THE STEREOCHEMISTRY OF ENZYMIC REACTIONS AT PROCHIRAL CENTRES

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ABSTRACT

The steric course of decarboxylation of L-ornithine to yield putrescine, catalysed by L-ornithine decarboxylase (E.C. 4.1.1.17) of *E. coli*, and of L-arginine to yield agmatine, catalysed by L-arginine decarboxylase (E.C. 4.1.1.19) of *E. coli*, is investigated by deuterium labelling. Replacement of the carboxyl group by a solvent derived proton occurs with retention of configuration in each case.

In conflict with an earlier report, incubation of cadaverine in deuterium oxide in the presence of L-lysine decarboxylase (E.C. 4.1.1.18) of *B. cadaveris* did not lead to entry of deuterium into the α-position of cadaverine. Likewise, L-ornithine decarboxylase did not catalyse exchange of the α-hydrogen of putrescine, nor did L-arginine decarboxylase catalyse such an exchange in agmatine.

The stereochemistry of hydrogen abstraction in the conversion of cadaverine into Δ'-piperidine, of putrescine into Δ'-pyrroline, and of agmatine into 4-guanidinobutanal, catalysed by hog kidney diamine oxidase (E.C. 1.4.3.6) is investigated. The *Si*-hydrogen from C-1 of the substrate is removed while the *Re*-hydrogen from C-1 of the substrate is maintained at the sp^2 carbon atom of each of the products.

The diamine oxidase catalysed oxidative deamination of cadaverine takes place without detectable isotope effect, while an intramolecular primary hydrogen-deuterium isotope effect ($k_{H_{Si}}/k_{D_{Si}} \approx 4$) is observed in the diamine oxidase catalysed oxidation of putrescine.

DEDICATED TO

the memory of my father-in-law,

Ron Cowley

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CHAPTER 1

INTRODUCTION

Enzymes, the biological catalysts, effect chemical transformations within living organisms.[1-3] Constructed from the much smaller units, amino acids, each enzyme constitutes a chiral matrix, a catalyst of superb perfection,[4,5] whereby one molecule is processed into another in such a way that each step is under precise steric control.[2,6,7] One manifestation of this control is the enormous rate enhancements characteristic of so many enzyme-catalysed reactions.[8,9] Stereospecificity is another manifestation of such control.

The stereospecificity exhibited by enzymes in the reactions that they catalyse can take several forms. The ability of enzymes to make chiral distinctions between two enantiomeric forms of a substrate molecule has been recognised since the time of Pasteur.[10] However, it was not apparent until the middle of this century that enzymes are also capable of prochiral distinctions.[11] This very subtle aspect of enzyme reaction stereospecificity, chiral recognition of the stereoheterotopic groups or atoms at prochiral centres, is only revealed through investigations which employ the technique of isotopic labelling. Investigations of this type have revealed, as enunciated by Sir John W. Cornforth[12], that

> "...*enzymic reactions occur by stereospecific mechanisms* regardless of whether this specificity determines the structure of the product."

Enzymes that catalyse the decarboxylation of L-α-amino acids are found in microorganisms, in mammalian tissues and in plants.[13] These enzymes show a high degree of substrate specificity for individual amino acids. The reaction that is catalysed involves the conversion of a chiral centre within the L-α-amino acid, $R\text{-}CH(NH_2)CO_2H$, into a prochiral centre within the corresponding amine, $R\text{-}CH_2NH_2$. The stereochemistry of the replacement of a carboxyl group by a proton in the course of this reaction has been determined in several instances, by a variety of methods. The results of these investigations, discussed in Section 1.4, have revealed an apparent stereochemical consistency among the amino acid decarboxylases[14] which is referred to later in Chapter 4.

The amine oxidases which also form a widely distributed group of enzymes catalyse the oxidation of primary amines to aldehydes.[15-17] These enzymes are of low substrate specificity, each acting upon a wide range of substrates. Enzymic oxidative deamination involves the abstraction of a proton from the prochiral centre adjacent to an amino group within the primary amine, $R\text{-}CH_2NH_2$, to generate an sp^2 centre within the corresponding aldehyde, RCHO. The stereochemistry of hydrogen abstraction, in the course of the reaction mediated by several amine oxidases and with a wide variety of substrates, has been determined. The methods that have been employed and the results obtained are summarised in Section 1.5.

This thesis reports an investigation of the stereochemistry of both these reaction types. The stereochemistry of decarboxylation of the basic amino acids, L-ornithine and L-arginine, catalysed by the respective bacterial α-decarboxylases is investigated using deuterium labelling. Chapter 2 describes how the products of these two enzymic reactions, putrescine (1,4-diaminobutane) and agmatine (1-amino-4-guanidinobutane), respectively, chirally deuteriated at C-1, were prepared and how their relative configurations were established.

Determination of the stereochemistry of the reaction mediated by hog kidney diamine oxidase is described in Chapter 3. This was established by ^2H NMR analysis of the products obtained when enantiomeric samples of (1-^2H)cadaverine of known configuration served as substrate. Evidence in support of the assumption that the absolute stereochemistry of the reaction catalysed by hog kidney diamine oxidase is the same, whatever the substrate, is presented. ^2H NMR was then employed to determine the labelling pattern of the products obtained by oxidative deamination of the chiral samples of (1-^2H)putrescine and of (1-^2H)-agmatine to establish their absolute configuration, based on this assumption (Chapter 3). However, a kinetic isotope effect was observed in the diamine oxidase catalysed reaction with putrescine as substrate, but none with cadaverine as substrate. It was therefore necessary to confirm the configurational assignments by an independent method. Chapter 4 describes how the absolute stereochemistry of the enantiomeric samples of (1-^2H)putrescine and (1-^2H)agmatine was confirmed by chemical correlation with a standard of known configuration, thereby establishing

the steric course of decarboxylation. Chapter 5 presents a summary of these results.

1.1 HISTORICAL DEVELOPMENT OF THE CONCEPT OF PROCHIRALITY

Stereochemical selectivity was first observed in living organisms by Louis Pasteur in 1858.[18,19] Much to his astonishment and pleasure he was able to demonstrate that, when the grey mold *Penicillium glaucum* was grown in the presence of racemic tartaric acid, only the dextrorotatory species was fermented; the levorotatory species remained intact. At that time, Pasteur recognised that the optical activity associated with many organic substances was a reflection of molecular dissymmetry.[18,20] Even so, the relationship between such molecules, in terms of their three dimensional structure, as well as the relation to the observed stereoselectivity in a biochemical transformation could not be grasped until the theory of the tetrahedral structure of carbon was proposed by van't Hoff and Le Bel in 1874.[21] After this structural theory had become well established, Emil Fischer in his pioneering work on sugar stereochemistry provided clear evidence that it is the exact three dimensional structure of a molecule which determines whether or not an enzyme will act upon it.[22] It was observed that certain yeast preparations effected the hydrolysis of methyl α-D-glucopyranoside as well as maltose, but not of methyl β-D-glucopyranoside. By means of an illustration, Fischer suggested that enzyme and substrate (in this case a glucoside) must fit each other like "lock and key" if catalytic activity is to occur.[22]

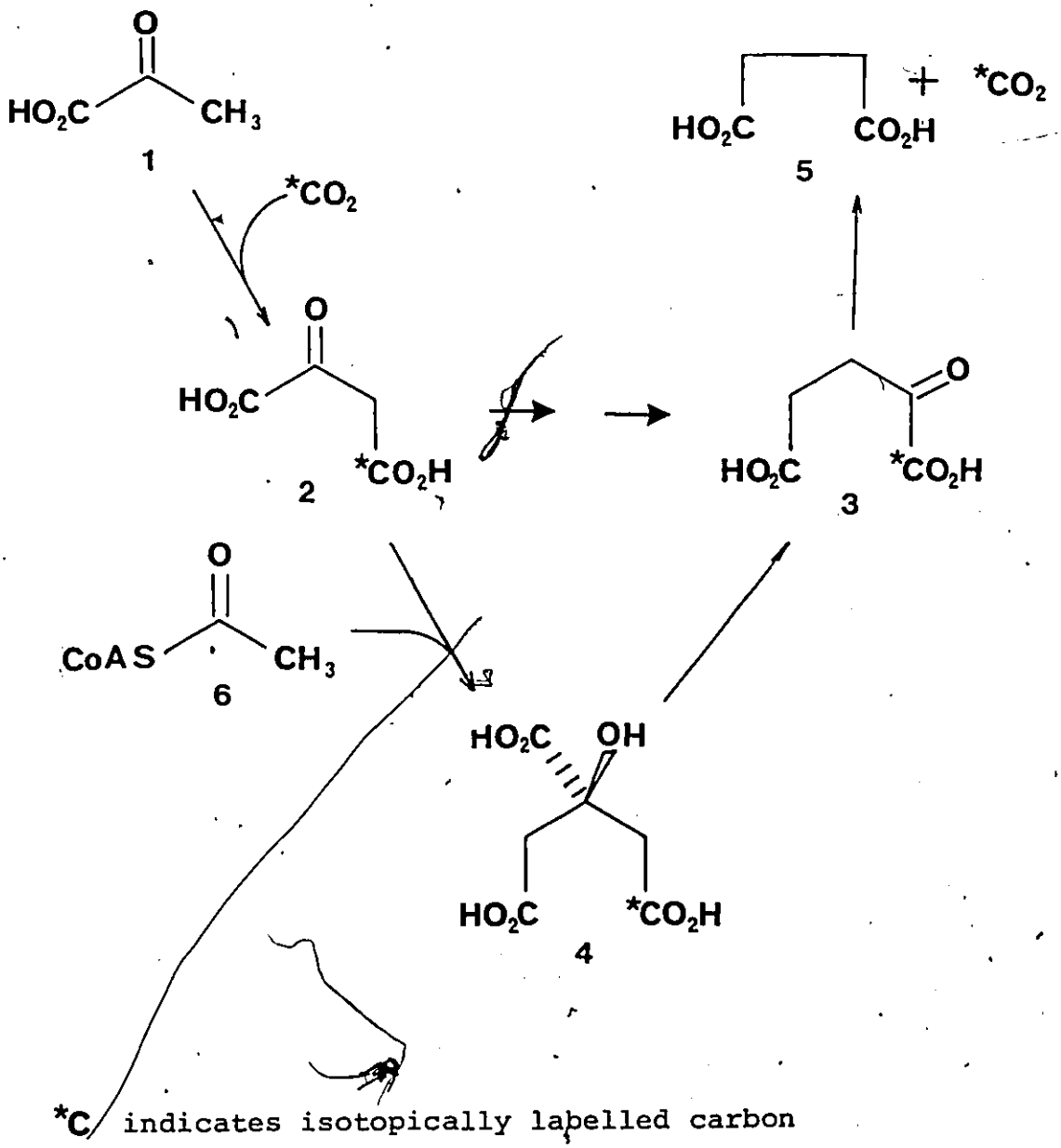
At that time, the chemical nature of enzymes was unknown. Indeed, the term "enzyme", coined by Kuhne[23] in 1878, literally translates "in yeast". However, it was not until the development of improved enzyme isolation and purification techniques in the 1920s that the chemical constitution of enzymes became apparent; and it was concluded that enzymes consisted of protein.[24] The first convincing evidence was reported by Sumner[25] in 1926. A crystalline sample of the enzyme urease was obtained from extracts of jack bean and the catalytic activity of the protein was demonstrated. Shortly thereafter, crystalline pepsin, trypsin and chymotrypsin were isolated, firmly establishing the protein nature of enzymes.[26] As a result of this discovery, it was also recognised that such protein molecules were responsible for the stereochemical specificity so often observed in biological systems.[27]

A consistent feature, revealed in all enzyme-catalysed reactions, is the specificity.[28] The early work of Pasteur, Fischer and others uncovered only a single aspect of this specificity. It is now known that enzymes are capable of distinguishing among structural and stereochemical features of their substrates, that each enzyme mediates a specific type of reaction, and that they are capable of chiral and prochiral distinctions in the course of that reaction. Such specificity undoubtedly arises from the exact chiral environment provided by the enzyme which allows the appropriate orientation of the reacting species, throughout the whole process of catalysis.[4-7]

The ability of enzymes to make prochiral distinctions was first observed shortly after the discovery of the stable and radioactive isotopes of carbon.[29] Indeed, the results of isotopic tracer experiments

were quite unexpected and, on the basis of such experiments, it became necessary, in 1941, to question the role of citric acid in the biochemical process which is now known as the "Tricarboxylic Acid Cycle". Preparations of minced pigeon liver were exposed to $^{11}CO_2$ or $^{13}CO_2$ in the presence of unlabelled pyruvic acid (1), and labelled oxaloacetic acid (2) and α-ketoglutaric acid (3) were isolated. Degradation of the labelled sample of α-ketoglutarate indicated that only the α-carboxyl group contained isotopic carbon[30,31] (Scheme 1). At that time, there was considerable evidence that citric acid (4) was an intermediate in the biochemical transformation of oxaloacetate into α-ketoglutarate.[32-34] However, the results from the experiments with isotopically labelled carbon dioxide were taken as sufficient evidence that citric acid, a "symmetrical molecule", could not possibly be an intermediate. It was reasoned that if it were an intermediate, both carboxyl groups of α-ketoglutarate should be equally labelled since the two carboxymethylene groups of citric acid are "chemically identical" (when unlabelled).

The fallacy in the interpretation of these experiments was pointed out by Ogston in 1948.[11] Ogston argued that "...it is possible that an asymmetric enzyme which attacks a symmetrical compound can distinguish between its identical groups," and illustrated how this might occur by suggesting a "three-point combination" between the symmetrical substrate and an enzyme surface. Ogston made reference to aminomalonic acid which, as in the case of citrate, had been excluded as an intermediate in a biochemical pathway, the conversion of L-serine into glycine, based on similar, erroneous interpretations of isotopic tracer experiments.[35]



Scheme 1. Enzymic formation of isotopically labelled α-ketoglutaric acid.

Ogston's hypothesis was soon tested and confirmed, at least in principle, by Potter and Heidelberger[36] and Lorber *et al.*[37] who clearly demonstrated that citric acid must be a precursor of α-ketoglutarate. Enzymatically formed carbon-14 labelled citrate (4), derived from $^{14}CO_2$ or [4-^{14}C]oxaloacetate (2), was first isolated from the liver preparations and purified. The sample of citrate was then reincubated with a fresh preparation to yield α-ketoglutarate labelled exclusively in the carboxyl carbon atom adjacent to the keto group, as revealed by oxidative degradation to succinic acid (5) and radioactive carbon dioxide (Scheme 1).

Even though citric acid was reinstated as an intermediate in the "Tricarboxylic Acid Cycle", the idea that a symmetrical molecule could behave in an unsymmetrical fashion created much confusion at that time. The difficulties that were experienced with this concept have recently been reflected upon by Barbara Vennesland[38]:

> "In a mood of desperation, I sat down with Ogston's paper and took some stick and ball models to assist my thinking. Ogston's paper didn't help, though I read it several times attentively. I thought he was implying a substrate might stay stuck to the enzyme, and that did not help one bit. While I was staring bleakly at the models, they suddenly told me the answer, but not in words, just in a picture. What happened in that moment of sudden insight seemed to be that I was suddenly graced with the ability to see the asymmetric carbon atom as van't Hoff had originally seen it. The feeling I experienced was a curious combination of exhilarating, sweet relief (because there was no contradiction between the two sets of experiments) and total

> dismay: 'Oh you idiot, why haven't you realised that before.' There were quite a few of us who had experiences similar to mine after the appearance of Ogston's paper."

The concept of "three-point combination" referred to by Ogston[11] was merely meant as an illustration to demonstrate how an enzyme "might" differentiate between two identical groups within a molecule[39] and is not a necessary requirement for the occurrence of such distinctions. Whether or not a distinction between two identical groups within a molecule is possible depends on the symmetry of that molecule. By considering the structural requirements necessary for the occurrence of such distinctions, it was first recognised by Hirschman[40-42] that a distinction between identical groups within a molecule is only possible if the groups cannot be interchanged by rotation about an axis of rotation (C_n). Hirschman suggested that the differentiation of chemically identical groups within a molecule, which lacks rotational symmetry, may be effected through the formation of diastereomeric transition states.

In 1966, Hanson introduced the concept of prochirality.[43] He proposed that a molecule possessing a tetrahedral centre of the type, R_1R_2CAA, in which the two substituents, A, are constitutionally identical (*e.g.* as in citric acid), should be defined as "prochiral" since such a centre can mentally be converted into a chiral centre if one of the pair of identical substituents is considered to be different from the other. A system of specification of such a pair of identical substituents was developed and is now in common usage (see Section 1.2).

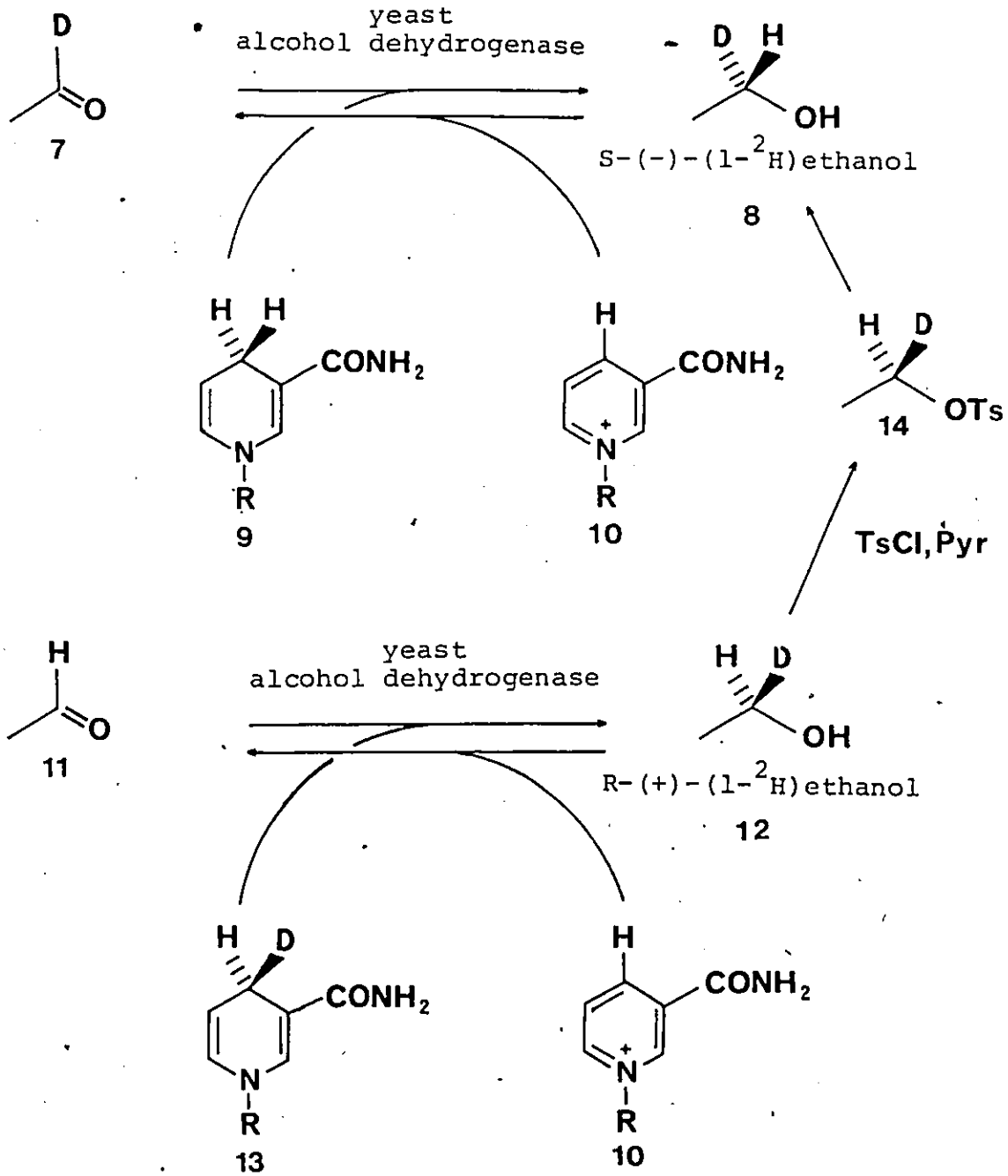
The structural relationships between groups and their molecular environments (topic relationships),[44-49] as well as in isolation (morphic relationships),[49,50] have now been examined in considerable detail. Two identical (homomorphic) groups which occupy identical superimposable environments (*i.e.* interchangeable by a C_n operation) are termed homotopic, those in nonsuperimposable environments are heterotopic. Groups which bear a heterotopic relationship may either be constitutionally heterotopic or stereoheterotopic. If two stereoheterotopic groups are interchangeable by a rotation-reflection operation (S_n, where $S_1 = \sigma$), they are termed enantiotopic; if the groups cannot be interchanged by any symmetry operation, they are diastereotopic. Enantiotopic groups are only distinguishable under chiral conditions[40,41] by interaction with a chiral reagent or within a chiral environment. Diastereotopic groups are, in principle, distinguishable by any reagent[42] (either achiral or chiral), or by physical observation under achiral or chiral conditions. It is important to emphasise, however, that, although a pair of homomorphic groups within a molecule may, in principle, be distinguishable in a chemical reaction under a certain set of conditions as determined by examination of the symmetry of that molecule, the magnitude of the difference, or even whether such a difference will be substantial enough to be observable, cannot, in general, be determined *a priori*.[44]

It should be clear from the above discussion that the two carboxymethylene groups of citric acid bear an enantiotopic relationship. It is also not too surprising that an enzyme, which functions as a chiral catalyst and a chiral reagent, as well as providing a chiral environment, is capable of effecting a distinction between these groups. This was shown

by the experiments, referred to earlier, involving the use of radioactive carbon dioxide[36] and labelled oxaloacetate (2).[37] These experiments not only revealed the occurrence of such a stereospecific distinction, but also indicated that a specimen of citric acid (4), rendered chiral due to isotopic substitution, was produced from [4-^{14}C]oxaloacetate (2). Thus in the course of enzymic synthesis of citrate, addition of acetyl coenzyme A (6) occurs stereospecifically at only one of the two stereoheterotopic faces of the carbonyl carbon (C-2) of oxaloacetate (*cf.* Scheme 1). The stereochemistry of these reactions has since been fully elucidated and is the subject of several reviews.[10,51]

Shortly after the discovery of prochirality, Westheimer, Vennesland and their colleagues provided clear evidence that enzymes were also capable of making stereospecific distinctions between the enantiotopic hydrogen atoms at prochiral methylene groups.[51] This was achieved by deuterium labelling (Scheme 2).[52] A sample of (1-^{2}H)ethanol (8) was obtained by enzymic reduction of (1-^{2}H)acetaldehyde (7), catalysed by yeast alcohol dehydrogenase (E.C. 1.1.1.1) in the presence of coenzyme NADH (nicotinamide adenine dinucleotide) (9). When the deuteriated sample of ethanol (8) was reincubated with the enzyme, in the presence of the oxidized form of the coenzyme, NAD^{+} (10), (1-^{2}H)acetaldehyde (7) was obtained without any detectable loss of deuterium.

In another experiment it was shown that the dehydrogenase catalysed the reversible formation of a sample of (1-^{2}H)ethanol (12) from unlabelled acetaldehyde (11) and the reduced form of the deuteriated coenzyme, NAD^{2}H (13).[53] When the tosylate (14) of this sample of (1-^{2}H)-ethanol (12) was displaced with hydroxide ion in an S_N2 process (*i.e.*



Scheme 2. Enzymic synthesis and chemical correlation of the enantiomers of (1-^{2}H)ethanol.

involving complete inversion of configuration at C-1), the resulting sample of (1-^{2}H)ethanol (8) gave (1-^{2}H)acetaldehyde (7) on incubation with alcohol dehydrogenase and unlabelled NAD^+ (10) (Scheme 2). The conclusion was inescapable: enzymic oxidation of ethanol to acetaldehyde is accompanied by stereospecific removal of one of the enantiotopic hydrogens from C-1. Conversely, yeast alcohol dehydrogenase is capable of distinguishing between the two stereoheterotopic faces of the carbonyl carbon of acetaldehyde. Thus the two samples of (1-^{2}H)ethanol ((8) and (12)) were enantiomeric, and it was subsequently shown[54] that the sample (8), obtained by enzymic reduction of (1-^{2}H)acetaldehyde is optically active ($[\alpha]_{589}$ - 0.28°). However, it was not until ten years later that the absolute configuration of a sample of the R-(+)-enantiomer (12) was established by unambiguous chemical synthesis.[55]

Since these early investigations, the stereochemistry of enzymic reactions has been investigated with increasing intensity. The results of many of these investigations are discussed in detail in several excellent books[56-58] and review articles.[10,12,14,38,49,51,59-66] Stereospecificity, as complete as the methods of examination can reveal, has been observed in most enzyme-catalysed reactions. Indeed, an apparent lack of stereospecificity in the course of the formation of product from substrate provides valuable mechanistic information and points to the involvement of a non-enzyme directed step or a tortiosymmetric intermediate.[64-66]

1.2 SPECIFICATION OF STEREOHETEROTOPIC GROUPS AND FACES

A system for specifying the stereoheterotopic groups or atoms at prochiral centres, based on the Cahn, Ingold and Prelog system of stereochemical nomenclature for chiral centres,[67] was introduced in 1966 by Hanson.[43] If one of the two homomorphic substituents (A) at a prochiral centre (R_1R_2CAA) is arbitrarily assigned a higher priority[67] than the other, the "imaginary" chiral centre ($R_1R_2CA^1A^2$, where A^1 is assigned a higher priority than A^2), so generated, will either have the $\underline{\underline{R}}$- or $\underline{\underline{S}}$-configuration, depending upon the relative priorities of the two heteromorphic substituents (R_1 and R_2). The stereoheterotopic group or atom, assigned a higher priority (A^1), is designated *pro*-$\underline{\underline{R}}$ (or $A^1_{\underline{\underline{R}}}$) or *pro*-$\underline{\underline{S}}$ (or $A^1_{\underline{\underline{S}}}$), respectively. Conversely, the group or atom assigned a lower priority (A^2) is specified as *pro*-$\underline{\underline{S}}$ (or $A^2_{\underline{\underline{S}}}$) or *pro*-$\underline{\underline{R}}$ (or $A^2_{\underline{\underline{R}}}$), respectively.

The stereoheterotopic faces of a trigonal centre ($R_1R_2R_3C$) are assigned similarly. The three heterotopic substituents are ordered according to the priority rules;[67] and, depending upon their relative priorities, the corresponding faces, above and below the plane defined by $R_1R_2R_3C$, are specified by the descriptors *re* (or *Re*) and *si* (or *Si*).[43,49]

More recently, an alternative system of specification was proposed by Prelog and Helmchen.[68] In this system the prochiral tetrahedral centre (R_1R_2CAA) is considered to consist of two chiral simplexes, divided by a plane of symmetry (see, for example, Fig. 1-19 in ref. 59). The two half-spaces defined by the plane of symmetry are labelled *Re* or *Si*. Depending upon the relative priorities of the substituents, R_1, R_2

and A,[67] allows the two stereoheterotopic substituents to be designated as A_{Re} or A_{Si}, respectively. Homomorphic groups or atoms designated as A_{Re} and A_{Si} in the Prelog-Helmchen system[68] correspond to those specified as $A_{\underline{R}}$ and $A_{\underline{S}}$, respectively, in the Hanson system,[49] only when the stereoheterotopic groups or atoms are of either lowest or highest priority.

The merits of these two approaches have been discussed.[48,49,68,69] Although the Hanson system,[43] since refined by Hirschman and Hanson,[45,47,49] has been more widely employed,[69] the Prelog-Helmchen descriptors[68] have enjoyed recent use,[58] particularly for the designation of the sterohetero-topic hydrogens at prochiral methylene groups (*e.g.* ref. 70). The Prelog-Helmchen system is used throughout this thesis.

1.3 DETERMINATION OF CONFIGURATION OF PROCHIRAL METHYLENE GROUPS, RENDERED CHIRAL DUE TO ISOTOPIC SUBSTITUTION

Many biochemical processes lead to the formation or destruction of a prochiral methylene group.[59,71,58] These enzyme-catalysed transformations involve stereospecific hydrogen transfer, either to or from a carbon atom within achiral or chiral molecules. To probe the stereochemistry of such processes requires the use of deuterium or tritium labelling. This is achieved by employing appropriately labelled substrates, which in the course of the enzymic reaction afford a prochiral methylene group, rendered chiral by isotopic substitution within the product. Alternatively, the methylene group, chirally labelled with deuterium or tritium, within the substrate is employed for the stereochemical

investigation of reactions involving hydrogen abstraction. The stereochemistry of these reactions is then determined by configurational assignment of the chirally labelled methylene group within the product or substrate, respectively.

The absolute configuration of isotopically engendered chiral methylene groups can be determined in several ways. These include chemical synthesis involving reactions of established or determinable stereochemistry and correlation with standards of known configuration.[59,72]

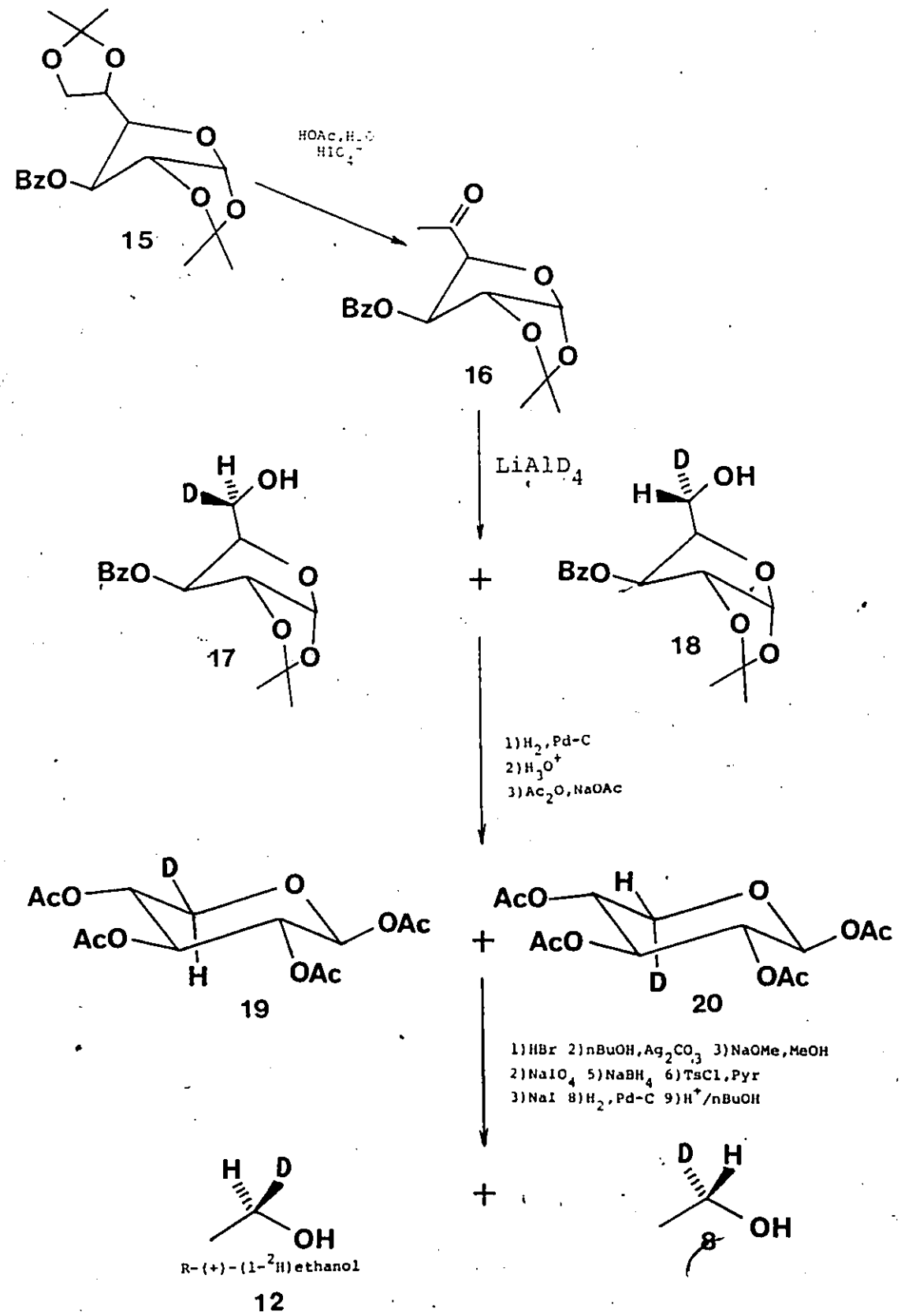
When the chirality of the methylene group is due to deuterium substitution, virtually complete deuterium enrichment can be attained. The absolute configuration of the chiral deuteriated centre can be determined by comparison with standards of known chirality by means of physicochemical methods[73] such as optical rotatory dispersion (ORD)[74] and nuclear magnetic resonance (NMR)[73-77] spectroscopy. Assignment of configuration can also be effected by enzymic methods,[72] which involve enzymes of known stereospecificity.

By contrast, when the chirality of the methylene group is due to tritium substitution, only a very small fraction of the molecules which comprise the labelled material will carry tritium. Since there is a risk of radiochemical hazard, this isotope is only employed at tracer levels.[78] Configurational analysis is achieved, in this case, by employing enzymic reactions of known stereochemistry.[59,72]

It was referred to earlier that yeast alcohol dehydrogenase mediates reversible, stereospecific hydrogen transfer from C-1 of ethanol to C-4 of NAD^+ (see Scheme 2). Loewus *et al.*[52] had employed deuterium to probe the steric course of this reaction and obtained

enantiomeric samples of (1-^{2}H)ethanol. Although the absolute configuration of the chiral samples of (1-^{2}H)ethanol was not known at that time, this was later established when a specimen of known configuration was obtained by unambiguous chemical synthesis.[55] The chirality of the samples of (1-^{2}H)ethanol, those of enzymic origin and that obtained by chemical synthesis, was linked by comparison of their optical activity.[54,55]

A sample of R-(+)-(1-^{2}H)ethanol (12) was prepared by Lemieux and Howard[55] as outlined in Scheme 3. The key step in this synthesis involved stereoselective reduction of the aldehyde (16) with lithium aluminium deuteride to yield, as the major product, the R-(5-^{2}H)xylofuranoside derivative (17). The chirality of the deuteriated methylene group was determined by ^{1}H NMR spectroscopy, following the conversion of the deuteriated alcohols, (17) and (18), into the corresponding β-D-(5-^{2}H)-xylopyranose tetraacetates, (19) and (20), respectively. The ^{1}H NMR signals due to the equatorial (*Re*-) and axial (*Si*-) hydrogens at C-5 of β-D-xylopyranose tetraacetate (unlabelled (19), conformation shown in Scheme 3) had earlier been assigned to a pair of doublets at 4.13 ($J_{4a,5e}$ 4.2 Hz) and 3.54 ($J_{4a,5a}$ 8.2 Hz) ppm, respectively.[79] When the deuteriated product ((19) + (20)) was examined,[55] the deuterium enrichment at the 5-*Re*-position (65%) was greater than that at the 5-*Si*-position (35%) indicating that the product was comprised of a 30% enantiomeric excess of the R-(5-^{2}H)-species (19). This result also indicated that reduction of (16) had occurred preferentially from the *Re*-face of the aldehyde. The mixture of (5-^{2}H)xylopyranose tetraacetates was then subjected to degradation, involving reactions which did not affect the chiral integrity of the deuteriated methylene group, to



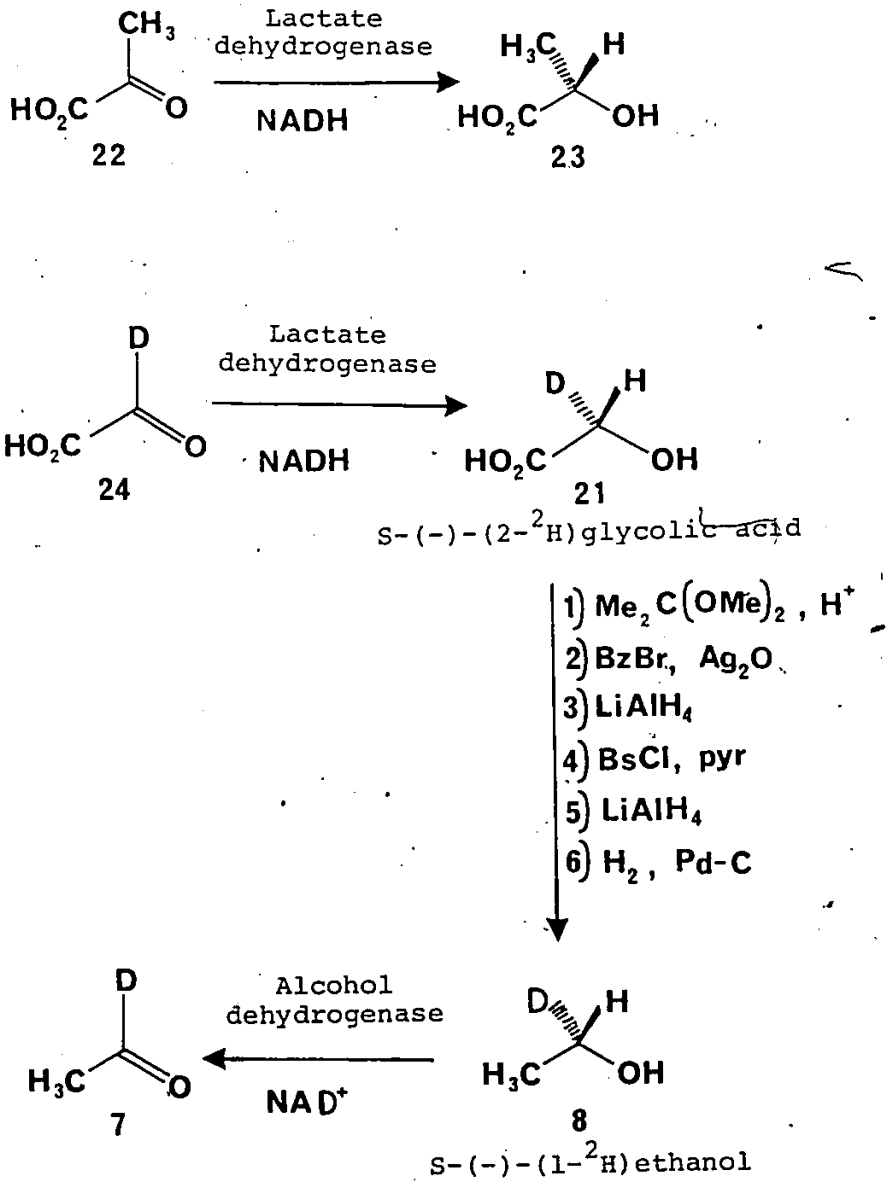
Scheme 3. Chemical synthesis of (+)-(1-^{2}H)ethanol (12).

afford a dextrorotatory specimen ($[\alpha]_{589}$ + 0.66°) of predominantly R-(1-^{2}H)ethanol (12) (Scheme 3).

The assignment of the R-configuration to dextrorotatory (1-^{2}H)-ethanol (12) provided clear evidence that the levorotatory specimen (8) obtained by enzymic reduction of (1-^{2}H)acetaldehyde (7) has the S-configuration and that alcohol dehydrogenase promotes stereospecific transfer of hydrogen from C-4 of NADH (9) to the *Re*-face of the aldehydic carbon of acetaldehyde to furnish the *Re*-hydrogen of ethanol (Scheme 2).

The configuration of levorotatory (1-^{2}H)ethanol (8) was subsequently confirmed by Weber and Arigoni[80] by correlation with S-(-)-(2-^{2}H)glycolic acid (21) as shown in Scheme 4. It had previously been shown that muscle lactic acid dehydrogenase (E.C. 1.1.1.27) which normally catalysed the reduction of pyruvic acid (22) to L-lactic acid (23) in the presence of NADH, acts upon (2-^{2}H)glyoxylic acid (24) to afford the levorotatory enantiomer of (2-^{2}H)glycolic acid (21)[81,82] ($[\alpha]_{250}$ - 27°).[83] The absolute configuration of (-)-(2-^{2}H)glycolate (21) was unequivocally established by neutron diffraction techniques[82] and, thus serves as a primary configurational standard.[59,72]

Reduction of S-(-)-(1-^{2}H)glycolic acid (21) gave S-(1-^{2}H)-ethanol (8) (Scheme 4). This specimen of S-(1-^{2}H)ethanol (8) on treatment with yeast alcohol dehydrogenase in the presence of NAD^+ gave (1-^{2}H)acetaldehyde (7), establishing its identity with the levorotatory specimen which had previously been obtained by the reverse process[54] (*cf.* Scheme 2).

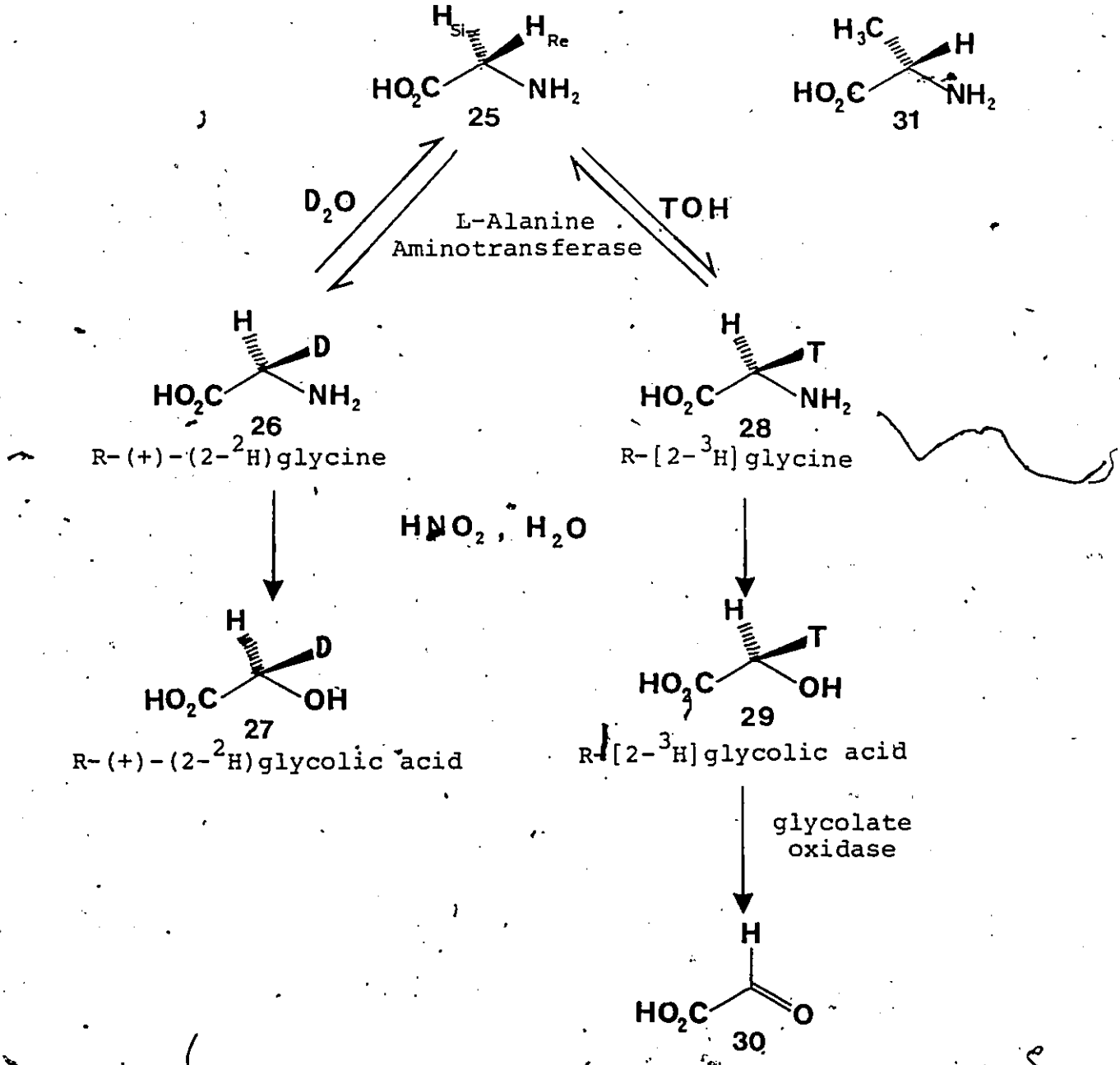


Scheme 4. Stereochemical correlation of (-)-(1-^{2}H)ethanol (8) with S-(2-^{2}H)glycolic acid (21).

In the present context, chiral (1-^{2}H)amines, in which one or the other of the two enantiotopic hydrogens at the prochiral methylene group adjacent to nitrogen is replaced by deuterium, are obtained by enzymic decarboxylation of the corresponding L-α-amino acids (Chapter 2). As will be seen in Chapters 3 and 4, the configurational analysis of the enantiomeric (1-^{2}H)amines ultimately rests on stereochemical correlation with [2-^{3}H]- or (2-^{2}H)glycine of known absolute configuration.

Chiral samples of (2-^{2}H)- and [2-^{3}H]glycine have been prepared by enzymic reactions and by chemical synthesis. The chirality, as well as the configurational purity, of stereospecifically labelled glycines was determined by several methods. One of these methods involved the direct correlation of the configuration of a specimen of (2-^{2}H)glycine with that of the primary configurational standard, R-(2-^{2}H)glycolic acid (27) (Scheme 5).[84-86]

Besmer and Arigoni[84,85] obtained an enantiomeric sample of (2-^{2}H)glycine (26) by incubation of unlabelled glycine (25) with L-alanine aminotransferase (E.C. 2.6.1.2) in deuterium oxide. This preparation took advantage of the observation that L-alanine aminotransferase, which normally catalyses the transfer of the amino group from L-alanine to α-ketoglutaric acid, mediates stereospecific exchange of hydrogen from C-2 of glycine with the medium.[86,87] The sample of (2-^{2}H)-glycine (26) was optically active and showed a plane positive ORD curve ($[\alpha]_{238}$ + 36.7°).[85] On treatment with nitrous acid (+)-(2-^{2}H)glycine (26) gave (+)-(2-^{2}H)glycolic acid (27) which was determined to be the R-enantiomer by comparison of its ORD curve with that of an authentic specimen. Based on the well grounded assumption that the nitrous acid



Scheme 5. Stereochemical correlation of (+)-(2-^{2}H)glycine (26) with R-(2-^{2}H)glycolic acid (27) and of 2-^{3}H glycine (28) with R-[2-^{3}H]glycolic acid (29).

deamination reaction occurs with retention of configuration,[86,88,89] the dextrorotatory sample of (2-^{2}H)glycine (26) was assigned the R-configuration.

