-aminopropyl)quinoline. Samples (40 mg) of R-(1-^{2}H)-, S-(1-^{2}H)-, and (1,1-$^2H_2$)cadaverine dihydrochloride (Expts 1, 2 and 3a, respectively), obtained from the appropriate incubation with L-lysine decarboxylase, were incubated in separate experiments with diamine oxidase, as described under (i) above. The samples of 3-(3′-aminopropyl)quinoline dihydrochloride obtained (2–7 mg) in each of the three experiments were identical, except for the presence of ^{2}H at C-2 and/or C-3′, with authentic unlabelled material, as revealed by ^{1}H and ^{2}H NMR spectroscopy.

Another sample of (1,1-$^2H_2$)cadaverine (30 mg) dihydrochloride (Expt 3b) was incubated with diamine oxidase, as described under (ii), above.

The major product, 3-(3′-aminopropyl)quinoline (**5**) dipicrate (7 mg), m.p. 205–208° gave an ^{1}H NMR spectrum (in DMSO-$^2H_6$) which indicated the absence of ^{1}H from C-2 (δ 9.30, 0.5 H) and from C-3′ (δ 3.16, 3H, two H at C-1′, one H at C-3′).

The minor product, 2,3-tetramethylene-1,2-dihydroquinazolinium (**6**) picrate (*ca* 1 mg) showed a ^{1}H

†1 U will oxidize 1.0 μmole putrescine per hr at pH 7.2 at 37°.

‡1 U will decompose 1.0 μmole hydrogen peroxide per min at pH 7.0 at 25°.

NMR spectrum (in DMSO-$^2H_6$–^2H_2O, 10:1) similar to that of unlabelled material, except that the signal at 5.75 ppm (H-2) corresponded to *ca* 0.5 H. The signal due to H-4′ (*ca* 4.0 ppm) was unresolved, due to the presence of a large overlapping signal of ^{2}HOH.

4-Aminobutanal (**8**). *Incubation of putrescine with hog kidney diamine oxidase and isolation of the product as 2,3-trimethylene-1,2-dihydroquinazolinium* (**11**) *picrate.* A soln containing putrescine dihydrochloride (Sigma) (40 mg), *o*-aminobenzaldehyde (24 mg), diamine oxidase (Sigma, Grade II) (200 mg) and catalase (Sigma) (50 μg) in phosphate buffer (0.1 M, pH 7.2, 20 ml) was incubated in a constant temp water bath at 36° for 6 hr. The pH of the mixture was then adjusted to pH 4–4.5 with conc hydrochloric acid and the soln concentrated (*ca* 15 ml) by heating on a steam bath under a stream of N_2. The precipitated protein was removed by centrifugation and filtration of the supernatant soln through Celite. A satd soln of picric acid in MeOH (0.15 ml) was added, with swirling, to the clear yellow soln and the product, which precipitated when the mixture was cooled at 0° overnight, was collected and recrystallized from MeOH–water, yield 14 mg. The m.p. (159–160°) and ^{1}H NMR spectrum (DMSO-$^2H_6$) were identical with those of the dihydroquinazolinium picrate prepared from ornithine.

Enzymic conversion of (^{2}H)*putrescine into* (^{2}H)-2,3-*trimethylene*-1,2-*dihydroquinazolinium picrate.* Samples (30 mg) of R-(−)-(1-^{2}H)-, S-(+)-(1-^{2}H)-, and (1,1-$^2H_2$)putrescine dihydrochloride (Expts 4, 5, and 6, respectively) were incubated with diamine oxidase in the presence of *o*-aminobenzaldehyde to give samples of the dihydroquinazolinium picrate (9–15 mg) (see above), which were labelled with deuterium at C-2 and/or C-3′, as determined by ^{2}H and ^{1}H NMR spectroscopy.

Incubation of agmatine (**12**) *with hog kidney diamine oxidase and isolation of the product, 4-guanidinobutanal* (**13**), *as its dinitrophenylhydrazone* (*DNP*) *derivative* (**15**). A potassium phosphate buffer soln (0.05 M, pH 7.2, 40 ml) containing agmatine sulfate (111 mg, 0.012 M), diamine oxidase (Sigma, Grade II) (243 mg), and beef liver catalase (Sigma) (*ca* 150 μg) was incubated at 36° in a constant temp water bath. The course of the reaction was followed by TLC (silica gel; 1-BuOH–AcOH–water (5:1:1)) which showed the formation of a single oxidation product (Sakaguchi positive) at R_f 0.35 (agmatine, R_f 0.06).

When oxidation was complete (*ca* 23 hr), the enzymic mixture was acidified (pH 4) with 10% HCl, heated on a steam bath for 30 min, cooled to room temp, and its pH adjusted to pH 6 with NaOHaq (2% w/v). EtOH (8 ml) was added and the precipitated protein was removed by filtration through Celite. The filtrate was evaporated to dryness *in vacuo* and the residue was dissolved in ^2H_2O (*ca* 1 ml). ^{1}H NMR (^2H_2O), δ 1.7 (4H, s (br), H-2,3), 3.2 (2H, m (br), H-4), 5.2 (1H, s (br), H-1); ^{13}C NMR (^2H_2O, 60,000 transients), δ 22.8 (C-3), 35.4 (C-2), 48.2 (C-4), 83.8 (C-1), 156.5 (−NH(C=NH)NH_2). The ^{1}H and ^{13}C NMR spectra suggest that the product of oxidative deamination, 4-guanidinobutanal, occurs in aqueous soln as its hydrate (**14**).