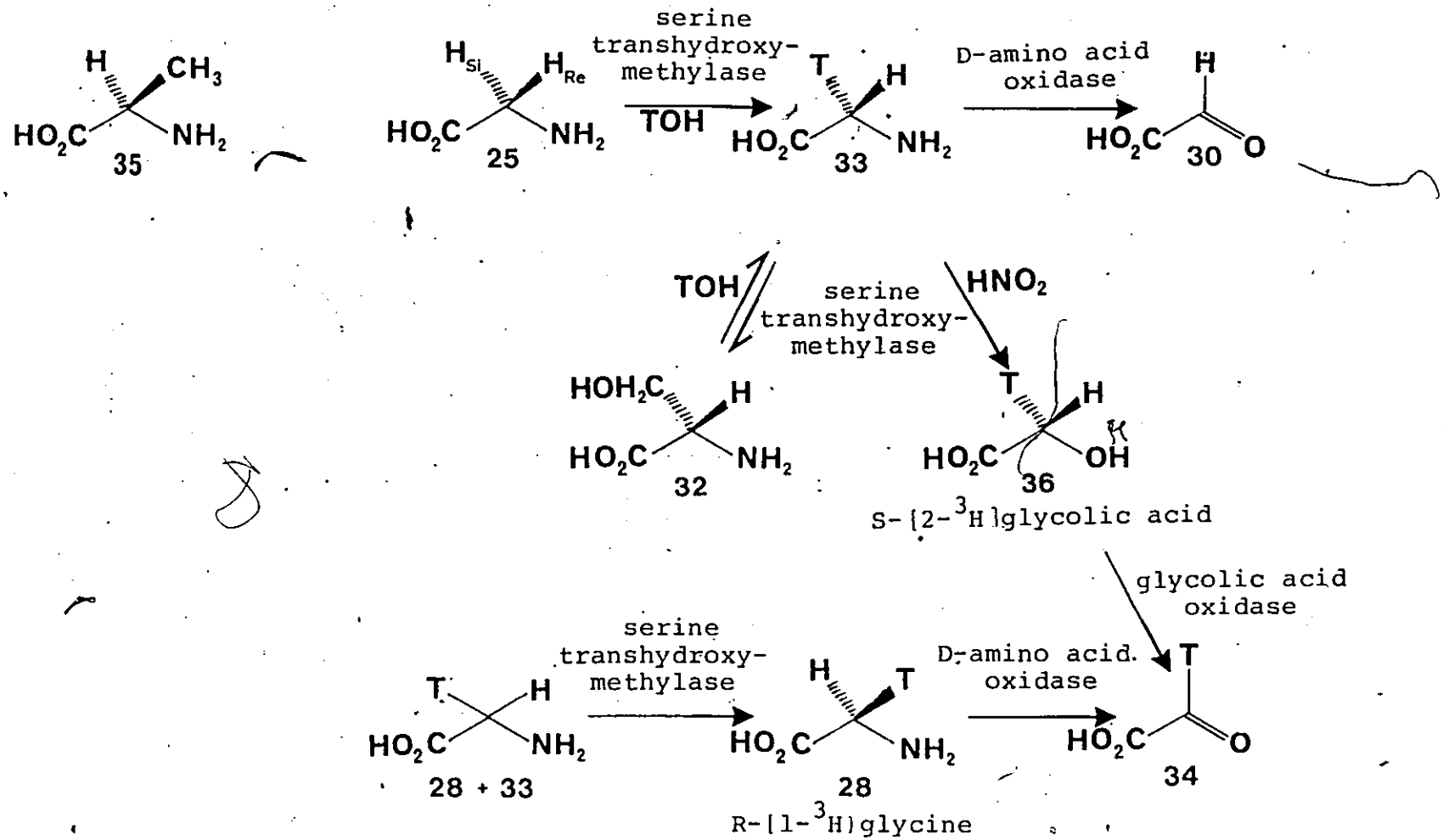
The assignment of the R-configuration to (+)-(2-^{2}H)glycine served to establish that L-alanine aminotransferase promotes stereospecific labilisation of the *Re*-hydrogen of glycine. The steric environment of the *Re*-hydrogen of glycine (25) corresponds to that of the α-hydrogen of L-alanine (31), which is lost in the course of the normal enzymic reaction (*cf.* ref. 87).

A method for the stereochemical analysis of [2-^{3}H]glycines was based on this correlation, and involved enzymic assay with glycolic acid oxidase (E.C. 1.1.1.26).[85,86]

A specimen of R-[2-^{3}H]glycine (28) was obtained by the action of L-alanine aminotransferase on unlabelled glycine (25) in tritiated water, which was then converted, as before, by treatment with nitrous acid, into R-[2-^{3}H]glycolic acid (29) (Scheme 5).[86] Previous work by Rose and his colleagues[81,82] had demonstrated that the glycolic acid oxidase catalysed oxidation of glycolic acid to glyoxylic acid (30) takes place with stereospecific removal of the *Re*-hydrogen. When the specimen of tritiated glycolate (29) was treated with this enzyme, complete loss of label occurred in the course of the oxidation, to yield unlabelled glyoxylic acid (30). The result served to confirm the configurational assignment of the R-[2-^{3}H]glycolic acid (29) as well as that of the R-[2-^{3}H]glycine (28).

The configurational assignment of samples of tritiated glycine has been determined by direct enzymic assay with D-amino acid oxidase (E.C. 1.4.3.3).[87,90,91] D-Amino acid oxidase catalyses the oxidative deamination of a wide range of D-amino acids (but not L-amino acids),[92] as well as of glycine,[93] to the corresponding 2-oxoacids. This enzymic reaction was first employed by Akhtar and Jordan[90] to assign absolute configuration to enantiomeric samples of [2-^{3}H]glycine which were obtained by an exchange reaction, catalysed by serine transhydroxymethylase (E.C. 2.1.2.1) (Scheme 6).

The serine transhydroxymethylase catalysed reaction involves the reversible transformation of glycine (25) and formaldehyde into L-serine (32).[94] In the absence of formaldehyde, the enzyme catalyses exchange with the medium of one of the hydrogens from C-2 of glycine.[95] Employing the latter conditions, Akhtar and Jordan[90,91] prepared samples of the two enantiomers of [2-^{3}H]glycine. One of the enantiomers (33) was obtained from glycine (25) by incubation in tritiated water; the other (28) was obtained from racemic [2-^{3}H]glycine by incubation in unlabelled water. The two enantiomers of [2-^{3}H]glycine were then treated with D-amino acid oxidase. Stereospecific loss of tritium occurred in the course of the oxidation of the enantiomer (33) to yield unlabelled glyoxylic acid (30), whereas oxidation of the enantiomer (28) led to complete retention of tritium in the product, [1-^{3}H]glyoxylic acid (34). The steric course of the oxidation of glycine (25) to glyoxylic acid (30) mediated by D-amino acid oxidase had not been determined. Akhtar and Jordan[90] assumed, on the basis of a comparison of the relative steric environments of the two enantiotopic hydrogens of glycine (25) with that



Scheme 6. Stereospecificities of D-amino acid oxidase and serine transhydroxymethylase.

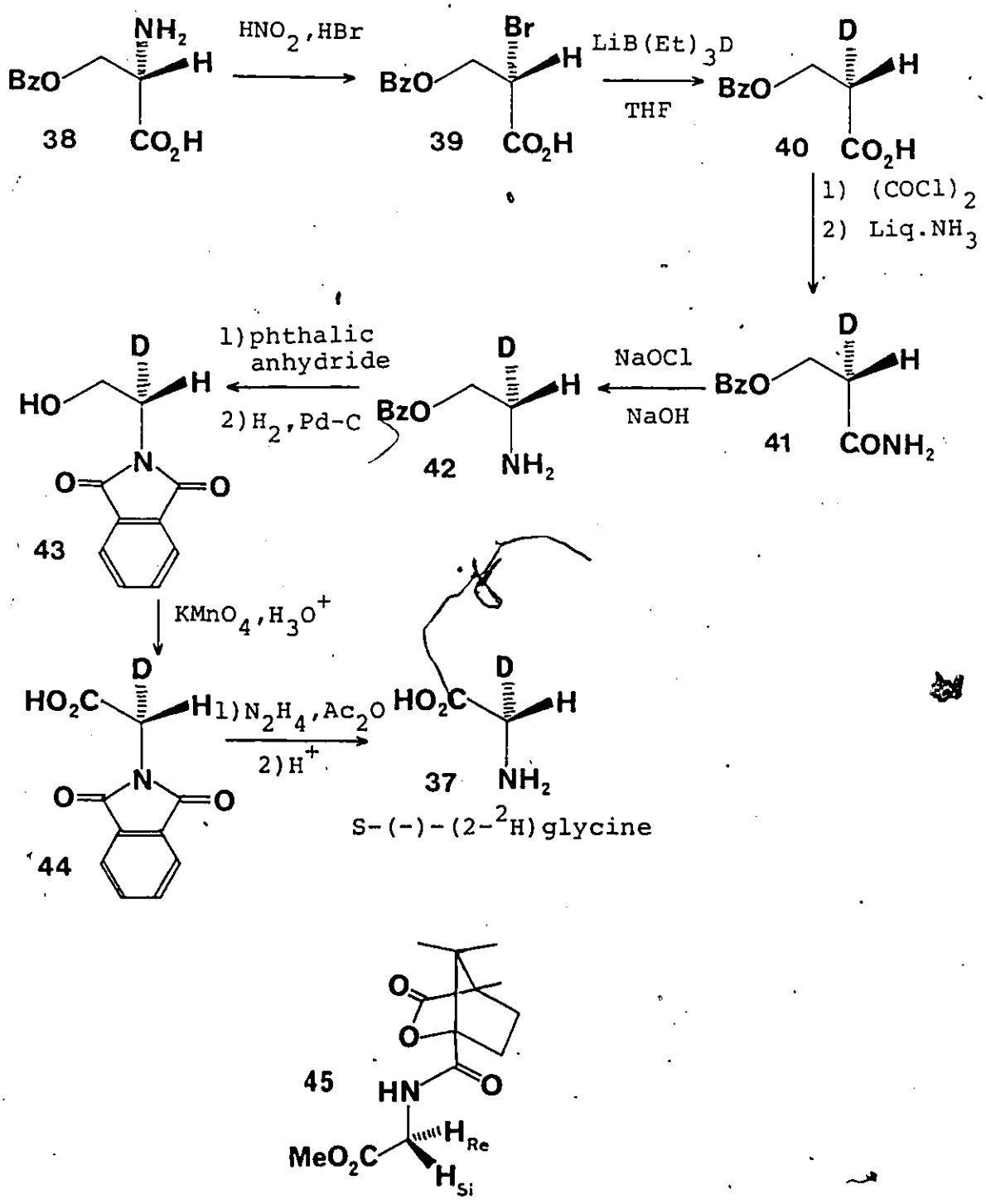
of the α-hydrogen of D-alanine (35), that the reaction would involve stereospecific removal of the *Si*-hydrogen. Based on this assumption the two enantiomers of [2-^{3}H]glycine, (33) and (28), were assigned the S- and the R-configuration, respectively (Scheme 6).

The stereospecificity of D-amino acid oxidase is verified by experiments carried out by Wellner[87] and Besmer and Arigoni.[86]

Wellner[87] prepared a sample of [2-^{3}H]glycine (33) by the action of serine transhydroxymethylase on L-serine (32) in tritiated water. Employing D-amino acid oxidase, the chirality of this sample was found to be the same as that of [2-^{3}H]glycine (33), obtained similarly from unlabelled glycine (25), *via* exchange: oxidation of both samples gave unlabelled glyoxylic acid (30). In an independent investigation, the sample of [2-^{3}H]glycine (33) derived from L-serine (32) was correlated with S-[2-^{3}H]glycolic acid (36).[86] Nitrous acid deamination of the [2-^{3}H]glycine (33) gave a sample of [2-^{3}H]glycolate (36) which, on treatment with glycolic acid oxidase, afforded [2-^{3}H]glyoxylic acid (34). Since oxidation of glycolic acid, catalysed by glycolic acid oxidase, involves stereospecific removal of the *Re*-hydrogen,[81,82] and reaction with nitrous acid takes place with retention of configuration, it follows that the sample of [2-^{3}H]glycine (33) has the S-configuration. These stereochemical correlations (summarised in Scheme 6) not only provide firm evidence that oxidation of glycine, catalysed by D-amino acid oxidase, is accompanied by stereospecific loss of the *Si*-hydrogen, but also demonstrate that serine transhydroxymethylase mediates the stereospecific release of the *Si*-hydrogen from C-2 of glycine.

More recently an entirely chemical synthesis of R- and S-(2-^{2}H)-glycine of very high configurational purity was reported by Armarego *et al.*[96] The synthetic sequence employed for the preparation of S-(-)-(2-^{2}H)glycine (37) is shown in Scheme 7. S-*O*-Benzylserine (38) was converted into the S-bromide (39) by treatment with nitrous acid in the presence of hydrobromic acid. Reductive debromination of the bromide (39) with lithium triethyl borodeuteride led to the stereospecific introduction of deuterium to give S-(-)-3-benzyloxy(2-^{2}H)propionic acid (40). The stereochemistry of both these reactions was confirmed in separate experiments. Most interesting is the reductive debromination step which occurred with retention of configuration. This was established by chemical correlation of the (-)-3-benzyloxy(2-^{2}H)propionic acid (40) with S-(+)-(2-^{2}H)propionic acid of known configuration.[97,98] The acid (40) was then converted into the amide (41) and, following a Hoffmann rearrangement which occurred with retention of configuration, S-(+)-2-benzyloxy(1-^{2}H)ethylamine (42) was obtained. The S-(1-^{2}H)amine (42) gave, by reactions not involving the chiral deuteriated centre, S-(-)-(2-^{2}H)glycine (37) ($[\alpha]_{238}$ - 35.0°).[96]

The enantiomeric purity of the sample of S-(2-^{2}H)glycine (37) was established by ^{1}H NMR spectroscopy. The method involved prior conversion of glycine into the methyl camphanoylamide derivative (45), in which the enantiotopic hydrogens at C-2 of glycine are rendered diastereotopic. In the presence of the shift reagent, Eu(dpm)$_3$, the ^{1}H NMR signal (in C^2HCl_3) due to the *Re*-hydrogen occurred at higher field than that due to the *Si*-hydrogen.



Scheme 7. Chemical synthesis of S-(-)-(2-^{2}H)glycine (37).

This method was first employed by Gerlach and Zagalak[75] to distinguish between the enantiotopic hydrogens at C-3 of an achiral primary alcohol by ^1H NMR spectroscopy. In this investigation the ^1H NMR spectra (in CCl_4) of the camphanate esters of several chiral primary (1-^2H)-alcohols of known configuration were examined in the presence of $Eu(dpm)_3$. A consistent feature among all the camphanate esters examined was the observation that the chemical shift corresponding to the *Re*-hydrogen occurs at higher field than that corresponding to the *Si*-hydrogen. Such consistency is also observed for the methylene hydrogens adjacent to nitrogen in the camphanoylamide derivative of methyl 4-aminobutyrate,[99] as well as in the dicamphanoylamide derivative of cadaverine.[100] As suggested by Gerlach and Zagalak,[75] the consistent occurrence of the *Re*-hydrogen at higher field probably arises because the derivatives adopt similar conformations in the complex with $Eu(dpm)_3$. A recent report, however, indicates that, in the absence of a shift reagent, the chemical shift of the 3-*Si*-hydrogen of 3-camphanoylamidopropionic acid occurs at higher field than the 3-*Re*-hydrogen.[101]

1.4 STEREOCHEMISTRY OF ENZYMIC DECARBOXYLATION

The L-amino acid decarboxylases are a widely occurring group of enzymes which catalyse the decarboxylation of L-α-amino acids into the corresponding primary amines.[13] These enzymes exhibit a very high degree of substrate specificity for the individual amino acids.

In the early 1940s, Gale and his co-workers[102] demonstrated the occurrence of six inducible α-decarboxylases in cell-free extracts of *Escherichia coli* and showed that the reactions catalysed by several of

these enzymes are pyridoxal phosphate dependent. Distinct bacterial enzymes from *E. coli*, as well as from other bacteria, which mediate the specific decarboxylation of tyrosine, lysine, glutamic acid, histidine, arginine and ornithine were characterised.[102] Later investigations revealed other specific amino acid decarboxylases in several species of bacteria.[13] Included among the substrates for which these α-decarboxylases are specific were L-*S*-adenosylmethionine[103,104] and *meso*-2,6-diaminopimelic acid.[105] The enzyme specific for the latter substrate acts only at the α-carbon of diaminopimelate possessing D-(*i.e.* R-) chirality.[106] In addition to the inducible bacterial L-ornithine and L-arginine decarboxylases,[13] distinct, constitutive (biosynthetic) enzymes[107,108] which catalyse the same reactions have also been isolated and characterised. The inducible and constitutive enzymes are similar, in that they both require pyridoxal phosphate for catalytic activity; but they differ in other catalytic properties, such as the optimum pH of reaction.[109,110]

Decarboxylase activity is also detected in plants and mammalian tissue.[13,111,112] Although few plant enzymes have been isolated, several mammalian L-α-amino acid decarboxylases, specific for glutamic acid,[113] ornithine[114,115] and *S*-adenosylmethionine[115] have been obtained in essentially pure form. An aromatic L-amino acid decarboxylase which catalyses the decarboxylation of L-tyrosine, L-tryptophan and L-phenylalanine as well as other aromatic amino acids has been isolated from mammalian tissue;[116] the enzyme from hog kidney has been purified to homogeneity.[117]

It was first demonstrated[118] almost thirty years ago that the reaction catalysed by three of these enzymes (L-tyrosine decarboxylase (E.C. 4.1.1.25) from *Streptococcus faecalis*; L-lysine decarboxylase (E.C. 4.1.1.18) from *Bacillus cadaveris*; L-glutamate decarboxylase from *Clostridium welchii* and from *E. coli*) takes a stereochemically defined course. Mandeles, Koppelman and Hanke[118] showed that the reaction mediated by these bacterial α-decarboxylases in deuterium oxide leads to the introduction of a single solvent deuterium atom at the prochiral centre adjacent to nitrogen in the respective primary amine products. When one of the deuteriated amines, 4-amino(4-^{2}H)butyric acid (46), the product obtained by decarboxylation of L-glutamic (47) acid in ^2H_2O, was reincubated with L-glutamate decarboxylase in ^1H_2O, complete loss of deuterium was observed in the reisolated amine. In another experiment a sample of 4-amino(4-^{2}H)butyric acid (48) was obtained from L-(2-^{2}H)glutamic acid (49) by enzymic decarboxylation in ^1H_2O: the integrity of the deuterium label in this sample was maintained, even after prolonged incubation with the decarboxylase. It was concluded from these experiments that "...the monodeuterioamine which is formed by these actions [enzymic decarboxylation of either the L-amino acid in ^2H_2O or the L-(2-^{2}H)amino acid in ^1H_2O] is one of a pair of enantiomorphs."

Later, the actual stereochemical course of these reactions was determined. The conversions of L-tyrosine into tyramine,[119] of L-lysine into cadaverine,[120] and of L-glutamic acid into 4-aminobutyric acid,[121] each catalysed by the appropriate enzyme, all take place with net retention of configuration, as shown by means of isotopic labelling with deuterium or tritium. Thus, decarboxylation of L-tyrosine (50) in ^2H_2O

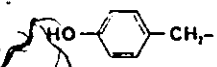
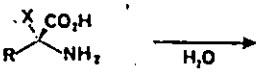
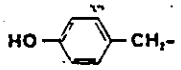
yields R-(1-^{2}H)tyramine (51),[119,122] and of L-glutamic acid (47) in ^2H_2O yields R-4-amino(4-^{2}H)butyric acid (46).[121] Similarly, decarboxylation of L-[2-^{3}H]lysine (52) in water generates S-[1-^{3}H]cadaverine (53), whereas L-lysine (54) in tritiated water yields R-[1-^{3}H]cadaverine (55)[120] (Table 1). The chirality of the products was determined by kinetic,[119] enzymic[120] or chemical[121] correlation with compounds of known stereochemistry.

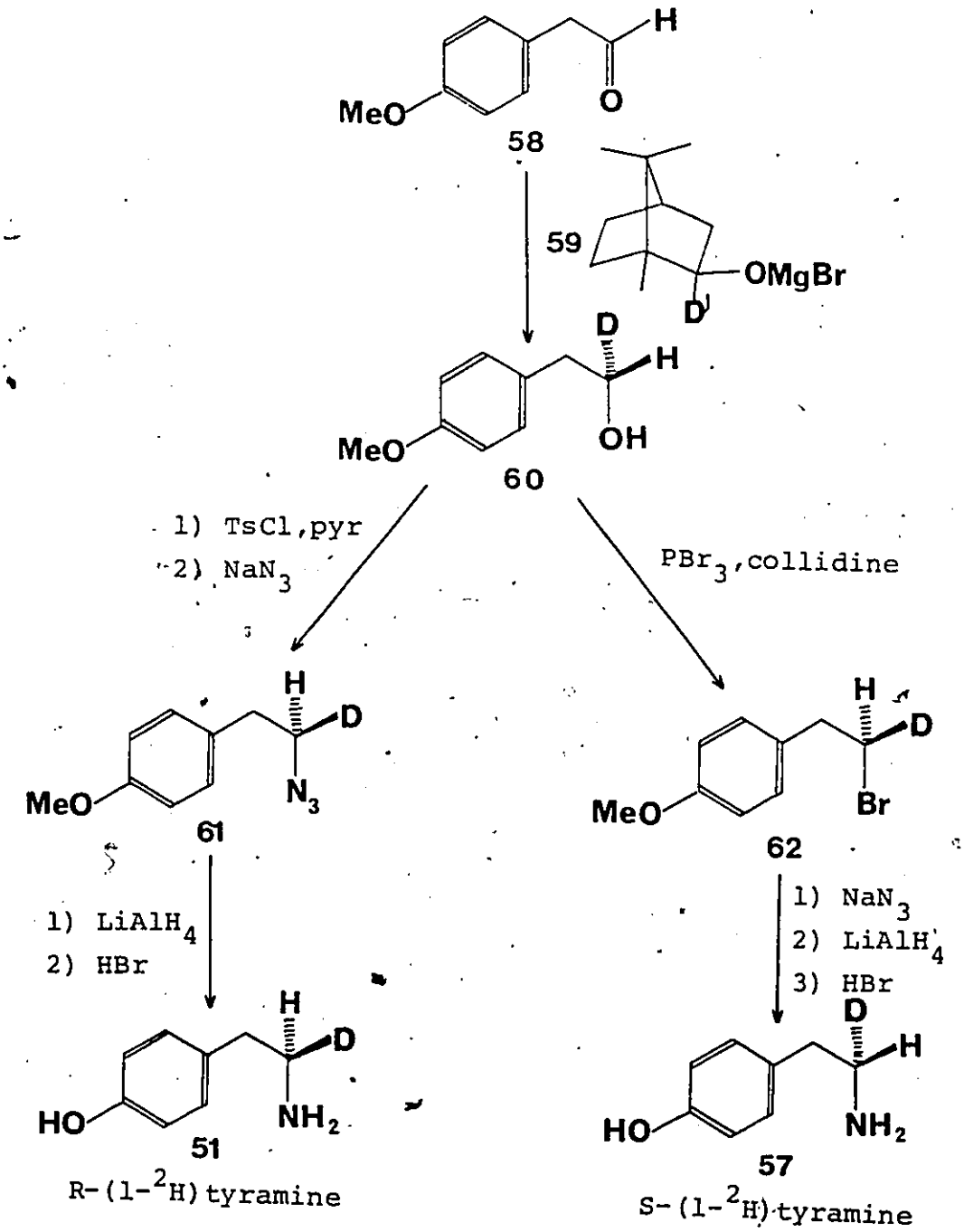
The absolute configuration of the samples of (1-^{2}H)tyramine obtained by enzymic decarboxylation was determined by comparison of the relative rates of enzymic oxidation, mediated by rat liver mitochondrial monoamine oxidase (E.C. 1.4.3.4), with synthetic standards of known configuration.[119,122]

Belleau and Burba[119] prepared R- and S-(1-^{2}H)tyramine by the synthetic sequence outlined in Scheme 8. The key step in this reaction scheme was the stereoselective Meerwein-Pondorff type reduction of *p*-methoxyphenylacetaldehyde (58) with (2-^{2}H)isobornyloxymagnesium bromide (59) to afford S-(-)-2-*p*-methoxyphenyl(1-^{2}H)ethanol (60) ($[\alpha]_{589}$ - 1.44°). This method of reduction had previously been employed by Streitwieser and his colleagues for the preparation of chiral specimens of (1-^{2}H)-benzyl alcohol[123] and (1-^{2}H)butanol.[124] Consideration of the two possible product determining transition states for reduction had allowed assignment of configuration of these products, based on the assumption that the transition state of least steric crowding would be preferred.[125]

The S-(1-^{2}H)alcohol (60) was employed to obtain both enantiomers of (1-^{2}H)tyramine. Treatment of the toluene sulfonate ester of (60) with sodium azide, under conditions where complete inversion of

Table 1. Chiral (1-^{2}H)- or [1-^{3}H]amines derived from L-tyrosine, L-lysine and L-glutamic acid by enzymic decarboxylation.

Enzyme	R	X	Amino acid	Chirally labelled amine	Ref.
					
L-tyrosine decarboxylase		D	L-tyrosine (50)	R-(1-^{2}H)tyramine (51)	119
L-lysine decarboxylase	$H_2NCH_2(CH_2)_2CH_2-$	T	L-lysine (54)	R-[1-^{3}H]cadaverine (55)	120
L-glutamate decarboxylase	$HO_2CCH_2CH_2-$	D	L-glutamic acid (47)	R-4-amino(4-^{2}H)-butyric acid (46)	121
					
L-tyrosine decarboxylase		D	L-(2-^{2}H)tyrosine (56)	S-(1-^{2}H)tyramine (57)	119
L-lysine decarboxylase	$H_2NCH_2(CH_2)_2CH_2-$	T	L-[2-^{3}H]lysine (52)	S-[1-^{3}H]cadaverine (53)	120
L-glutamate decarboxylase	$HO_2CCH_2CH_2-$	D	L-(2-^{2}H)glutamic acid (49)	S-4-amino(4-^{2}H)-butyric acid (48)	118 (*cf.* 121)



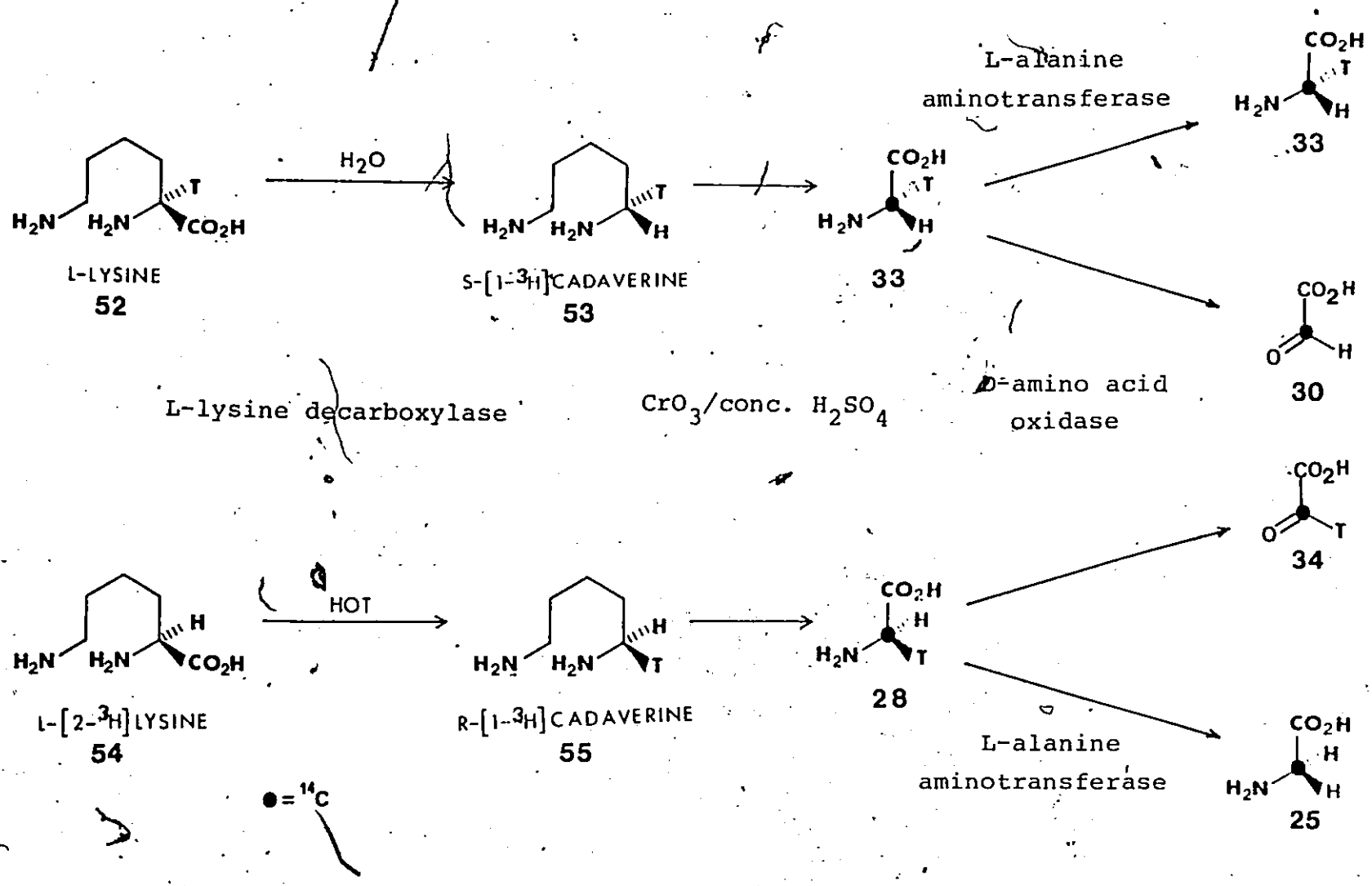
Scheme 8. Synthesis of the enantiomers of (1-^{2}H)tyramine.

configuration had been demonstrated in other similar reactions,[126] afforded the R-(1-^{2}H)azide (61) which, following reduction and demethylation, gave R-(1-^{2}H)tyramine (51). Conversion of the S-(1-^{2}H)alcohol (60) into the bromide (62) of opposite configuration, by treatment with phosphorus tribromide under S_N2 conditions,[127,128] followed by the azide displacement reaction, gave S-(1-^{2}H)tyramine (57).

When the two synthetic samples of (1-^{2}H)tyramine were subjected to enzymic oxidation, catalysed by the rat liver monoamine oxidase, the rate of oxidation of S-(1-^{2}H)tyramine (57) was approximately twice that of R-(1-^{2}H)tyramine (52).[119]

The mechanism of the reaction involving nucleophilic displacement of the tosyl group by azide ion, employed by Belleau and Burba[119] for the preparation of (61) (Scheme 8), has recently been investigated.[129] Apart from direct S_N2 displacement, aryl participation was found[129] to make a significant contribution (~30%) to the outcome of the reaction. Although this finding does not affect the overall configurational assignments of the synthetic samples of (1-^{2}H)tyramine prepared by Belleau and Burba,[119] it does indicate that they were of low configurational and isotopic purity.

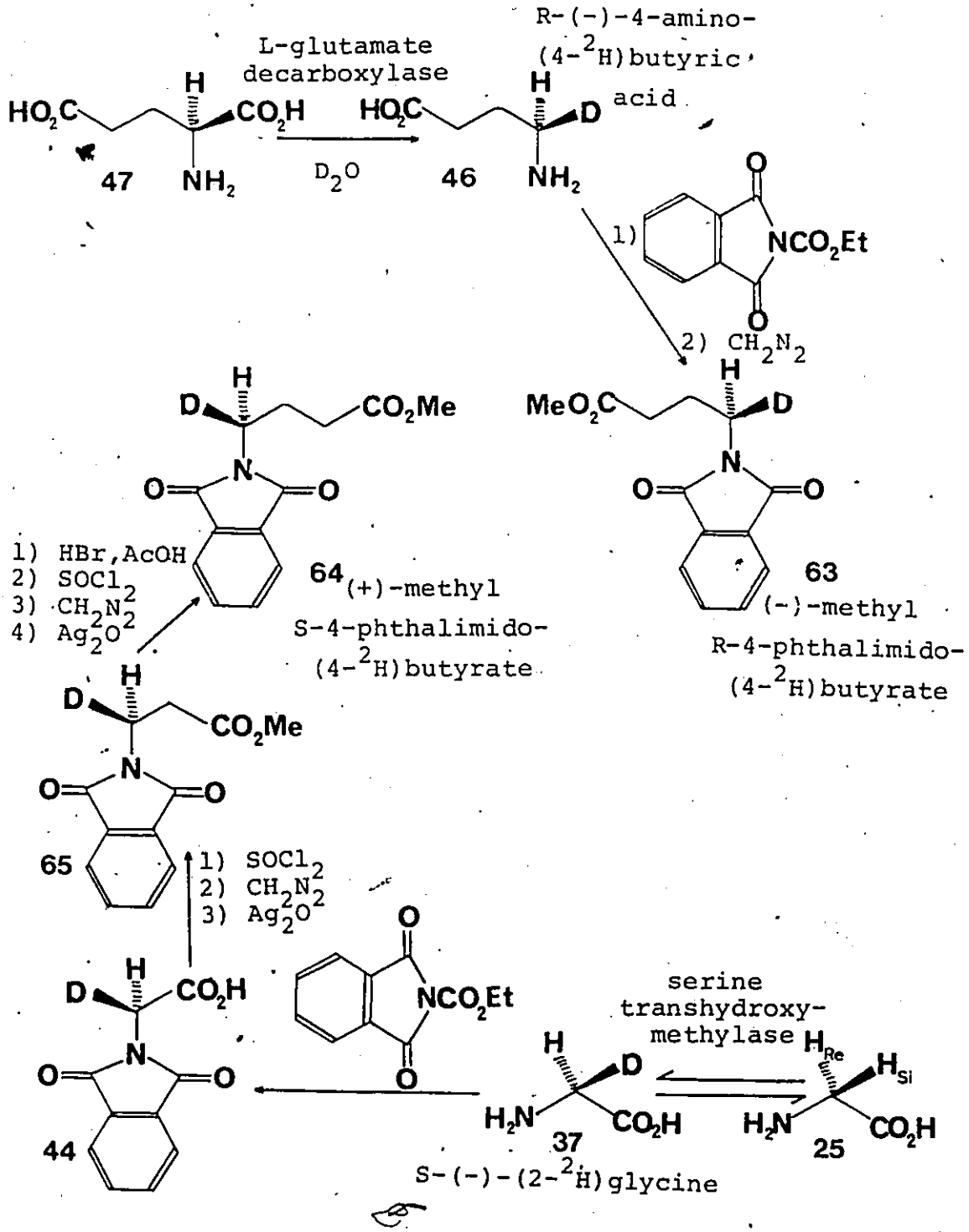
The stereochemistry of the reaction catalysed by L-lysine decarboxylase was determined by radiochemical tracer methods. As outlined in Scheme 9, Leistner and Spenser[120] determined the chirality of the two enantiomers of [1-^{3}H]cadaverine, that (53) obtained by decarboxylation of L-[2-^{3}H]lysine (52), and that (55) obtained by decarboxylation of L-lysine (54) in the presence of tritiated water, by stereochemical correlation with the corresponding enantiomers of [2-^{3}H]glycine. This was



Scheme 9. Stereochemical correlation of the enantiomers of [1-^{3}H] cadaverine with the corresponding enantiomers of [2-^{3}H] glycine.

achieved by chromic acid oxidation, under conditions which did not lead to significant exchange of label with the medium. By taking advantage of the known opposed stereospecificities of D-amino acid oxidase and L-alanine aminotransferase, referred to earlier (Section 1.3), the resulting specimens of [2-^{3}H]glycine were unambiguously assigned the S- and R-configuration, respectively. Thus, when each of the two specimens of [2-^{3}H]glycine was treated, together with [2-^{14}C]glycine as internal standard, with D-amino acid oxidase, the ^{3}H/^{14}C ratio was maintained in the oxidation product (34) derived from the R-[2-^{3}H, 2-^{14}C]glycine (28), whereas almost all the tritium, relative to carbon-14, was absent in the product (30) derived from the S-[2-^{3}H, 2-^{14}C]glycine (33). Conversely, incubation of the two intermolecularly doubly labelled samples with L-alanine amino transferase, in the absence of an amino group acceptor, resulted in almost complete loss of tritium relative to carbon-14 from the R-[2-^{3}H, 2-^{14}C]glycine (28), but not from S-[2-^{3}H, 2-^{14}C]glycine (33).

The absolute configuration of 4-amino(4-^{2}H)butyric acid (46) was determined by correlation with that of the R-enantiomer of (2-^{2}H)glycine. The stereochemical correlation employed by Yamada and O'Leary[121] is outlined in Scheme 10. Levorotatory 4-amino(4-^{2}H)butyric acid (46) obtained from L-glutamic acid (47) by enzymic decarboxylation in deuterium oxide was converted into (-)-methyl-4-phthalimido(4-^{2}H)butyrate (63). The specific optical rotation of this sample ($[\alpha]_{340}$ - 6.23°) was compared directly with that of an authentic sample of (+)-methyl S-4-phthalimido(4-^{2}H)butyrate (64) ($[\alpha]_{340}$ + 6.23°) which had been synthesised from S-(2-^{2}H)glycine. Since it was found that the specific rotations

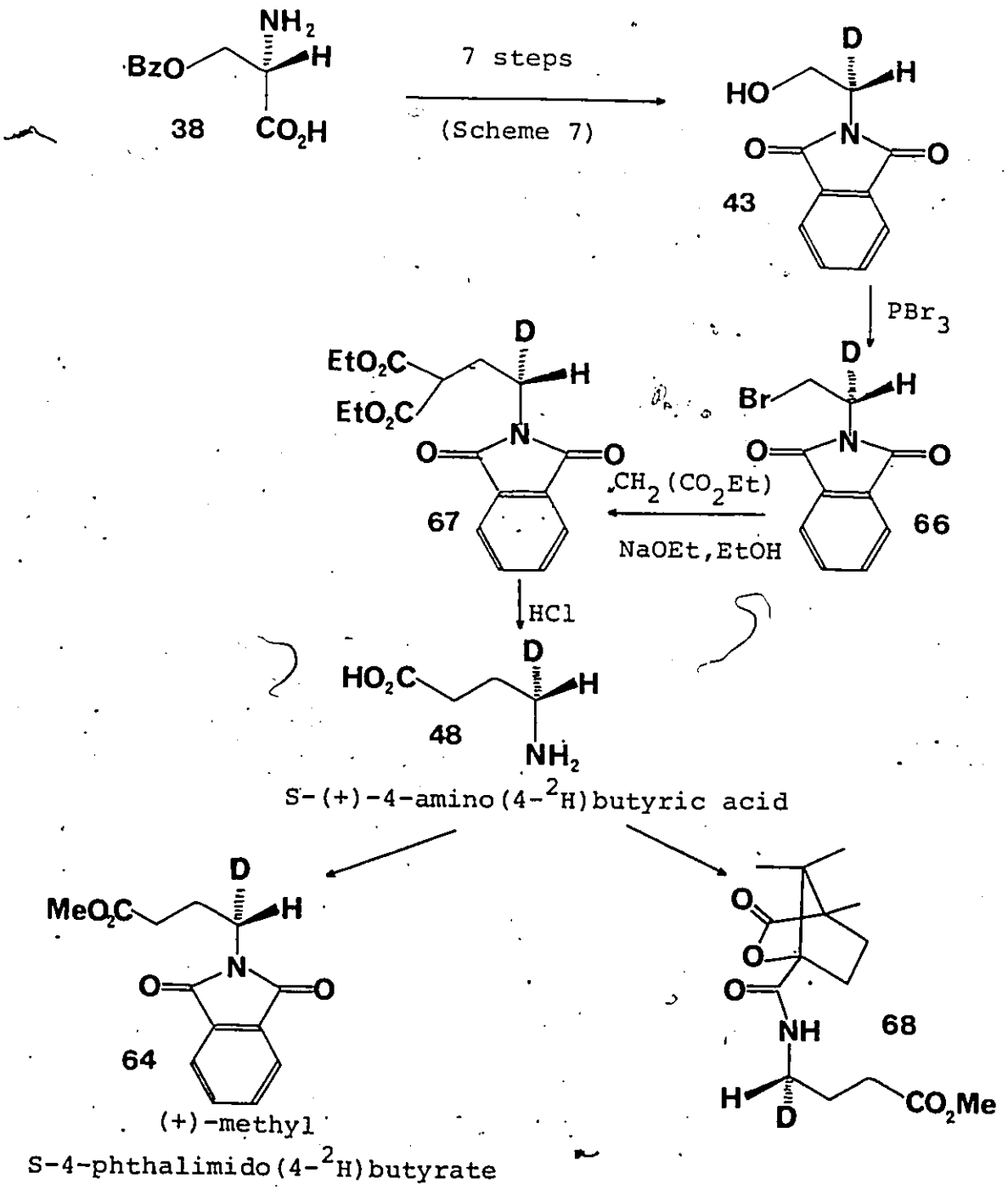


Scheme 10. Stereochemical correlation of 4-amino(4-^{2}H)-butyric acid (46) with S-(2-^{2}H)glycine (37).

of the two samples were of equal magnitude but of opposite sign, it followed that the two samples were enantiomeric, that the sample (63) and therefore also (-)-4-amino(4-^{2}H)butyric acid (46) possessed the R-configuration.

The S-enantiomer of (2-^{2}H)glycine was prepared by stereospecific exchange of the *Si*-hydrogen of glycine, mediated by serine transhydroxymethylase, in deuterium oxide (*cf.* Scheme 6). The phthalimido derivative (44) was then converted by means of a double Wolff rearrangement, *via* (+)-methyl S-3-phthalimido(3-^{2}H)propionate (65), into the (+)-methyl S-4-phthalimido(4-^{2}H)butyrate (64). The Wolff rearrangement is expected to occur with retention of configuration,[130] even at a migrating primary carbon,[131] which allowed assignment of configuration to the butyrate (64) based on the known configuration of S-(2-^{2}H)glycine.[85-87,90,91]

Methyl S-4-phthalimido(4-^{2}H)butyrate (64) has recently been synthesised by an independent method (Scheme 11). Santaniello and coworkers[99] prepared S-(+)-2-phthalimido(2-^{2}H)ethanol (43) in seven steps from L-*O*-benzylserine (38) following the procedure which had been employed by Armarego *et al.*[96] for the synthesis of S-(2-^{2}H)glycine (37) (Scheme 7). By reactions not involving the chiral deuteriated centre, chain extention was effected by addition of diethyl malonate to the bromide (66) and, following hydrolysis, S-(-)-4-amino(4-^{2}H)butyric acid (48) was obtained. This was then converted into methyl S-4-phthalimido-(4-^{2}H)butyrate (64) which showed a specific optical rotation at the sodium-D line which was identical with that of the sample obtained by Yamada and O'Leary.[121] The configurational purity of S-4-amino(4-^{2}H)-butyric acid (48) was determined from the ^{1}H NMR spectrum of the methyl



Scheme 11. Chemical synthesis of S-(+)-4-amino(4-^{2}H)butyric acid (48).

camphanoylamide derivative (68) in the presence of $Eu(dpm)_3$, which showed the absence of the signal corresponding to the *Si*-hydrogen at the methylene group adjacent to nitrogen. Employing this method of configurational analysis, Santaniello *et al.*[99] confirmed that decarboxylation of L-glutamic acid, catalysed by L-glutamate decarboxylase of *E. coli*, to afford 4-aminobutyric acid takes place with net retention of configuration. Further confirmation that this reaction occurs with net retention of configuration was very recently obtained by a radiochemical tracer method.[132] The reaction catalysed by L-glutamate decarboxylase from a mammalian source (rat brain) has also been shown to take a similar steric course.[133]

Net retention of configuration in the decarboxylation of L-tyrosine[129] and of L-lysine,[134,135,100] catalysed by bacterial enzymes, was later confirmed by independent methods and was found also in the reactions catalysed by enzymes in plant systems.[129]

The stereochemistry of the reactions catalysed by several other L-amino acid α-decarboxylases has been investigated quite recently. Net retention was demonstrated also in the decarboxylation of L-histidine[136,137] and of L-*S*-adenosylmethionine,[138] catalysed by bacterial enzymes, L-histidine decarboxylase (E.C. 4.1.1.22) from *Lactobacillus* 30a and from *Clostridium welchii*, and *S*-adenosylmethionine decarboxylase from *E. coli*, respectively.

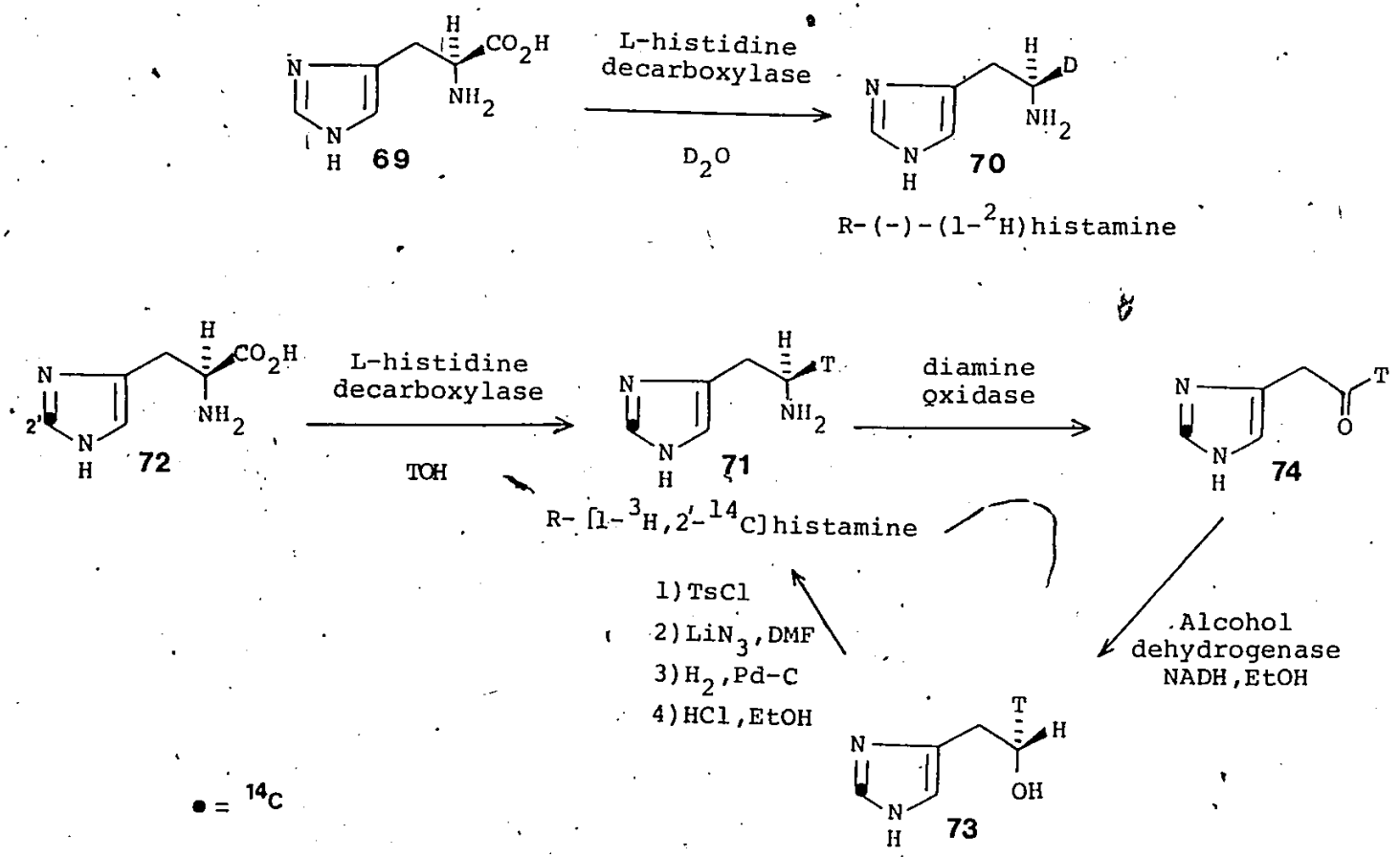
The stereochemistry of the decarboxylation of L-histidine (69) catalysed by the bacterial enzyme from *Lactobacillus* 30a was first investigated by Chang and Snell[139] in 1968. By carrying out the reaction in deuterium oxide, a levorotatory specimen of (1-^{2}H)histamine (70) was

obtained, which, by comparison of the sign of the optical rotation with that of other, similar deuteriated compounds (*i.e.* (-)-(1-^{2}H)butylamine), was tentatively assigned the R-configuration. Later, the stereochemical course of this reaction was firmly established by radiochemical tracer techniques (Scheme 12),[136] and by correlation of (-)-(1-^{2}H)histamine (70) with R-(1-^{2}H)tyramine (51).[137]

Battersby and his co-workers[136] employed tritium as the chiral marker to obtain R-[1-^{3}H, 2'-^{14}C]histamine (71) from L-[2'-^{14}C]histidine (72) by enzymic decarboxylation in tritiated water. Similarly, S-[1-^{3}H, 2'-^{14}C]histamine was obtained from L-[2-^{3}H, 2'-^{14}C]histidine in unlabelled water. The absolute configuration of the [1-^{3}H]histamines was determined by a further enzymic reaction.