After removal of the ^2H_2O *in vacuo*, the residue was redissolved in water (5 ml) and evaporated to dryness. It was then extracted with EtOH (3 × 2 ml). The combined EtOH extracts were evaporated to dryness and a soln of dinitrophenylhydrazine in ethanolic phosphoric acid (0.1 M, 5 ml) was added. The soln was heated at reflux for 30 min and after cooling at 0° overnight the yellow–orange DNP-phosphate derivative precipitated (82 mg). ^{1}H NMR (^2H_2O), δ 1.88 (2H, quin., J 6.8 Hz, H-3), 2.46 (2H, d of t, J_1 6.8 Hz, J_2 5.1 Hz, H-2), 3.26 (2H, t, J 6.8 Hz, H-4), 7.68 (1H, d, J 9.8 Hz, ArH), 7.74 (1H, t, J 5.1 Hz, H-1), 8.23 (1H, d of d, J_1 9.8 Hz, J_2 2.7 Hz, ArH), 9.03 (1H, d, J 2.7 Hz, ArH); ^{13}C NMR (DMSO-$^2H_6$, 35 mg/ml, 30,000 transients), δ 25.3 (C-3), 29.4 (C-2), 116.5, 123.3, 129.0, 130.1, 136.9, 144.9 (aromatic C atoms), 154.3 (C-1), 157.2 (−NH(C=NH)NH_2). The ^{13}C signal arising from C-4 was presumably buried under the DMSO-$^2H_6$ septet centered at 39.6 ppm.

Part of the DNP-phosphate (15 mg) was dissolved in hot ethanolic hydrogen chloride, and after cooling to 0°, the hydrochloride salt precipitated as yellow–orange needles which were recrystallized from EtOH, yield 5 mg; m.p. 95–96°. The ^{1}H NMR spectrum (^2H_2O) of the DNP-hydrochloride was identical with that of the phosphate.

Incubation of deuteriated samples of agmatine with diamine oxidase. Incubation of R-(−)-(1-^{2}H)- and S-(+)-(1-^{2}H)agmatine sulfate with diamine oxidase, in separate experiments, and isolation of the products of oxidative deamination as the DNP-phosphate derivatives was carried out as described above.

Experiment 7. The DNP-phosphate (25 mg) obtained from R-(−)-(1-^{2}H)agmatine sulfate (33 mg) showed a ^{1}H NMR spectrum (^2H_2O) similar to that of unlabelled material except for the absence of the triplet at 7.74 ppm (H-1) and the presence of a triplet (J 6.8 Hz) centred at 2.46 ppm (H-2), instead of a double triplet. The ^{2}H NMR spectrum (H_2O, 4.8 mg/ml, 3484 transients) of this sample showed one signal at 7.6 ppm (br) as well as the signal due to natural abundance deuterium in water at 4.5 ppm.

Experiment 8. The ^{1}H NMR spectrum (^2H_2O) of the DNP-phosphate (32 mg) obtained from S-(+)-(1-^{2}H) agmatine sulfate (41 mg) was identical with that of unlabelled material and no signals were observed in its ^{2}H NMR spectrum (H_2O, 7.4 mg/ml, 14612 transients) other than the one due to natural abundance deuterium in water.

Instrumental Methods

Measurement of optical activity. Optical rotation was measured by means of a Perkin–Elmer 241 MC polarimeter equipped with a mercury emission lamp, using a 1 dm polarimeter tube. At least 10 readings were taken at each mercury emission line. Mean values and 95% confidence limits are reported.

^{1}H-NMR *spectra* were recorded at ambient temp on a Varian EM 390 spectrometer or a Bruker WH90 pulsed Fourier transform spectrometer, each operating at 90 MHz, or a Bruker WP80 pulsed Fourier transform spectrometer at 80 MHz. Chemcial shifts are reported as ppm downfield from DSS for samples in ^2H_2O solution or from internal TMS for samples in DMSO-$^2H_6$ or $^2HCCl_3$ solns.

^{2}H-NMR *spectra* were recorded on a Bruker WH90 pulsed Fourier transform spectrometer operating at 13.82 MHz at ambient temp., with broad band proton noise decoupling. Spectra were obtained of samples in aqueous or dimethyl sulfoxide solution in 10 mm (o.d.) sample tubes, using ^2H_2O as external lock. The signal due to natural abundance ^{2}H in ^{2}HOH or ^{2}H-DMSO served as internal standard. Chemical shifts are downfield from perdeuteriotetramethylsilane.

^{13}C-NMR *spectra* were recorded on a Bruker WP80 spectrometer operating at 20 MHz in the pulsed Fourier transform mode with complete proton decoupling. Spectra were obtained on samples in ^2H_2O, $^2HCCl_3$ or DMSO-$^2H_6$, in 10 mm (o.d.) sample tubes. Chemical shifts are reported as ppm downfield from external TMS.

Mass spectra were determined on a Micromass 7070F double focussing mass spectrometer, operating with electron impact (EI) or chemical ionization (CI), by direct injection of the samples. Methane at 5×10^{-5} mm torr was the reagent gas for CI. The isotopic content in deuterium enriched samples was calculated as described by Biemann.[54]

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