Other experiments had shown that the diamine oxidase (E.C. 1.4.3.6) from pea seedlings mediates stereospecific removal of the *Si*-hydrogen from the methylene group adjacent to nitrogen in the course of the oxidation of several primary amines to the corresponding aldehydes (Section 1.5). When the diamine oxidase was employed for stereochemical analysis, together with horse liver alcohol dehydrogenase (E.C. 1.1.1.1), reduction of the oxidation product gave the corresponding primary alcohol. Thus, the ^{3}H/^{14}C ratio was largely maintained within the product (73), when the specimen of [1-^{3}H, 2'-^{14}C]histamine (71), obtained from L-[2'-^{14}C]histidine (72) by decarboxylation in tritiated water, was subjected to stereochemical analysis.

To confirm that the diamine oxidase promotes stereospecific removal of the *Si*-hydrogen when histamine serves as substrate, a synthetic sample of R-[1-^{3}H, 2'-^{14}C]histamine (71) was prepared from [1-^{3}H, 2'-^{14}C]-



Scheme 12. Stereochemistry of (1-^{2}H)- and [1-^{3}H]histamine, derived by enzymic decarboxylation in isotopically labelled water.

histaminol (73), the aforementioned product of stereochemical analysis. This sample of [1-^{3}H]histaminol (73) had been derived from the imidazole-[1-^{3}H]acetaldehyde (74) by stereospecific reduction mediated by alcohol dehydrogenase. Since horse liver alcohol dehydrogenase was known to catalyse stereospecific transfer of hydrogen from NADH to the *Re*-face of the aldehyde carbon of a wide variety of substrates[56] (*cf.* Scheme 2), the alcohol (73) was assigned the S-configuration.

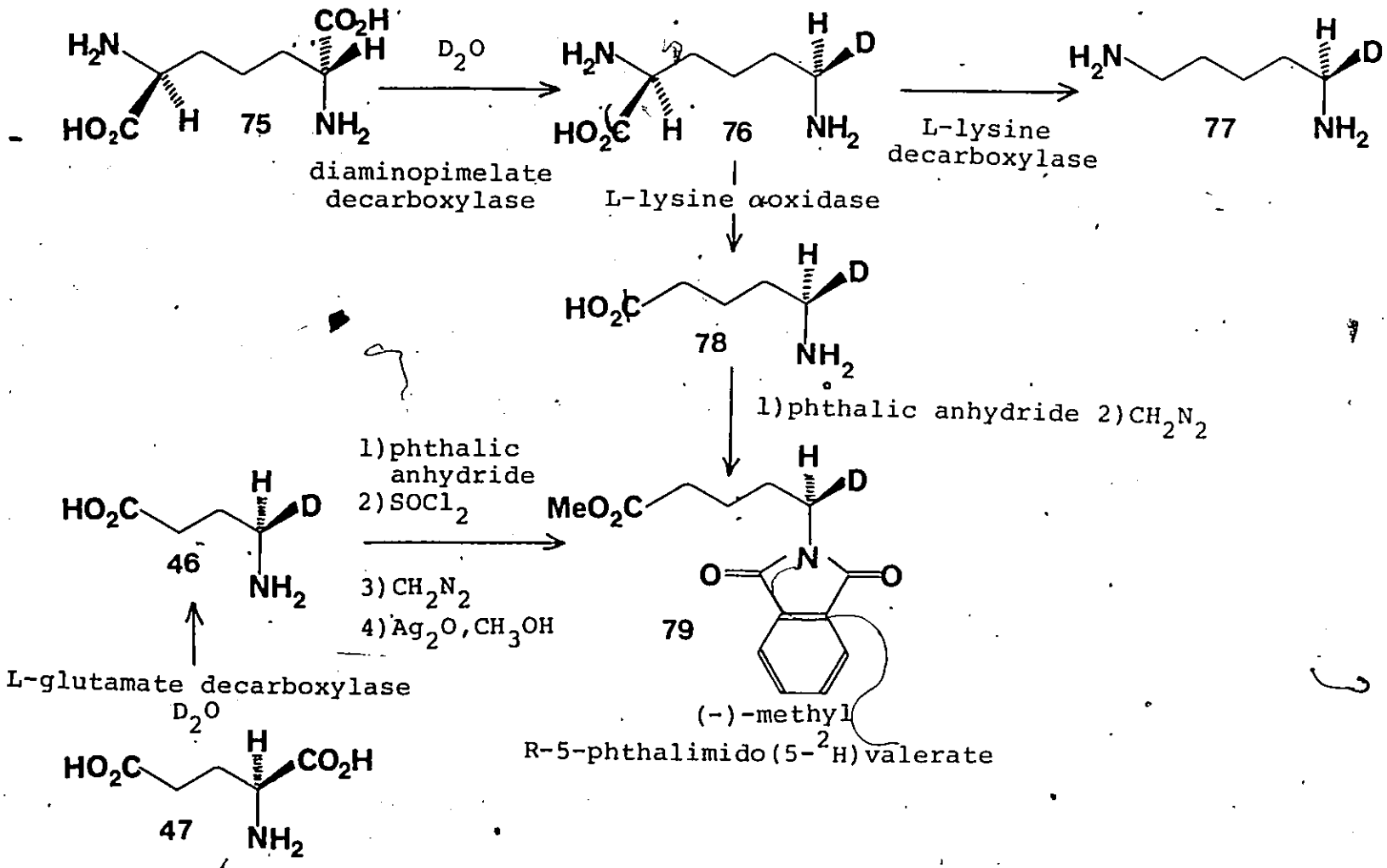
The ^{3}H/^{14}C ratio was also largely retained when the synthetic specimen of R-[1-^{3}H, 2'-^{14}C]histamine was subjected to the stereochemical assay conditions which established its identity with that of the specimen obtained by enzymic decarboxylation.

The method of stereochemical analysis developed by Battersby and co-workers was applied to establish a similar steric course in the reaction, catalysed by mammalian L-histidine decarboxylase (from fetal rats),[140] and, also to reinvestigate the stereochemistry of decarboxylation of L-tyrosine,[129] of L-glutamic acid,[132] and of L-lysine,[135] catalysed by the specific bacterial α-decarboxylases. Similarly, decarboxylation of L-tyrosine catalysed by mammalian aromatic L-amino acid decarboxylase (E.C. 4.1.1.28) (from hog kidney) was shown, by this method, to occur with net retention of configuration.[136] Net retention in the decarboxylation of L-tryptophan, catalysed by the mammalian aromatic L-amino acid decarboxylase was also recently demonstrated by an analogous method involving monoamine oxidase (E.C. 1.4.3.4).[141]

Recently, the decarboxylation of *meso*-α,ε-diaminopimelic acid (75) to L-lysine (54), catalysed by *meso*-α,ε-diaminopimelate

decarboxylase (E.C. 4.1.1.20) of *Bacillus sphaericus* has been shown to occur with net inversion of configuration.[142] This enzyme catalyses the final step in the biosynthesis of L-lysine in bacteria[13] and differs from other amino acid α-decarboxylases in that decarboxylation occurs at the chiral centre of the substrate which has the D-(*i.e.* R-)configuration.[106] The chirality of the deuteriated centre of L-(6-^{2}H)lysine (76) obtained from *meso*-α,ε-diaminopimelic acid (75) by enzymic decarboxylation in deuterium oxide, was determined[142] by stereochemical correlation with R-4-amino(4-^{2}H)butyric acid (46),[121] and with R-(1-^{2}H)cadaverine (77)[120,134] (Scheme 13).

The L-lysine-α-oxidase catalysed oxidation of L-(6-^{2}H)lysine (76) gave, under the reaction conditions employed,[143] 5-amino(5-^{2}H)-valeric acid (78) which was converted into the (-)-*N*-phthaloyl methyl ester (79) ($[\alpha]_{589}$ - 0.92°). The sign of the specific optical rotation of this sample was found to be the same as that of an authentic specimen of (-)-methyl R-5-phthalimido(5-^{2}H)valerate ($[\alpha]_{589}$ - 0.95°) which had been derived from R-4-amino(4-^{2}H)butyric acid (46) *via* a Wolff rearrangement. It followed that the sample of L-(6-^{2}H)lysine possessed the R-configuration at C-6. The inference was confirmed by conversion of the L-(6-^{2}H)lysine (76) into R-(1-^{2}H)cadaverine (77). Configurational assignment of the deuteriocadaverine was effected with pea seedling diamine oxidase.[134]



Scheme 13. Stereochemical correlation of L-(6-^{2}H)lysine (76) with R-4-amino-(4-^{2}H)butyric acid (46) and R-(1-^{2}H)cadaverine (77).

1.5 STEREOCHEMISTRY OF ENZYMIC OXIDATIVE DEAMINATION OF PRIMARY AMINES

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)) catalyse the oxidation of primary amines to aldehydes.[15-17,144,145] 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.

The classification of the two groups of amine oxidases was proposed by Zeller[146] in 1940, according to their substrate specificities. It was later found, however, that certain monoamine oxidases catalysed the oxidation of some long chain aliphatic diamines which were not acted upon by diamine oxidase.[17] Similarly, other investigations indicated that oxidation of several monoamines was efficiently catalysed by diamine oxidase.[15] The results of these investigations led to the proposal of a new system of classification based on inhibition studies,[147-149] and more recently, since the co-factor requirements of the two groups of enzymes have been partially established, they are often referred to as the copper-containing amine oxidases (diamine oxidases) and the FAD-containing amine oxidases (monoamine oxidases).[150]

The absolute stereochemistry of the abstraction of a hydrogen atom from the prochiral methylene group adjacent to nitrogen 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

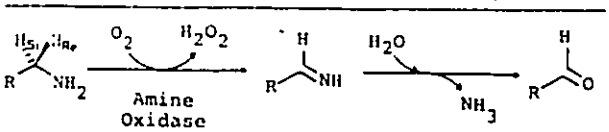
compounds of known chirality. The results of these investigations are summarised in Table 2.

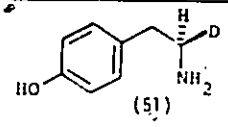
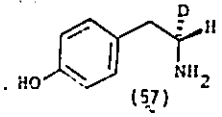
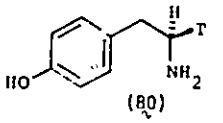
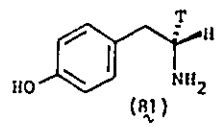
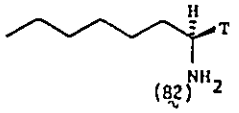
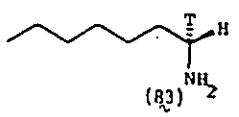
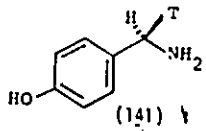
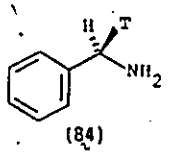
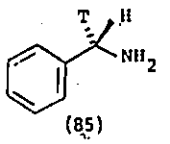
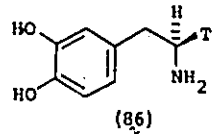
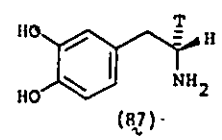
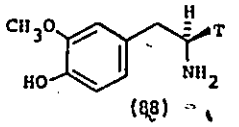
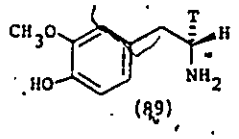
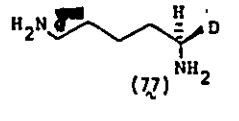
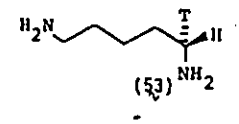
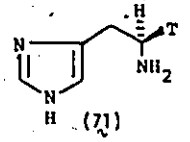
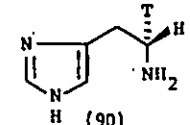
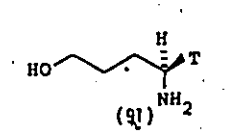
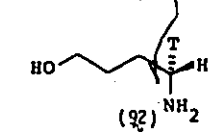
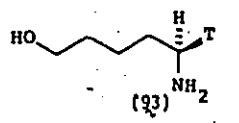
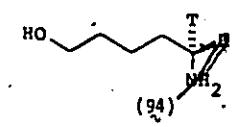
Rat liver mitochondrial monoamine oxidase, acting upon tyramine, was shown, by a kinetic method, to catalyse removal of the *Re*-hydrogen. The rate of oxidation of S-(1-^{2}H)tyramine (57) was found to be 2.3 times faster than that of R-(1-^{2}H)tyramine, (51) indicating a kinetic isotope effect $k_{H_{Re}}/k_{D_{Re}}$ of 2.3.[119,122] The enantiomeric samples of (1-^{2}H)-tyramine had been obtained by enzymic decarboxylation of L-(2-^{2}H)tyrosine (56), and of L-tyrosine (50) in ^2H_2O. The observed isotope effect in the course of enzymic oxidation not only served to establish the stereochemistry of hydrogen abstraction, but led to the determination of the absolute configuration of the two (1-^{2}H)tyramines: a kinetic isotope effect of similar magnitude was observed in the oxidation of S-(1-^{2}H)-, and of R-(1-^{2}H)tyramine, of established configuration, obtained by chemical synthesis (Scheme 8).

Abstraction of the *Re*-hydrogen in the course of the reaction catalysed by mitochondrial monoamine oxidase has recently been verified by radioactive tracer methods, employing S- and R-[1-^{3}H]tyramine[129] as well as S- and R-[1-^{3}H]heptylamine.[151]

Plasma amine oxidase, on the other hand, appears to catalyse the reaction of opposite chirality: radioactivity was retained in the product (*p*-hydroxybenzaldehyde) obtained in the oxidation of *p*-hydroxy-R-[1-^{3}H]benzylamine (84), catalysed by bovine plasma amine oxidase.[152] Similarly, the $^3H/^{14}C$ ratio was maintained in the product when R-[1-^{3}H, 1-^{14}C]benzylamine (85) served as substrate of this enzyme, whereas most of the tritium relative to carbon-14 was lost from S-[1-^{3}H,1-^{14}C]-

Table 2. Reported stereospecificity of hydrogen abstraction in the oxidation of [1-^{2}H]- or [1-^{3}H]amines, catalysed by amine oxidases.



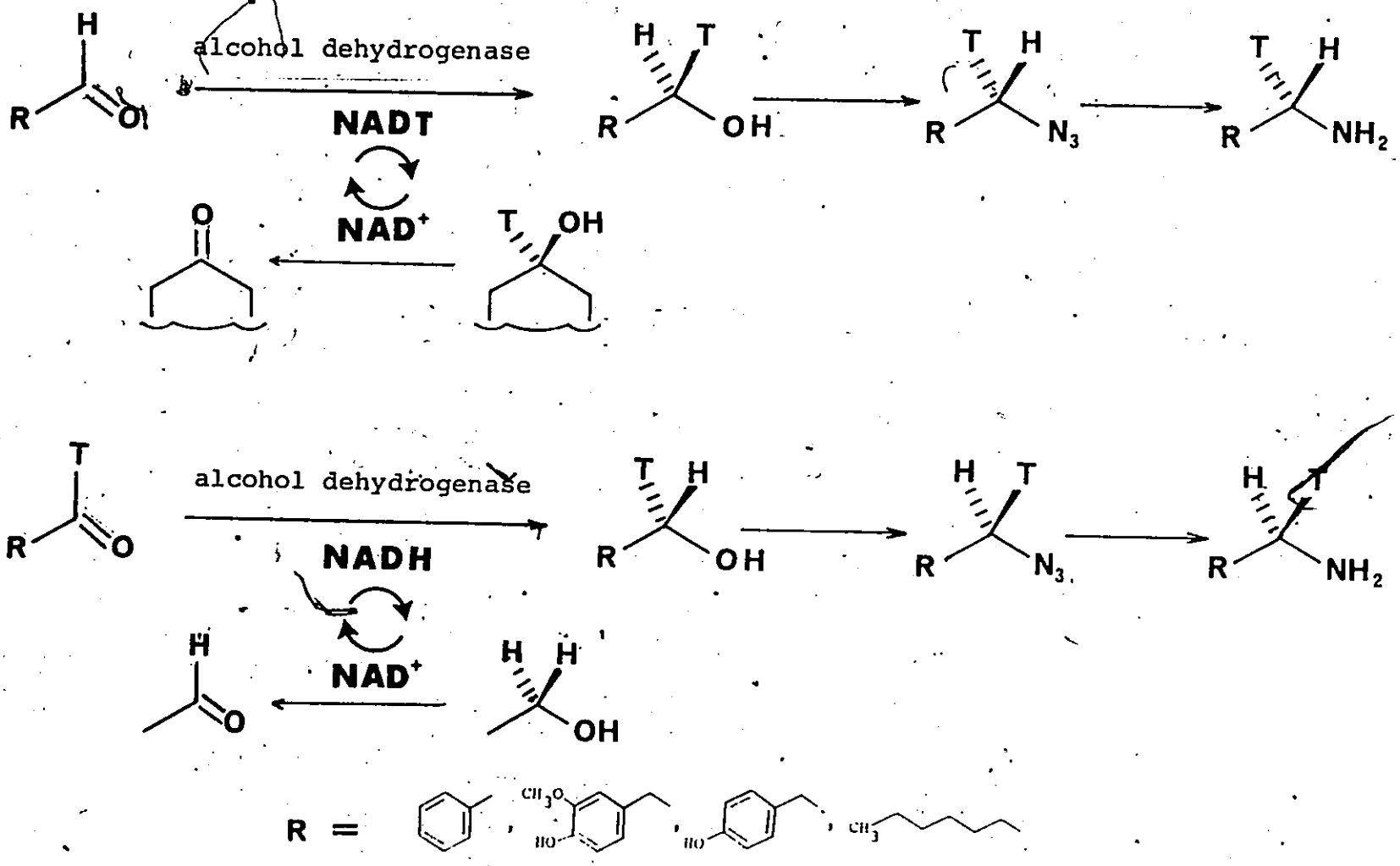
Substrates	Enzyme	Stereospecificity	Ref(s).
 (51)  (57)	rat liver monoamine oxidase	H_{Re}	119,122
 (80)  (81)	rat liver monoamine oxidase	H_{Re}	129
	pea seedling diamine oxidase	H_{Si}	129
 (82)  (83)	rat liver monoamine oxidase	H_{Re}	151
	pea seedling diamine oxidase	H_{Si}	132
 (141)	bovine plasma amine oxidase	H_{Si}	152
 (84)  (85)	bovine plasma amine oxidase	H_{Si}	153
	pea seedling diamine oxidase	H_{Si}	155
 (86)  (87)	bovine plasma amine oxidase	non-stereospecific	154
 (88)  (89)	pea seedling diamine oxidase	H_{Si}	156
 (77)  (53)	pea seedling diamine oxidase	H_{Si}	134
 (71)  (90)	pea seedling diamine oxidase	H_{Si}	136
 (91)  (92)	pea seedling diamine oxidase	H_{Si}	132 (*cf.* 121)
 (93)  (94)	pea seedling diamine oxidase	H_{Si}	135 (*cf.* 120,134)

benzylamine (86).[153] Thus, the *Si*-hydrogen had been removed in each case. Oxidation of dopamine catalysed by bovine plasma amine oxidase, however, appears to be non-stereospecific.[154] The $^{3}H/^{14}C$ ratio was largely maintained (80-92%) in the products when R-[1-^{3}H,1-^{14}C]-, S-[1-^{3}H,1-^{14}C]-, as well as RS-(*i.e.* racemic)-[1-^{3}H,1-^{14}C]dopamine served as substrates.

The stereochemistry of the reaction catalysed by diamine oxidase from pea seedlings has also been determined. With R- and S-[1-^{3}H]-benzylamine as substrates, it was shown[155] that the *Si*-hydrogen is abstracted in the course of enzymic oxidation. More recently it was established that the *Si*-hydrogen is abstracted in the course of the oxidation, catalysed by pea seedling diamine oxidase, of R-[1-^{3}H]- and S-[1-^{3}H]-3-*O*-methyldopamine,[156] R-(1-^{2}H)- and S-[1-^{3}H]cadaverine,[134] R-[1-^{3}H]- and S-[1-^{3}H]tyramine,[129] R-[1-^{3}H]- and S-[1-^{3}H]histamine[136] and S-1-amino[1-^{3}H]heptane[132] (Table 2).

Many of these investigations were undertaken by Battersby and his co-workers at Cambridge (*cf.* Table 2). The two enantiomers of [1-^{3}H]benzylamine,[155] [1-^{3}H]-3'-*O*-methyldopamine,[156] and [1-^{3}H]tyramine,[129] as well as S-1-amino[1-^{3}H]heptane[132] were prepared by analogous synthetic sequences employing reactions of known stereochemistry. This sequence is outlined in Scheme 14.

The key step leading to chiral primary amines of very high configurational purity[157] involved stereospecific reduction, catalysed by horse liver or yeast alcohol dehydrogenase, of either the unlabelled aldehyde (in the presence of [4-^{3}H]-NADH) or the [1-^{3}H]aldehyde (in the



Scheme 14. Synthesis of enantiomeric [1-^{3}H]amines employing alcohol dehydrogenase for the chiral introduction of label.

presence of unlabelled NADH) (*cf.* Scheme 2). A coupled redox system was employed in which ethanol served as the hydride donor, while [1-^{3}H]cyclohexanol[157] or [1-^{3}H]cyclopentanol[156] served to deliver tritium. The two chiral [1-^{3}H]alcohols, so obtained, were then converted into suitable derivatives and treated with azide ion under S_N2 conditions, which gave, following reduction, the [1-^{3}H]amines of opposite chirality.

R-[1-^{3}H]Histamine (71) was prepared similarly.[136] Both enantiomers of [1-^{3}H]histamine were also derived by enzymic decarboxylation (Section 1.4).

It follows from other work of the Cambridge group that oxidation of chiral specimens of [4-^{3}H]butan-1-ol,[132] and of [5-^{3}H]pentan-1-ol[135] also occur with loss of hydrogen from the *Si*-position (at C-4 and C-5, respectively). The tritiated amines had been obtained from the corresponding enantiomeric samples of 4-amino[4-^{3}H]butyric acid and [1-^{3}H]-cadaverine, respectively, which in turn had been derived by enzymic decarboxylation of L-glutamic acid, and of L-lysine. Decarboxylation of both these L-amino acids has been shown, by independent methods, to occur with retention of configuration (Section 1.4).

1.6 OBJECTIVES OF THE PRESENT INVESTIGATION

When the present investigation was initiated, the stereochemistry of the enzymic decarboxylation of ornithine and of arginine was unknown. Decarboxylation of ornithine yields the diamine, putrescine. This diamine is an important intermediate in the biosynthesis of the polyamines, spermidine and spermine,[112,158,159] and of a large number of plant

alkaloids, *e.g.* the pyrrolidine, pyrrolizidine and tropane alkaloids.[160-162] L-Arginine is a protein amino acid and is closely related to L-ornithine, both chemically and metabolically. Arginine is a key intermediate in the formation of naturally occurring guanidines.[163] Agmatine is the decarboxylation product of arginine. In microorganisms[158] and also in higher plants[112,164] agmatine is an intermediate in the formation of putrescine. In mammalian systems putrescine is formed directly from ornithine by decarboxylation.[111,158]

Cadaverine is another biologically important diamine and is derived from lysine by decarboxylation. Decarboxylation of L-lysine forms a key step in the biosynthesis of many plant alkaloids.[160-162]

Putrescine and cadaverine are the normal physiological substrates of diamine oxidase.[15,17] In plant systems the oxidation products of these substrates are intermediates in the biosynthesis of the pyrrolidine and piperidine alkaloids.[160-162,165] In bacteria and mammalian systems, diamine oxidase is involved in the catabolism of these diamines.[158,159]

When the present investigation was undertaken, the stereochemistry of the reaction catalysed by the diamine oxidase from pea seedlings had been determined with benzylamine as substrate.[155] The stereochemistry of the reactions catalysed by diamine oxidase with its normal substrates, cadaverine and putrescine, as well as histamine and agmatine, had not been investigated. The diamine oxidase from hog kidney was commercially available. Although the reaction catalysed by this enzyme was claimed to be stereospecific,[166] the absolute stereochemistry of hydrogen abstraction was unknown.

The objective of the present investigation was to determine the absolute stereochemistry of the hog kidney diamine oxidase catalysed reactions with its physiological substrates. This investigation required enantiomeric samples of cadaverine, putrescine and agmatine, chirally deuteriated or tritiated at C-1 (*cf.* Section 1.5). Enzymic decarboxylation provided the means of obtaining such chirally labelled amines (*cf.* Section 1.4). Using deuterium as the chiral marker, ^{2}H NMR spectroscopy was employed to investigate the stereospecificity of hydrogen abstraction in the reaction catalysed by hog kidney diamine oxidase (Chapter 3). Since the stereochemistry of the enzymic decarboxylation of L-lysine to cadaverine had previously been determined,[120] enantiomeric samples of (1-^{2}H)cadaverine, of known chirality, were readily obtainable. The steric course of decarboxylation of L-ornithine to putrescine, catalysed by L-ornithine decarboxylase, and of L-arginine to agmatine, catalysed by L-arginine decarboxylase, had not been investigated. These enzymic reactions provided enantiomeric samples of (1-^{2}H)putrescine and (1-^{2}H)agmatine, but of unknown absolute configuration (Chapter 2). Determination of the steric course of the reactions leading to the formation of the chiral samples of (1-^{2}H)putrescine and (1-^{2}H)agmatine was a further objective of the present work. The results of these investigations are presented in the subsequent chapters of this thesis.

CHAPTER 2

CHIRAL (1-^{2}H)AMINES BY ENZYMIC DECARBOXYLATION OF THE BASIC AMINO ACIDS

2.1 INTRODUCTION

The stereochemistry of the replacement of the carboxyl group of an α-amino acid by a solvent proton, in the course of the reaction catalysed by an L-amino acid α-decarboxylase, can be determined if the prochiral centre that is generated is rendered chiral by isotopic substitution. If the decarboxylation of the amino acid is carried out in the presence of isotopically labelled water (^{3}HOH or ^2H_2O), one of the enantiomers of the amine will be generated. The other enantiomer arises when a 2-labelled sample ([2-^{3}H]- or (2-^{2}H)-) of the L-α-amino acid undergoes decarboxylation in aqueous solution.

Assignment of the absolute configuration of the two enantiomers will then establish the absolute stereochemistry of decarboxylation. The methods that have been employed by others to determine the absolute configuration of the isotopically engendered chiral centre within the 1-labelled samples ([1-^{3}H]- or (1-^{2}H)-) of the enantiomeric amines were referred to in Chapter 1 (Section 1.4).

When tritium was employed as tracer, stereochemical analysis was effected by means of enzymes of known stereospecificity, by very sensitive radioisotope counting techniques.[120,129,132-136,138,140,141,168] Since only very few molecules comprising the product of enzymic

decarboxylation were labelled with tritium (*i.e.* were [1-^{3}H]amines), and were therefore also chiral, physicochemical methods, such as ORD, NMR (however, see ref. 169) and mass spectrometry, which are of lower sensitivity, were inapplicable for configurational assignment.

When deuterium was employed as the marker,[99,100,118,119,121,134,137,139,142] the enantiomeric samples of the (1-^{2}H)amines were readily obtained at a high level of deuterium enrichment and were not only chiral but also optically active (subject to the sensitivity of the detection instruments). The chirality of the (1-^{2}H)amines was determined by physicochemical techniques, by the classical method of measuring optical activity followed by assignment of configuration by comparison of this activity with standards of known configuration,[121,137,139,142] as well as by NMR methods.[99,100] Alternatively, configurational assignment was achieved by means of enzymes of known stereospecificity, by taking advantage of an observed kinetic isotope effect,[119,122] and by employing mass spectrometry for the detection of label.[134,142].

In the present investigation, deuterium is employed as the chiral marker to determine the stereochemistry of the enzymic decarboxylations of ornithine and arginine, catalysed by the appropriate bacterial α-decarboxylase. The absolute configuration of the resulting chiral samples of (1-^{2}H)putrescine and (1-^{2}H)agmatine is determined by an enzymic assay employing hog kidney diamine oxidase and ^{2}H NMR spectroscopy (Chapter 3), as well as by adaptation of the classical approach to configurational assignment, measurement of optical activity and comparison with standards of known stereochemistry (Chapter 4).

In this chapter, the preparation of optically active, enantiomeric samples of (1-^{2}H)putrescine and of (1-^{2}H)agmatine, and the determination of their relative configurations is described. Chiral samples of (1-^{2}H)cadaverine of known configuration[120] are also prepared and the observed sign of rotation of these samples is compared with that of the corresponding samples of (1-^{2}H)putrescine and (1-^{2}H)agmatine.

2.2 METHODS AND RESULTS

Enantiomeric chirally deuteriated samples of putrescine, of agmatine and of cadaverine were obtained by decarboxylation of L-ornithine, catalysed by the inducible form of L-ornithine decarboxylase (E.C. 4.1.1.17, L-ornithine carboxylyase) of *E. coli*,[170,171] of L-arginine, catalysed by the inducible form of L-arginine decarboxylase (E.C. 4.1.1.19, L-arginine carboxylyase) of *E. coli*,[170, 172-174] and of L-lysine, catalysed by L-lysine decarboxylase (E.C. 4.1.1.18, L-lysine carboxylyase) of *B. cadaveris*,[175,176] respectively. Incubation of unlabelled amino acid with the appropriate L-amino acid decarboxylase in ^2H_2O afforded one enantiomer of the corresponding (1-^{2}H)amine. Similarly, incubation of the (2-^{2}H)amino acid in ^1H_2O afforded the other enantiomer. The details of these experiments are presented in Table 3.

The incubation conditions adopted for the preparation of the (1-^{2}H)amines were similar to those previously employed by others for the estimation of amino acid decarboxylase activity.[174] High substrate concentrations, together with long incubation times (1-2 days), which were required for preparative scale reactions, led to the formation of the products in good yield. The three bacterial α-decarboxylases

ble 3. Conditions for incubation of amino acids with α-decarboxylases to yield chiral (1-^{2}H)amines.

Substrate			Enzyme		Method			Incubation time	Product		
	^{2}H content[a] (atom %)	conc. (mM)		conc. (U mL^{-1})		^{2}H content (atom %)	pH(^{2}H)	(h)		^{2}H content (atom %)	
DL-lysine (95)	-	35	L-lysine decarboxylase	2.1	^2H_2O	>98	6.0	48	R-(-)-(1-^{2}H)cadaverine (77) dihydrochloride	≥95[a]	$^2H_1$[b]
DL-(2-^{2}H)lysine (96)	*ca.* 95 $^2H_1$	35	L-lysine decarboxylase	2.6	^1H_2O	-	6.0	40	S-(+)-(1-^{2}H)cadaverine (97) dihydrochloride	≥95[a]	$^2H_1$[b]
L-ornithine (98)	-	20	L-ornithine decarboxylase	0.17	^2H_2O	>98	5.0	40	(-)-(1-^{2}H)putrescine (100) dihydrochloride	98.7 ± 0.3	$^2H_1$[c]
DL-ornithine (99)	-	40								99.7 ± 0.3	$^2H_1$[c]
DL-(2-^{2}H)ornithine (101)	*ca.* 98 $^2H_1$ *ca.* 92 $^2H_1$	40	L-ornithine decarboxylase	0.17	^1H_2O	-	5.0	20	(+)-(1-^{2}H)putrescine (102) dihydrochloride	97.2 ± 0.3 93.1 ± 0.9	$^2H_1$[c] $^2H_1$[c]
L-arginine (103)	-	20	L-arginine decarboxylase	1.6	^2H_2O	>98	5.2	30-40	(-)-(1-^{2}H)agmatine (104) sulfate	≥98[a]	$^2H_1$
L-(2-^{2}H)arginine (105)	*ca.* 85 $^2H_1$	20	L-arginine decarboxylase	1.6	^1H_2O	-	5.2	40	(+)-(1-^{2}H)agmatine (106) sulfate	*ca.* 85[a]	$^2H_1$
L-(2-^{2}H)arginine (105)	*ca.* 92 $^2H_1$	20	L-arginine decarboxylase	1.6	^1H_2O	-	5.2	40	(+)-(1-^{2}H)agmatine (106) sulfate	*ca.* 92[a]	$^2H_1$

[a] Deuterium content determined by ^{1}H NMR.

[b] Monodeuteriation ($^2H_1$) only determined by m.s. of the N,N'-dibenzoyl derivatives.

[c] Deuterium content determined by C.I. m.s.

employed in this investigation were commercially available (Sigma Chemical Co.) as partially purified, water soluble powders and were used without further purification.

2.2.1 Preparation of the enantiomers of (1-^{2}H)cadaverine, (1-^{2}H)putrescine and (1-^{2}H)agmatine

Enantiomeric samples of (1-^{2}H)cadaverine of known absolute configuration[120] were obtained by decarboxylation of L-lysine, catalysed by L-lysine decarboxylase. R-(-)-(1-^{2}H)Cadaverine (77) dihydrochloride was obtained by decarboxylation of the L-component of DL-lysine (95) in ^2H_2O. S-(+)-(1-^{2}H)Cadaverine (97) dihydrochloride was obtained by decarboxylation of the L-component of DL-(2-^{2}H)lysine (96) in ^1H_2O. The two samples of (1-^{2}H)cadaverine showed specific optical rotations of equal magnitude but of opposite sign. The ORD curves of the two samples are shown in Figure 1.

Decarboxylation of either L-ornithine or the L-component of DL-ornithine in deuterium oxide, and of DL-(2-^{2}H)ornithine in water, catalysed by L-ornithine decarboxylase, afforded the enantiomers of (1-^{2}H)putrescine. Ornithine (98) in ^2H_2O gave (-)-(1-^{2}H)putrescine (100) dihydrochloride, (2-^{2}H)ornithine (101) in ^1H_2O gave (+)-(1-^{2}H)-putrescine (102) dihydrochloride. The ORD curves of the two samples are shown in Figure 2.

The enantiomers of (1-^{2}H)agmatine were obtained similarly from L-arginine, catalysed by L-arginine decarboxylase. L-arginine (103) in ^2H_2O gave (-)-(1-^{2}H)agmatine (104) sulfate, L-(2-^{2}H)arginine (105)

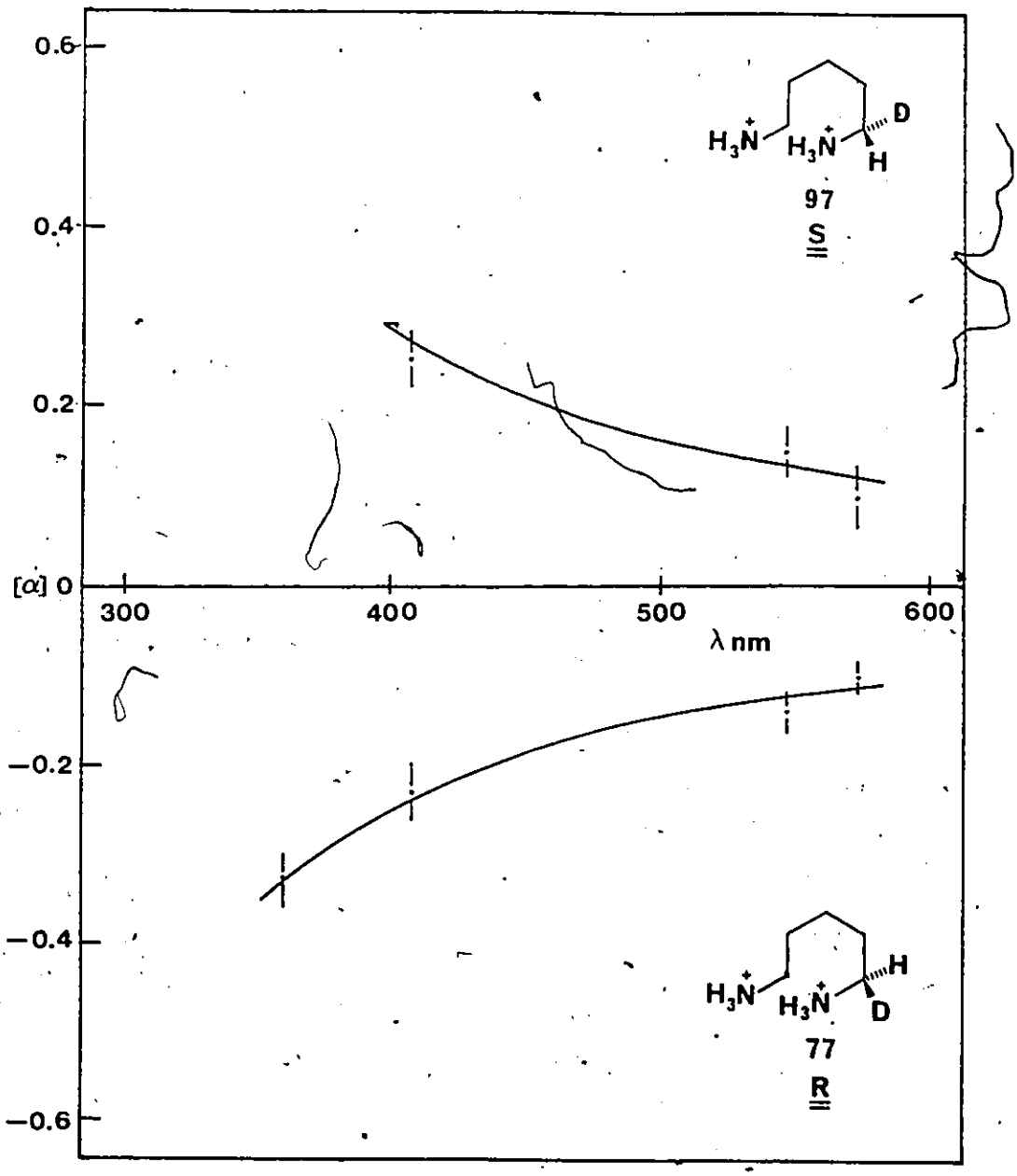
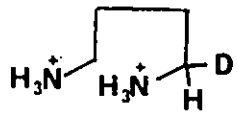


Figure 1. Optical rotatory dispersion (ORD) curves of S-(+)- and R-(-)-(1-^{2}H)cadaverine dihydrochloride.



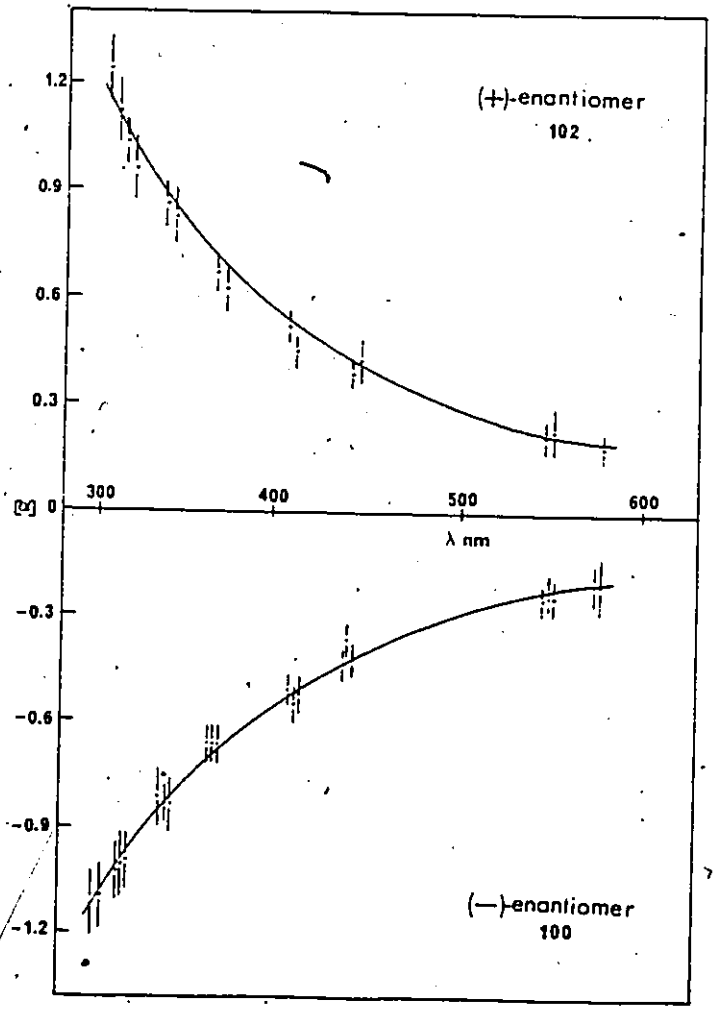


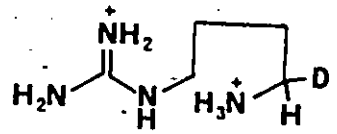
Figure 2. ORD curves of (+)- and (-)-(1-^{2}H)putrescine dihydrochloride. The samples of the (+)-enantiomer (102) were prepared by decarboxylation of the L-component of DL-(2-^{2}H)ornithine (101) (value plotted on the left, for each wavelength) and by hydrolysis of (+)-(1-^{2}H)agmatine (106) sulfate (value on the right). The samples of the (-)-enantiomer (100) were prepared by decarboxylation in ^2H_2O of L-ornithine (98) (value on the left) and of the L-component of DL-ornithine (99) (value in the centre), and by hydrolysis of (-)-(1-^{2}H)-agmatine (104) sulfate (value on the right).

in ^1H_2O gave (+)-(1-^{2}H)agmatine (106) sulfate. The ORD curves of the enantiomeric (1-^{2}H)agmatine samples are shown in Figure 3.

Alkaline hydrolysis with sodium hydroxide in ethanol led to quantitative conversion of agmatine into putrescine. (-)-(1-^{2}H)Agmatine (104) sulfate afforded (-)-(1-^{2}H)putrescine (100) dihydrochlpride (Expt. 9), (+)-(1-^{2}H)agmatine (106) sulfate afforded (+)-(1-^{2}H)putrescine (102) dihydrochloride (Expt. 10) (Table 4). Within experimental error, the ORD curves of the (1-^{2}H)putrescine samples, so obtained, were coincident with those of the samples obtained by enzymic decarboxylation (Figure 2).

The deuterium enrichment of the samples of the chiral (1-^{2}H)-amines was determined either by ^{1}H NMR or by mass spectrometry (Table 3). It was found that each of the amines was monodeuteriated.

A previous report[118] had indicated that L-lysine decarboxylase mediates exchange of the α-protons of cadaverine with the medium. Incubation of cadaverine, in deuterium oxide solution 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 (Section 6.2), 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. The mass spectrum of the sample of cadaverine, reisolated as the *N*,*N*'-dibenzoyl derivative, was identical with that of unlabelled material (Section 6.2.1.2).



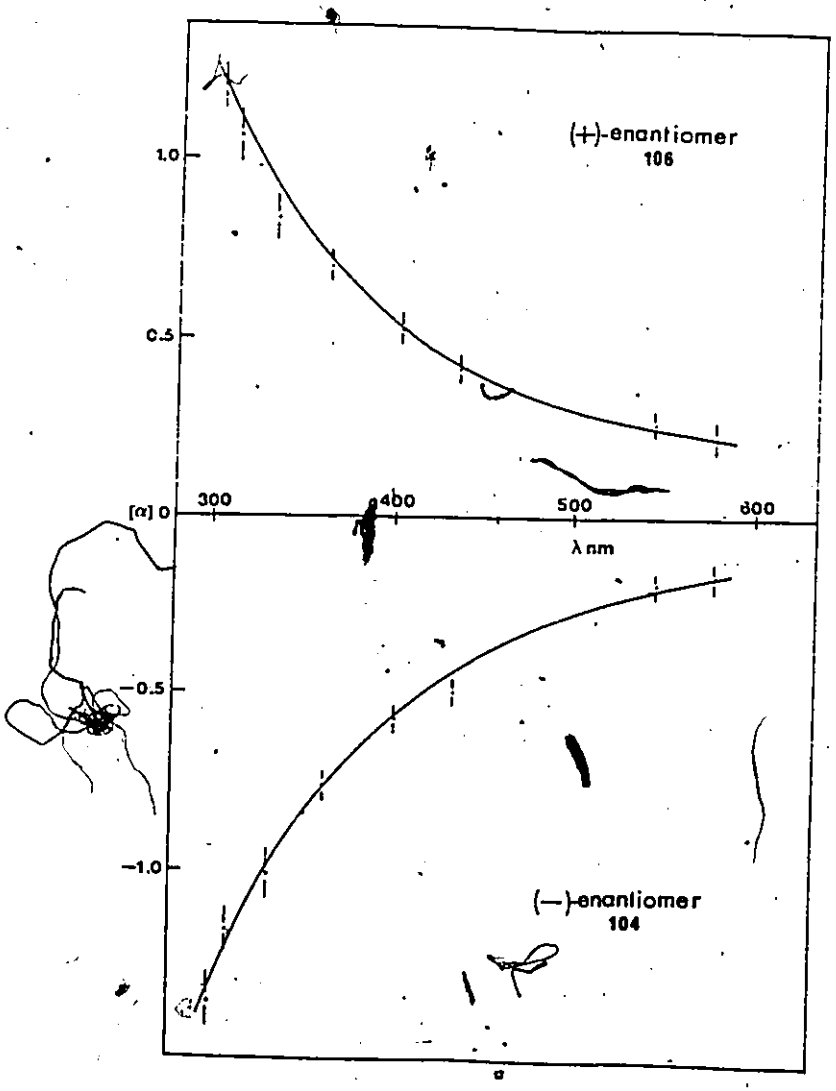


Figure 3. ORD curves of (+)- and (-)-(1-^{2}H)agmatine sulfate.

Table 4. Conversion of (1-^{2}H)agmatine into (1-^{2}H)putrescine.

Expt. No.	Reactant			Product		
		^{2}H content[a] (atom %)	Weight (mg)		^{2}H content[b] (atom %)	Weight (mg)
9	(-)-(1-^{2}H)agmatine (104) sulfate	≥98 $^2H_1$	65	(-)-(1-^{2}H)putrescine (100) dihydrochloride	99.4 ± 0.4 $^2H_1$	41
10	(+)-(1-^{2}H)agmatine (106) sulfate	*ca.* 92 $^2H_1$	40	(+)-(1-^{2}H)putrescine (102) dihydrochloride	91.7 ± 0.8 $^2H_1$	22

[a] Determined by ^{1}H NMR.

[b] Determined by C.I. m.s.

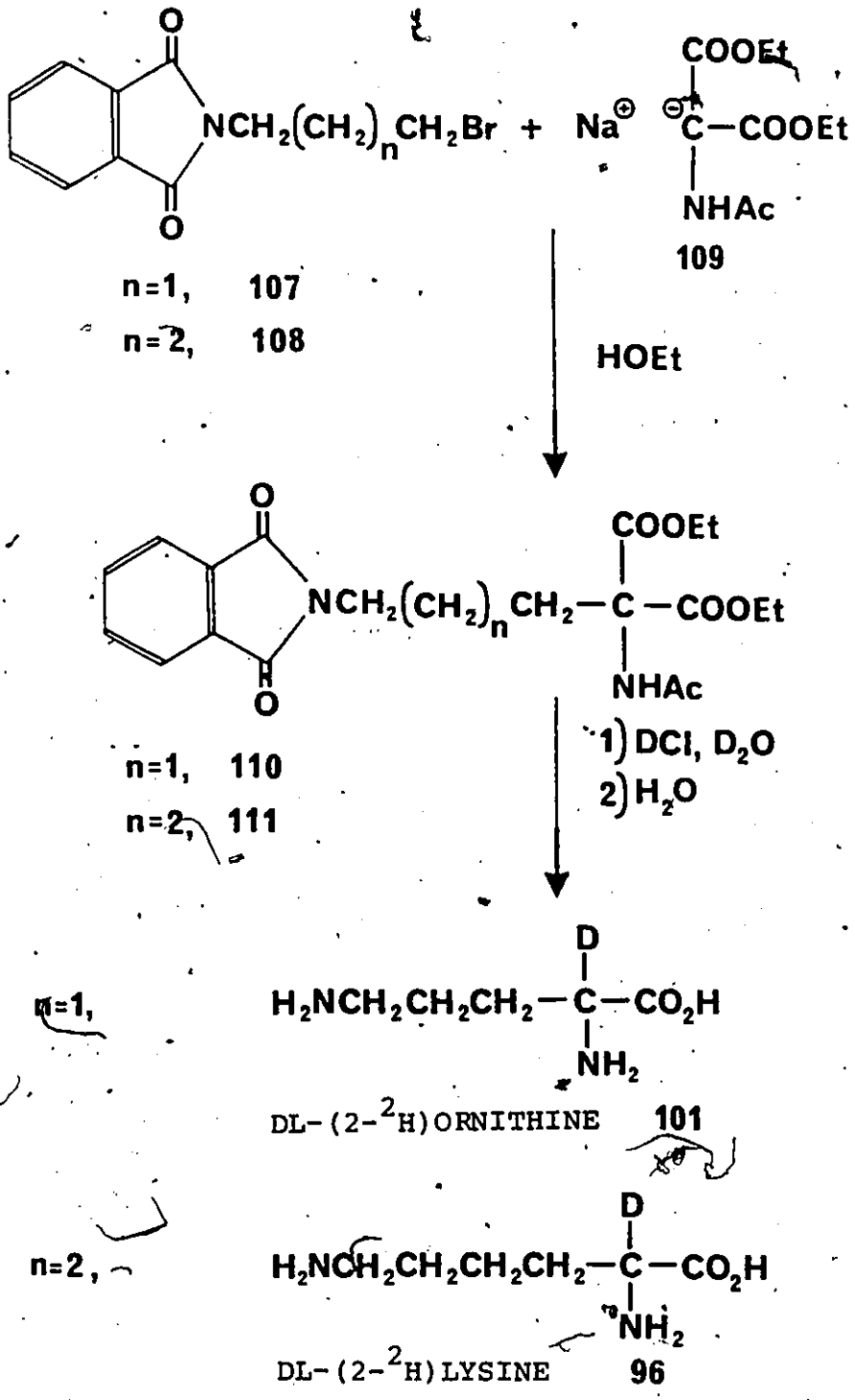
2.2.2 Synthesis of (2-^{2}H)amino acids

The basic amino acids, specifically deuteriated at the carbon adjacent to the carboxyl group (*i.e.* C-2), which were required for this investigation were prepared by the reaction sequences outlined in Schemes 15 and 16.

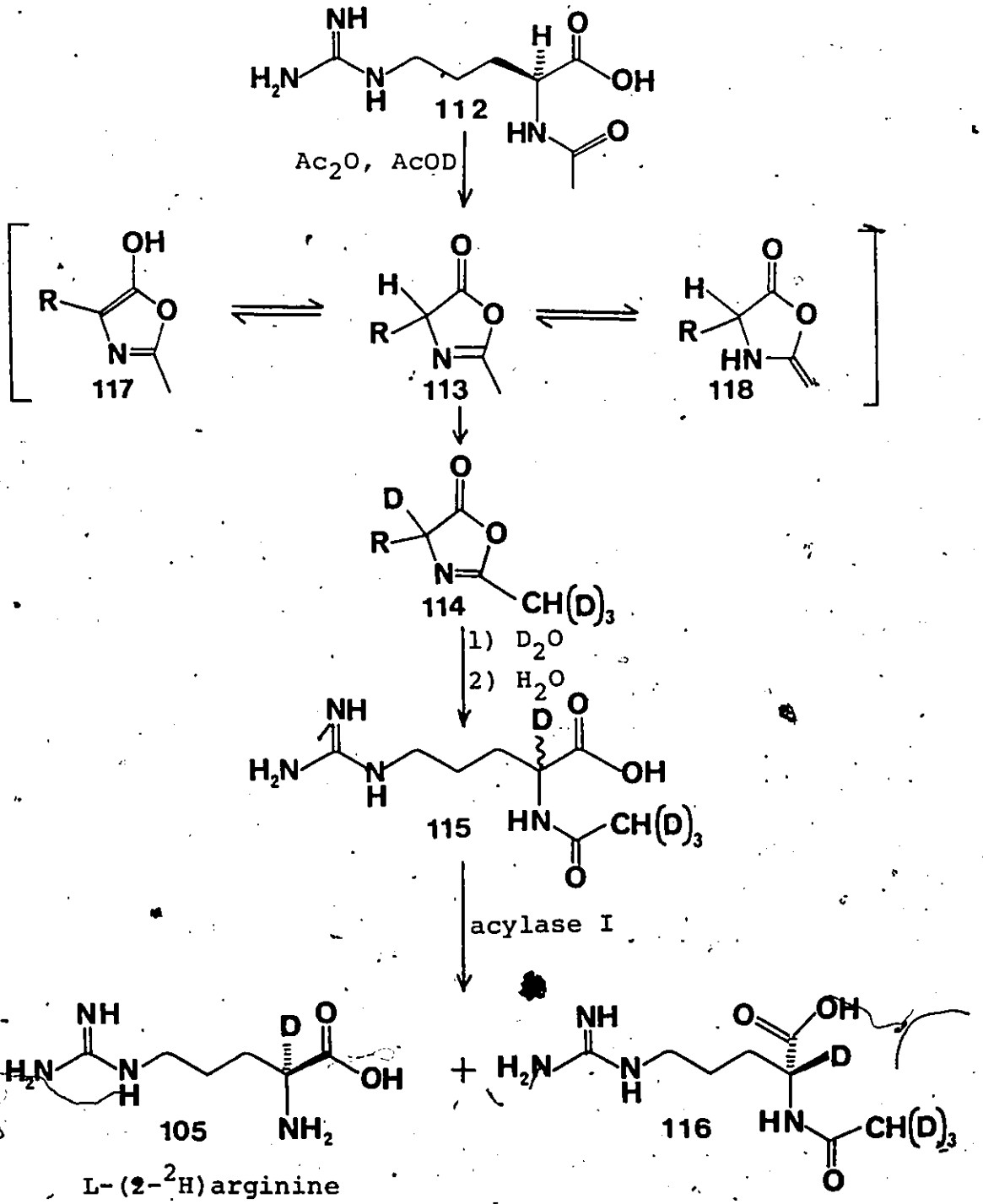
DL-(2-^{2}H)Ornithine (101) and DL-(2-^{2}H)lysine (96) were prepared in an analogous fashion by adaptation of a conventional synthetic route.[177] Reaction of *N*-(3-bromopropyl)phthalimide (107) and of *N*-(4-bromobutyl)phthalimide (108) with sodium diethyl acetamidomalonate (109) in ethanol afforded 2-acetamido-2-(4-phthalimidopropyl)malonate (110) and the corresponding butylmalonate (111), respectively. Acid catalysed hydrolysis of the aminomalonates (110) and (111) with concomitant decarboxylation in deuterium oxide (> 98 atom % ^{2}H) led to the introduction of deuterium into C-2 of the respective products, DL-(2-^{2}H)-ornithine (101) monohydrochloride and DL-(2-^{2}H)lysine (96) monohydrochloride. Samples of the two deuteriated diamino acids prepared in this way were ≥ 92 atom % deuteriated at C-2, as determined by ^{1}H NMR spectroscopy (Table 3). The ^{2}H NMR spectra of the products further indicated that the label was confined solely to the C-2 position.

The racemic samples of (2-^{2}H)ornithine and (2-^{2}H)lysine were subsequently used for the preparation of the corresponding samples of (1-^{2}H)putrescine and (1-^{2}H)cadaverine, respectively (Section 2.2.1) without prior resolution into the L- and D-enantiomers.

L-(2-^{2}H)Arginine (105) was prepared from α-*N*-acetyl-L-arginine (112) by exchange, *via* the related oxazolone (113), followed by



Scheme 15. Synthesis of DL-(2-^{2}H)ornithine (101) and DL-(2-^{2}H)lysine (96).



Scheme 16. Synthesis of L-(2-^{2}H)arginine (105).

resolution of the racemate (115) employing acylase I (E.C. 3.5.1.4) (*cf.* ref. 178). Treatment of α-*N*-acetyl-L-arginine (112) with acetic anhydride in deuterioacetic acid (*ca.* 98 atom % *O*-^{2}H), followed by ring opening of the oxazolone (114), afforded racemic α-*N*-acetyl-(2-^{2}H)arginine (115). The ^{1}H NMR spectrum (^2H_2O) of the deuteriated α-*N*-acetylarginine not only indicated deuteriation at C-2 (δ 4.20), but also revealed that the protons of the methyl group of the *N*-acetyl moiety (δ 2.05) had also exchanged with the medium. The introduction of deuterium into these two positions involves acid catalysed interconversion of the three tautomeric forms of the oxazolone, as depicted in Scheme 16: hydrogen-deuterium exchange at C-2 arises from interconversion of the oxazolone (113) and the oxazoline (117), whereas exchange of the methyl protons arises from interconversion of the oxazolone (113) and the enamine (118). Similar exchange of the *N*-acetyl methyl protons of oxazolones derived from other amino acids has been observed.[136]

L-(2-^{2}H)Arginine was readily obtained from the racemic deuteriated α-*N*-acetyl derivative.[179,180] Stereospecific hydrolysis of the L-enantiomer of deuteriated α-*N*-acetyl-DL-arginine (115), catalysed by hog kidney acylase I, afforded configurationally pure samples of L-(+)-(2-^{2}H)arginine (105) monohydrochloride and (-)-α-*N*-acetyl-D-(2-^{2}H)arginine (116). L-(2-^{2}H)Arginine showed a plane positive ORD curve which was coincident with that of an authentic unlabelled specimen (Section 6.1.3.3). The samples of deuterioarginine were labelled exclusively at C-2 (by ^{2}H NMR) to an extent of 85 and 92 atom % ^{2}H (by ^{1}H NMR).

2.3 DISCUSSION

The stereochemistry of the reactions catalysed by L-ornithine decarboxylase and by L-arginine decarboxylase had not been investigated when the present work was undertaken. Since enzymic decarboxylation of several other L-amino acids was known to take place stereospecifically (Section 1.4), it was expected that the analogous enzyme-catalysed reactions of ornithine and of arginine would also occur stereospecifically. When deuterium labelling was employed to probe the stereochemistry of these two reactions, chiral samples of (1-^{2}H)putrescine and of (1-^{2}H)-agmatine were obtained.

2.3.1 Determination of the relative configurations of the enantiomers of (1-^{2}H)putrescine and (1-^{2}H)agmatine

Decarboxylation of L-ornithine (98) in deuterium oxide, catalysed by L-ornithine decarboxylase of *E. coli*, afforded one of the enantiomers of (1-^{2}H)putrescine (100). The dihydrochloride of this enantiomer was optically active and showed a plane negative ORD curve. Similarly, the levorotatory enantiomer of (1-^{2}H)putrescine (100) dihydrochloride was obtained by incubation of DL-ornithine (99) with the decarboxylase in deuterium oxide. The ORD curve of this specimen was identical with that of the specimen obtained directly from L-ornithine (Figure 2).

The other enantiomer of (1-^{2}H)putrescine (102) was obtained by enzymic decarboxylation of the L-component of DL-(2-^{2}H)ornithine (101) in unlabelled water. The ORD curve of this enantiomer was plane positive (Figure 2). The unreacted D-component of the (2-^{2}H)substrate was

reisolated from the enzymic reaction mixture. The sample of D-(2-^{2}H)-ornithine monohydrochloride, so obtained, exhibited a plane negative ORD curve which was the mirror image of that of an authentic sample of L-(+)-ornithine monohydrochloride (Section 6.2.2.1.2). Thus the D-(*i.e.* R-)-enantiomer of ornithine does not serve as substrate of the enzyme.

It follows that L-ornithine decarboxylase mediates stereospecific decarboxylation of L-(*i.e.* S-)-ornithine: reaction of L-ornithine in ^2H_2O yields the (-)-enantiomer of (1-^{2}H)putrescine; reaction of L-(2-^{2}H)ornithine in ^1H_2O, the (+)-enantiomer.

L-Arginine decarboxylase promotes the stereospecific decarboxylation of L-arginine. The two enantiomers of (1-^{2}H)agmatine were obtained by the action of L-arginine decarboxylase of *E. coli* on L-arginine in deuterium oxide, and on L-(2-^{2}H)arginine, respectively. The sample of agmatine sulfate derived from L-arginine (103) in ^2H_2O was the (-)-enantiomer (104), that derived from L-(2-^{2}H)arginine (105) in ^1H_2O was the (+)-enantiomer (106). The ORD curves of the two enantiomers of (1-^{2}H)agmatine (Figure 3) showed a mirror image relationship indicating that they were of opposite configuration and also of similar configurational purity.

It was a simple matter to establish the relative configuration of the chiral samples of (1-^{2}H)putrescine and (1-^{2}H)agmatine. The unlabelled amines bear only a single structural variation: agmatine possesses a 4-guanidino substituent whereas putrescine is the corresponding diamine. To convert agmatine into putrescine, methods to effect hydrolytic cleavage of the guanidino group of agmatine were investigated.

Alkaline hydrolysis with barium hydroxide[181,182] or with aqueous sodium hydroxide,[183] conditions which had previously been employed to convert L-arginine into L-ornithine[181,182] and DL-ornithine,[183] respectively, led to incomplete conversion of agmatine into putrescine. When hydrolysis with sodium hydroxide in aqueous ethanol was attempted, quantitative conversion of agmatine to putrescine was achieved (Section 6.3.1). Hydrolysis of (-)-(1-^{2}H)agmatine (104) sulfate with ethanolic sodium hydroxide cleaved the guanidino group, while the chiral integrity at C-1 was maintained, to yield (-)-(1-^{2}H)putrescine (100) dihydrochloride (Expt. 9). Similarly, (+)-(1-^{2}H)agmatine (106) sulfate gave (+)-(1-^{2}H)-putrescine (102) dihydrochloride (Expt. 10) (Table 4). The ORD curves of the two enantiomeric samples of (1-^{2}H)putrescine were identical with those of the corresponding enantiomeric samples derived from L-ornithine and L-(2-^{2}H)ornithine by enzymic decarboxylation (Figure 2).

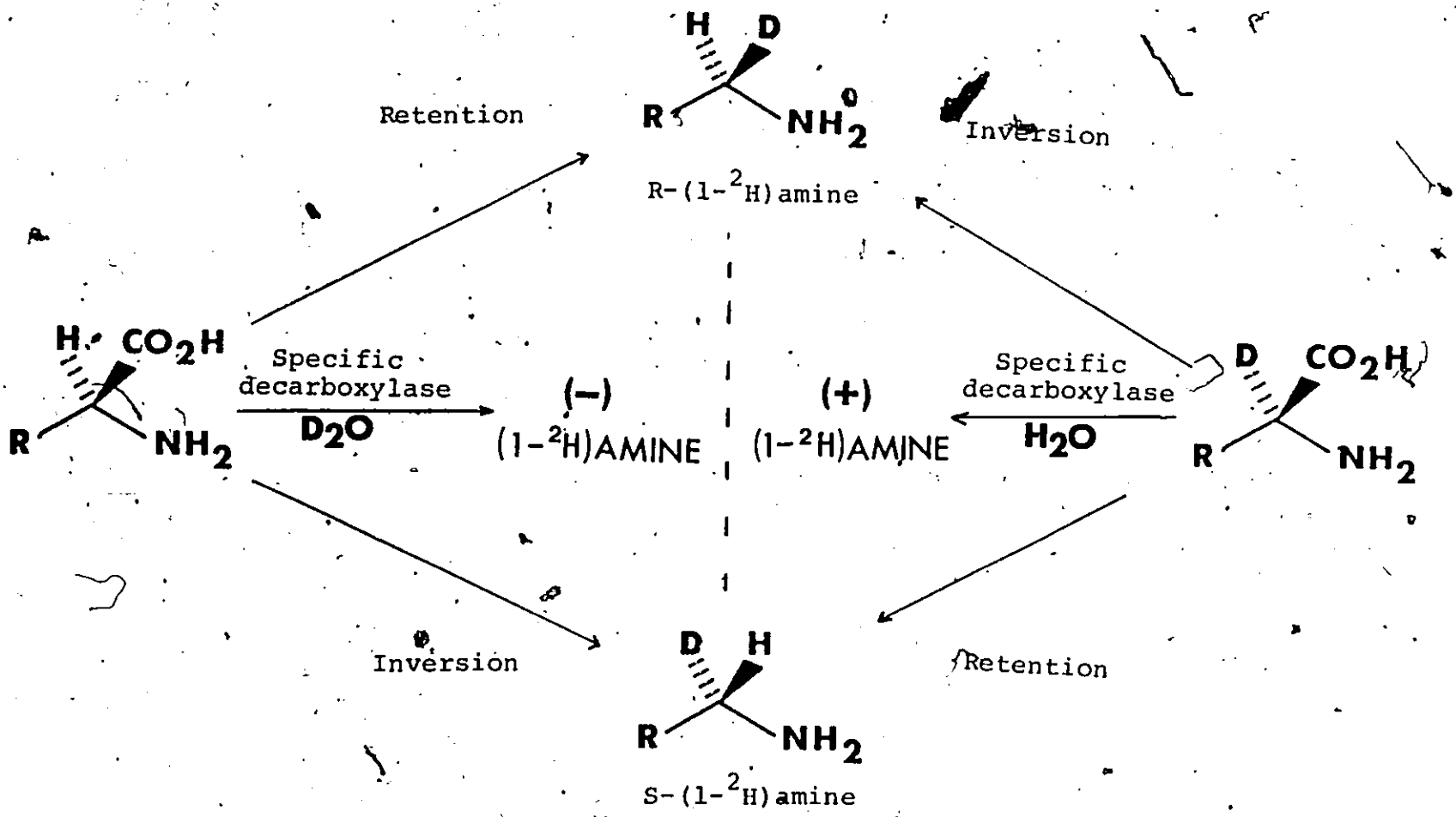
It follows that the levorotatory samples of (1-^{2}H)putrescine dihydrochloride and of (1-^{2}H)agmatine sulfate have the same configuration at C-1. Since these two samples were derived from L-ornithine, and from L-arginine, respectively, by decarboxylation in ^2H_2O, it follows further that the steric course of the decarboxylation of L-ornithine, catalysed by L-ornithine decarboxylase, is identical with that of the decarboxylation of L-arginine, catalysed by L-arginine decarboxylase.

Congruently, the dextrorotatory samples of (1-^{2}H)putrescine dihydrochloride and of (1-^{2}H)agmatine sulfate correspond in configuration at C-1. Each was derived from the corresponding L-(2-^{2}H)amino acid. Thus, the decarboxylations catalysed by L-ornithine decarboxylase and

by L-arginine decarboxylase take the same stereochemical course. Either both reactions proceed with net retention of configuration or they both proceed with net inversion.

If decarboxylation of the L-amino acids were to occur with net retention of configuration, the (-)-(1-^{2}H)amines would possess the R-configuration at C-1, while the (+)-(1-^{2}H)amines would possess the S-configuration. If, on the other hand, decarboxylation were to occur with net inversion of configuration, the (-)-(1-^{2}H)amines would possess the S-configuration at C-1, while the (+)-(1-^{2}H)amines would possess the R-configuration (Scheme 17).

The analogous decarboxylation of L-lysine to yield cadaverine, catalysed by L-lysine decarboxylase, has been shown to take place with net retention of configuration. By employing tritium as the chiral marker, Leistner and Spenser[120] demonstrated that the enantiomeric samples of [1-^{3}H]cadaverine, obtained by enzymic decarboxylation of L-lysine (54) in tritiated water, and of L-[2-^{3}H]lysine (52) in unlabelled water, were R-[1-^{3}H]cadaverine (55) and S-[1-^{3}H]cadaverine (53), respectively, by correlation with [2-^{3}H]glycine of known configuration (Section 1.4, Scheme 9). When deuterium is employed as the chiral marker, enantiomeric samples of (1-^{2}H)cadaverine, of known configuration, can be obtained similarly. Thus R-(1-^{2}H)cadaverine (77) was prepared from the L-component[175] of DL-lysine (95) by decarboxylation in ^2H_2O, and S-(1-^{2}H)cadaverine (97) was prepared from the L-component of DL-(2-^{2}H)-lysine (96) by decarboxylation in ^1H_2O. The two enantiomeric samples of (1-^{2}H)cadaverine were optically active. Comparison of the magnitude of



$$R = NH_2CH_2CH_2CH_2- \quad , \quad NH_2(C{=}NH)NHCH_2CH_2CH_2-$$

Scheme 17. Derivation of enantiomeric samples of (1-^{2}H)putrescine and (1-^{2}H)agmatine.

their respective rotations leads to the inference that they were of comparable configurational purity (Figure 1).

Each of the chiral samples of (1-^{2}H)putrescine, (1-^{2}H)agmatine and (1-^{2}H)cadaverine possesses optical activity. This optical activity therefore provides a method of distinguishing between the two enantiomeric forms of the respective (1-^{2}H)amines, according to the sign of their rotation or rotatory dispersion. The sample of (1-^{2}H)cadaverine dihydrochloride derived from L-lysine (by decarboxylation in ^2H_2O) is levorotatory, that derived from L-(2-^{2}H)lysine is dextrorotatory. Since the stereochemistry of the reaction, from which the two enantiomeric samples of (1-^{2}H)cadaverine were obtained, is known, the sign of rotation provides the means of recognizing the configuration of a given enantiomer. Thus, the levorotatory (1-^{2}H)cadaverine dihydrochloride has the R-configuration, and the dextrorotatory (1-^{2}H)cadaverine dihydrochloride has the S-configuration.

Since the ORD curves of the two enantiomers of (1-^{2}H)putrescine (Figure 2), and of (1-^{2}H)agmatine (Figure 3) each bear a mirror image relationship, the samples are of opposite chirality.[184] The stereochemistry of the reactions by which these enantiomeric (1-^{2}H)amines were prepared has not been established. Therefore, the sign of rotation only provides a means of identifying the enantiomer (*e.g.* the levorotatory enantiomer) obtained by a given set of reaction conditions (*i.e.* decarboxylation of the L-amino acid in ^2H_2O). The absolute configuration of the chiral deuteriated centre within the (1-^{2}H)amines cannot be inferred from the sign of rotation.

Several theories have been advanced which do permit assignment of absolute configuration, but are based on calculation of rotatory dispersion curves from an assumed molecular geometry.[185] Empirical relationships such as the octant rule[186,187] have allowed definitive conclusions to be reached regarding the conformation or absolute configuration of molecules from their ORD or circular dichroism (CD) spectra. Such studies have recently focussed on molecules which owe their chirality solely to isotopic substitution.[188] However, unambiguous assignment of configuration is only possible for conformationally rigid molecular systems.

A direct correlation between the observed sign of rotation and the absolute configuration of acyclic molecules has been based on differences of the polarizabilities of the four substituents at the chiral centre.[189] Application of such a method was successful in correctly predicting the absolute configuration of (-)-(1-^{2}H)ethanol.[190] However, configurational assignments made by this method are not unequivocal and must be confirmed by independent methods. Assignment of configuration has also been made by comparison of the optical activity associated with closely related compounds. Determination of configuration by such comparisons is not always reliable. Enzymically prepared (-)-(1-^{2}H)-ethanol (8)[54] (Scheme 2) was first assigned the R-configuration on the basis of a comparison of the sign of rotation with that of R-(-)-(1-^{2}H)-butanol, of known configuration.[54,192] This assignment was later proven to be incorrect: levorotatory (1-^{2}H)ethanol has the S-configuration[55,80] (Section 1.3).

It is interesting to note in this context that each of the salts of the samples of (1-^{2}H)putrescine, (1-^{2}H)agmatine and (1-^{2}H)cadaverine derived by enzymic decarboxylation of the corresponding L-amino acids in deuterium oxide, show plane negative ORD curves. The levorotatory samples of (1-^{2}H)putrescine dihydrochloride and (1-^{2}H)agmatine sulfate were shown to be of the same configuration. The levorotatory sample of (1-^{2}H)cadaverine dihydrochloride is known to have the R-configuration. Since putrescine and cadaverine are structurally similar, differing only by a methylene group, it would be tempting to assume that the (1-^{2}H)-diamines of the same sign of rotation are also of the same configuration. Congruently, the salts of the three deuteriated amines derived by enzymic decarboxylation of the corresponding L-(2-^{2}H)amino acids are dextrorotatory, suggesting a similar comparison.

To base the configurational assignments of the samples of (1-^{2}H)-putrescine solely on this analogy, however, would clearly be unsound. It has been observed that the S-enantiomers of (1-^{2}H)ethanol[54] and (1-^{2}H)-propanol[191] show rotations of opposite sign, whereas the S-enantiomers of (1-^{2}H)propanol[191] and (1-^{2}H)butanol[124] show rotations of the same sign. The structural difference between both of these pairs of molecules also consists of a single methylene group. Similarly, *n*-butylamine, cadaverine and 4-aminobutyric acid are structurally similar in the vicinity of the methylene group adjacent to nitrogen. However, S-(1-^{2}H)-butylamine is levorotatory,[126] whereas S-(1-^{2}H)cadaverine dihydrochloride is dextrorotatory. On the other hand, the R-enantiomers of (1-^{2}H)-cadaverine dihydrochloride and 4-amino(4-^{2}H)butyric acid[121] are both

levorotatory. It would appear from these examples that no obvious correlation exists between the observed sign of rotation and the absolute configuration of the chiral deuteriated centres. The chirality of the centres in these compounds must be established separately.

2.3.2 Investigation of enzyme mediated exchange of the α-hydrogens of cadaverine, putrescine and agmatine

Several bacterial L-amino acid α-decarboxylases have been reported to catalyse stereospecific exchange of one of the hydrogen atoms of the methylene group adjacent to nitrogen of the amine which forms the normal decarboxylation product (Table 5). The occurrence of such an exchange reaction was first observed by Mandeles, Koppelman and Hanke.[118] A sample of 4-amino(4-^{2}H)butyric acid (46) had been prepared by the action of L-glutamate decarboxylase on L-glutamic acid (47) in ^2H_2O. When the sample of (46) was reincubated with the decarboxylase in ^1H_2O, the reisolated amine showed the complete absence of deuterium. In another experiment, the enantiomeric sample of 4-amino(4-^{2}H)butyric acid (48), which had been prepared by enzymic decarboxylation of L-(2-^{2}H)glutamic acid (49), was similarly incubated with the decarboxylase. In this experiment, deuterium was maintained in the reisolated amine (48). The results of the two incubation experiments not only indicated the occurrence of exchange with the medium of one of the enantiotopic hydrogens at C-4 of 4-aminobutyric acid, but also provided the first evidence that enzymic decarboxylation takes place stereospecifically[118] (Section 1.4).

L-Glutamate decarboxylase was later shown to catalyse decarboxylation of L-glutamic acid to 4-aminobutyric acid with retention of

Table 5. Reported hydrogen/deuterium (or tritium) exchange at the α-methylene group of biogenic amines, catalysed by the appropriate amino acid decarboxylase.

Enzyme (source)	Substrate Amine	Reported Exchange	Reported No exchange
Lysine decarboxylase (*B. cadaveris*)	Cadaverine	118	-
Glutamate decarboxylase (*E. coli*)	4-Aminobutyrate	118	193
Tyrosine decarboxylase (*S. faecalis*)	Tyramine	129	-
Histidine decarboxylase (*Lactobacillus* 30a) (*C. welchii*)	Histamine	136 136	139
S-Adenosylmethionine decarboxylase (*E. coli*)	*S*-Adenosyl-*S*-methyl-3-thiopropylamine	-	138

configuration,[121] but the hydrogen deuterium exchange reaction could not be confirmed.[193] Prolonged incubation of a highly purified preparation of L-glutamate decarboxylase with 4-aminobutyric acid in deuterium oxide did not lead to the incorporation of ^{2}H at C-4.[193] On the basis of this result, it was suggested[193] that the enzyme preparation that had previously been employed by Mandeles *et al.*[118] was probably contaminated with 4-aminobutyrate aminotransferase. A bacterial ω-aminotransferase has recently been shown[194] to mediate stereospecific removal of the 4-*Re*-hydrogen of 4-aminobutyric acid.

Stereospecific enzyme-mediated exchange with the medium of the *Re*-hydrogen from the α-carbon of tyramine[129] and of histamine[136] has recently been demonstrated by Battersby and his co-workers. Incubation of [1-^{14}C]tyramine and of [2'-^{14}C]histamine, in tritiated water, with bacterial L-tyrosine decarboxylase and L-histidine decarboxylase, respectively, led to the incorporation of ^{3}H into the 1-*Re*-position of the reisolated amines.[129,136] The chirality of the reisolated doubly labelled samples of R-[1-^{3}H]tyramine and R-[1-^{3}H]histamine was determined by enzymic analysis with rat liver monoamine oxidase[129] and pea seedling diamine oxidase,[136] respectively (*cf.* Section 1.5, Table 2). The conversions of L-tyrosine into tyramine[119,129] and of L-histidine into histamine,[136,137] each catalysed by the respective bacterial enzyme, have both been shown to take place with net retention of configuration (Section 1.4). Since it is the *Re*-hydrogen at C-1 of tyramine and of histamine which occupies the same steric environment as that of the carboxyl group within the corresponding L-(*i.e.* S-)amino acids, and it is this hydrogen which was stereospecifically labilised on incubation

of the two amines with the respective decarboxylases, it was inferred[129] that the exchange reaction occurs at the active site of the decarboxylases by a reversal of the final step of the decarboxylation process.*

The incubation experiments with bacterial L-histidine decarboxylase from *Lactobacillus* 30a and from *C. welchii* in tritiated water resulted in very low incorporations of tritium into the α-position of histamine which was taken as an indication that the rate of the hydrogen-tritium exchange reaction was very slow.[136] An earlier attempt to demonstrate the analogous exchange reaction in deuterium oxide, in the presence of the enzyme from *Lactobacillus*, had not led to detectable incorporation of the label into histamine.[139]

Isotopic tracer methods have been employed to investigate whether *S*-adenosylmethionine decarboxylase mediates a similar exchange reaction.[138] When R-*S*-adenosyl-*S*-methyl-3-thio[1-^{3}H]propylamine, obtained by enzymic decarboxylation of *S*-adenosylmethionine in ^3HOH, was reincubated with the decarboxylase in ^1H_2O, no exchange of tritium from the 1-*Re*-position of the amine with the medium was detectable.

Mandeles *et al.*[118] had also reported that the action of L-lysine decarboxylase (from *B. cadaveris*) on cadaverine in ^2H_2O leads to incorporation of deuterium into the amine. In the other cases, in which the occurrence of an enzyme-catalysed exchange process has been demonstrated, the hydrogen which is labilised is the same as that which is introduced

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* The mechanism of action of the L-amino acid decarboxylases is discussed in Section 4.3.

during enzymic decarboxylation (*i.e.* the *Re*-hydrogen).[129,136] The sample of R-(1-^{2}H)cadaverine, required for the present investigation, was prepared by decarboxylation of L-lysine, catalysed by the decarboxylase from *B. cadaveris*, in deuterium oxide. Even though this reaction leads, in the first instance, to R-(1-^{2}H)cadaverine, prolonged incubation might have led to significant further conversion to a dideuteriated species, chirally labelled at both α-methylene groups (*i.e.* RR-(1,5-$^2H_2$)cadaverine), if enzyme mediated stereospecific hydrogen-deuterium exchange had taken place. The sample of R-(1-^{2}H)cadaverine (77) that was obtained (Table 3), however, was monodeuteriated and not dideuteriated. But, in view of the conflicting reports concerning the occurrence of hydrogen exchange in the presence of L-glutamate decarboxylase, experiments were carried out to obtain independent evidence concerning the possibility of exchange of the α-hydrogens of cadaverine. Experiments were also conducted to investigate the possibility of exchange of the α-hydrogens of putrescine and agmatine in the presence of the decarboxylases specific for L-ornithine and L-arginine, respectively.

Prolonged incubation, in deuterium oxide solution (99.8 atom % ^{2}H), of cadaverine with L-lysine decarboxylase, of putrescine with L-ornithine decarboxylase, and of agmatine with L-arginine decarboxylase, followed by reisolation of the bases, yielded samples whose ^{1}H NMR spectra were indistinguishable from those of the starting materials. The mass spectrum of the reisolated *N*,*N*'-dibenzoyl derivative of cadaverine was identical with that of unlabelled material. No deuterium was detectable in the ^{2}H NMR spectrum of the reisolated sample of agmatine sulfate.

Thus, exchange of protium by deuterium was not detectable under the conditions used to prepare the $\underline{R}_r$-(1-^{2}H)cadaverine (77), as well as the (-)-enantiomers of (1-^{2}H)putrescine (100) and (1-^{2}H)agmatine (104). Within the limits of detection, the chiral samples of (1-^{2}H)cadaverine and (1-^{2}H)putrescine were indeed monodeuteriated (Table 3). 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, 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. m.s.) showed monodeuteriation only.

These results also indicate that the commercial preparations of the α-decarboxylases, employed in the present investigation, did not contain other enzyme activity (*e.g.* aminotransferases) which might have led to labilisation of the α-protons of the amines.

CHAPTER 3

THE STEREOSPECIFICITY OF HOG KIDNEY DIAMINE OXIDASE

3.1 INTRODUCTION

The oxidation of a primary amine to an aldehyde is catalysed by the amine oxidases. The stereochemistry of the hydrogen abstraction process, in the conversion of the prochiral methylene group adjacent to nitrogen within the primary amine, $R\text{-}CH_2NH_2$, into the sp^2 centre within the aldehyde, R-CHO, can be determined if chiral samples, of known absolute chirality, of the 1-labelled ([1-^{3}H]- or (1-^{2}H)-) amine is employed as substrate. One of the enantiomers of the 1-labelled amine will lead to product which, by stereospecific loss of protium, retains the label at the sp^2 centre. The other enantiomer will lead to product which, by stereospecific loss of the label, retains protium at the sp^2 centre. Measurement of the conservation or release of label within the products derived from the two enantiomeric samples permits determination of the absolute stereochemistry of the process.

The stereochemistry of the reactions catalysed by the amine oxidases from rat liver mitochondria (monoamine oxidase),[119,122,129,151] pea seedlings (diamine oxidase)[129,132,134,136,155,156] and bovine plasma,[152,153] has been determined in several instances, with a wide variety of substrates. The results of these investigations were referred to in Section 1.4 (*cf.* Table 2). It has been reported that the reaction

catalysed by the diamine oxidase from hog kidney also takes place with stereospecific release of one of the enantiotopic hydrogens from C-1 of histamine,[166] although the absolute stereochemistry of this process was not determined. The stereospecificity exhibited by hog kidney diamine oxidase, in the reactions which it catalyses, was unknown.

The preparation of enantiomeric samples of (1-^{2}H)cadaverine, (1-^{2}H)putrescine and (1-^{2}H)agmatine was described in Chapter 2. Hog kidney diamine oxidase catalyses the oxidative deamination of each of these amines to yield the corresponding aldehyde. In this chapter, the reactions of the deuteriated samples of the three amines are investigated. The absolute stereochemistry of the hydrogen abstraction process in the reaction catalysed by hog kidney diamine oxidase is determined by employing the enantiomers of (1-^{2}H)cadaverine, of known chirality, as substrates. Assignment of absolute configuration to the enantiomeric samples of (1-^{2}H)-putrescine and (1-^{2}H)agmatine is then made on the assumption that the stereochemical course of the reaction with cadaverine as substrate is the same as that with putrescine and agmatine as substrates.

3.2 METHODS AND RESULTS

The enantiomeric samples of (1-^{2}H)cadaverine, (1-^{2}H)putrescine and (1-^{2}H)agmatine were prepared by enzymic decarboxylation of the corresponding L-amino acids. The method of preparation is discussed in Chapter 2.

R-(-)-(1-^{2}H)Cadaverine (77) dihydrochloride (Expt. 1), (-)-(1-^{2}H)-putrescine (100) dihydrochloride (Expt. 4) and (-)-(1-^{2}H)agmatine (104) sulfate (Expt. 7) were derived from L-lysine, L-ornithine and L-arginine,

respectively, by decarboxylation in deuterium oxide. S-(+)-(1-^{2}H)-Cadaverine (97) dihydrochloride (Expt. 2), (+)-(1-^{2}H)putrescine (102) dihydrochloride (Expt. 5) and (+)-(1-^{2}H)agmatine (106) sulfate (Expt. 8) were derived from L-(2-^{2}H)lysine, L-(2-^{2}H)ornithine and L-(2-^{2}H)arginine, respectively, by decarboxylation in unlabelled water (Table 3, Section 2.2).

Samples of (1,1-$^2H_2$)cadaverine (119) dihydrochloride (Expt. 3) and of (1,1-$^2H_2$)putrescine (120) dihydrochloride (Expt. 6) (each > 90% perdeuteriated at one of the terminal carbon atoms) were prepared similarly, by enzymic decarboxylation of the L-component of DL-(2-^{2}H)lysine (96) (Section 6.2.1.1.3) and of the L-component of DL-(2-^{2}H)ornithine (101) (Section 6.2.2.1.3) in deuterium oxide.

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 and 8) were incubated with hog kidney diamine oxidase (E.C. 1.4.3.6 diamine: oxygen oxidoreductase (deaminating)). The details of these experiments are presented in Table 6.

The incubation in Experiments 1-6 was carried out in the presence of *o*-aminobenzaldehyde which serves to trap[195,196] the oxidation products which, themselves, are difficult to isolate,[196,197] since they tend to trimerise at physiological pH.[198,199,200,201]

The product of the enzymic oxidation of cadaverine (121), 5-aminopentanal (122), in equilibrium with Δ^1-piperideine (123), is trapped by *o*-aminobenzaldehyde (124) to yield, as the major product, 3-(3'-aminopropyl)quinoline (125) which is isolated as the dipicrate or the

Table 6. Incubation of labelled substrates with diamine oxidase.

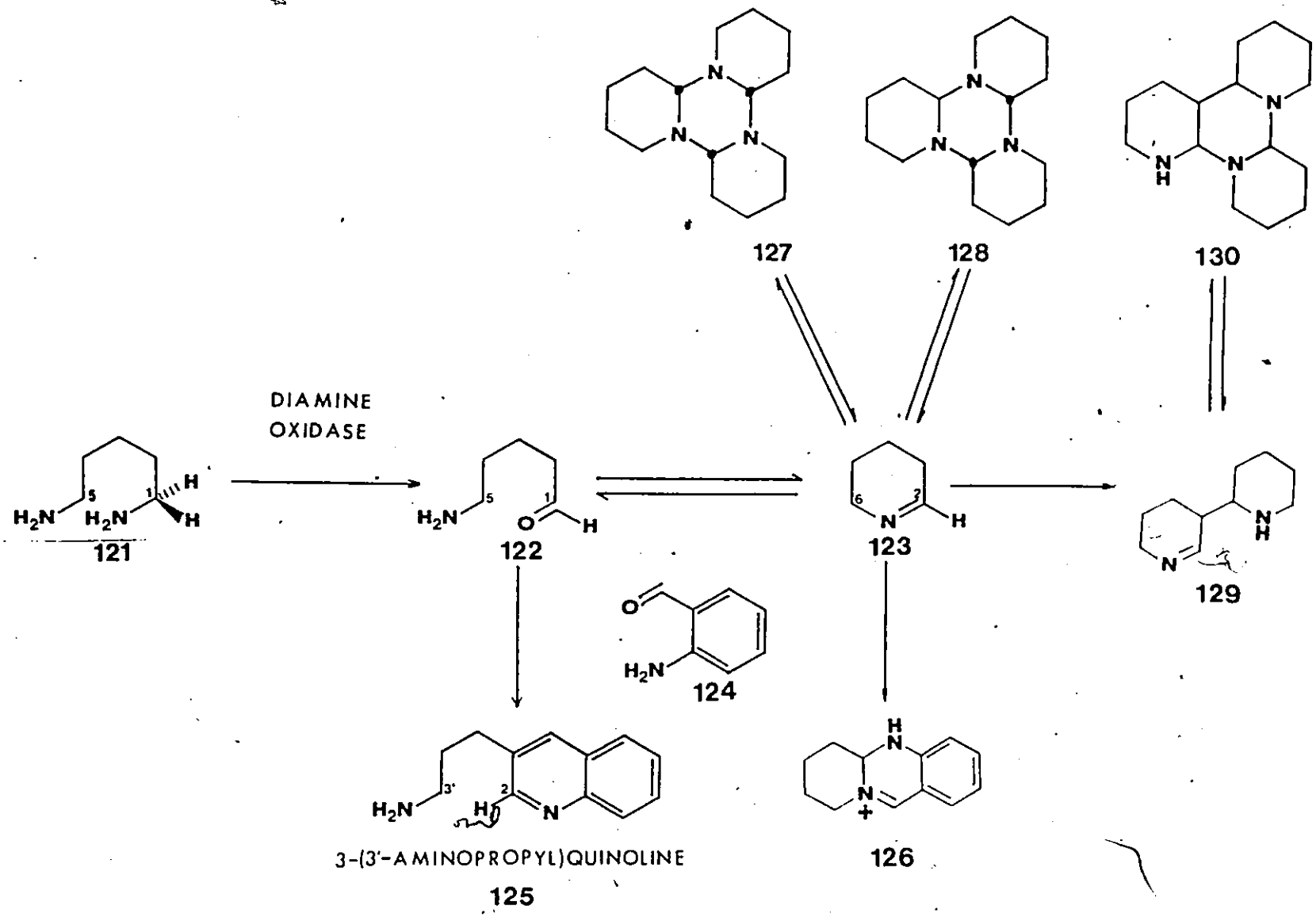
Expt. No.	Substrate				Diamine oxidase	*o*-aminobenz aldehyde	Incubation time
		^2H content (atom %)	Weight (mg)	Conc. (*mM*)	Conc. (mg mL^{-1})	Conc. (*mM*)	(h)
1	R-(-)-(1-^2H)cadaverine dihydrochloride	≥95 ($^2H_1$)	40	7.6	7.3	8.4	40
2	S-(+)-(1-^2H)cadaverine dihydrochloride	≥95 ($^2H_1$)	40	7.4	7.2	8.8	39
3 (a)	(1,1-$^2H_2$)cadaverine dihydrochloride	*ca.* 93 ($^2H_2$)	40	7.6	7.4	9.1	39
(b)	(1,1-$^2H_2$)cadaverine dihydrochloride	*ca.* 93 ($^2H_2$)	30	9.3	10.1	9.8	6
4 (a)	(-)-(1-^2H)putrescine dihydrochloride	99.7 ($^2H_1$)	30	9.3	10.4	8.4	6
(b)	(-)-(1-^2H)putrescine dihydrochloride	98.7 ($^2H_1$)	22	13.4	9.6	10.0	6
5	(+)-(1-^2H)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	(-)-(1-^2H)agmatine sulfate	≥98 ($^2H_1$)	33	12.1	6.7	-	25
8	(+)-(1-^2H)agmatine sulfate	*ca.* 85 ($^2H_1$)	41	12.0	6.6	-	26

dihydrochloride.[198] A second product, 2,3-tetramethylene-1,2-dihydroquinazolinium ion (126), isolated as the picrate,[197,198] is formed in lower yield (Scheme 18). The oxidation product of putrescine (131), 4-aminobutanal (132), in equilibrium with Δ'-pyrroline (133), is trapped as 2,3-trimethylene-1,2-dihydroquinazolinium ion (134), isolated as the picrate,[197,199,202] while 3-(2'-aminoethyl)quinoline (135) is formed as a minor product (Scheme 19).

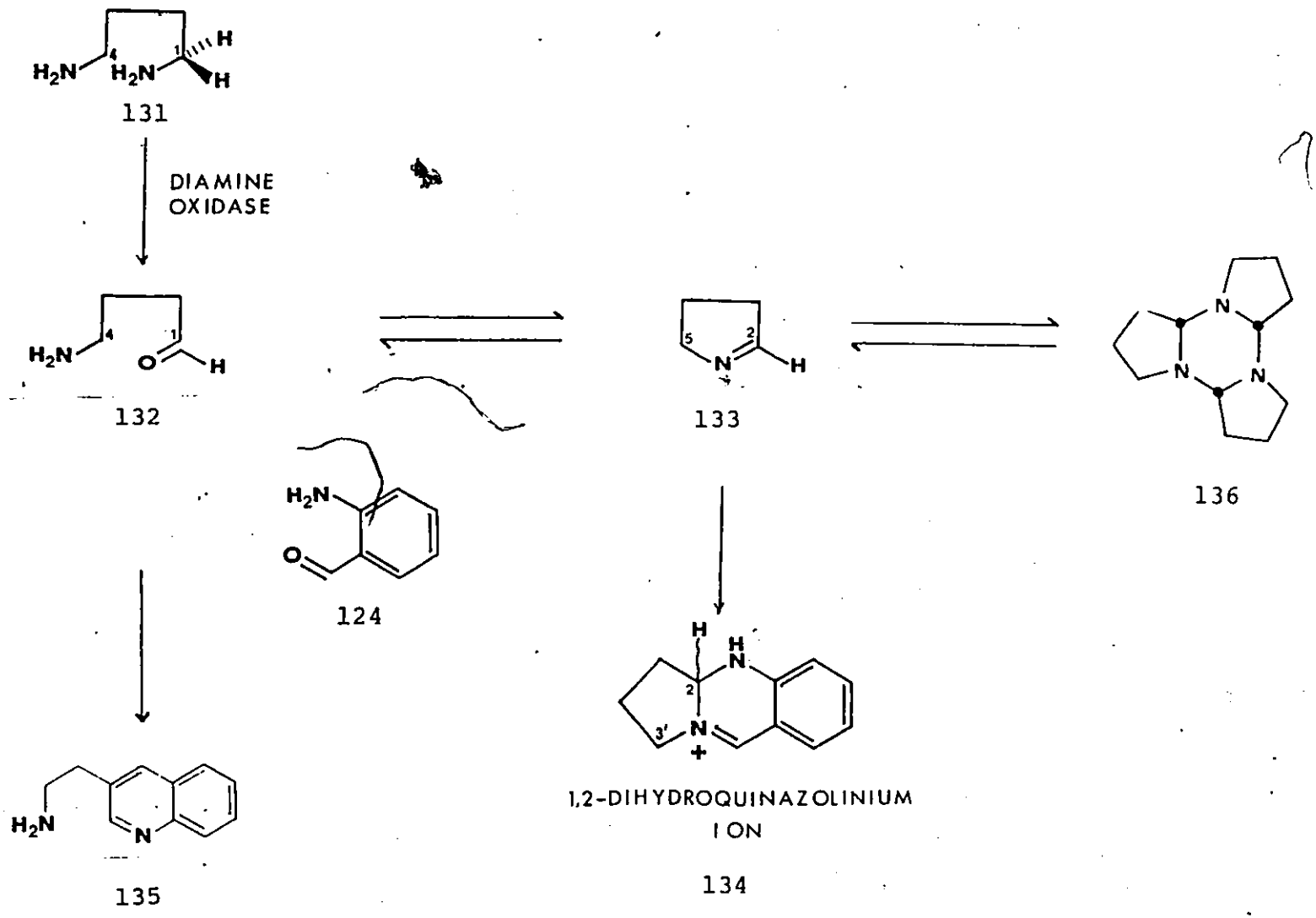
The samples of 3-(3'-aminopropyl)quinoline dihydrochloride, obtained from the incubation, with diamine oxidase, of the three deuteriated cadaverines (Expts. 1-3) (Table 7) and the samples of 2,3-trimethylene-1,2-dihydroquinazolinium picrate, obtained similarly from the three deuteriated putrescines (Expts. 4-6) (Table 8) each contained deuterium. The location of the deuterium in these samples was determined by ^2H NMR spectroscopy.

Assignment of the NMR signals was facilitated by the chemical synthesis of (2-^2H)-3-(3'-aminopropyl)quinoline dihydrochloride (δ 8.8) and of (2-^2H)-2,3-trimethylene-1,2-dihydroquinazolinium picrate (δ 5.3), by oxidation of DL-(2-^2H)lysine (*cf.* ref. 203) and DL-(2-^2H)ornithine (*cf.* ref. 199), respectively, with *N*-bromosuccinimide, to yield (1-^2H)-5-aminopentanal and (1-^2H)-4-aminobutanal, respectively, which were trapped, as before, as their *o*-aminobenzaldehyde adducts (Sections 6.4.1.1.2 and 6.4.1.2.2).

The samples of 3-(3'-aminopropyl)quinoline (125) dihydrochloride obtained from the incubation with diamine oxidase of R-(1-^2H)cadaverine (Expt. 1) and (1,1-$^2H_2$)cadaverine (Expt. 3a), each showed two signals in



Scheme 18. Products of the incubation of cadaverine (121) with diamine oxidase.



Scheme 19. Products of the incubation of putrescine (131) with diamine oxidase.

Table 7. Products of the incubation of cadaverine (121) with diamine oxidase.

Expt. No.	Substrate	Product (125): 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 (77) dihydrochloride	2	2.9, 8.8 [(1.0 ± 0.2)/1]	103 ± 19
2	S-(+)-(1-^{2}H)cadaverine (97) dihydrochloride	5	2.9	50 ± 19
3 (a)	(1,1-$^2H_2$)cadaverine (119) dihydrochloride	7	2.9, 8.8 [(2.2 ± 0.4)/1]	74 ± 11
(b)	(1,1-$^2H_2$)cadaverine (119) dihydrochloride	7		75 ± 11

Table 8. Products of the incubation of putrescine (131) with diamine oxidase.

Expt. No.	Substrate	Product (134)		
		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)	(-)-(1-^{2}H)putrescine (100) dihydrochloride	15	4.1, 5.2 [(1.2 ± 0.1)/1]	100 ± 9
(b)	(-)-(1-^{2}H)putrescine (100) dihydrochloride	7	4.1, 5.2 [(1.0 ± 0.3)/1]	
5	(+)-(1-^{2}H)putrescine (102) dihydrochloride	10	4.1	82 ± 4
6	(1,1-^{2}H$_2$)putrescine (120) dihydrochloride	12	4.1, 5.2 [(7.0 ± 0.5)/1]	90 ± 8

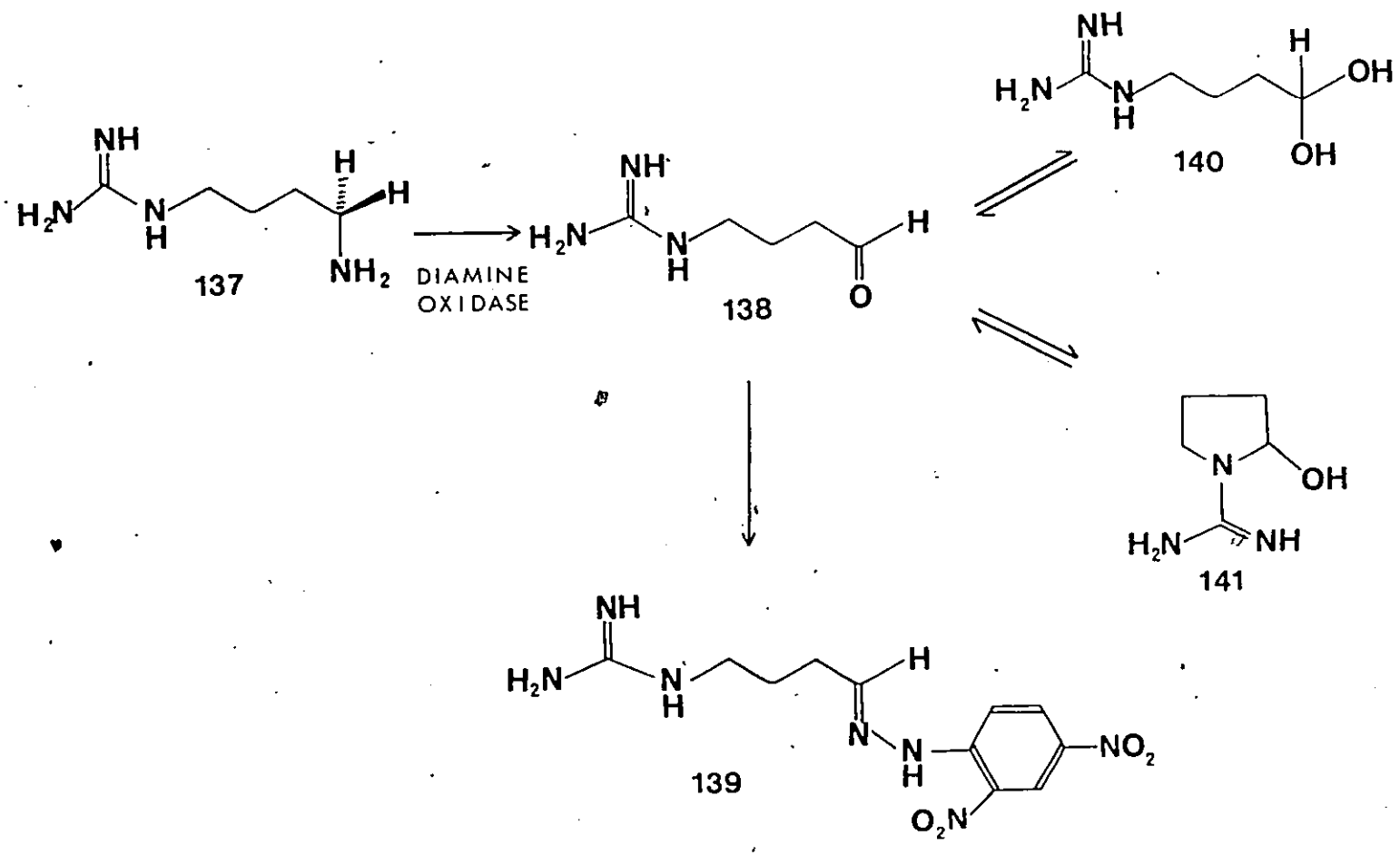
their ^2H NMR spectra (δ 8.8 and δ 2.9). The sample of (125) obtained from S-(1-^2H)cadaverine showed only a single signal (δ 2.9) in its ^2H NMR spectrum. It follows that oxidative deamination of cadaverine (121) to 5-aminopentanal (122), catalysed by diamine oxidase, is accompanied by removal of the *Si*-hydrogen.

The samples of 2,3-trimethylene-1,2-dihydroquinazolinium (134) picrate obtained from the incubation of (-)-(1-^2H)putrescine (Expt. 4) and (1,1-$^2H_2$)putrescine (Expt. 6) each showed two signals in their ^2H NMR spectra (δ 5.2 and δ 4.1) whereas only one ^2H NMR signal (δ 4.1) was observed in the spectrum of the sample of (134) obtained from (+)-(1-^2H)-putrescine (Expt. 5).

Correspondingly, in the oxidation of agmatine (137) to 4-guanidinobutanal (138)[196] which was isolated as the phosphate salt of its 2,4-dinitrophenylhydrazone (139) (Scheme 20), deuterium is preserved in the oxidation product of (-)-(1-^2H)agmatine sulfate (Expt. 7) but is lost in the formation of the product from (+)-(1-^2H)agmatine sulfate (Expt. 8) (Table 9).

If hog kidney diamine oxidase mediates stereospecific removal of the *Si*-hydrogen from C-1 of putrescine (131) and agmatine (137), as it does with cadaverine (121), it follows that deuterium occupies the 1-*Re*-position of the levorotatory samples of (1-^2H)putrescine and of (1-^2H)agmatine. Congruently, deuterium occupies the 1-*Si*-position of the dextrorotatory samples of (1-^2H)putrescine and of (1-^2H)agmatine.

While the enzymic reaction with the three substrates thus takes a qualitatively similar stereochemical course, a quantitative difference in



Scheme 20.. Product of the incubation of agmatine (137) with diamine oxidase.

Table 9. Products of the incubation of agmatine (137) with diamine oxidase.

Expt. No.	Substrate	Product (139)		
		4-guanidinobutanal-2',4'-dinitrophenylhydrazone phosphate		
		Yield (mg)	^{2}H NMR signals (ppm)	Retention of deuterium (%) (determined by ^{1}H NMR)
7	(-)-(1-^{2}H)agmatine (104) sulfate	25	7.6	100 ± 5
8	(+)-(1-^{2}H)agmatine (106) sulfate	32	-	0 ± 5

the reactions of cadaverine on the one hand and of putrescine on the other is revealed by the ^2H 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 with a deuterium retention of 75% (Expt. 3a, Table 7), the corresponding product from (1,1-$^2H_2$)putrescine showed a deuterium enrichment at the two deuteriated positions which gives rise to relative areas 7:1 for the signals at δ 4.1 and 5.2 with a deuterium retention of almost 90% (Expt. 6, Table 8). This observation indicated the occurrence of a primary intramolecular hydrogen-deuterium isotope effect in the course of the oxidation of (1,1-$^2H_2$)-putrescine, but not (1,1-$^2H_2$)cadaverine.

3.3 DISCUSSION

3.3.1 ^2H NMR as a probe of the stereochemistry of enzymic oxidative deamination

Hog kidney diamine oxidase catalyses the oxidative deamination of a wide range of primary amines, including diamines, agmatine and arylalkyl amines, to the corresponding aldehydes.[167,204-207] The aliphatic diamines, cadaverine (121) and putrescine (131) are amongst substrates of the enzyme which have the lowest Michaelis constant (K_M)[205] and the highest oxidation rate (V_{max}).[206,207] Agmatine (137) is also readily attacked and is oxidised at a rate which is *approx.* half that of the aliphatic diamines.[206]

The stereochemistry of the removal of an α-hydrogen atom in this reaction may be determined using as substrates chiral amines of known

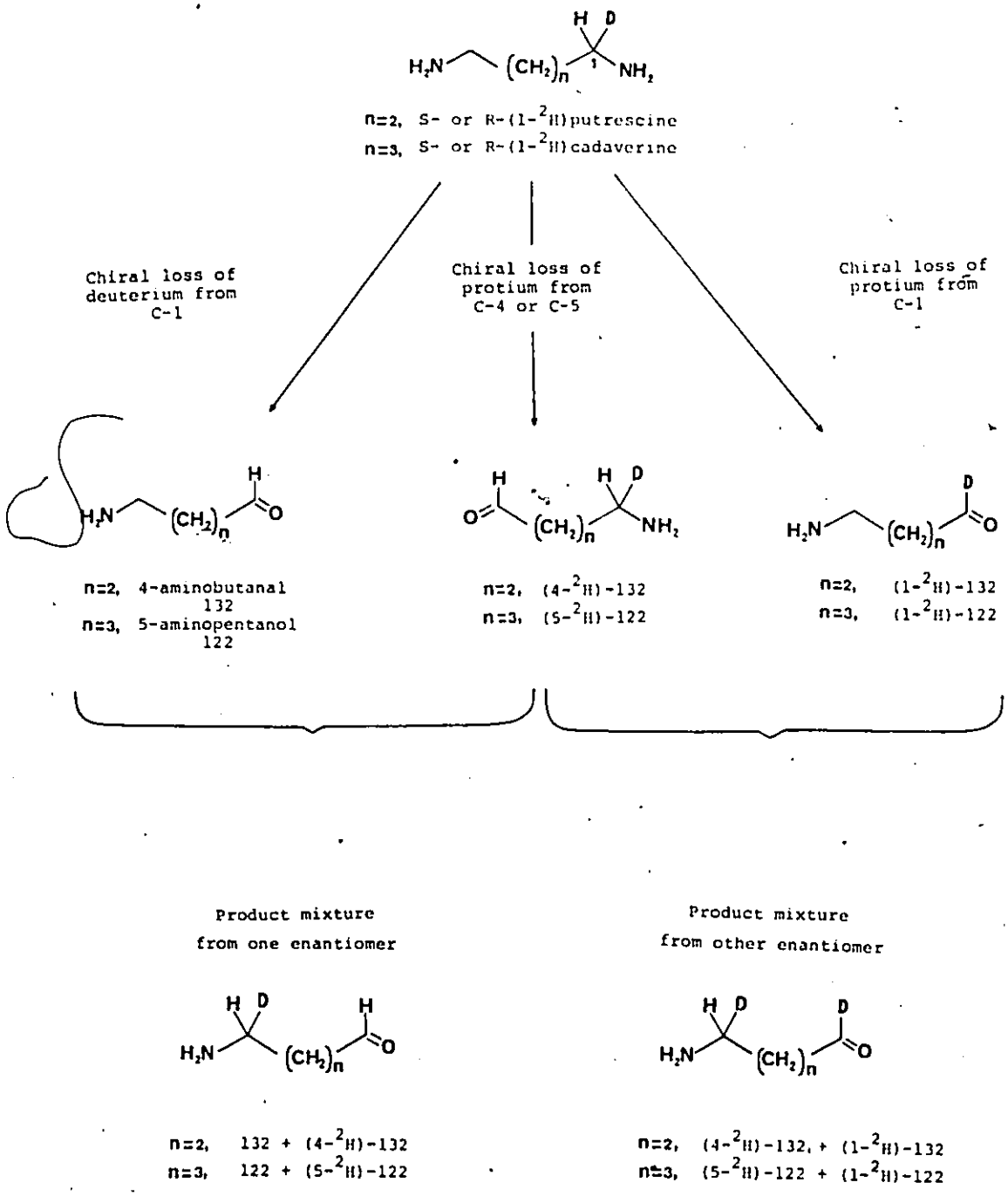
chirality, in which one or the other of the two enantiotopic hydrogen atoms on the carbon adjacent to the reacting primary amino group is replaced by deuterium or tritium. Enantiomeric, chirally labelled samples of (1-^{2}H)cadaverine, (1-^{2}H)putrescine and (1-^{2}H)agmatine were available (Chapter 2). Thus, with deuterium as the label, the deuterium content of the products of the enzyme-catalysed process serves as a diagnostic indicator of the steric course of the reaction. Since starting materials with deuterium enrichment approaching 100 atom % at the desired position can be used, the deuterium content as well as its location within the products can be determined by ^{1}H or ^{2}H NMR spectroscopy.

When chirally labelled (1-^{2}H)amines, which bear a single reactive amino group (*e.g.* (1-^{2}H)agmatine), serve as substrates, one enantiomer, by stereospecific loss of deuterium, yields a product which is devoid of label, whereas the other enantiomer, by stereospecific loss of protium, yields a product which retains label. If the chirality of the (1-^{2}H)substrate is known, determination of the presence or absence of deuterium in the product is sufficient to establish, unequivocally, the chirality of the process which leads to the products. Alternatively, if the chirality of the process is known, but that of the (1-^{2}H)substrate is not, determination of the presence or absence of deuterium in the product provides a definitive answer to the question of the chirality of the (1-^{2}H)substrate.

By contrast, when the aliphatic diamines, rendered chiral by deuterium substitution at only one of the two equivalent methylene groups adjacent to nitrogen (*i.e.* (1-^{2}H)cadaverine or (1-^{2}H)putrescine), serve as substrates, the reaction will not lead to deuterium-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 deuterium.

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 (Scheme 21). Thus the presence or absence of deuterium within the product will not be a diagnostic criterion for the stereochemical course of the reaction, or for the stereochemistry of the (1-^{2}H)substrate. Quantitative determination of deuterium within the product will be necessary in order to obtain the required information. A further problem arises if the reaction is accompanied by a substantial isotope effect (*cf.* ref. 135 and 168). Measurement of the extent of retention of label within the product would, in this case, not yield information concerning the



Scheme 21. Distribution of label within the products of incubation of enantiomeric samples of (1-^{2}H)-putrescine and of (1-^{2}H)cadaverine with diamine oxidase.

stereochemistry of hydrogen 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 deuterium within the products becomes mandatory.

The distribution of deuterium within the products can be determined very accurately by ^{2}H NMR spectroscopy.[169,208,209] Since deuterium exhibits a significant electric quadrupole moment, its relaxation behaviour is dominated by a quadrupole exchange mechanism.[208,210] This results in shorter relaxation times compared to those of other nuclei commonly employed in NMR investigations (*e.g.* ^{1}H, ^{13}C, ^{15}N).[211] As a consequence of the short ^{2}H-relaxation times,[211] combined with the absence of a nuclear Overhauser enhancement (when spectra are obtained with proton decoupling),[208] the relative extent of enrichment within partially deuteriated molecules can be determined accurately by integration.[169,208] Quantitative determination of deuterium enrichment at a specific site may be determined by reference to a suitable internal standard or, alternatively, this information can, in some cases, be obtained from the corresponding ^{1}H NMR spectrum.

Assignment of the ^{2}H NMR signals arising from deuteriation at specific sites within a labelled molecule is often facilitated by comparison with the corresponding ^{1}H NMR signals (observed in the ^{1}H NMR spectrum of the unlabelled molecule), since, apart from a small isotope effect, the chemical shift values of ^{2}H-resonances are the same as those of the analogous ^{1}H-resonances.[208] Unambiguous assignment of either the ^{1}H- or ^{2}H-resonances, corresponding to protium or deuterium, respectively, at a position within the molecule, can be achieved if a labelled

standard, in which the position of interest is specifically labelled with deuterium, is available by chemical synthesis.

^{2}H NMR suffers from two major limitations: spectral crowding and poor resolution.[169] These limitations are the result of the quadrupole moment and the low magnetogyric ratio associated with ^{2}H nuclei.[208] Since the magnetogyric ratio of deuterium is significantly less than that of protium, the chemical shift dispersion of ^{2}H-resonances is only a fraction (*i.e.* 15%) of that of the corresponding ^{1}H-resonances.[208] Extensive line broadening of ^{2}H-resonances, due to the dominance of deuterium quadrupole relaxation,[169,208] often accompanies this effect which results in poorly resolved ^{2}H NMR signals. These limitations can be overcome by obtaining spectra at higher magnetic field or in the presence of lanthanide shift reagents. In the present investigation, the problem of poor resolution was avoided by employing suitable derivatives of the products of enzymic reaction (labelled samples of 5-aminopentanal and 4-aminobutanal (Scheme 21)), in which the chemical shifts of the ^{2}H NMR signals, corresponding to the two deuteriated centres, were well separated.

3.3.2 Isolation of enzymic reaction products

Enzymic oxidative deamination of the aliphatic diamines, cadaverine (1$\underset{\sim}{2}$1) and putrescine (1$\underset{\sim}{3}$1), yields the corresponding ω-aminoaldehydes, 5-aminopentanal (1$\underset{\sim}{2}$2) and 4-aminobutanal (1$\underset{\sim}{3}$2), which are in equilibrium with their cyclic imines, Δ'-piperideine (1$\underset{\sim}{2}$3) and Δ'-pyrroline (1$\underset{\sim}{3}$3), respectively[195,197,212] (Schemes 18 and 19).

The action of diamine oxidase on cadaverine has been shown[195,197] to lead to reaction products which accumulate as a mixture of at least

three isomeric trimers (Scheme 18). The chemistry of Δ'-piperideine has been extensively investigated by Schöpf and his co-workers.[198,213-215] Reversible trimerization of Δ'-piperideine (123) gives rise to α- and β-tripiperideine ((127) and (128), respectively[216]) which differ only in the relative configuration of the three chiral methine carbon atoms. A second, structural isomer, *iso*-tripiperideine (130) appears to arise *via* tetrahydroanabasine (129). *iso*-Tripiperideine (130) possesses four chiral centres and may comprise a mixture of as many as 16 stereoisomers (8 enantiomeric pairs). The constitution, configuration and conformation of the three trimers has recently been investigated by ^{13}C NMR spectroscopy.[216]

Δ'-Pyrroline also undergoes polymerization under the enzymic reaction conditions[195,200,201] (Scheme 19). This imine has been reported to be difficult to isolate since it is susceptible to air oxidation at pH values of greater than 7.[199] At pH values of less than 7, Δ'-pyrroline (133) exists in equilibrium with the symmetrical trimer (136) (*i.e.* C_3 symmetry),[201,217] analogous to α-tripiperideine (127).

Due to the complexity of the enzymic reaction mixtures derived from cadaverine (Scheme 18) and putrescine (Scheme 19), it was therefore necessary to isolate each of the oxidation products, 5-aminopentanal and 4-aminobutanal, respectively, as a suitable derivative. There were two requirements for such a derivative: (i) when samples of the (1-^2H)-diamines serve as substrates of diamine oxidase, the distinctiveness of the two potentially deuteriated positions within the reaction products, the corresponding ω-aminoaldehyde or its cyclic imine, must be retained

within the derivatised products (*cf.* Section 3.3.1, Scheme 21); (ii) the ^2H NMR signals due to deuterium substitution at the two corresponding positions within the derivatised products should be well separated. Also desirable was a derivative which would be produced *in situ* by reaction with a reagent, which could be added to the enzyme incubation mixture without affecting the activity of diamine oxidase.

The reactions of Δ'-piperideine (123) and Δ'-pyrroline (133) with *o*-aminobenzaldehyde (124) to yield the corresponding quinoline ((125) and (135), respectively) and dihydroquinazolinium ((126) and (134), respectively) derivatives had previously been investigated.[198,199,202] *o*-Aminobenzaldehyde had also been employed to trap the reaction products obtained by enzymic oxidation of cadaverine and putrescine.[195,197] This reagent has also been used for the spectrophotometric determination of diamine oxidase activity:[218,219] with putrescine (131) as the substrate, Δ'-pyrroline (133) is trapped *in situ* as the 2,3-trimethylene-1,2-dihydroquinazolinium ion (134). *o*-Aminobenzaldehyde does not significantly inhibit diamine oxidase activity.[218] This was the reagent employed in the present investigation for the isolation of the enzymic reaction products.

Under the conditions adopted for incubation of the diamines with diamine oxidase (Table 6), 5-aminopentanal (122) (⇌ Δ'-piperideine (123)) was trapped with *o*-aminobenzaldehyde (124) to afford 3-(3'-aminopropyl)-quinoline (125) as the major product (Scheme 18), whereas 4-aminobutanal (132) (⇌ Δ'-pyrroline (133)) afforded the dihydroquinazolinium adduct (134) as the major product (Scheme 19). The integrity of the two potentially deuteriated positions within each of these products (*i.e.* C-2 and

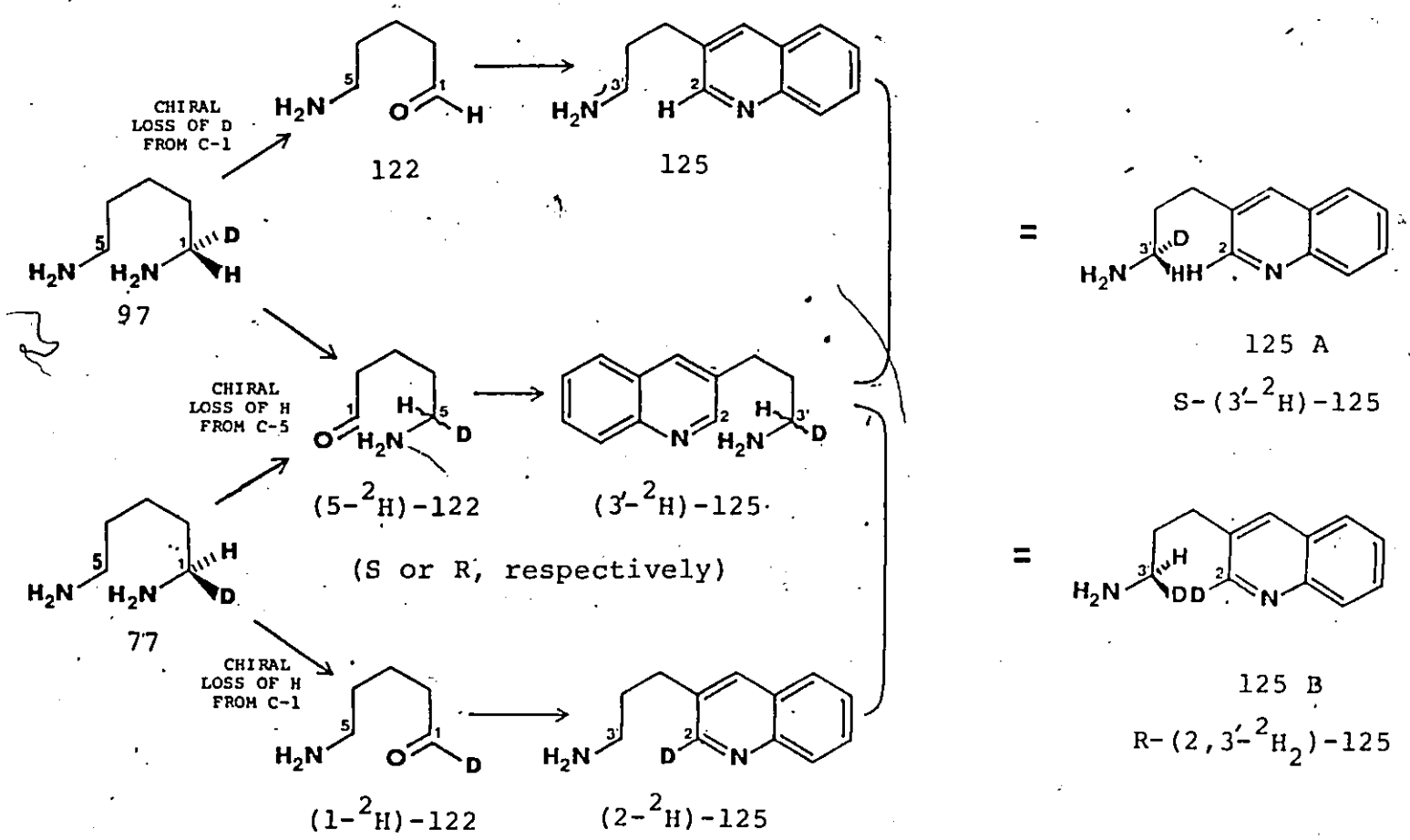
C-3' in both (125) and (134)) was maintained when samples of (1-^{2}H)cadaverine and (1-^{2}H)putrescine served as substrates: the ^{2}H NMR signals corresponding to deuterium substitution at the two positions were fully resolved (Sections 3.3.3 and 3.3.4).

Enzymic oxidative deamination of agmatine (137) affords 4-guanidinobutanal (138)[196] (Scheme 20). Incubation of agmatine with hog kidney diamine oxidase led to quantitative oxidation of the amine (Section 6.4.2.2.3). The ^{1}H NMR spectrum (^2H_2O) of the oxidation product showed three poorly resolved signals which, by integration, corresponded to the protons at C-1 (δ 5.2), C-4 (δ 3.2) and C-2,3 (δ 1.7). The absence of a signal in the region characteristic of aldehydic protons (> 9 ppm),[220] and the occurrence of a signal at 5.2 ppm, suggests that the product exists in aqueous solution as the hydrated aldehyde (140) or, possibly, as a cyclic hydrated imine, *e.g.* (141). Corroboration of this inference was obtained from the ^{13}C NMR spectrum of the product: no signal was present in the aldehyde carbon region (*ca.* 200 ppm),[221] while the signal attributed to C-1 occurred at 83.8 ppm. The product of enzymic oxidation of agmatine (137) was subsequently isolated as the 2,4-dinitrophenylhydrazone derivative (139) (Scheme 20) which was identified from its ^{1}H and ^{13}C NMR spectra. The products resulting from the incubation of the samples of (1-^{2}H)agmatine with diamine oxidase were similarly isolated, as their dinitrophenylhydrazone derivatives (Section 3.3.5).

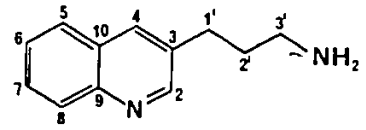
3.3.3 The stereochemistry of the dehydrogenation of cadaverine, catalysed by hog kidney diamine oxidase

Incubation of S-(1-^{2}H)- and R-(1-^{2}H)cadaverine with hog kidney diamine oxidase afforded deuteriated samples of 5-aminopentanal (122) which were trapped with *o*-aminobenzaldehyde to yield deuteriated samples of 3-(3'-aminopropyl)quinoline (125) (Scheme 22). The two samples were isolated as their dihydrochloride salts and ^{2}H NMR spectroscopy was employed to determine the location of deuterium within the two derivatives (Table 7). In order to assign the ^{2}H NMR signals of the deuteriated quinoline derivatives, the ^{1}H NMR spectra of samples of the unlabelled derivative and of derivatives of known isotope distribution were examined.

A specimen of 3-(3'-aminopropyl)quinoline (125) was obtained from cadaverine (121), by enzymic dehydrogenation, and was found to be identical with an authentic specimen prepared[198] from α-tripiperideine (127) (Section 6.4.1.1.1, *cf.* Scheme 18). The ^{1}H NMR spectrum of the free base (125) in ^2H_2O (Figure 4) showed two low field doublets at δ 8.63 (J 1.8 Hz) and δ 8.27 (J ≈ 1.8 Hz), each corresponding to one proton. These signals were readily assigned to the protons at C-2 and C-4 of the quinoline ring system. The five line pattern centered at δ 2.93 (relative area 4) arising from two overlapping triplets (J ≈ 7.5 Hz) was assigned to the protons at C-1' and C-3' of the 3-aminopropyl side chain. The chemical shifts corresponding to each of these protons (H-2, H-4, H-1', H-3') were sensitive to changes in p^{2}H. When the ^{1}H NMR spectrum of the dihydrochloride salt was recorded in ^2H_2O, the difference in chemical shift between the two doublets arising from H-2 and H-4 was only 0.07 ppm; the signals due to H-1' and H-3' were indistinguishable and



Scheme 22. Products of the incubation of S-(1-^{2}H)- and R-(1-^{2}H)cadaverine with diamine oxidase.



125

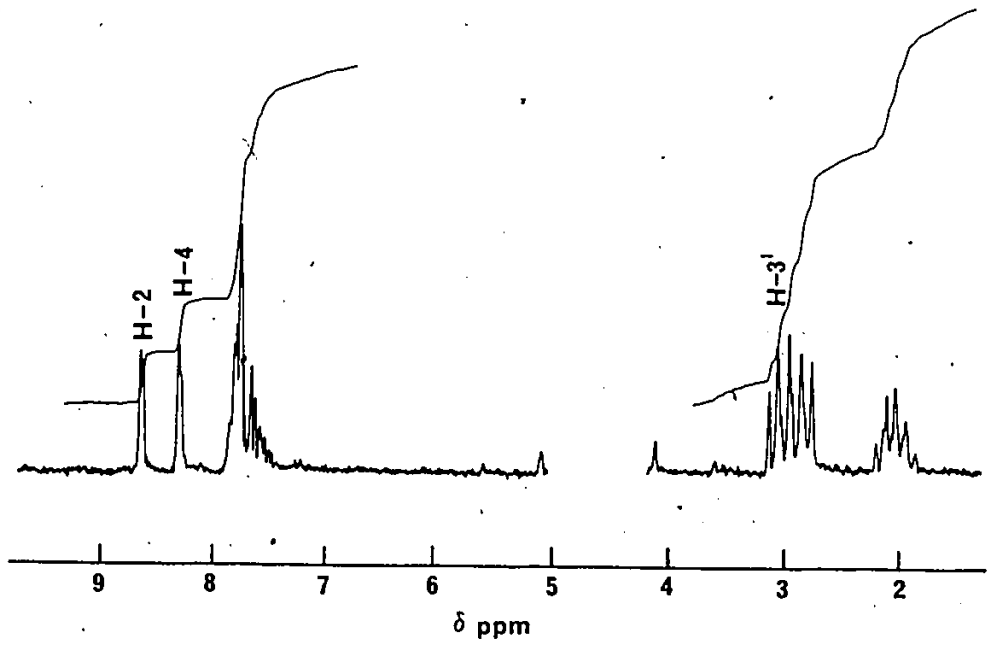


Figure 4. ^{1}H NMR spectrum of 3-(3'-aminopropyl)quinoline (125) (in $^{2}H_2O$).

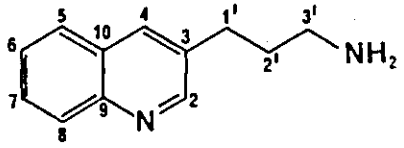
gave the appearance of a triplet centred at δ 3.05. At p^{2}H *ca.* 5, however, the spectrum of the dihydrochloride was similar to that of the free base (in ^2H_2O) (Table 10). The signals due to H-2 and H-4 were separated by 0.32 ppm, whereas the protons at C-1' and C-3' gave rise to a five line pattern. Unambiguous assignment of the signals arising from H-2 and H-3' was made using compounds of known isotope distribution.

A sample of 3-(3'-aminopropyl)-(2-^{2}H)quinoline (125D) was prepared by *N*-bromosuccinimide oxidation of (2-^{2}H)lysine, followed by condensation of the product, (1-^{2}H)-5-aminopentanal with *o*-aminobenzaldehyde (Section 6.4.1.1.2). The ^{1}H NMR spectrum (^2H_2O, p^{2}H ~ 5) of the dihydrochloride of (2-^{2}H)-(125) showed virtually complete absence of the signal at δ 8.68 which was present in the spectrum of the unlabelled specimen. This established that the signal at lowest field (*i.e.* δ 8.68) in the spectrum of the quinoline (125) corresponds to H-2.

A sample of 3-(3'-aminopropyl)quinoline dihydrochloride (125C) intermolecularly doubly labelled with deuterium at C-2 and C-3' was obtained by incubation of (1,1-$^2H_2$)cadaverine with diamine oxidase in the presence of *o*-aminobenzaldehyde (Expt. 3a). The ^{1}H NMR spectrum (^2H_2O, p^{2}H ~ 5) of this sample (125C) showed the two signals at δ 8.32 (H-4) and δ 8.68 (H-2) in a ratio of 2:1. The intensity of the downfield portion (δ 2.95, t) of the five line pattern centred at δ 2.90 was *approx.* half that of the upfield portion (δ 2.85, t), indicating that the triplet at δ 2.95 corresponded to the protons at C-3' (*cf.* Table 10).

The ^{2}H NMR spectra of these samples of 3-(3'-aminopropyl)quinoline dihydrochloride ((125C) and (125D)), as well as those of the two samples ((125A) and (125B)) derived from S-(1-^{2}H)- and R-(1-^{2}H)cadaverine are shown in Figure 5.

Table 10. ^1H NMR data for 3-(3'-aminopropyl)quinoline (125) and its dihydrochloride salt.



3-(3'-aminopropyl)-quinoline (solvent)	δ (ppm)[a]					
	H-2	H-4	Aromatic-H	H-1'	H-2'	H-3'
Free base (^2H_2O)	8.63 d $J_{2,4}$ 1.8 Hz	8.27[b] d $J_{2,4}$ ~ 1.8 Hz	7.46-8.00	2.83[c] t $J_{1',2'}$ 7.5 Hz	1.93 quin $J_{1',2'} \approx J_{2',3'}$	3.04[c] t $J_{2',3'}$ 7.5 Hz
Dihydrochloride (^2H_2O)	8.89 d $J_{2,4}$ 1.8 Hz	8.82[b] d $J_{2,4}$ ~ 1.8 Hz	7.61-8.15	3.05[d] m -	2.02 quin J 7.4 Hz	3.05[d] m -
Dihydrochloride (^2H_2O, p^{2}H 5)	8.68[e] s	8.32[f] s	7.42-8.02	2.85[c] t $J_{1',2'}$ 7.2 Hz	1.94 quin $J_{1',2'} \approx J_{2',3'}$	2.95[c] t $J_{2',3'}$ 7.2 Hz

[a] Relative to DSS.
[b] Poorly resolved.
[c] Two overlapping triplets.
[d] Apparent triplet.
[e] Width at half height, w ½ = 6 Hz.
[f] w ½ = 4 Hz.

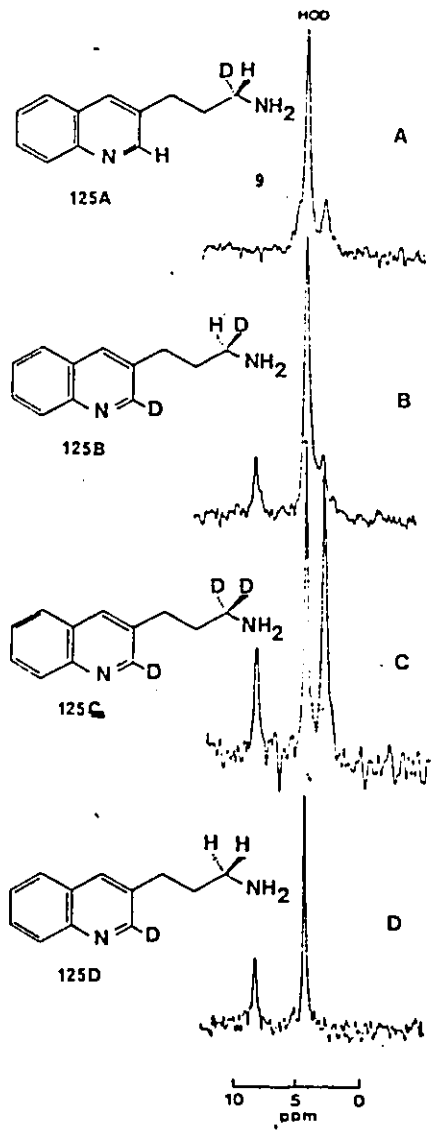


Figure 5. Proton decoupled ^2H NMR spectra of deuterium labelled samples of 3-(3'-aminopropyl)quinoline (125) dihydrochloride: A (125A), derived from S-(1-^2H)cadaverine (by enzymic oxidative deamination) (5 mM, 56628 transients); B (125B), derived similarly from R-(1-^2H)cadaverine (5 mM, 57000 transients); C (125C), derived similarly from (1,1-$^2H_2$)cadaverine (13 mM, 3475 transients); D (125D), obtained from DL-(2-^2H)lysine by chemical oxidation (8 mM, 4808 transients).

The ^2H NMR spectrum of the sample of 3-(3'-aminopropyl)-(2-^2H)-quinoline (125D) showed a single peak at δ 8.8 (Figure 5, spectrum D) (apart from the signal at δ 4.5 due to the natural abundance of deuterium in water). The ^2H NMR spectrum of the quinoline derivative (125C) derived from (1,1-$^2H_2$)cadaverine (Expt. 3a) showed two signals at δ 2.9 and δ 8.8, of relative areas 2:1, due to deuterium at C-3' and C-2, respectively (Figure 5, spectrum C).

The quinoline derivative of the sample of 5-aminopentanal derived from S-(1-^2H)cadaverine contained deuterium only at C-3' of the side chain (δ 2.9, Figure 5, spectrum A), that derived from R-(1-^2H)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, Figure 5, spectrum B). Thus, the 5-aminopentanal, derived from S-(1-^2H)cadaverine, was a mixture of (5-^2H)-5-aminopentanal and nondeuteriated 5-aminopentanal, whereas the product from R-(1-^2H)cadaverine was a mixture of (5-^2H)-5-aminopentanal and (1-^2H)-5-aminopentanal. Deuterium had been stereospecifically lost from S-(1-^2H)-cadaverine, protium from R-(1-^2H)cadaverine (Scheme 22).

It follows that diamine oxidase from hog kidney mediates the stereospecific removal of the *Si*-hydrogen from C-1 of cadaverine, and that the product of oxidative deamination, 5-aminopentanal, in equilibrium with Δ'-piperideine, retains the *Re*-hydrogen at the sp^2 carbon.

This stereospecificity corresponds to that of the analogous reactions, catalysed by the copper-amine oxidases (E.C. 1.4.3.6)[150] from pea seedlings and bovine plasma (Table 2, Section 1.5). The hog kidney enzyme is also a copper-amine oxidase.[222-224] The FAD-containing enzyme, rat liver mitochondrial monoamine oxidase (E.C. 1.4.3.4),[150] by contrast,

catalyses the reaction of opposite chirality: oxidative deamination of tyramine[119,122,129] and heptylamine[151] takes place with loss of the *Re*-hydrogen from the carbon adjacent to the amino group.

The diamine oxidase from pea seedlings has been shown to catalyse stereospecific removal of the 1-*Si*-hydrogen in the course of the oxidation of every substrate so far investigated (Table 2). The stereochemical consistency with such a wide range of substrates would appear to justify the assumption that, in the reaction catalysed by this enzyme, oxidation of every substrate is accompanied by loss of the *Si*-hydrogen. Indeed, configurational assignments of the absolute stereochemistry of chiral samples of 4-amino[4-^{3}H]butan-1-ol,[132] of 5-amino[5-^{3}H]pentan-1-ol,[135] of 3-methylthio[1-^{3}H]propylamine[138] and, very recently, of [1-^{3}H]-putrescine,[168] have been made on the basis of this assumption.

Similarly, the assumption that mitochondrial rat liver monoamine oxidase catalyses removal of the *Re*-hydrogen not only from tyramine[119, 122,129] and heptylamine[151] but also from other substrates, has served as the basis of configurational assignment of chiral samples of [α-^{3}H]-tryptamine.[141] The validity of such an assumption would therefore appear to be well accepted. Based on this assumption, that hog kidney diamine oxidase not only mediates stereospecific removal of the *Si*-hydrogen from C-1 of cadaverine, but also of putrescine and of agmatine, configurational assignments of the chiral samples of (1-^{2}H)putrescine and of (1-^{2}H)-agmatine can be made.

3.3.4 Configurational assignment of the enantiomeric samples of (1-^{2}H)putrescine

Incubation of the enantiomeric samples of (1-^{2}H)putrescine, obtained by enzymic decarboxylation of L-ornithine, with diamine oxidase produced deuteriated samples of Δ'-pyrroline (133) which were trapped with *o*-aminobenzaldehyde (124) to yield deuteriated samples of 2,3-trimethylene-1,2-dihydroquinazolinium (134) picrate. The location of deuterium in the samples of the dihydroquinazolinium derivative was determined by ^{2}H NMR spectroscopy (Table 8, Figure 6).

The oxidation of putrescine catalysed by diamine oxidase is expected to occur with stereospecific loss of the 1-*Si*-hydrogen (Section 3.3.3). Thus, oxidation of the S-enantiomer of (1-^{2}H)putrescine by stereospecific loss of the *Si*-deuterium leads to a sample of Δ'-pyrroline (133) enriched in deuterium exclusively at C-5, together with nondeuteriated (133). The dihydroquinazolinium derivative (134) obtained from this (5-^{2}H)-Δ'-pyrroline will be deuteriated exclusively at C-3' of the trimethylene ring. Oxidation of the R-enantiomer of (1-^{2}H)putrescine by stereospecific retention of the *Re*-deuterium but loss of the *Si*-hydrogen from C-1, leads to a sample of Δ'-pyrroline, intermolecularly doubly labelled at C-2 and C-5. This, in turn, yields an intermolecularly labelled dihydroquinazolinium adduct, deuteriated at C-3', as well as at C-2 of the dihydroquinazolinium ring system (Scheme 23).

Assignment of the NMR signals due to ^{1}H and ^{2}H at these positions was facilitated by chemical synthesis of (2-^{2}H)-(134). This was achieved by oxidative decarboxylation of (2-^{2}H)ornithine (101) with *N*-bromosuccinimide to afford (2-^{2}H)-Δ'-pyrroline which was then trapped, as before, with *o*-aminobenzaldehyde.

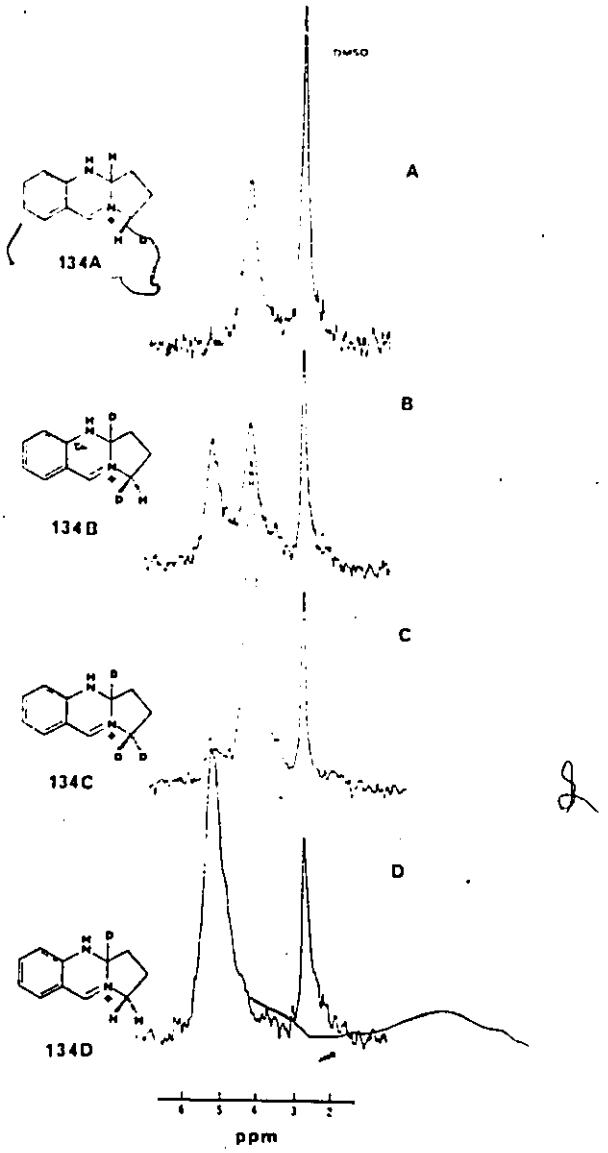
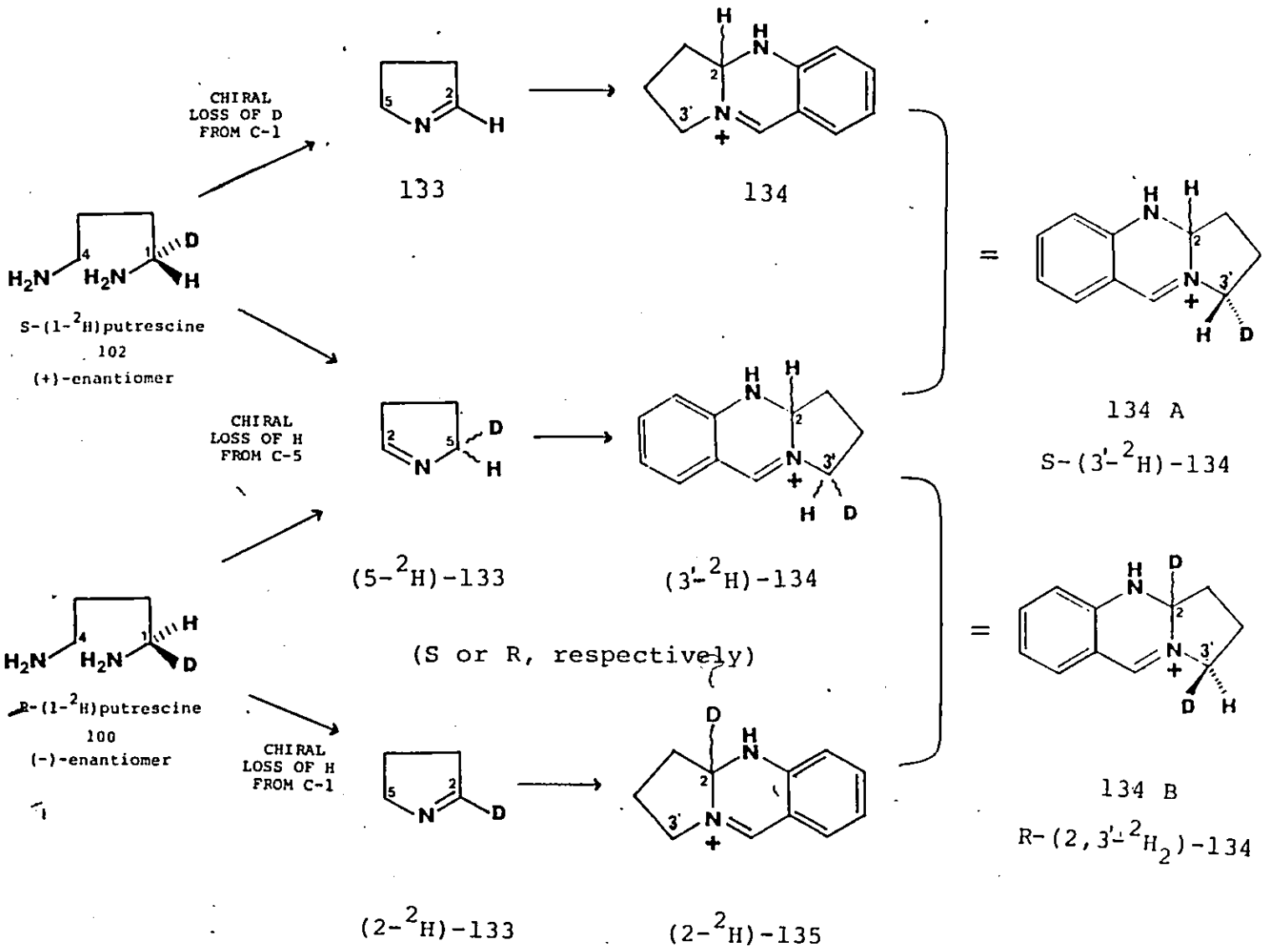


Figure 6. Proton decoupled ^2H NMR spectra of deuterium labelled samples of 2,3-trimethylene-1,2-dihydroquinazolinium (134) picrate: A (134A), derived from (+)-(1-^2H)putrescine dihydrochloride (by enzymic oxidative deamination) (13 mM, 15140 transients); B (134B), derived similarly from (-)-(1-^2H)putrescine dihydrochloride (13 mM, 10000 transients); C (134C), derived similarly from (1,1-$^2H_2$)putrescine dihydrochloride (26 mM, 4824 transients); D (134D), obtained from DL-(2-^2H)ornithine by chemical oxidation (20 mM, 5000 transients).



Scheme 23. Products of the incubation of the (+)- and (-)-enantiomers of (1-^{2}H)-putrescine dihydrochloride with diamine oxidase.

The ^1H NMR spectra of unlabelled samples of 2,3-trimethylene-1,2-dihydroquinazolinium picrate derived from putrescine, by enzymic oxidation, and from ornithine, by oxidative decarboxylation with *N*-bromosuccinimide, were identical (Figure 7). The signals assigned to the protons at C-3' and C-2 appeared at δ 4.12 (t, $J_{2',3'}$ 6.8 Hz) and δ 5.20 (t, $J_{2,1'}$ 6.0 Hz), respectively. The downfield signal (δ 5.20) was absent in the ^1H NMR spectrum of the sample of 2,3-trimethylene-(2-^2H)-1,2-dihydroquinazolinium (1̃34D) picrate. The ^2H NMR spectrum of (2-^2H)-(1̃34) showed a single peak at δ 5.1 (Figure 6, spectrum D) (apart from a signal at δ 2.8 due to the natural abundance of ^2H in dimethylsulfoxide).

The assignment of these NMR signals was further verified by examination of the ^2H NMR spectrum of a sample of (2,3',3'-$^2H_3$)-(1̃34) (*i.e.* (1̃34C)) which was obtained by enzymic dehydrogenation of (1,1-$^2H_2$)putrescine (Expt. 6, Table 8). The ^2H NMR spectrum of this sample showed two signals at δ 4.1 and δ 5.2 (relative areas*, 7:1) corresponding to deuteriation at C-3' and C-2, respectively (Figure 6, spectrum C).

The ^2H NMR spectra of the dihydroquinazolinium derivatives obtained from the two enantiomeric samples of (1-^2H)putrescine are shown in Figure 6 (spectra A and B). The dihydroquinazolinium derivative (1̃34A) derived from (+)-(1-^2H)putrescine (1̃02) dihydrochloride (Expt. 5) contained deuterium only at C-3' of the trimethylene ring (δ 4.1, Figure 6,

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* The relative areas of these signals are discussed in Section 3.3.6.

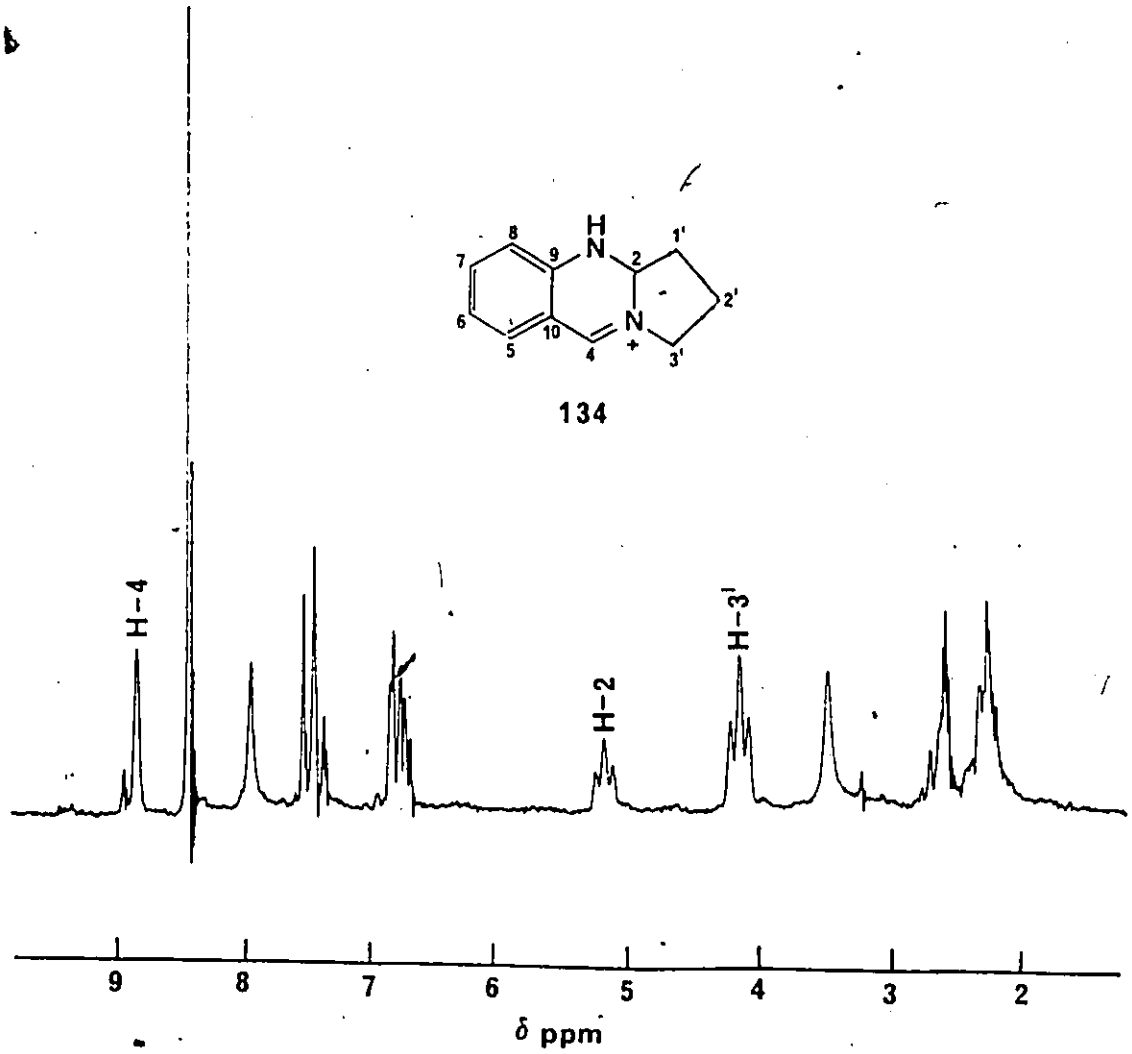


Figure 7. ^{1}H NMR spectrum of 2,3-trimethylene-1,2-dihydroquinazolinium (134) picrate.

spectrum A) while that (134B) derived from (-)-(1-^{2}H)putrescine (100) dihydrochloride (Expt. 4) contained deuterium at C-3', as well as at C-2' of the dihydroquinazolinium ring system (δ 4.1 and δ 5.2, Figure 6, spectrum B). Thus, the deuteriated Δ'-pyrroline derived from the sample of (+)-(1-^{2}H)putrescine was a mixture of (5-^{2}H)-Δ'-pyrroline and nondeuteriated (134), while the product derived from the sample of (-)-(1-^{2}H)-putrescine was a mixture of (5-^{2}H)-Δ'-pyrroline and (2-^{2}H)-Δ'-pyrroline. Deuterium had been stereospecifically lost from the (+)-enantiomer, and protium from the (-)-enantiomer.

If hog kidney diamine oxidase mediates stereospecific removal of the *Si*-hydrogen when putrescine is the substrate, as it does when cadaverine is the substrate, it follows that deuterium occupies the *Si*-position of (+)-(1-^{2}H)putrescine, while protium occupies the *Si*-position of (-)-(1-^{2}H)putrescine. (+)-(1-^{2}H)Putrescine is the S-enantiomer, (-)-(1-^{2}H)putrescine, the R-enantiomer (Scheme 23). Thus, it was S-(1-^{2}H)putrescine which was derived from L-(2-^{2}H)ornithine, by enzymic decarboxylation in ^1H_2O, and it was R-(1-^{2}H)putrescine which was derived from unlabelled L-ornithine, by enzymic decarboxylation in ^2H_2O. Replacement of the carboxyl group of L-ornithine by a solvent proton in the course of decarboxylation, catalysed by L-ornithine decarboxylase of *E. coli* occurs with retention of configuration (*cf.* Scheme 17, Section 2.3.1).

3.3.5 Configurational assignment of the enantiomeric samples of (1-^{2}H)agmatine

Incubation of (-)-(1-^{2}H)agmatine (104) sulfate with hog kidney diamine oxidase (Expt. 7) yielded a sample of 4-guanidinobutanal (138)

(isolated as the phosphate salt of the 2',4'-dinitrophenylhydrazone (139)) which contained deuterium at the aldehyde carbon atom as revealed by examination of its ^2H NMR spectrum. The product (139) derived from the enantiomeric (+)-(1-^2H)agmatine (106) sulfate (Expt. 8) was free of deuterium: no signal due to deuterium in the product was detectable by ^2H NMR (Table 9).

The ^1H NMR spectra of the two dinitrophenylhydrazones confirm the inference that deuterium is maintained in one of the products but not in the other (Figure 8). The downfield region of the spectrum of the aldehyde dinitrophenylhydrazone from (+)-(1-^2H)agmatine sulfate (Figure 8, spectrum A) shows a proton triplet, centred at δ 7.74 ($J_{1,2}$ 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, coincides with the downfield line of a doublet, δ 7.68 ($J_{5',6'}$ 9.8 Hz), due to H-6' of the aromatic nucleus. This triplet is missing entirely in the spectrum of the sample derived from (-)-(1-^2H)agmatine (Figure 8, spectrum B). Thus, this sample is completely deuteriated at C-1.

Complete retention of deuterium in the product derived from one enantiomer of (1-^2H)agmatine (104), and complete loss of deuterium in the product derived from the other enantiomer of (1-^2H)agmatine (106), provides clear evidence that, in the course of the conversion of agmatine to 4-guanidinobutanal, hog kidney diamine oxidase mediates stereospecific release of one of the two enantiotopic hydrogens from the carbon adjacent to the reacting amino group. The degree of stereospecificity is as complete as the methods of detection can reveal. Thus, on the assumption that the reaction occurs with stereospecific removal of the *Si*-hydrogen

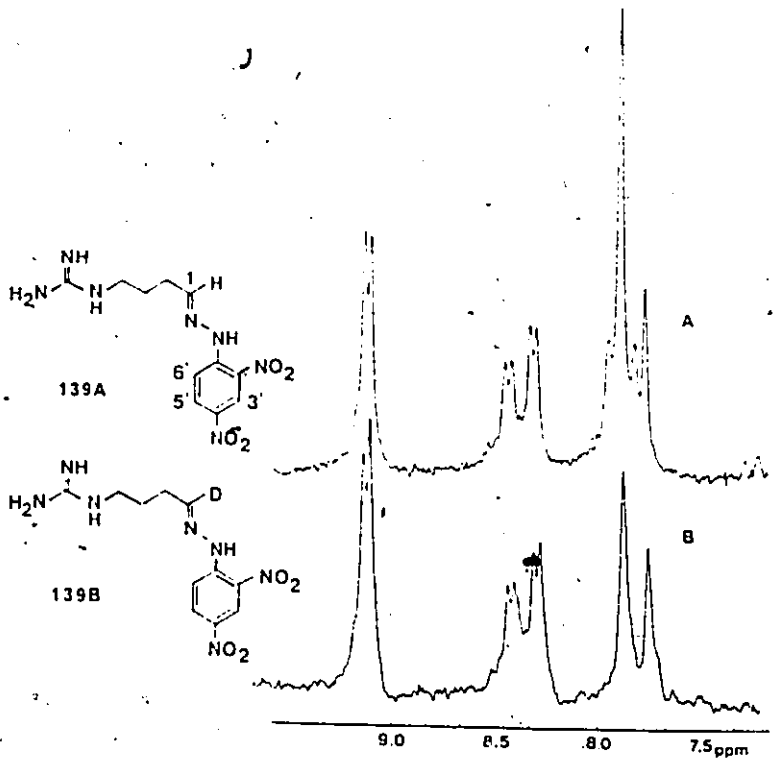


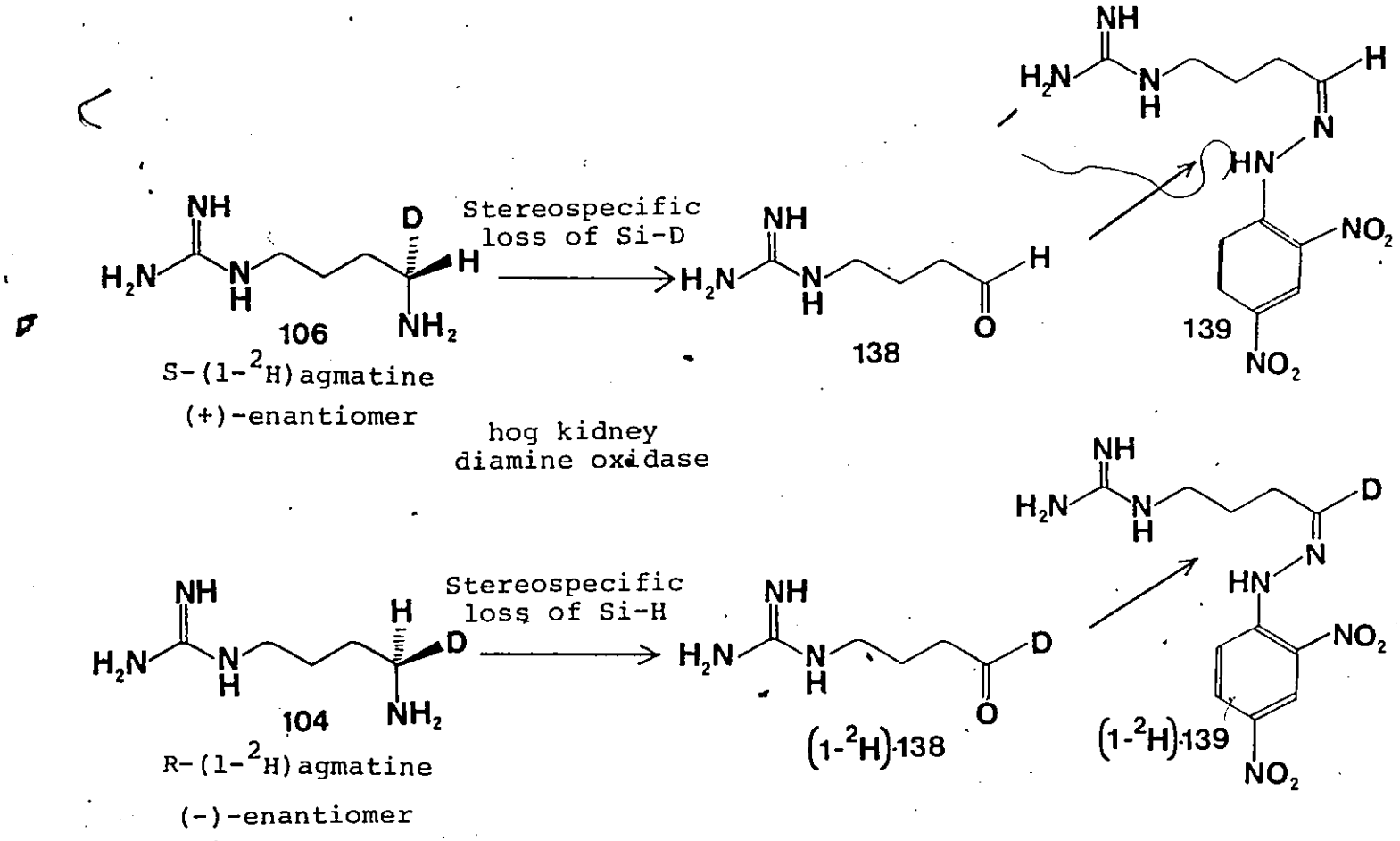
Figure 8. Downfield region of the ^1H NMR spectra of samples of 4-guanidinobutanal dinitrophenylhydrazone (139), derived from deuterium labelled samples of agmatine by enzymic oxidative deamination. A (139A), derived from (+)-(1-^2H)agmatine sulfate. B (139B), derived from (-)-(1-^2H)agmatine sulfate. 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'), 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).

(Section 3.3.3), it follows that (-)-(1-^{2}H)agmatine (104) sulfate is the R-enantiomer and that (+)-(1-^{2}H)agmatine (106) sulfate is the S-enantiomer (Scheme 24).

The sample of (-)-(1-^{2}H)agmatine was obtained from L-arginine by decarboxylation in ^2H_2O. The sample of (+)-(1-^{2}H)agmatine was obtained from L-(2-^{2}H)arginine in ^1H_2O. Since deuterium occupies the *Re*-position in the levorotatory sample and the *Si*-position in the dextrorotatory sample, it follows that enzymic decarboxylation of L-arginine to yield agmatine, catalysed by L-arginine decarboxylase of *E. coli*, occurs with net retention of configuration (*cf.* Scheme 17, Section 2.3.1).

3.3.6 Occurrence of an intramolecular isotope effect in the enzymic oxidation of (1-^{2}H)putrescine

The results discussed in previous sections of this chapter provide unequivocal evidence that the reaction catalysed by hog kidney diamine oxidase, with cadaverine, putrescine and agmatine as substrates, occurs with a very high degree of stereospecificity. Indeed, 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 (Figure 5, spectra A and B) and of (1-^{2}H)putrescine (Figure 6, spectra A and B) show that the stereochemical course of the reaction is highly exacting with both substrates. Even so, comparison of the ^{2}H NMR spectra of the corresponding products obtained from (1,1-$^2H_2$)cadaverine (Figure 5, spectrum C) and from (1,1-$^2H_2$)-putrescine (Figure 6, spectrum C) reveals that the extent of reaction at the deuterium labelled site, relative to that at the unlabelled site may be significantly different with the two substrates.



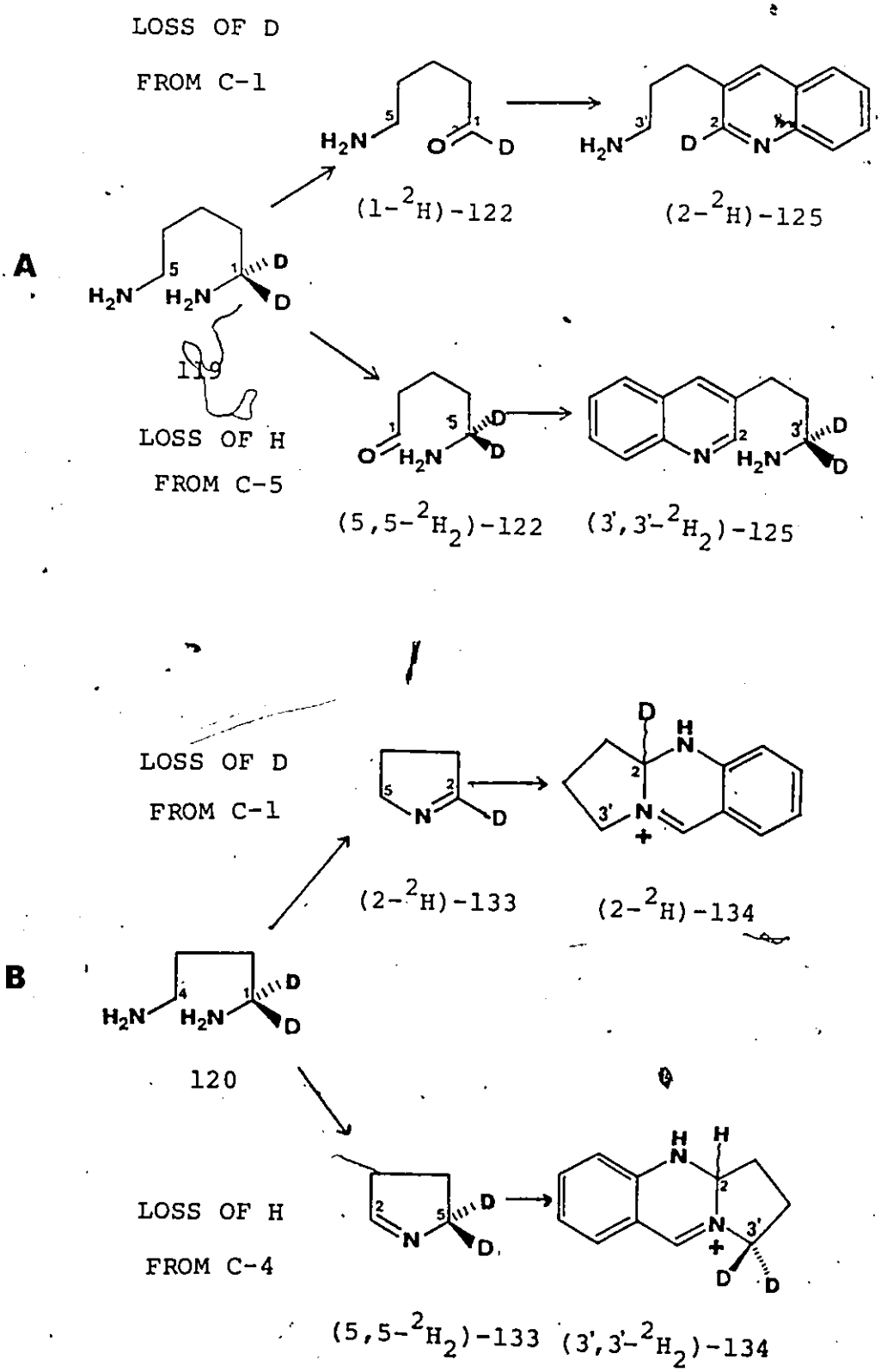
Scheme 24. Products of the incubation of the (+)- and (-)-enantiomers of (1-^{2}H)-agmatine sulfate with diamine oxidase.

The product (125C) from (1,1-$^2H_2$)cadaverine showed deuterium enrichment at C-3' (δ 2.9) which was approximately twice that at C-2 (δ 8.8) (Expt. 3a, Table 7; Figure 5, spectrum D), whereas the product (134C) from (1,1-$^2H_2$)putrescine, which bears deuterium at the corresponding positions, showed deuterium enrichment at C-3' (δ 4.1) which was seven times greater than that at C-2 (δ 5.2) (Expt. 6, Table 8; Figure 6, 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 (Scheme 25). 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 ^2H NMR spectrum of the quinoline or the quinazolinium derivative which is isolated.

The ^2H NMR spectrum of the sample of 3-(3'-aminopropyl)quinoline (125) dihydrochloride derived from (1,1-$^2H_2$)cadaverine (119) (Expt. 3a, Table 7; Figure 5, spectrum C) indicates the presence of a mixture of the dideuteriated species, (3',3'-$^2H_2$)-(125) (δ 2.9, relative area 2.2 (± 0.4)) and the monodeuteriated species, (2-^2H)-(125) (δ 8.8, 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 (119) to an equal extent (Scheme 25, A).

By contrast, the ^2H NMR spectrum of the sample of 2,3-trimethylene-1,2-dihydroquinazolinium (134) picrate derived from (1,1-$^2H_2$)putrescine (120) (Expt. 6, Table 8; Figure 6, spectrum C) indicates preferential formation of the dideuteriated species, (3',3'-$^2H_2$)-(134) (δ 4.1, relative



Scheme 25. Products derived from the diamine oxidase catalysed oxidation of (1,1-^{2}H$_2$)cadaverine (119) (A), and (1,1-^{2}H$_2$)putrescine (120) (B).

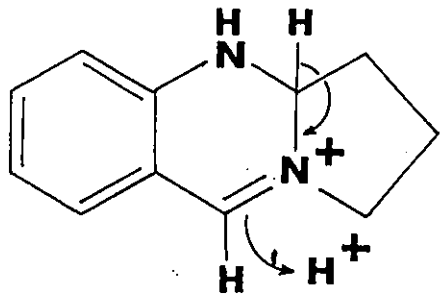
area 7.0 (± 0.5)) over the monodeuteriated species, (2-^{2}H)-(1$\underset{\sim}{3}$4) (δ 5.2, relative area 1), by a factor of *ca.* 3.5.

Two obvious possibilities must be considered for the predominance of the dideuteriated product, (3',3'-$^2H_2$)-(1$\underset{\sim}{3}$4), over the monodeuteriated product, (2-^{2}H)-(1$\underset{\sim}{3}$4), in the product mixture.

The first possibility is that, as in the case of (1,1-$^2H_2$)-cadaverine, enzymic oxidation of (1,1-$^2H_2$)putrescine 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)-(1$\underset{\sim}{3}$4), by solvent mediated exchange, *e.g. via* tautomerization (Scheme 26). This would lead to unlabelled (1$\underset{\sim}{3}$4) from the monodeuteriated species, (2-^{2}H)-(1$\underset{\sim}{3}$4), but would not affect deuterium enrichment in the dideuteriated species, (3',3'-$^2H_2$)-(1$\underset{\sim}{3}$4). The net effect of such a process would thus be to change the ratio of dideuteriated to monodeuteriated (1$\underset{\sim}{3}$4) 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 protium-deuterium isotope effect, protium being released more readily (from C-4) than deuterium is released (from C-1) in the course of oxidation of (1,1-$^2H_2$)putrescine (1$\underset{\sim}{2}$0) (Scheme 25, B).

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 (1$\underset{\sim}{3}$4C). The relative areas of the signals assigned to the protons at C-2 (δ 5.20) and C-3' (δ 4.12) correspond to 0.76 and 0.50 H atoms, respectively. This



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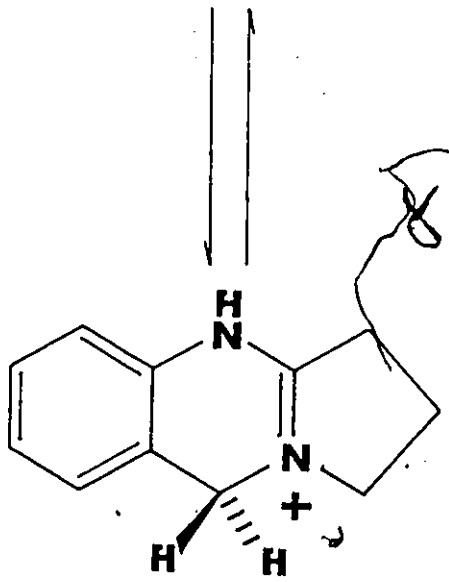

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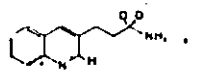
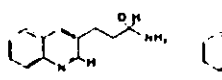
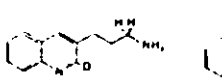
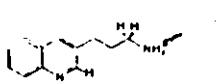
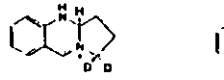
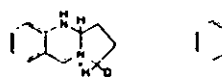
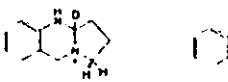
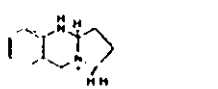
Scheme 26. Tautomerization of the 2,3-trimethylene-1,2-dihydroquinazolinium ion (134).

indicates that the product consists of 24% (2-^{2}H)-(1$\underset{\sim}{34}$) and 74% (3',3'-$^2H_2$)-(1$\underset{\sim}{34}$) (Table 11, Expt. 6). Since these two species account for approximately 98% of the product, and since non-deuterated (1$\underset{\sim}{34}$) contributes less than 1%, loss of deuterium by tautomerization of the dihydroquinazolinium ion cannot be significant under the conditions of the experiment.

This inference is further supported by the results of Experiments 4a and 4b. The sample of dihydroquinazolinium salt (1$\underset{\sim}{34}$B), derived from (-)-(1-^{2}H)putrescine (1$\underset{\sim}{00}$) dihydrochloride, consisted of approximately equal amounts of (3'-^{2}H)-(1$\underset{\sim}{34}$) and (2-^{2}H)-(1$\underset{\sim}{34}$), as determined from the ^{2}H NMR spectrum (Table 8, Expts. 4a and 4b). The ^{1}H NMR spectrum of (1$\underset{\sim}{34}$B) (Table 11, 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)-(1$\underset{\sim}{34}$), derived from (-)-(1-^{2}H)putrescine dihydrochloride, 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)-(1$\underset{\sim}{34}$), which is derived, under similar conditions (Table 6, 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 catalysed oxidation of (1,1-$^2H_2$)putrescine, it can be concluded that the observed high ratio is the result of isotope discrimination between the two symmetry-equivalent, *i.e.* homotopic, but isotopically distinct sites of the substrate, in the course of the enzymic oxidation of (1,1-$^2H_2$)putrescine (Scheme 25, B). The mole ratio of the two components of the product, (3',3'-$^2H_2$)-(1$\underset{\sim}{34}$)/(2-^{2}H)-(1$\underset{\sim}{34}$), derived from

Table 11. Analysis of the ^1H NMR spectra of the ^2H-labelled samples of 3-(3'-aminopropyl)quinoline and 2,3-trimethylene-1,2-dihydroquinazolinium picrate obtained in Experiments 1-6.

Expt. No.	Product	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'-$^2H_2$)- (125)	(3'-^{2}H)- (125)	(2-^{2}H)- (125)	(125)
1[b]	(125B)	0.51 ± 0.04	1.51 ± 0.18[d]		49(± 18)	49(± 4)	2(± 18)
2[b]	(125A)	1.00 ± 0.05	1.53 ± 0.18[d]		47(± 18)		53(± 18)
3(a)[b]	(125C)	0.49 ± 0.05	1.10 ± 0.17[d]	41(± 18)	8(± 20)	51(± 5)	-[g]
3(b)[c]	(125C)	0.50 ± 0.04	1.07 ± 0.20[d]	43(± 20)	7(± 22)	50(± 4)	-[g]
	2,3-trimethylene-1,2-dihydroquina-zolinium picrate						
				(3',3'-$^2H_2$)- (134)	(3'-^{2}H)- (134)	(2-^{2}H)- (134)	(134)
4(a)[e]	(134B)	0.50 ± 0.03	1.50 ± 0.08		50(± 8)	50(± 3)	0(± 9)
5[e]	(134A)	1.00 ± 0.03	1.20 ± 0.04		80(± 4)		20(± 4)
6[e]	(134C)	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 (in ^2H_2O, p^{2}H 5) assigned to H-2 and H-3' appeared at 8.68 (s) and 2.95 (t, $J_{2',3'}$ 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 (in DMSO-$^2H_6$) assigned to H-2 and H-3' appeared at 9.30 (d, $J_{2,4}$ 1.2 Hz) and 3.16 (t, $J_{2',3'}$ 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, $J_{2',3'}$ 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 ^1H NMR signals due to H-2 and H-3'.

[g] The contribution of the unlabelled species (125) was taken to be zero.

[h] The contribution of the unlabelled species (134) found to be < 1%, was taken to be zero.

(1,1-$^2H_2$)putrescine (120) (91% $^2H_2$)* indicates an intramolecular isotope effect, $k_H/k_D \simeq 4$ (Table 12, Expt. 6). By contrast, oxidation of (1,1-$^2H_2$)-cadaverine (119) (*ca.* 93% $^2H_2$)* leads to a product containing (3',3'-$^2H_2$)-(125) and (2-^2H)-(125) in a ratio 1:1, indicating a negligible intramolecular isotope effect (Table 12, Expt. 3a).

Thus, whereas a primary intramolecular isotope effect is observed in the diamine oxidase catalysed reaction with (1,1-$^2H_2$)putrescine as substrate, but not with (1,1-$^2H_2$)cadaverine as substrate, it is evident that the rate-limiting step in the hydrogen abstraction process with each of the two substrates is not the same. This finding weakens the assumption that the stereochemistry of hydrogen abstraction in the oxidation of putrescine is the same as that in the oxidation of cadaverine (Section 3.3.3) since not only the rate-limiting step, but also the mechanism, might be different with each of the two substrates.

The reaction catalysed by plasma amine oxidase has recently been shown[154] to have a different stereochemical outcome with homologous substrates, which differ by only one methylene group. With *p*-hydroxybenzylamine[152] and benzylamine[153] as substrates, bovine plasma amine oxidase mediates stereospecific removal of the *Si*-hydrogen from the carbon adjacent to nitrogen. With enantiomeric samples of [1-^3H]dopamine ((86) and (87), Table 2) as substrates, the enzyme-mediated hydrogen

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* The contribution of the mono- and dideuteriated components to the products derived solely from the (1,1-$^2H_2$)-species of the substrates is discussed in Section 3.3.6.1.

Table 12. Intramolecular isotope effect in the oxidation of (1-^{2}H)- and (1,1-$^2H_2$)cadaverine and putrescine, catalysed 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 7 and 8).

[b] Determined from the relative area of the ^{1}H NMR signals corresponding to H-2 and H-3' (from Table 11).

[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 (Section 3.3.6.1).

[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 (Section 3.3.6.1).

abstraction process appears to be non-stereospecific and a substantial hydrogen-tritium isotope effect is expressed.[154] To explain these results, it was suggested[154] that the dehydrogenation reaction, with dopamine as substrate, occurs by two competing mechanisms.

Hog kidney diamine oxidase exhibits absolute stereospecificity in its reactions with agmatine, putrescine and cadaverine as substrates. In Chapter 2 of this thesis, it was demonstrated that the dextrorotatory samples of (1-^{2}H)agmatine and (1-^{2}H)putrescine are of the same configuration. Similarly, the levorotatory samples of these (1-^{2}H)amines are of the same configuration (Section 2.3.1). Since hog kidney diamine oxidase catalyses stereospecific removal of deuterium from the dextrorotatory enantiomers and of protium from the levorotatory enantiomers, it follows that the reaction stereospecificity exhibited by this enzyme is the same with both agmatine and putrescine as substrates. It would seem very unlikely that hog kidney diamine oxidase catalyses the reaction of opposite chirality with cadaverine as substrate. Even so, to put to rest any doubt, the configurational assignments of the enantiomeric samples of (1-^{2}H)agmatine were confirmed by an independent method (discussed in Chapter 4). This provided conclusive evidence that deuterium occupies the 1-*Si*-position in the dextrorotatory sample and the 1-*Re*-position in the levorotatory sample. Thus, hog kidney diamine oxidase catalyses stereospecific removal of the *Si*-hydrogen from C-1 of all three substrates even though oxidation of (1,1-$^2H_2$)putrescine, but not of (1,1-$^2H_2$)cadaverine, is subject to a primary intramolecular hydrogen-deuterium isotope effect.

Correspondingly, a primary intramolecular isotope effect is observed in the oxidation of S-(1-^{2}H)putrescine, but none in the oxidation of S-(1-^{2}H)cadaverine.

The ^{1}H NMR spectrum of the product (134A) of the oxidation of S-(1-^{2}H)putrescine indicates the presence of two components, (3'-^{2}H)-(134) and unlabelled (134), in the approximate ratio 4:1 (Table 11, Expt. 5). Thus, the *Si*-proton at C-4 of S-(1-^{2}H)putrescine is abstracted in preference to the *Si*-deuteron at C-1. On the other hand, the composition of the product (125A) from S-(1-^{2}H)cadaverine (Table 11, Expt. 2) indicates the absence of an isotope effect (Table 12, Expt. 2).

Intramolecular isotope effects are not observed in the reactions of the R-enantiomers of (1-^{2}H)putrescine and (1-^{2}H)cadaverine. The ^{2}H and ^{1}H NMR spectra of the products, (134B) and (125B), obtained from the enzymic oxidation of R-(1-^{2}H)putrescine (Expts. 4a and 4b) and of R-(1-^{2}H)-cadaverine (Expt. 1), respectively, indicate equal molar amounts of the two monodeuteriated components in each of the products (Tables 7, 8 and 11). α-Secondary hydrogen-deuterium isotope effects, k_H'/k_D', in the range 1.1-1.3 have been observed in other enzyme-catalysed hydrogen abstraction processes.[225-227] Although the reactions of the R-(1-^{2}H)diamines take place without detectable intramolecular isotope effect, the occurrence of small α-secondary effects cannot be ruled out due to the sensitivity of the methods used to determine the ratio of the two components in the reaction products (Table 12).

Thus, an intramolecular isotope effect ($k_{H_{Si}}/k_{D_{Si}} \approx 4$) is only observed in the reactions catalysed by hog kidney diamine oxidase when S-(1-^{2}H)- and (1,1-^{2}H$_2$)putrescine serve as substrates (Table 12). An

intramolecular isotope effect of similar magnitude (k_H/k_D = 3.91-4.75) in the hog kidney diamine oxidase catalysed oxidation of (1,1-$^2H_2$)putrescine was recently reported by Callery *et al.*[228] In this investigation, the contribution of the monodeuteriated species, (2-^{2}H)-Δ'-pyrroline, and of the dideuteriated species, (5,5-$^2H_2$)-Δ'-pyrroline, to the oxidation product was determined by analysis of the *N*-trifluoroacetyl-2-cyanopyrrolidine derivative by mass spectrometry.

The magnitude of hydrogen-deuterium isotope effects observed in enzymic reactions are known to be sensitive to variation in experimental conditions.[226,229,230] Since cadaverine (Expts. 1, 2 and 3a) and putrescine (Expts. 4, 5 and 6) had been incubated with diamine oxidase under slightly different conditions (Table 6), the reaction with (1,1-$^2H_2$)-cadaverine was repeated (Table 6, Expt. 3b) employing conditions similar to those used in the oxidation of (1,1-$^2H_2$)putrescine (Table 6, Expt. 6). The product, 5-aminopentanal (122), was trapped with *o*-aminobenzaldehyde, and the picrate salts of the quinoline derivative (125), as well as the dihydroquinazolinium ion (126) were isolated (Scheme 18). In agreement with the earlier results these products again contained mono- and dideuteriated species in the ratio 1:1, confirming the absence of an intramolecular isotope effect (Tables 11 and 12, Expt. 3b).

Thus, an intramolecular isotope effect is not observed with cadaverine as the substrate of hog kidney diamine oxidase. Similarly, oxidation of cadaverine, catalysed 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-^{3}H]cadaverine (53) in the presence of [1-^{14}C]cadaverine.[134] The resulting Δ^1-piperideine (143), which was

trapped as pelletierine (144), retained 55 (± ?)* % ^{3}H, relative to ^{14}C, indicating little, if any, preference for removal of the *Si*-protium from C-5 over that of the *Si*-tritium from C-1 of cadaverine (Scheme 27).

The oxidation of S-[1-^{3}H]cadaverine, together with [1-^{14}C]-cadaverine, catalysed by pea seedling diamine oxidase, was recently reinvestigated.[135] 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-^{3}H]cadaverine (53) served as the substrate, the pentan-1,5-diol (146) was essentially free of tritium, relative to ^{14}C. The 5-aminopentan-1-ol (94), on the other hand, retained almost all the tritium (95 ± 5%) relative to ^{14}C. This was interpreted[135] to indicate a large isotope effect in the oxidation of the S-[1-^{3}H]cadaverine (53). In view of the result reported by Gerdes and Leistner,[134] it is more likely that the retention of tritium, relative to ^{14}C, in the sample of S-5-amino[5-^{3}H,1,5-$^{14}C_2$]pentan-1-ol (94) was the result of a substantial intermolecular isotope effect in its further oxidation to [1-^{14}C]pentan-1,5-diol (146), rather than the consequence of an intramolecular isotope effect in its generation from S-[1-^{3}H,1-^{14}C]cadaverine (53) (Scheme 27).

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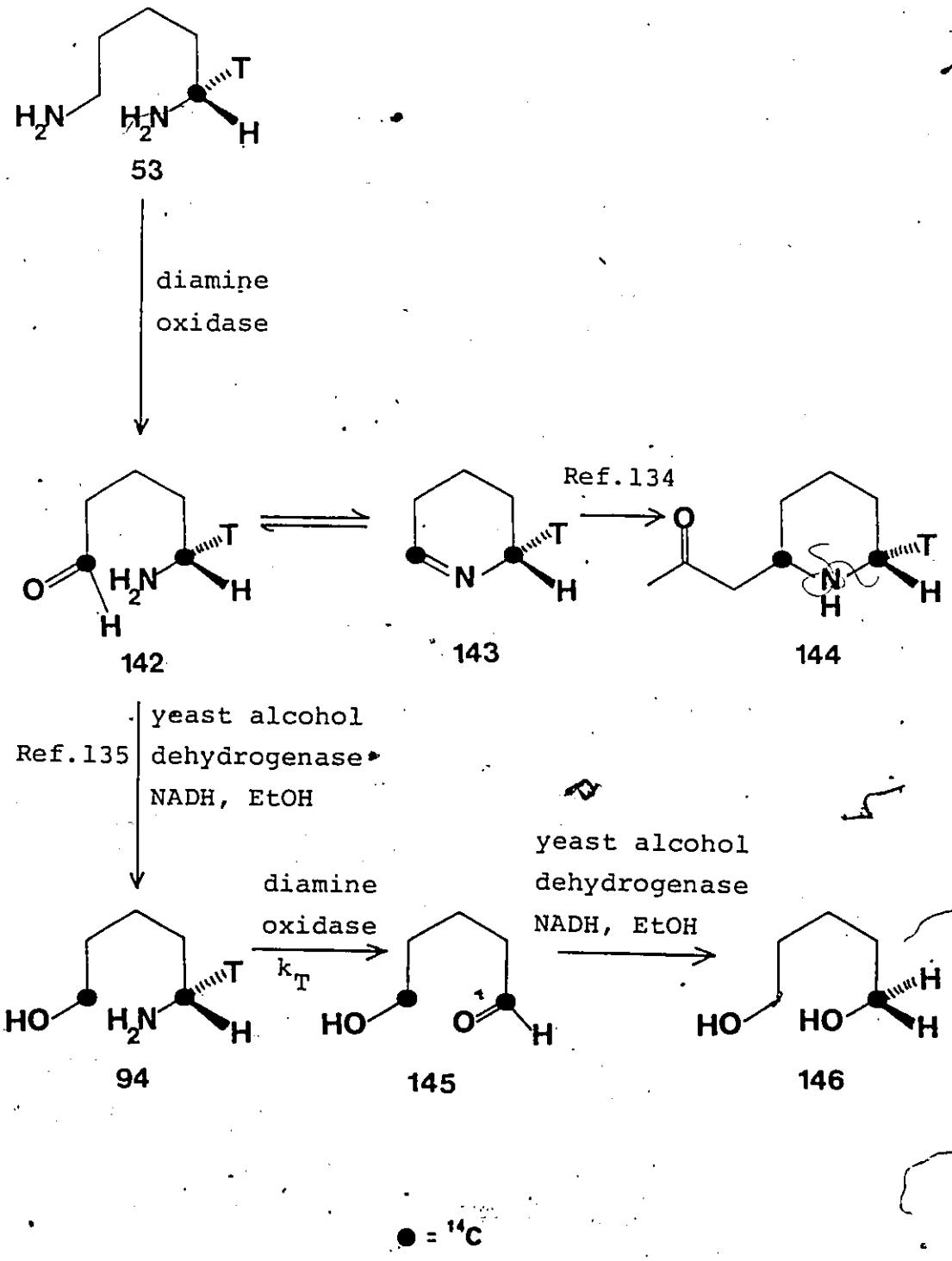
* The confidence limits of the counting data were not given; the result[134] was taken to indicate a loss of 50% of the tritium, relative to ^{14}C, *i.e.* a reaction not accompanied by an isotope effect.



Scheme 27. Reported products of isolation from the incubation of S-[1-^{3}H] cadaverine (53) with pea seedling diamine oxidase.

Intramolecular isotope effects in enzyme catalysed hydrogen abstraction in substrates with C_{2v} symmetry have been investigated in a number of instances[231-233] 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.[234] This model is adaptable to the C_{2v} case. If hydrogen abstraction were irreversible, the intramolecular isotope effect is a function of the relative rate of this hydrogen 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 catalysed oxidation of S-(1-^{2}H)putrescine. If equilibration of labelled and unlabelled regions is slow, relative to C-H (or C-^{2}H) 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 the variation in intramolecular isotope effects appears to arise from changes in the relative contribution of the individual rate constants to the overall kinetic scheme, rather than from intrinsic differences in the rate-limiting step,[226,230,234] mechanistic inferences cannot be based on the observed differences in the behaviour of putrescine and cadaverine as substrates of hog kidney diamine oxidase.

It is noteworthy in this context that the values of intermolecular isotope effects in reactions catalysed 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)[228] 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, the hydrogen-deuterium isotope effect was found to be sensitive to variation in pH and temperature.[235] Values for k_H/k_D ranged from 4.8 to 1.8 for pH values from pH 6 to pH 8, at 20°C. The isotope effect on the kinetic parameters V_{max}/K_M varied from 2.1 at pH 7.0 and 40°C to 5.2 at pH 6 and 20°C. The isotope effect on V_{max} was less sensitive to changes in pH and temperature, varying from 1.6 to 2.8. Similar values for intermolecular isotope effects have been observed in the reactions catalysed by monoamine oxidase from rat liver[119,122,236] (k_H/k_D = 2.3), and from rabbit liver[237] (k_H/k_D = 2), as well as by hog plasma monoamine oxidase[238] (k_H/k_D = 2.8).

3.3.6.1 Determination of the composition of the enzymic oxidation products obtained from samples of the (1,1-$^2H_2$)diamines

The samples of (1,1-$^2H_2$)putrescine and (1,1-$^2H_2$)cadaverine were prepared by enzymic decarboxylation of the corresponding (2-^2H)amino acids in deuterium oxide. Since the (2-^2H)amino acids were not fully deuteriated at C-2 and enzymic decarboxylation in ^2H_2O led to incomplete deuterium incorporation into the α-position of the amines, the samples of the (1,1-$^2H_2$)diamines also contained monodeuteriated species.

The sample of (1,1-$^2H_2$)putrescine (Expt. 6) was found to be composed of 91.0 ± 1.0% dideuteriated amine and of 9.0 ± 1.0% monodeuteriated amines, *i.e.* R- and S-(1-^2H)putrescine, by mass spectrometry (Section

6.2.2.1.3). Since this sample was prepared from a specimen of DL-ornithine which was *ca.* 92% monodeuteriated at C-2 (by ^1H NMR), enzymic decarboxylation of the L-component of the specimen of DL-(2-^2H)ornithine in ^2H_2O must have led to incorporation of *ca.* 98.9 atom % ^2H from the medium into the 1-*Re*-position* of putrescine. It follows that the sample consisted of 91% (1,1-$^2H_2$)-, 7.9% R-(1-^2H)-, 1.0% S-(1-^2H)- and 0.1% non-deuteriated putrescine.

Oxidative deamination of the sample of (1,1-$^2H_2$)putrescine, catalysed by hog kidney diamine oxidase, led to a product (134C) which consisted of a mixture of mono- and dideuteriated species (Table 11, Expt. 6). Given the above composition of the sample of putrescine, the contribution of the two species, (3',3'-$^2H_2$)-(134) and (2-^2H)-(134), derived solely from (1,1-$^2H_2$)putrescine (120), to the product can be determined from either the ^2H or ^1H NMR spectra: 73.3 ± 6.8% (3',3'-$^2H_2$)-(134) and 17.7 ± 1.0% (2-^2H)-(134), determined from the relative areas of the ^2H NMR signals corresponding to ^2H enrichment at C-3' and C-2 of (134C) (Table 7, Expt. 6); and, 72.4 ± 7.2 % (3',3'-$^2H_2$)-(134) and 18.6 ± 0.5% (2-^2H)-(134), determined from the relative areas of the ^1H NMR signals assigned to the protons at C-3' and C-2 of (134C) (Table 11, Expt. 6). These values for the contribution of the two species to the product were used for the calculation of the values of the intramolecular isotope effect reported in Table 12.

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* Since the enzymic decarboxylation of L-ornithine to putrescine takes place with net retention of configuration (Sections 3.3.4 and 4.3), the solvent derived deuteron occupies the 1-*Re*-position.

The deuterium content of the samples of (1,1-$^2H_2$)cadaverine (Expts. 3a and 3b) was not accurately known (*ca.* 95% dideuteriated at one of the methylene groups by ^1H NMR). The sample of (1,1-$^2H_2$)cadaverine was obtained from the L-component of DL-(2-^2H)lysine (*ca.* 95 atom % ^2H at C-2 as determined by ^1H NMR) by enzymic decarboxylation in ^2H_2O (> 98 atom % ^2H) (Section 6.2.1.1.3). The contribution of the chiral monodeuteriated species to the sample can be calculated if the efficiency of deuterium incorporation into the product of decarboxylation is estimated. If it is assumed that decarboxylation of the specimen of L-(2-^2H)lysine leads to incorporation of 98 atom % ^2H* from the medium into the 1-*Re*-position[120] of the diamine, the sample will be composed of 93% (1,1-$^2H_2$)-, 5% R-(1-^2H), and 2% S-(1-^2H)cadaverine. Taking this to be the composition of the samples of (1,1-$^2H_2$)cadaverine employed in experiments 3a and 3b, the relative contribution of the dideuteriated, (3',3'-$^2H_2$)-(125), and the monodeuteriated, (2-^2H)-(125), species to the product (125C) derived exclusively from the (1-1,-$^2H_2$)-species (119) was determined (Table 12).

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* The enzymic decarboxylation of L-ornithine and L-arginine under similar conditions in ^2H_2O was found to lead to incorporation of > 98 atom % ^2H into the products (Table 3, Expts. 4, 7, 9 and 11).

CHAPTER 4

THE STEREOCHEMISTRY OF THE ENZYMIC DECARBOXYLATION OF L-ARGININE AND L-ORNITHINE

4.1 INTRODUCTION

Evidence has been presented which demonstrates that the reactions catalysed by the bacterial L-ornithine and L-arginine decarboxylases take place stereospecifically (Chapter 2). By employing deuterium to probe the stereochemistry of these enzymic reactions, optically active enantiomeric samples of (1-^{2}H)putrescine and (1-^{2}H)agmatine were obtained. Configurational analysis of the chiral samples of (1-^{2}H)putrescine and (1-^{2}H)agmatine by the enzymic method described in Chapter 3 led to the inference that the replacement of the carboxyl group by a proton in the enzymic conversions of L-ornithine to putrescine and of L-arginine to agmatine occurs with net retention of configuration.

The determination of absolute configuration by the enzymic method was based on the assumption that the stereochemistry of the reaction, catalysed by hog kidney diamine oxidase, with putrescine and agmatine as substrates was the same as that with cadaverine as substrate. The stereochemistry of this reaction had been determined with enantiomeric samples of (1-^{2}H)cadaverine, of known configuration, as substrate. However, the occurrence of a primary kinetic isotope effect in the reaction, catalysed by hog kidney diamine oxidase, when (1,1-$^2H_2$)putrescine served as substrate, but not when (1,1-^{2}H)cadaverine served as substrate, indicated

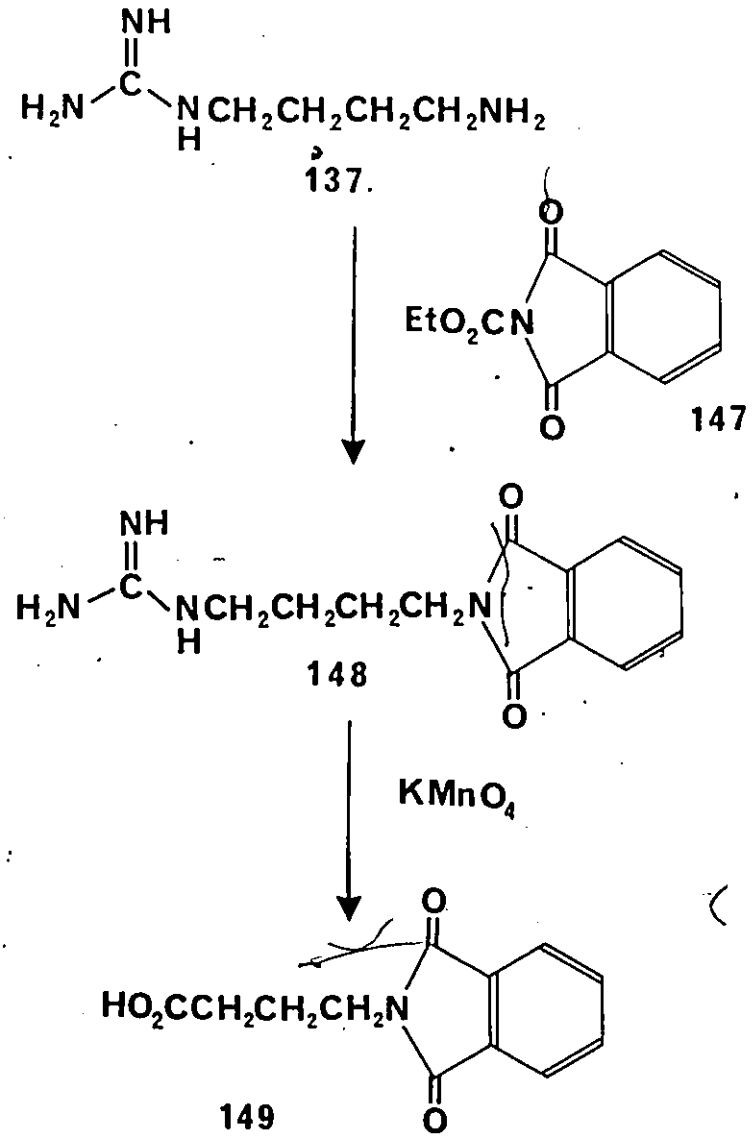
that the rate-limiting step of the hydrogen abstraction process with putrescine as substrate was not the same as that with cadaverine as substrate. Since there was the possibility that the mechanism of the hydrogen abstraction process was also different with the two substrates, it would have been unsound to base the assignments of absolute chirality solely on the use of this assumption.

The optical activity exhibited by the chiral samples of (1-^{2}H)-putrescine and (1-^{2}H)agmatine provided an independent method for configurational assignment. This method is described in this chapter and involves chemical correlation of the chiral samples of (1-^{2}H)agmatine with 4-phthalimido(4-^{2}H)butyric acid of known stereochemistry.

4.2 METHODS AND RESULTS

The preparation of the enantiomeric samples of (1-^{2}H)agmatine and of (1-^{2}H)putrescine is described in Chapter 2 of this thesis.

(-)-(1-^{2}H)Agmatine (104) sulfate and (+)-(1-^{2}H)agmatine (106) sulfate were obtained, respectively, by decarboxylation of L-arginine (103) in ^2H_2O and of L-(2-^{2}H)arginine (105) in ^1H_2O, catalysed by L-arginine decarboxylase of *E. coli* (Table 3, Section 2.2). The two enantiomers of (1-^{2}H)agmatine were converted in two steps into the corresponding enantiomers of 4-phthalimido(4-^{2}H)butyric acid. Treatment of agmatine (137) with *N*-carbethoxyphthalimide (147)[239] gave 4-phthalimidobutylguanidine (148) which, on oxidation with potassium permanganate, afforded 4-phthalimidobutyric acid (149) (Scheme 28).



4-phthalimidobutyric acid

Scheme 28. Synthesis of 4-phthalimidobutyric acid (149) from agmatine (137).

The sample of (-)-(1-^{2}H)agmatine (104) sulfate gave (-)-4-phthalimido(4-^{2}H)butyric acid (150) (Expt. 11). The sample of (+)-(1-^{2}H)agmatine (106) sulfate gave (+)-4-phthalimido(4-^{2}H)butyric acid (151) (Expt. 12). The ORD curves of the enantiomeric 4-phthalimido(4-^{2}H)butyric acids are shown in Figure 9.

A sample of the (-)-4-phthalimido(4-^{2}H)butyric acid (150) was converted into (-)-methyl 4-phthalimido(4-^{2}H)butyrate (63) by treatment with diazomethane.

An authentic specimen of (-)-methyl R-4-phthalimido(4-^{2}H)butyrate was obtained from M.H. O'Leary, University of Wisconsin. This sample had been prepared by methylation of (-)-4-phthalimido(4-^{2}H)butyric acid which had been obtained by decarboxylation in ^2H_2O of L-glutamic acid, catalysed by L-glutamate decarboxylase from *E. coli*, and had been shown[121] to have R-chirality by direct correlation with an authentic specimen, prepared from chiral (2-^{2}H)glycine by a series of reactions which did not affect the stereochemistry at the chiral centre (Scheme 10).

The sample of (-)-methyl 4-phthalimido(4-^{2}H)butyrate (63) derived from (-)-(1-^{2}H)agmatine sulfate (104) and the sample of (-)-methyl R-4-phthalimido(4-^{2}H)butyrate supplied by M.H. O'Leary showed similar plane negative ORD curves (Figure 10).

4.3 DISCUSSION

In Chapter 2 of this thesis, evidence was presented which demonstrates that the optically active samples of (1-^{2}H)putrescine and (1-^{2}H)-agmatine of the same sign of rotation correspond in configuration at C-1

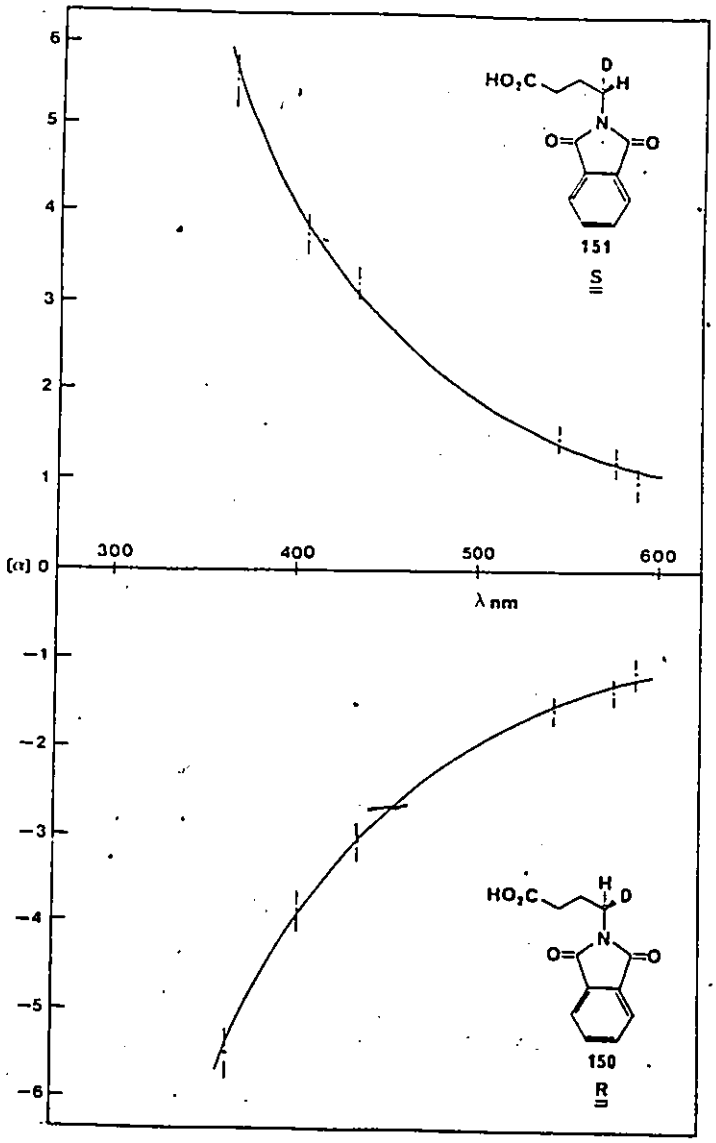


Figure 9. ORD curves of (+)- and (-)-4-phthalimido(4-^{2}H) butyric acid.

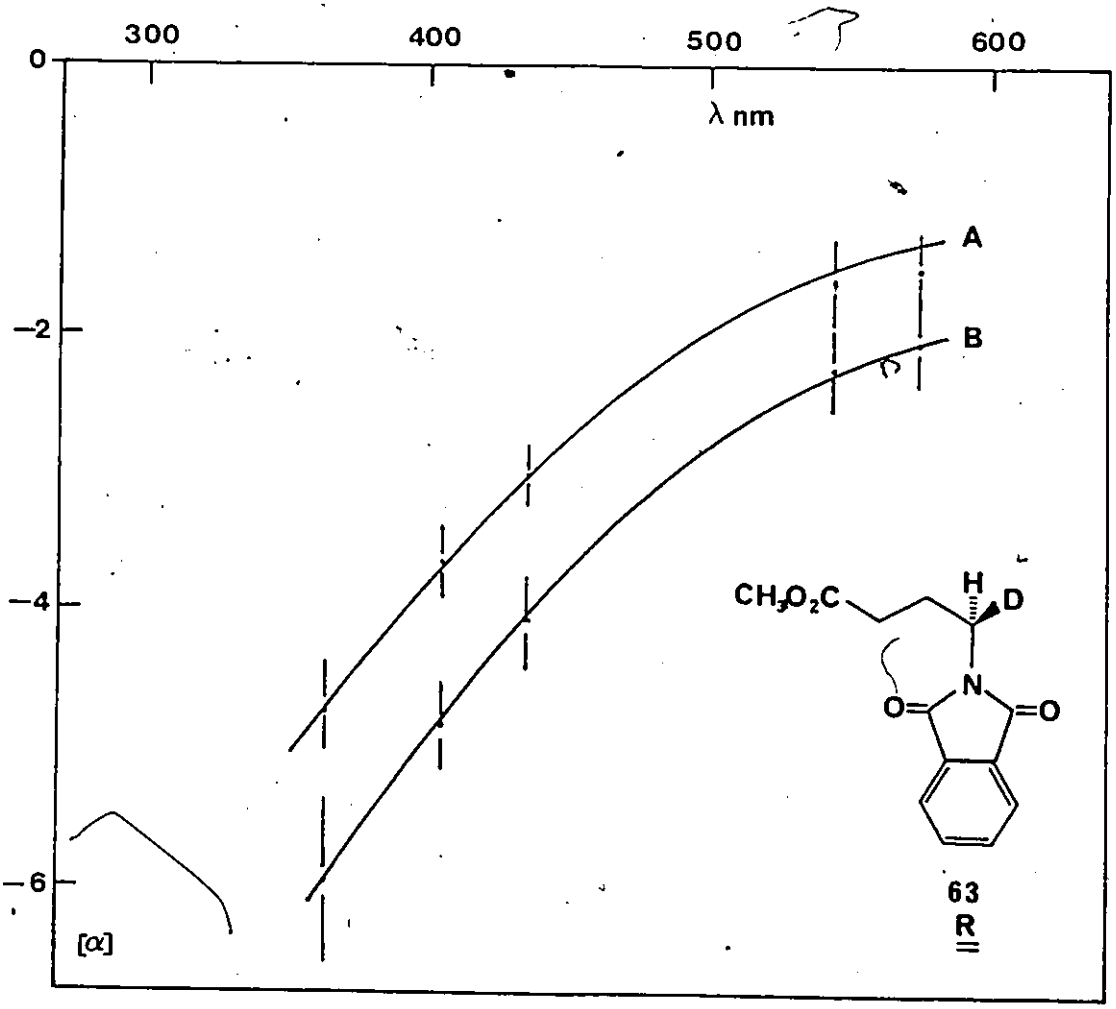
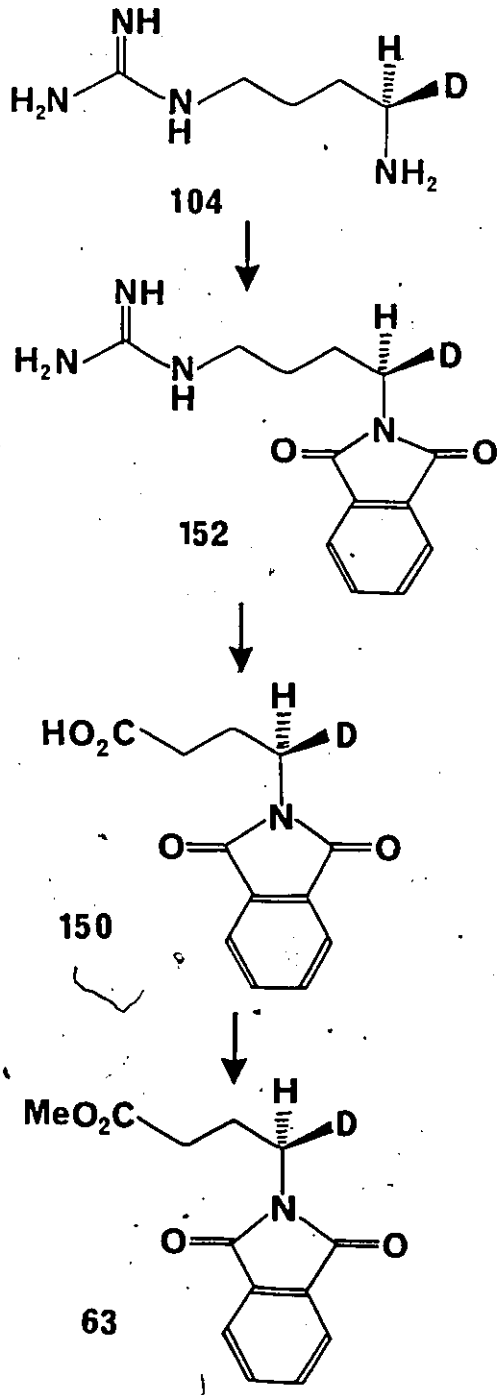


Figure 10. ORD curves of (-)-methyl 4-phthalimido(4-^{2}H)butyrate (63).
Curve A: Sample supplied by M.H. O'Leary.
Curve B: Sample obtained from (-)-(1-^{2}H)agmatine (104) sulfate.

(Expts. 9 and 10, Table 4). Thus, stereochemical correlation of either (1-^{2}H)agmatine or (1-^{2}H)putrescine with a suitable configurational standard would allow assignment of absolute configuration to both of the (1-^{2}H)amines.

(1-^{2}H)Agmatine rather than (1-^{2}H)putrescine was chosen for chemical correlation with a known standard in order to avoid possible complications due to the C_{2v} symmetry of putrescine.

(1-^{2}H)Agmatine was converted into the 1-*N*-phthaloyl derivative. The primary amino group was thus afforded protection from oxidative attack. The guanidino group was then removed by oxidation with potassium permanganate, yielding 4-phthalimido(4-^{2}H)butyric acid whose stereochemical integrity had been maintained. By this reaction sequence (-)-(1-^{2}H)-agmatine (104) sulfate afforded (-)-4-phthalimido(4-^{2}H)butyric acid (150), and (+)-(1-^{2}H)agmatine (106) sulfate afforded (+)-4-phthalimido(4-^{2}H)-butyric acid (151). The two enantiomers of 4-phthalimido(4-^{2}H)butyric acid showed ORD curves of equal magnitude but of opposite sign (Figure 9). The levorotatory enantiomer of 4-phthalimido(4-^{2}H)butyric acid (150) ($[\alpha]_{589}$ - 1.69°) has been shown to have the R-configuration at C-4.[121] Thus, R-(-)-4-phthalimido(4-^{2}H)butyric acid (150) ($[\alpha]_{589}$ - 1.11°, methanol) was obtained from (-)-(1-^{2}H)agmatine (104) sulfate. Since conversion of the sample of (-)-(1-^{2}H)agmatine (104) sulfate into (-)-4-phthalimido(4-^{2}H)butyric acid (150) involved a reaction sequence which did not affect the chirality of the deuteriated centre, it follows that the levorotatory sample of (1-^{2}H)agmatine (104) has the R-configuration at C-1 (Scheme 29).



(-)-methyl R-4-phthalimido(4-^{2}H)butyrate

Scheme 29. Conversion of (-)-(1-^{2}H)agmatine (104) sulfate into (-)-methyl 4-phthalimido(4-^{2}H)butyric acid (63).

Correspondingly, S-(+)-4-phthalimido(4-^{2}H)butyric acid (151) ($[\alpha]_{589}$ + 1.13°, methanol) was obtained from (+)-(1-^{2}H)agmatine (106) sulfate. This indicates that the dextrorotatory sample of (1-^{2}H)agmatine (106) has the S-configuration at C-1.

Confirmation of these stereochemical assignments was obtained by direct comparison of the ORD curve of the methyl ester of the sample of 4-phthalimido(4-^{2}H)butyric acid, prepared from the sample of (-)-(1-^{2}H)-agmatine sulfate, with that of an authentic specimen.

A sample of (-)-4-phthalimido(4-^{2}H)butyric acid (150) was converted into the corresponding methyl ester, (-)-methyl 4-phthalimido-(4-^{2}H)butyrate (63) (isotopic enrichment (m.s.) 98.2 ± 0.3 atom % $^2H_1$). This derivative was compared directly with an authentic specimen of (-)-methyl 4-phthalimido-R-(4-^{2}H)butyrate (isotopic enrichment 92.5 ± 0.4 atom % $^2H_1$, 4.7 ± 0.4% $^2H_2$), which had been prepared in the laboratory of M.H. O'Leary, University of Wisconsin.[121] The absolute configuration of this sample ultimately rests on chemical correlation with that of R-(2-^{2}H)glycine (26)[121] (Section 1.4, Scheme 10).

The two samples of (-)-methyl 4-phthalimido(4-^{2}H)butyrate (63), that from (-)-(1-^{2}H)agmatine (104) sulfate and that supplied by O'Leary, both showed plane negative ORD curves (Figure 10). Since (-)-methyl 4-phthalimido(4-^{2}H)butyrate has the R-configuration, the (-)-(1-^{2}H)-agmatine sulfate from which it was obtained, by a reaction sequence which did not affect the chiral centre, also has the R-configuration (Scheme 29, no change in ligand priorities around the chiral centre).

Since this R-(-)-(1-^{2}H)agmatine (104) sulfate had been obtained from L-arginine (103) by decarboxylation in ^2H_2O, catalysed by the

inducible L-arginine decarboxylase (E.C. 4.1.1.19) from *E. coli* the reaction catalysed by this enzyme proceeds with net retention of configuration.

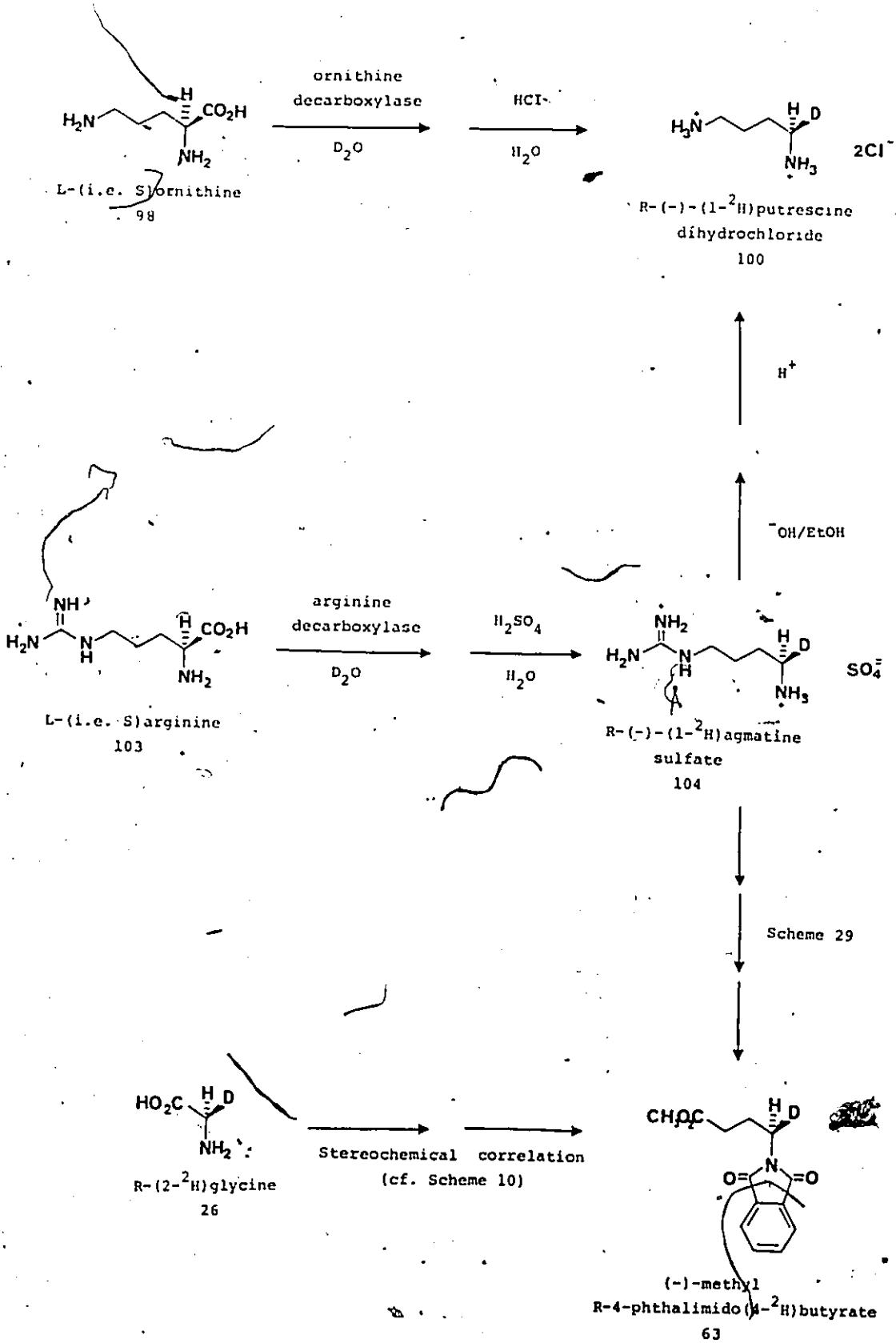
Since R-(-)-(1-^{2}H)agmatine (104) sulfate yielded (-)-(1-^{2}H)-putrescine (100) dihydrochloride in a reaction which does not affect the chiral centre, this compound is R-(-)-(1-^{2}H)putrescine dihydrochloride.

Since the R-(-)-(1-^{2}H)putrescine (100).dihydrochloride had been obtained from L-ornithine (98) by decarboxylation in ^2H_2O, catalysed by the inducible L-ornithine decarboxylase (E.C. 4.1.1.17) from *E. coli*, the reaction catalysed by this enzyme proceeds with net retention of configuration.

These configurational relationships are summarised in Scheme 30.

The stereochemical course of the reactions catalysed by these enzymes has very recently been determined by independent methods.[100,168] In agreement with the results presented in this thesis, the conversions of L-ornithine to putrescine and of L-arginine to agmatine were reported by Orr and Gould,[100] and by Robins[168] to occur with net retention of configuration.

Using deuterium to probe the stereochemistry of these reactions, Orr and Gould[100] employed the NMR method of Gerlach and Zagalak[75] (Section 1.3) to assign absolute configuration to the enantiomeric samples of (1-^{2}H)putrescine, which had been obtained from L-ornithine, by enzymic decarboxylation, and from (1-^{2}H)agmatine, by hydrolysis. This method involved ^{1}H NMR analysis of the biscamphanoylamide derivatives in the presence of the shift reagent, Eu(fod)$_3$. Determination of the chirality



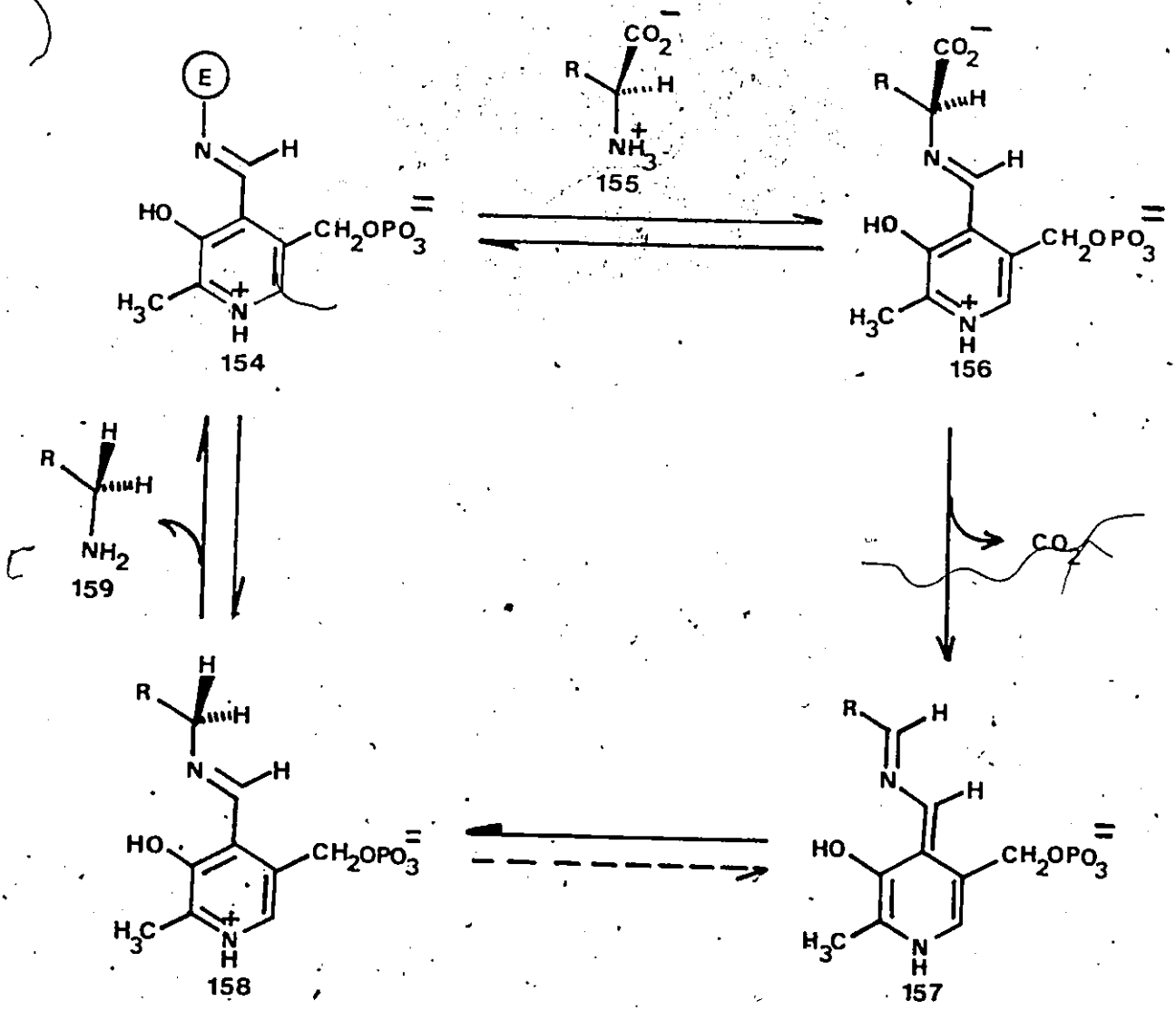
Scheme 30. Stereochemical interrelationship of L-ornithine, L-arginine, and their conversion and degradation products.

of the two enantiomers of (1-^{2}H)putrescine was based on the assumption that the ^{1}H NMR signal corresponding to the α-*Si*-hydrogens of putrescine (*i.e.* H_{Si} at C-1 and C-4) occurs at lower field than that corresponding to the α-*Re*-hydrogens. In the ^{1}H NMR spectrum of the biscamphanoylamide derivative of cadaverine, the signal arising from the α-*Si*-hydrogens was found to occur at lower field.[100]

Robins[168] employed radioactive tracer methods to investigate the stereochemistry of these reactions. The enantiomeric samples of [1-^{3}H]-putrescine derived from L-ornithine or L-arginine, *via* [1-^{3}H]agmatine, were subjected to stereochemical analysis with pea seedling diamine oxidase in the presence of yeast alcohol dehydrogenase. This method was similar to that employed by Battersby *et al.*[135] for the configurational assignment of samples of [1-^{3}H]cadaverine (*cf.* Scheme 27). It was assumed that pea seedling diamine oxidase mediates stereospecific removal of the *Si*-hydrogen from C-1 of putrescine.

The decarboxylation of L-ornithine to putrescine in intact plants (*Nicotiana tabacum*) has also been shown to take place with net retention of configuration.[240,241]

The bacterial L-ornithine and L-arginine decarboxylases,[171,173] as well as many other amino acid α-decarboxylases,[13] require pyridoxal 5'-phosphate for catalytic activity. The mechanism for the mode of action of the pyridoxal 5'-phosphate dependent α-decarboxylases, proposed independently by Westheimer[242] and Metzler *et al.*,[243] involves initial formation of an enzyme-bound Schiff's base (156) between the prosthetic group (154)[13] and the amino acid substrate (155) (Scheme 31). According to the stereoelectronic theory advanced by Dunathan,[244,245] the enzyme



Scheme 31. Mechanism for the mode of action of the pyridoxal 5'-phosphate dependent amino acid decarboxylases.

imposes conformational constraints upon the amino acid-pyridoxal phosphate complex (156) which lead to the carboxyl group being aligned orthagonally to the plane of the extended conjugated system (*e.g.* conformation shown for (156) in Scheme 31). These conformational restraints allow maximum orbital overlap between the breaking σ-bond and the π-system during the decarboxylation step.[245] Decarboxylation of (156) produces the intermediate imine (157), which then undergoes protonation at the α-carbon to yield, *via* the Schiff's base (158), the amine product (159).

Protonation of the intermediate imine (157) can, in principle, occur from either side of the planar conjugated system. Since the pyridoxal phosphate dependent α-decarboxylase catalysed decarboxylations of L-ornithine,*,[100,168] L-arginine,*,[100,168] L-lysine,[120,134,135] L-tyrosine,[119,129] L-glutamic acid,[99,121,132,133] L-tryptophan[141] and L-histidine[140] have all been shown to take place with net retention of configuration (Section 1.4), it would appear that protonation occurs only from the side of the planar imine (157) which corresponds to that from which the carboxyl group is liberated (Scheme 31). Such stereochemical consistency is also observed in the reactions catalysed by other pyridoxal phosphate dependent enzymes (*e.g.* aminotransferases)[14,245,246] and it has been suggested[14,246] that all bond-making and bond-breaking processes in pyridoxal phosphate dependent enzyme-catalysed reactions occur on the same face of the substrate-coenzyme complex. Decarboxylation

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* Results of the present investigation.

of *meso*-α,ε-diaminopimelic acid, catalysed by the pyridoxal phosphate dependent enzyme, diaminopimelic acid decarboxylase, however, takes place with net inversion of configuration[142] (Section 1.4).

The bacterial amino acid α-decarboxylases specific for L-histidine[247,248] and S-adenosyl-L-methionine[249] are not pyridoxal phosphate dependent but require enzyme bound pyruvate for catalytic activity. A mechanism for the mode of action of the pyruvate containing decarboxylases, analogous to that of the pyridoxal phosphate containing decarboxylases, has been proposed.[248,250] The decarboxylation of L-histidine[136,137] and of S-adenosyl-L-methionine,[138] catalysed by the appropriate pyruvate dependent decarboxylase, both occur with net retention of configuration.

CHAPTER 5

SUMMARY AND CONCLUSIONS

5.1 SUMMARY

Optically active enantiomeric samples of (1-^{2}H)putrescine, (1-^{2}H)agmatine and (1-^{2}H)cadaverine were prepared by enzymic decarboxylation. The dextrorotatory specimens of (1-^{2}H)putrescine (102), of (1-^{2}H)agmatine (106) and of S-(1-^{2}H)cadaverine (97) were obtained, respectively, by enzymic decarboxylation of L-(2-^{2}H)ornithine, catalysed by L-ornithine decarboxylase of *E. coli*, of L-(2-^{2}H)arginine, catalysed by L-arginine decarboxylase of *E. coli*, and of L-(2-^{2}H)lysine, catalysed by L-lysine decarboxylase of *B. cadaveris*. The levorotatory specimens of (1-^{2}H)putrescine (100), of (1-^{2}H)agmatine (104) and of R-(1-^{2}H)cadaverine (77) were obtained similarly, by decarboxylation of the corresponding unlabelled L-amino acids, catalysed by the appropriate α-decarboxylase in deuterium oxide.

The chiral samples of (1-^{2}H)putrescine and of (1-^{2}H)agmatine of the same sign of rotatory dispersion also corresponded in configuration at C-1. This was established by chemical conversion of the enantiomers of (1-^{2}H)agmatine into the corresponding enantiomers of (1-^{2}H)putrescine, which provided clear evidence that the decarboxylations of L-ornithine, catalysed by L-ornithine decarboxylase, and of L-arginine, catalysed by L-arginine decarboxylase, take the same stereochemical courses (Chapter 2).

The absolute stereochemistry of the hydrogen abstraction process in the reaction catalysed by hog kidney diamine oxidase was determined using as substrates the chiral samples of known chirality of (1-^{2}H)-cadaverine. The labelling patterns within the resulting deuteriated products were determined by ^{2}H NMR spectroscopy. Since enzymic oxidation was found to involve stereospecific loss of deuterium from C-1 of S-(1-^{2}H)cadaverine (97), and stereospecific loss of protium from C-1 of R-(1-^{2}H)cadaverine (77), it followed that hog kidney diamine oxidase mediates stereospecific removal of the *Si*-hydrogen from C-1 of cadaverine. Similar ^{2}H NMR methods were then employed to determine the labelling patterns within the products obtained, on incubation with hog kidney diamine oxidase, from the enantiomeric samples of (1-^{2}H)putrescine and (1-^{2}H)-agmatine. The results of these experiments indicated that enzymic oxidation of the (+)-(1-^{2}H)amines is accompanied by stereospecific removal of deuterium from C-1, whereas enzymic oxidation of the (-)-(1-^{2}H)amines is accompanied by stereospecific removal of protium from C-1. On the assumption that the absolute stereochemistry of the reaction catalysed by hog kidney diamine oxidase is the same with cadaverine, putrescine and agmatine as substrates, it was concluded that the dextrorotatory enantiomers of (1-^{2}H)putrescine dihydrochloride and of (1-^{2}H)agmatine sulfate possess the S-configuration, while the levorotatory enantiomers of the two (1-^{2}H)amines possess the R-configuration (Chapter 3).

A primary intramolecular hydrogen-deuterium kinetic isotope effect ($k_H/k_D \approx 4$) was observed in the diamine oxidase catalysed reaction of (1,1-$^2H_2$)putrescine (120), while oxidation of (1,1-$^2H_2$)cadaverine (119)

occurred without detectable intramolecular isotope effect. This observation made it necessary to question the basis of the assumption that the stereochemistry of the hydrogen abstraction process, catalysed by hog kidney diamine oxidase, was the same with both cadaverine and putrescine as substrates. The configurational assignments of the enantiomeric samples of (1-^{2}H)putrescine and (1-^{2}H)agmatine, made on this assumption, were confirmed by an independent method. This was achieved by stereochemical correlation of the enantiomers of (1-^{2}H)agmatine with the corresponding enantiomers of 4-phthalimido(4-^{2}H)butyric acid of known absolute chirality (Chapter 4). The chemical conversion of (-)-(1-^{2}H)agmatine (104) sulfate into R-(-)-4-phthalimido(4-^{2}H)butyric acid (150) by reactions which did not affect the chiral centre, led to the conclusion that the levorotatory (1-^{2}H)amine has R-chirality. Direct comparison of the ORD curve of (-)-methyl 4-phthalimido(4-^{2}H)butyrate (63), derived from (-)-(1-^{2}H)agmatine (104) sulfate, with that of an authentic specimen of (-)-methyl R-4-phthalimido(4-^{2}H)butyrate provided further evidence for this assignment. It followed that the levorotatory sample of (1-^{2}H)putrescine also has R-chirality, since hydrolytic cleavage of the guanidino group of (-)-(1-^{2}H)agmatine (104) sulfate afforded (-)-(1-^{2}H)putrescine (100) dihydrochloride. These stereochemical relationships firmly established that the decarboxylations of L-ornithine and L-arginine, catalysed by the respective bacterial decarboxylases, take place with net retention of configuration.

5.2 CONCLUSIONS

The objectives of this investigation were to determine the stereochemistry of the reactions catalysed by L-ornithine decarboxylase, L-arginine decarboxylase, and diamine oxidase. These objectives were accomplished.

Decarboxylation of L-ornithine to putrescine, catalysed by the inducible L-ornithine decarboxylase of *E. coli*, and of L-arginine to agmatine, catalysed by the inducible L-arginine decarboxylase of *E. coli*, takes place with net retention of configuration.

Oxidative deamination of cadaverine, of putrescine, and of agmatine, catalysed by hog kidney diamine oxidase, occurs with stereospecific removal of the *Si*-hydrogen from C-1 of each of the three substrates in the course of the conversion of the primary amino group to an aldehyde group.

The degree of stereospecificity of each of these enzyme-catalysed reactions is as complete as the methods of detection can reveal.

Oxidative deamination of S-(1-^{2}H)putrescine, catalysed by hog kidney diamine oxidase, exhibits a primary intramolecular hydrogen-deuterium kinetic isotope effect. No isotope effect is detectable in the course of the analogous oxidation of S-(1-^{2}H)cadaverine. This observation is interpreted in terms of the relative rate of hydrogen (or deuterium) abstraction compared to the rate of exchange, at the active site of the enzyme, of the two symmetry equivalent but isotopically distinct methylene groups of the substrates. Although this difference is observed in the diamine oxidase catalysed reactions of the two substrates, the

stereospecificity of the hydrogen abstraction with the two substrates is identical.

5.3 FUTURE INVESTIGATIONS

The methods employed in the present investigation are applicable to the solution of stereochemical problems associated with other enzymic reactions, and, more generally, to clarify stereochemical aspects of biosynthetic processes.

This work provides a method for the preparation of enantiomeric samples of (1-^{2}H)putrescine and (1-^{2}H)agmatine of known chirality. If tritium is employed as the chiral marker, similar methods can be employed for the preparation of the corresponding [1-^{3}H]amines of known configuration. The chirally labelled samples of putrescine and agmatine can therefore serve as configurational standards for stereochemical correlation with other molecules, rendered chiral due to isotopic substitution, but of unknown absolute configuration.

Oxidative deamination of *N*-methylputrescine to *N*-methyl-4-aminobutanal forms a key step in the biosynthesis of the pyrrolidine ring of nicotine.[160] The oxidation of *N*-methylputrescine in intact tobacco plants (*N. tabacum*) has recently been shown to occur with stereospecific removal of the *Si*-hydrogen from the carbon adjacent to the primary amino group.[241] A partially purified preparation of *N*-methylputrescine oxidase, the enzyme which is thought to catalyse this reaction, has been isolated from tobacco roots.[251] It would be of interest to examine the stereochemistry of the reactions mediated by this enzyme. If *N*-methylputrescine

oxidase is the enzyme responsible for the conversion of *N*-methylputrescine into *N*-methyl-4-aminobutanal during nicotine biosynthesis, stereospecific removal of the *Si*-hydrogen would be expected.[241] *N*-Methylputrescine oxidase also catalyses the oxidation of putrescine and cadaverine at appreciable rates.[251]

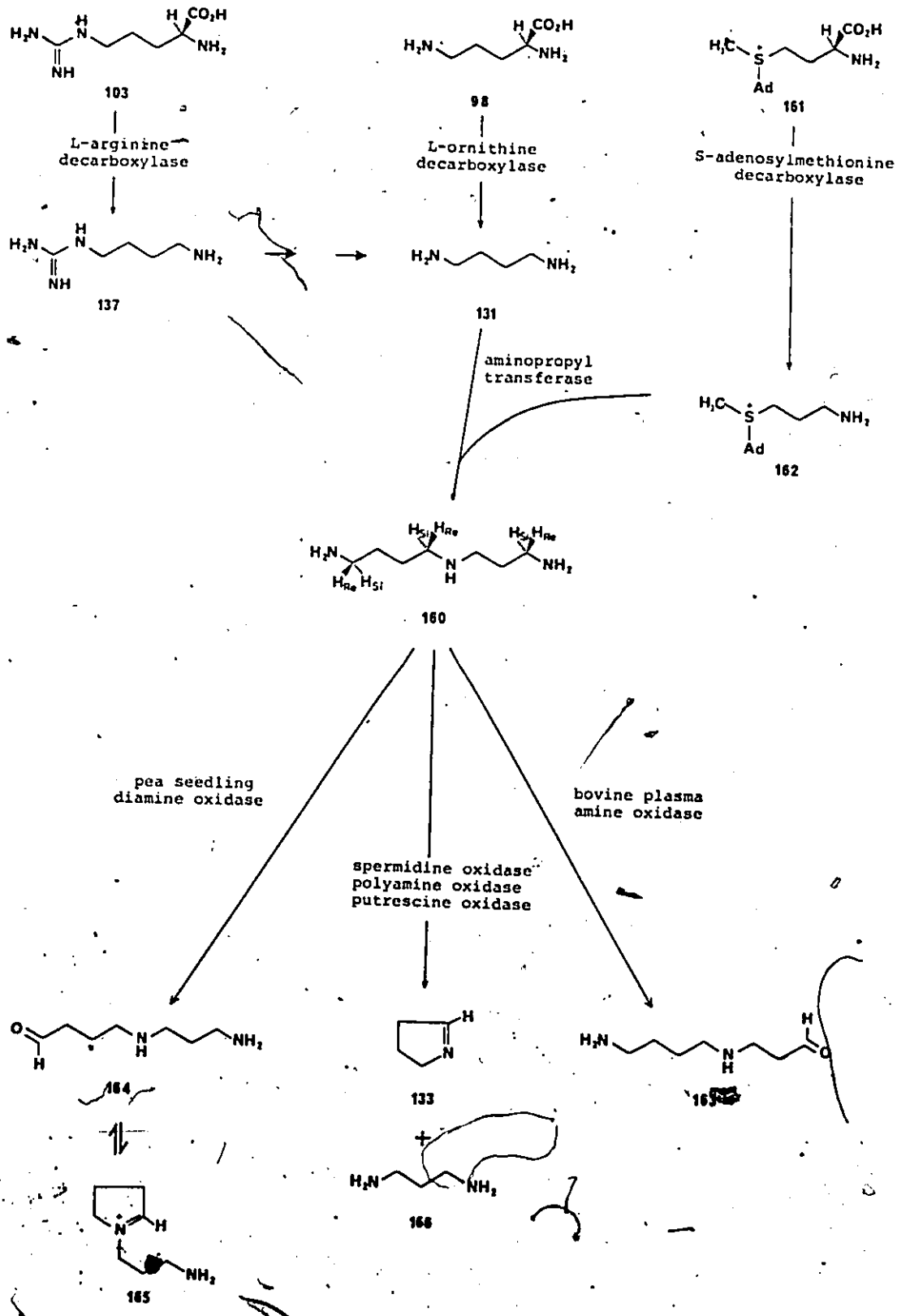
A putrescine oxidase (E.C. 1.4.3.4) which catalyses the oxidation of putrescine to 4-aminobutanal has been isolated and purified from *Micrococcus rubens*.[252-254] This enzyme is an FAD-containing amine oxidase and several of its molecular properties are similar to those of mitochondrial monoamine oxidase.[150] The monoamine oxidase from rat liver mitochondria mediates stereospecific removal of the *Re*-hydrogen from C-1 of tyramine[119,122,129] and 1-aminoheptane[151] in the course of the oxidation to the corresponding aldehyde. The stereochemistry of the reactions catalysed by other FAD-containing amine oxidases has not been investigated, whereas the copper-containing amine oxidases from pea seedling, bovine plasma (Table 2) and hog kidney (present investigation) catalyse the removal of the *Si*-hydrogen from C-1 of their substrates. The ^2H NMR methods employed in the present investigation can provide an answer to the question of the stereospecificity of the oxidative deamination of putrescine, catalysed by putrescine oxidase.

In addition to the inducible ("biodegradative") bacterial enzymes employed in the present investigation, distinct constitutive ("biosynthetic") L-ornithine and L-arginine decarboxylases have also been isolated and purified from *E. coli*.[109,110] Like their inducible counterparts, these constitutive enzymes require pyridoxal phosphate for catalytic

activity.[109,110] The constitutive enzymes are involved in the biosynthesis of putrescine in bacteria.[107,108] Putrescine is a precursor of the polyamines, spermidine (160) and spermine.[158] The *N*-propylamine moiety of spermidine (160) is derived from *S*-adenosylmethionine (161), following decarboxylation, catalysed by *S*-adenosylmethionine decarboxylase.[103,104] (Scheme 32). Decarboxylation of *S*-adenosylmethionine (161), catalysed by the enzyme from *E. coli*, has been shown[138] to occur with net retention of configuration. The stereochemistry of the decarboxylations catalysed by the constitutive L-arginine and L-ornithine decarboxylases has not been determined.

Highly purified preparations of mammalian ornithine decarboxylase from rat liver[114,255,256] and from rat prostate[115] have also been reported. The mammalian enzyme is also involved in the biosynthesis of spermidine (160).[158] Although the formation of putrescine (131) from arginine (103) *via* agmatine (137) has not been observed in mammalian tissue,[111,158,159] these reactions appear to be a major pathway leading to the formation of putrescine in higher plants.[112,158] L-Arginine decarboxylase has been isolated from barley[257] and *Lathyrus sativus*[258] seedlings. The steric course of the reactions catalysed by these enzymes has not been investigated.

Amine oxidases are involved in the degradative metabolism of polyamines.[112,158] Oxidation of one or the other of the two primary amino groups, as well as the secondary amino group of the 4-aminobutyl moiety of spermidine (160) has been observed with different enzymes (Scheme 32). Bovine plasma amine oxidase (spermine oxidase)[259] acts at



Scheme 32. Biosynthetic route leading to the formation of spermidine and its oxidation products derived by the action of amine oxidases.

the primary amine of the 3-aminopropyl group of spermidine (160) to yield *N*-(4-aminobutyl)-3-aminopropanal (163),[260] whereas pea seedling diamine oxidase acts at the primary amine of the 4-aminobutyl group of spermidine (160) to yield *N*-(3-aminopropyl)-4-aminobutanal (164)[196,261] which cyclizes to afford *N*-(3-aminopropyl)-Δ'-pyrrolinium ion (165) (*cf.* ref. 262). Spermidine oxidase from *Serratia marcescens*,[263,264] polyamine oxidase from oat seedlings,[265] as well as putrescine oxidase from *M. rubens*,[253] catalyse oxidation of the secondary amino group of the 4-aminobutyl moiety of spermidine (160) to yield Δ'-pyrroline (133) and 1,3-diaminopropane (166). The methods employed in the present investigation (Chapter 3) would provide the means of determining the stereochemistry of abstraction of a hydrogen atom from C-4 of spermidine in the reaction catalysed by the latter three enzymes. Enantiomeric samples of (4-^{2}H)spermidine could be prepared from the corresponding enantiomeric (1-^{2}H)putrescines or (1-^{2}H)agmatines by enzymic (*cf.* ref. 266) or chemical methods.

The stereochemistry of the oxidative deamination of spermidine (160) to *N*-(4-aminobutyl)-3-aminopropanal (163) would also merit investigation. Oxidation of *p*-hydroxybenzylamine[152] and benzylamine,[153] catalysed by bovine plasma amine oxidase, occurs with stereospecific removal of the *Si*-hydrogen, whereas the analogous oxidation of dopamine[154] is apparently nonstereospecific. Spermidine is the substrate most readily oxidised by this enzyme.[260]

CHAPTER 6

EXPERIMENTAL

6.1 PREPARATION OF (2-^{2}H)AMINO ACIDS

6.1.1 DL-(2-^{2}H)Lysine

6.1.1.1 Diethyl 2-acetamido-2-(4-phthalimidobutyl)malonate (111)

Diethyl acetamidomalonate (109) (Aldrich Chemical Co.) (2.17 g) was dissolved in hot dry ethanol* (10 ml) in a two-necked round bottom flask, fitted with a reflux condenser, calcium sulfate drying tube, and a pressure equalizing dropping funnel. A solution of sodium ethoxide (250 mg of sodium in 14 ml ethanol) was added and the resulting solution was heated at reflux while N-(4-bromobutyl)phthalimide (108) (Aldrich Chemical Co.) (2.83 g) in hot ethanol (14 ml) was added dropwise over 10 min. The reaction mixture was refluxed 20 h, and was then cooled to 0°C. 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 Section 6.1.1.2). Concentration of the filtrate *in vacuo* to one-half the original volume yielded additional product (1.0 g). Yield 90% from diethyl acetamidomalonate; m.p. 110-111°C (from 95% ethanol), ^{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),

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* Absolute ethanol purified by the Lund-Bjerrum method was used throughout.[267]

4.27 (4H, q, J 7.2 Hz), 6.74 (1H, s), 7.80 (4H, m); m.s., m/e 418 (M, 2%), 345 (100), 303 (89), 299 (80), 229 (97), 160 (42), 148 (23), 130 (44). Anal. Calcd. for $C_{21}H_{26}N_2O_7 \cdot \frac{1}{2}C_2H_5OH$: C, 59.85; H, 6.62; N, 6.35. Found: C, 59.65; H, 6.25; N, 6.41%.

6.1.1.2 DL-(2-^{2}H)Lysine (96) monohydrochloride (*cf.* ref. 177)

Diethyl 2-acetamido-2-(4-phthalimidobutyl)malonate (111) (2.77 g) was suspended in a solution of deuterium oxide containing deuterium chloride (38%, w/w in ^2H_2O, 25 ml) (Merck, Sharp and Dohme, 99.7 atom % ^{2}H) in a flask fitted with a reflux condenser and calcium sulfate drying tube. The mixture was heated at reflux for 18 h, cooled to 0°C, and diluted with water (10 ml). Phthalic acid, which precipitated, was filtered off and washed with water (2 x 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 deuterium. The residue, crude lysine dihydrochloride, was dissolved in hot 95% ethanol (8 ml), the solution was cooled to room temperature and filtered, and pyridine (*ca.* 600 mg) was added. DL-(2-^{2}H)lysine monohydrochloride which precipitated was recrystallized from aqueous ethanol. Yield 1.05 g; m.p. 259-260°C (decomp.) (lit. (nondeuteriated) m.p. 259-262°C[177]; 260-261°C[268]; 263-264°C[269]); ^{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 (< 0.05 H, 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).

6.1.2 DL-(2-^{2}H)Ornithine

6.1.2.1 Diethyl 2-acetamido-2-(3-phthalimidopropyl)malonate (110)

Diethyl acetamidomalonate (2.16 g) was reacted with *N*-(3-bromopropyl)phthalimide (107) (Aldrich) (2.69 g) under the same conditions that were used for the preparation of (111) (Section 6.1.1.1) to give the substitution product (110). Yield 2.85 g, 71%; m.p. 116-116.5°C (from 95% ethanol) (lit. m.p. 115-116°C[270]; 111-112°C[271]); ^{1}H NMR (C^2HCl_3), δ 1.20 (6H, t, J 7.2 Hz), 1:53 (2H, m), 2.00 (3H, s), 2.40 (2H, m), 3.67 (2H, t, J 7.2 Hz), 4.23 (4H, q, J 7.2 Hz), 6.80 (1H, s), 7.77 (4H, m).

6.1.2.2 DL-(2-^{2}H)Ornithine (101) monohydrochloride (*cf.* ref. 177)

Hydrolysis of diethyl 2-acetamido-2-(3-phthalimidopropyl) malonate (110) (784 mg) in deuterium chloride solution (38% w/w in ^2H_2O, 10 ml) and conversion of the product to its monohydrochloride as described for the preparation of (2-^{2}H)lysine (Section 6.1.1.2) afforded DL-(2-^{2}H)ornithine monohydrochloride (398 mg): m.p. 235-237°C (decomp.) (lit. (nondeuteriated) m.p. 232°C (decomp.)[183]; 222-224°C (decomp.)[271]; 215-216°C (decomp.)[177]). The ^{1}H NMR spectrum (^2H_2O) was similar to that of authentic unlabelled material and indicated that this sample was *ca.* 98% deuterium labelled at C-2: δ 1.90 (4H, m, H-3,4), 3.05 (2H, t, J 6.8 Hz, H-5), 3.80 (*ca.* 0.02 H, H-2). The ^{2}H NMR spectrum (H_2O, 107 *mM*, 200 transients) showed one signal at 3.5 ppm (relative to $^1H^2HO$ at 4.5 ppm).

In another experiment, hydrolysis of (110) (900 mg) gave a sample of DL-(2-^{2}H)ornithine monohydrochloride (330 mg) which was *ca.* 92% ^{2}H-labelled in the 2-position as determined by ^{1}H NMR.

6.1.3 L-(2-^{2}H)Arginine

6.1.3.1 α-*N*-Acetyl-L-arginine (112) dihydrate

L-Arginine monohydrochloride (4.24 g) was acetylated with acetic anhydride in aqueous sodium bicarbonate by a published procedure,[180] yielding α-acetyl-L-arginine dihydrate (4.10 g, 81%): m.p. 267-268°C (from water) (lit.[272] m.p. 266°C); ^{1}H NMR (^2H_2O), δ 1.73 (4H, m, H-3,4), 2.05 (3H, s, $COCH_3$), 3.23 (2H, t, J 6.8 Hz, H-5), 4.20 (1H, d of d, J_1 *ca.* 7.8 Hz, J_2 *ca.* 4.8 Hz, H-2); ORD, $[\alpha]_{289}$ - 10.1 ± 0.3°, $[\alpha]_{296}$ - 12.3 ± 0.3°, $[\alpha]_{302}$ - 14.1 ± 0.3°, $[\alpha]_{312}$ - 15.8 ± 0.3°, $[\alpha]_{334}$ - 16.6 ± 0.3°, $[\alpha]_{365}$ - 16.0 ± 0.3°, $[\alpha]_{405}$ - 13.6 ± 0.3°, $[\alpha]_{436}$ - 11.9 ± 0.3°, $[\alpha]_{546}$ - 7.6 ± 0.2°, $[\alpha]_{577}$ - 6.7 ± 0.1°, $[\alpha]_{589}$ - 6.3 ± 0.2° (*c* 2% in 1 *M* HCl, 25°C), (lit.[180] $[\alpha]_{589}$ - 7.0° (*c* 2% in 1 *M* HCl)).

6.1.3.2 α-*N*-Acetyl-DL-(2-^{2}H)arginine (115) dihydrate (*cf.* ref. 273)

α-*N*-Acetyl-L-arginine dihydrate (3.9 g) was dissolved in deuterium oxide (5 ml) and the solvent was evaporated *in vacuo*. This process was repeated three times to effect replacement of the exchangeable protons by deuterium. The residue was then recrystallized from deuterium oxide (3 ml) and deuterium oxide of crystallization was removed by drying over phosphorous pentoxide at 75°C for 24 h. The product, α-*N*-acetyl-arginine, in which all exchangeable protons had been replaced by ^{2}H,

m.p. 253-255°C (lit. (nondeuteriated)[273] m.p. 250°C), so obtained, was dissolved in deuterioacetic acid (25 ml) which had been prepared in the following way (*cf.* ref. 274).

A mixture of freshly distilled acetic anhydride (Fisher) (b.p. 137-138°C, 40 ml) and deuterium oxide (Merck, Sharp and Dohme, 99.7 atom % ^2H) (8 g) was stirred at room temperature under a stream of dry nitrogen until the solution was homogeneous (*ca.* 3 h). Distillation under anhydrous conditions gave deuterioacetic acid, $CH_3CO_2{}^2H$, b.p. 112-114°C. The ^1H NMR spectrum of a sample of the product indicated that it was > 98% deuterium labelled and contained a trace of acetic anhydride (*ca.* 1%).

Acetic anhydride (b.p. 137-138°C, 1.7 ml) was added to the solution of α-*N*-acetylarginine in deuterioacetic acid, and the mixture was heated at reflux under dry nitrogen. After 2 h deuterium oxide (0.4 ml) was added and heating at reflux was continued for 30 min. The solution was cooled to room temperature, acetic acid removed *in vacuo* and the residue, a yellow oil, was dissolved in water (5 ml). The solution was evaporated, the residue redissolved in water and the solution again evaporated. The oil was then redissolved in water (5 ml), the pH of the solution was adjusted to pH 8 with concentrated ammonium hydroxide, acetone (1 ml) was added and the mixture cooled to 0°C. The crystals which formed were filtered off, washed with cold water and dried *in vacuo* over phosphorus pentoxide. The sample of α-*N*-acetyl-DL-(2-^2H)-arginine (115) dihydrate (3.4 g) so obtained (optically inactive, m.p. 268-270°C (from water)), was 85-90% deuterium labelled at C-2 (^1H NMR). Deuterium was also present in the acetyl group.

6.1.3.3 L-(2-^{2}H)Arginine (105) monohydrochloride and α-*N*-acetyl-D-(2-^{2}H)arginine (116) dihydrate (*cf.* ref. 179,180)

The pH of a solution of α-*N*-acetyl-DL-(2-^{2}H)arginine dihydrate (3.2 g) in water (140 ml) was adjusted to pH 6.8 with hydrochloric acid (10% w/v). Hog kidney acylase I (Sigma, 6300 U/mg*, 8.6 mg) in water (20 ml) was added. The resulting solution was incubated at 36°C in a constant temperature water bath for 23 h. The pH of the enzymic reaction mixture was then adjusted to pH 4.8 with glacial acetic acid, Norite was added, and the mixture filtered through Celite. The clear filtrate was concentrated *in vacuo* (7 ml), and after cooling in a refrigerator for several days, α-*N*-acetyl-D-(2-^{2}H)arginine dihydrate, which crystallized, was filtered off and washed with water. Yield 1.4 g, 89%; m.p. 268-269°C (from water); ORD, $[\alpha]_{289}$ + 14.0 ± 0.3°, $[\alpha]_{296}$ + 16.9 ± 0.4°, $[\alpha]_{302}$ + 18.0 ± 0.4°, $[\alpha]_{312}$ + 19.4 ± 0.4°, $[\alpha]_{334}$ + 19.6 ± 0.4°, $[\alpha]_{365}$ + 17.9 ± 0.4°, $[\alpha]_{405}$ + 15.3 ± 0.3°, $[\alpha]_{436}$ + 13.2 ± 0.3°, $[\alpha]_{546}$ + 8.3 ± 0.2°, $[\alpha]_{577}$ + 7.2 ± 0.2°, $[\alpha]_{589}$ + 6.8 ± 0.2° (*c* 2.06% in 1 *M* HCl, 25°C).

The combined filtrate and washings containing L-(2-^{2}H)arginine (10 ml) were diluted with water (25 ml), sodium hydroxide solution (5% w/v) was added to pH 11, and the solution was cooled in an ice bath. Benzaldehyde (1.3 ml) was added and the mixture stirred at 0°C for 2 h. The benzylidine derivative which separated was filtered off and was washed with ethanol and ether. Yield 1.54 g, 93%; m.p. 198-199°C (decomp.) (lit. (nondeuteriated)[276] m.p. 206°-207°C (decomp.)).

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* 1 U will hydrolyse 1 μmole of *N*-acetyl-L-methionine/h. Hydrolysis of α-*N*-acetyl-L-arginine is *ca.* 60 times slower.[276]

The solution of the benzylidine derivative (1.53.g) in aqueous hydrochloric acid (2*M*, 12 ml) was heated (95°C) 20 min with stirring. After cooling to room temperature, the mixture was extracted with ether (2 x 5 ml) and the ether layers were discarded. The aqueous phase, containing the dihydrochloride of L-(2-^{2}H)arginine, was evaporated *in vacuo*. The residue was dissolved in hot 95% ethanol (5 ml), the solution cooled to room temperature, and filtered. L-(2-^{2}H)arginine monohydrochloride was precipitated from the filtrate by addition of pyridine (1.2 ml), and was recrystallized from aqueous ethanol. Yield 1.1 g, 91% from the benzylidine derivative; m.p. 222-223°C (lit. (nondeuteriated)[275] m.p. 220°C) ^{1}H NMR (^2H_2O), δ 1.74 (4H, m, H-3,4), 3.17 (2H, t, J 6.8 Hz, H-5), 3.72 (*ca.* 0.15H, H-2, *i.e.*, *ca.* 85% of ^{2}H); ORD, $[\alpha]_{296}$ + 94.5 ± 1.9°, $[\alpha]_{302}$ + 87.7 ± 1.9°, $[\alpha]_{312}$ + 75.6 ± 1.5°, $[\alpha]_{334}$ + 58.6 ± 1.2°, $[\alpha]_{365}$ + 43.3 ± 0.9°, $[\alpha]_{405}$ + 31.4 ± 0.6°, $[\alpha]_{436}$ + 25.3 ± 0.5°, $[\alpha]_{546}$ + 14.1 ± 0.3°, $[\alpha]_{577}$ + 12.4 ± 0.3°, $[\alpha]_{589}$ + 11.7 ± 0.2° (*c* 2.06% in water, 25°C). An authentic sample of unlabelled L-arginine monohydrochloride showed a plane positive ORD spectrum: $[\alpha]_{296}$ + 94.0 ± 1.9°, $[\alpha]_{302}$ + 87.2 ± 1.7°, $[\alpha]_{312}$ + 75.4 ± 1.5°, $[\alpha]_{334}$ + 58.6 ± 1.2°, $[\alpha]_{365}$ + 43.3 ± 0.9°, $[\alpha]_{405}$ + 31.5 ± 0.6°, $[\alpha]_{436}$ + 25.4 ± 0.5°, $[\alpha]_{546}$ + 14.0 ± 0.3°, $[\alpha]_{577}$ + 12.4 ± 0.3°, $[\alpha]_{589}$ + 11.7 ± 0.2° (*c* 2.07% in water, 25°C) (lit. $[\alpha]_{589}$ + 11.30° (*c* 5.0% in water, 25°C)[273], $[\alpha]_{589}$ + 12.2, + 12.3 (*c* 5.0% in water, 25°C)[275]).

A sample of L-(2-^{2}H)arginine monohydrochloride (67 mg) which was > 92% ^{2}H-labelled (by ^{1}H NMR) was obtained from α-*N*-acetyl-L-arginine under a similar set of reaction conditions (see above) except that the

^2H for ^1H exchange reaction was optimized by following the course of the reaction in perdeuterio-acetic acid-acetic anhydride solution by ^1H NMR.

6.2 PREPARATION OF (1-^{2}H)AMINES BY ENZYMIC DECARBOXYLATION

6.2.1 Cadaverine

6.2.1.1 Decarboxylation of lysine, catalysed 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 solutions, 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 deuterium oxide (3 x 5 ml) and evaporated to dryness to effect removal of exchangeable protons. The residue was then dissolved in 25 ml deuterium oxide (99.8 atom % ^2H, Stohler Isotopes) yielding a buffer solution, *ca.* 0.2 *M*, p^{2}H 6.

6.2.1.1.1 R-(-)-(1-^{2}H)Cadaverine (77) dihydrochloride by decarboxylation of the L-component of unlabelled DL-lysine in deuterium oxide solution (Experiment 1)

DL-Lysine monohydrochloride (Sigma) (152 mg) was twice dissolved in deuterium oxide (3 ml) and the solution evaporated to dryness. After being dried *in vacuo* overnight, the residue was dissolved in phosphate-^2H_2O buffer solution (0.2 *M*, p^{2}H 6, 24 ml) (see Section 6.2.1.1) and transferred into a flask containing L-lysine decarboxylase (Sigma, "Type

VI", 2.6 U/mg*) (24 mg). The flask, fitted with a calcium sulfate drying tube, was warmed at 36°C in a shaking constant temperature bath. After 48 h, this enzymic reaction mixture was acidified with 4 *M* hydrochloric acid, heated on a steam bath for 30 min and cooled to room temperature. The precipitated enzyme was removed by centrifugation, followed by filtration of the supernatant solutions through Celite. The filtrate was then lyophilysed, the residue dissolved in 10% (w/v) sodium hydroxide (3 ml), and the alkaline solution saturated with sodium chloride. (^{2}H)Cadaverine was extracted into 1-butanol (4 x 5 ml). The extract was acidified by addition of a solution of hydrogen chloride in butanol and the solution was then concentrated *in vacuo* (5 ml). R-(1-^{2}H)-Cadaverine dihydrochloride which precipitated was crystallized from 95% ethanol. 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 ± 0.02°, $[\alpha]_{407}$ - 0.23 ± 0.03°, $[\alpha]_{546}$ - 0.14 ± 0.01°, $[\alpha]_{579}$ - 0.10 ± 0.02° (*c* 4.92% in 0.1 *M* HCl, 26°C, uncorrected for incomplete deuteriation).

A sample of the dihydrochloride was converted into *N*,*N*'-dibenzoyl-R-(1-^{2}H)cadaverine: m.p. 132-133°C (from aqueous ethanol) (lit. (nondeuteriated) m.p. 129-131°C[277], 135°C[278]); m.s., 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°C, showed m.s., m/e 311 (M+1, 0.3%), 310 (M, 1.6), 205 (2.5), 189 (3.6), 188 (10.3), 105 (100), 77 (75).

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* 1 U will release 1.0 μmole carbon dioxide from L-lysine per minute at pH 6.0 at 37°C.

6.2.1.1.2 S-(+)-(1-^{2}H)Cadaverine (97) dihydrochloride by decarboxylation of the L-component of DL-(2-^{2}H)lysine (Experiment 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°C for 40 h, the enzymic reaction mixture was worked up as described above (Section 6.2.1.1.1) to give S-(1-^{2}H)cadaverine dihydrochloride (47 mg); ORD, $[\alpha]_{407}$ + 0.25 ± 0.03°, $[\alpha]_{546}$ + 0.15 ± 0.03°, $[\alpha]_{579}$ + 0.10 ± 0.03°, (*c* 3.17% in 0.1 *M* HCl, uncorrected for incomplete deuteriation). The ^{1}H 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°C; m.s., 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).

6.2.1.1.3 (1,1-$^2H_2$)Cadaverine (119) dihydrochloride by decarboxylation of the L-component of DL-(2-^{2}H)lysine in deuterium oxide solution (Experiment 3)

Decarboxylation of the L-component of DL-(2-^{2}H)lysine monohydrochloride (> 95% 2-^{2}H; 110 mg), catalysed 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 in Section 6.2.1.1.1, 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°C; m.s., 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).

6.2.1.2 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 solution of cadaverine free base (Aldrich) in ethanol at room temperature. The dihydrochloride precipitated on cooling; m.p. 255-257° (lit.[278] m.p. 255°C).

A solution of cadaverine dihydrochloride (23 mg) and L-lysine decarboxylase (Sigma, "Type II") (9 mg) in phosphate-^2H_2O buffer solution (0.2 *M*, p^{2}H 6, 10 ml) was incubated at 36°C under anhydrous conditions (*i.e.* exclusion of ^1H_2O). After 28 h the solution was acidified with concentrated hydrochloric acid, heated on a steam bath for 1 h, and the precipitated enzyme removed by centrifugation. The supernatant solution was lyophylised and exchangeable deuterium was removed by repeated solution of the residue in water (3 x 5 ml) followed by evaporation to dryness. The residue was then dissolved in water (2 ml). Part of the aqueous solution (1 ml) containing cadaverine was converted into the dibenzoyl derivative. Yield 23 mg; m.p. 129-131°C (from aqueous ethanol); ^{1}H NMR (C^2HCl_3), δ 1.3-1.8 (6H, m, H-2,3,4), 3.48 (4H, q, J *ca.* 6 Hz, H-1,5), 6.44 (2H, s (br), 2 NH), 7.3-7.5 (6H, m, ArH), 7.7-7.9 (4H, m, ArH); m.s., 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.

6.2.2 Putrescine

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

6.2.2.1.1 (-)-(1-^{2}H)Putrescine (100) dihydrochloride by decarboxylation of the L-component of unlabelled DL-ornithine in deuterium oxide solution (Experiment 4)

Anhydrous sodium carbonate (Analar) (111 mg) was added to a stirred solution of perdeuterioacetic acid (Merck, Sharp and Dohme 99.7 atom % ^{2}H) in deuterium oxide (Stohler Isotopes, 99.8 atom % ^{2}H) (0.2 *M*, 15 ml) under an atmosphere of dry nitrogen. After stirring for 1 h, DL-ornithine monohydrochloride (Aldrich) (98 mg), which had been stripped of exchangeable protons by repeated solution in ^2H_2O (3 x 5 ml) and evaporation to dryness, and L-ornithine decarboxylase (Sigma, 0.06 U/mg*) (45 mg) were dissolved in the buffered solution (*ca.* p^{2}H 5). The enzymic reaction mixture, in a flask fitted with a calcium sulfate drying tube, was agitated at 36°C for 40 h, and worked up as described for the decarboxylation of lysine (Section 6.2.1.1), to give (-)-(1-^{2}H)putrescine dihydrochloride (46 mg) which was recrystallized from 95% ethanol; ^{1}H NMR (^2H_2O), δ 1.77 (4H, m, H-2,3), 3.06 (*ca.* 3H, m, H-1,4); C.I. m.s., m/e 90 (M+H, 5%), 74 (5), 73 (M-NH_3, 100), 72 (2), (C.I. m.s. of authentic unlabelled material, m/e 89 (M+H, 5%), 73 (5), 72 (M-NH_3, 100), 71 (2));

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* 1 U will release 1.0 μmole carbon dioxide from L-ornithine per minute at pH 5.0 at 37°C.

99.7 ± 0.03% $^2H_1$ determined by C.I. m.s. The sample showed a plane negative ORD curve: $[\alpha]_{313}$ - 1.11 ± 0.10°, $[\alpha]_{334}$ - 0.83 ± 0.06°, $[\alpha]_{365}$ - 0.65 ± 0.06°, $[\alpha]_{405}$ - 0.56 ± 0.06°, $[\alpha]_{436}$ - 0.35 ± 0.04°, $[\alpha]_{546}$ - 0.24 ± 0.06°, $[\alpha]_{577}$ - 0.21 ± 0.08°, (*c* 3.39% in 0.1 *M* HCl, 26°C, uncorrected for incomplete deuteriation).

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. m.s., and gave ORD, $[\alpha]_{302}$ - 1.12 ± 0.11°, $[\alpha]_{313}$ - 1.03 ± 0.08°, $[\alpha]_{334}$ - 0.78 ± 0.09°, $[\alpha]_{365}$ - 0.66 ± 0.05°, $[\alpha]_{405}$ - 0.50 ± 0.04°, $[\alpha]_{436}$ - 0.43 ± 0.04°, $[\alpha]_{546}$ - 0.24 ± 0.05°, $[\alpha]_{577}$ - 0.20 ± 0.06°, (*c* 2.26% in 0.1 *M* HCl, 26°C, uncorrected for incomplete deuteriation).

6.2.2.1.2 (+)-(1-^{2}H)Putrescine (102) dihydrochloride by decarboxylation of the L-component of DL-(2-^{2}H)ornithine (Experiment 5)

A solution 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°C for 20 h. After workup, (+)-(1-^{2}H)putrescine dihydrochloride was obtained and recrystallized from 95% ethanol (yield 45 mg). The ^{1}H NMR spectrum (^2H_2O) was identical with that of (-)-(1-^{2}H)putrescine and the sample showed a plane positive ORD curve. C.I. m.s., m/e 90 (M+H, 3%), 74 (5), 73 ($M-NH_3$, 100), 72 (5); 97.2 ± 0.3% $^2H_1$ determined by C.I. m.s.

Another sample of (+)-(1-^{2}H)putrescine dihydrochloride obtained by enzymic decarboxylation of DL-(2-^{2}H)ornithine (*ca.* 92% ^{2}H) was 93.1 ± 0.9% mono-^{2}H labelled at C-1 (determined by C.I. m.s.); ORD,

$[\alpha]_{302}$ + 1.21 ± 0.09°, $[\alpha]_{313}$ + 0.98 ± 0.06°, $[\alpha]_{334}$ + 0.81 ± 0.06°, $[\alpha]_{365}$ + 0.64 ± 0.04°, $[\alpha]_{405}$ + 0.51 ± 0.04°, $[\alpha]_{436}$ + 0.30 ± 0.03°, $[\alpha]_{546}$ + 0.23 ± 0.03°, $[\alpha]_{577}$ + 0.20 ± 0.04°, (*c* 2.86% in 0.1 *M* HCl, 26°C, uncorrected).

Unreacted D-(2-^{2}H)ornithine was reisolated from the incubation mixture. The alkaline solution, remaining after extraction of the (1-^{2}H)putrescine into butanol, was neutralised with *conc.* hydrochloric acid, and was then applied to a column (25 x 1 cm) of Dowex 50-X8 (H^+ form). The column was washed with water (25 ml), hydrochloric acid (2 *M*, 25 ml) and water (25 ml). D-(2-^{2}H)Ornithine was eluted with ammonia (1 *M*, 25 ml), and was isolated as the monohydrochloride, as described earlier (*cf.* Section 6.1.1.2).

The ^{1}H and ^{2}H NMR spectra of the sample of D-(2-^{2}H)ornithine monohydrochloride (*ca.* 92% ^{2}H) were identical with those of the DL-(2-^{2}H)ornithine from which it was derived. ORD, $[\alpha]_{265}$ - 157.8 ± 7.9°, $[\alpha]_{280}$ - 114.8 ± 5.7°, $[\alpha]_{289}$ - 98.0 ± 4.9°, $[\alpha]_{296}$ - 87.3 ± 4.4°, $[\alpha]_{302}$ - 80.7 ± 4.0°, $[\alpha]_{313}$ - 69.2 ± 3.5°, $[\alpha]_{334}$ - 52.8 ± 2.6°, $[\alpha]_{365}$ - 39.3 ± 2.0°, $[\alpha]_{405}$ - 29.0 ± 1.5°, $[\alpha]_{436}$ - 23.4 ± 1.2°, $[\alpha]_{546}$ - 14.4 ± 0.8°, $[\alpha]_{577}$ - 12.9 ± 0.7°, $[\alpha]_{589}$ - 12.1 ± 0.8°, (*c* 0.63% in water, 25°C).

An authentic sample of unlabelled L-ornithine monohydrochloride gave the following ORD: $[\alpha]_{265}$ + 166.3 ± 3.3°, $[\alpha]_{280}$ + 124.9 ± 2.5°, $[\alpha]_{289}$ + 107.6 ± 2.2°, $[\alpha]_{296}$ + 96.1 ± 1.9°, $[\alpha]_{302}$ + 88.9 ± 1.8°, $[\alpha]_{313}$ + 78.1 ± 1.6°, $[\alpha]_{334}$ + 61.7 ± 1.2°, $[\alpha]_{365}$ + 45.1 ± 0.9°, $[\alpha]_{405}$ + 32.3 ± 0.7°, $[\alpha]_{436}$ + 26.1 ± 0.5°, $[\alpha]_{546}$ + 13.6 ± 0.4°,

$[\alpha]_{577}$ + 11.5 ± 0.3°, $[\alpha]_{589}$ + 11.8 ± 0.5°, (*c* 0.49% in water, 25°C) (lit.[285] $[\alpha]_{589}$ + 11.0° (*c* 5.5% in water, 23°C)).

6.2.2.1.3 (1,1-^{2}H)Putrescine (120) dihydrochloride by decarboxylation of the L-component of DL-(2-^{2}H)ornithine in deuterium oxide solution (Experiment 6)

Decarboxylation of the L-component of DL-(2-^{2}H)ornithine monohydrochloride (*ca.* 92% ^{2}H) (117 mg), catalysed by L-ornithine decarboxylase (40 mg) in perdeuterioacetate-^2H_2O buffer solution (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 in Section 6.2.2.1.1; ^{1}H NMR (^2H_2O), δ 1.78 (4H, m, H-2,3), 3.08 (*ca.* 2H, m, H-1,4); C.I. m.s., m/e 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. m.s.

6.2.2.2 Incubation of putrescine with ornithine decarboxylase in deuterium oxide solution

A solution 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°C under a nitrogen atmosphere. After 42 h a ^{1}H NMR spectrum of the reaction mixture indicated no apparent change in the signals due to putrescine: δ 1.77 (4H, m), 3.05 (*ca.* 4H, t (br)).

6.2.3 Agmatine

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

6.2.3.1.1 (-)-(1-^{2}H)Agmatine (104) sulfate by decarboxylation of L-arginine in deuterium oxide solution (Experiments 7, 9 and 11)

L-Arginine monohydrochloride (Eastman) (90 mg) was twice dissolved in deuterium oxide (3 ml) and evaporated to dryness to effect replacement of exchangeable protons with deuterium. 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* solution. The buffer had been prepared from perdeuterioacetic acid (0.2 *M* in ^2H_2O) and anhydrous sodium carbonate (8.36 mg/ml) (see Section 6.2.2.1.1). L-Arginine decarboxylase (Sigma; 2.9 U/mg (11 mg), Experiment 7; 0.7 U/mg (43 mg), Experiments 9 and 11)* was added and the resulting solution incubated at 36°C in a flask equipped with a calcium sulfate drying tube. After 30-40 h the incubation mixture was acidified (*ca.* pH 2) with concentrated 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 sodium hydroxide and then dissolved in 5% (w/v) sodium hydroxide solution (1-2 ml). The alkaline solution was saturated with sodium chloride and extracted with 1-butanol (4 x 3 ml). Evaporation of the butanol gave an oil which was redissolved in aqueous sulfuric acid (0.1 *M*, *ca.* 1 ml).

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* 1 U will release 1.0 μmole carbon dioxide from L-arginine per minute at pH 5.2 at 37°C.

After addition of methanol and cooling at 0°C overnight the crystalline sulfate of (-)-(1-^{2}H)agmatine separated and was recrystallized from water:methanol. Yield 72 mg; m.p. 238-240°C (lit. (nondeuteriated)[279] m.p. 236-239°C); ^{1}H NMR (^2H_2O), δ 1.66 (4H, m, H-2,3), 2.95 (*ca.* 1H, t (br), J *ca.* 6.8 Hz, H-1), 3.18 (2H, m, H-4); ORD, $[\alpha]_{302}$ - 1.37 ± 0.08°, $[\alpha]_{313}$ - 1.17 ± 0.07°, $[\alpha]_{334}$ - 1.01 ± 0.06°, $[\alpha]_{365}$ - 0.76 ± 0.04°, $[\alpha]_{405}$ - 0.57 ± 0.03°, $[\alpha]_{436}$ - 0.50 ± 0.03°, $[\alpha]_{546}$ - 0.19 ± 0.02°, $[\alpha]_{579}$ - 0.16 ± 0.03° (*c* 6.52% in water, 25°C, uncorrected for incomplete deuteriation); ^{2}H NMR (H_2O, 180 *M*, 100 transients) showed one signal at 2.9 ppm apart from that due to natural abundance ^{2}H in water 4.5 ppm).

6.2.3.1.2 (+)-(1-^{2}H)Agmatine (106) sulfate by decarboxylation of L-(2-^{2}H)arginine (Experiments 8, 10 and 12)

A solution of L-(2-^{2}H)arginine monohydrochloride (*ca.* 85% ^{2}H, Experiments 8 and 12; *ca.* 92% ^{2}H, Experiment 10) (0.02 *M*) and L-arginine decarboxylase (2.9 U/mg (19 mg), Experiment 8; 0.7 U/mg (75 mg), Experiments 10 and 12) in acetate buffer (0.2 *M*, pH 5.2, 35 ml) was incubated at 36°C for 40 h. The reaction mixture was worked up, as described in Section 6.2.3.1.1 to give (+)-(1-^{2}H)agmatine sulfate (100 mg). The m.p. and NMR spectra (^{1}H and ^{2}H) were similar to those obtained for (-)-(1-^{2}H)agmatine sulfate. The sample showed a plane positive ORD curve; $[\alpha]_{297}$ + 1.42 ± 0.11°, $[\alpha]_{302}$ + 1.21 ± 0.08°, $[\alpha]_{313}$ + 1.07 ± 0.07°, $[\alpha]_{334}$ + 0.84 ± 0.06°, $[\alpha]_{365}$ + 0.70 ± 0.04°, $[\alpha]_{405}$ + 0.53 ± 0.03°, $[\alpha]_{436}$ + 0.42 ± 0.03°, $[\alpha]_{546}$ + 0.27 ± 0.03°, $[\alpha]_{577}$ + 0.23 ± 0.04° (*c* 4.18% in water, 25°C, uncorrected for incomplete deuteriation).

6.2.3.2 Incubation of agmatine with L-arginine decarboxylase in deuterium oxide solution

A solution 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°C under a stream of dry nitrogen. After 92 h, paper chromatography (Whatman 3 MM, developed with 1-butanol:acetic acid:pyridine:water (4:1:1:2) showed only agmatine, R_f 0.36, as indicated by spraying separate chromatograms with ninhydrin (0.3% w/v; in 1-butanol:acetic acid, 97.3, v/v) and Sakaguchi reagent.[280] Agmatine sulfate was then reisolated from the enzymic reaction 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.

6.3 CONVERSION OF AGMATINE INTO PUTRESCINE

6.3.1 Hydrolysis of agmatine to putrescine

A solution of agmatine sulfate (55 mg) in ethanol:water (70:30, v/v, 3 ml) containing sodium hydroxide (10% w/v) was heated at reflux under a nitrogen atmosphere. When the reaction was complete (*ca.* 20 h) as revealed by paper chromatography (Whatman 3 MM, 1-butanol:acetic acid:pyridine:water, 4:1:1:2; visualized with ninhydin (0.3% w/v, in 1-butanol:acetic acid, 97:3, v/v); agmatine, R_f 0.36; putrescine, R_f 0.27), the reaction mixture was cooled to room temperature and acidified to pH 4

with 10% (w/v) hydrochloric acid. After evaporation of the solvent the residue was dissolved in sodium hydroxide solution (10% w/v, 3 ml), the solution saturated with sodium chloride and extracted with 1-butanol (3 x 3 ml). The butanol extract was acidified with HCl-butanol and concentrated *in vacuo* (4 ml). Putrescine dihydrochloride which separated was collected and recrystallized from aqueous ethanol. Yield 33 mg, 87%. The ^1H NMR was identical with that of authentic material.

6.3.2 Hydrolysis of (1-^2H)agmatine to (1-^2H)putrescine

6.3.2.1 Conversion of (-)-(1-^2H)agmatine (104) sulfate into (-)-(1-^2H)putrescine (100) dihydrochloride (Experiment 9)

Hydrolysis of (-)-(1-^2H)agmatine sulfate (65 mg), as described for unlabelled material (Section 6.3.1), gave (-)-(1-^2H)putrescine dihydrochloride (41 mg) which was twice recrystallized from aqueous ethanol: C.I. m.s., m/e 90 (M+H, 3%), 74 (5), 73 ($M-NH_3$, 100), 72 (2); 99.4 ± 0.4% ^2H, determined by C.I. m.s.; ORD, $[\alpha]_{302}$ - 1.07 ± 0.09°, $[\alpha]_{313}$ - 0.98 ± 0.08°, $[\alpha]_{334}$ - 0.84 ± 0.08°, $[\alpha]_{365}$ - 0.67 ± 0.05°, $[\alpha]_{405}$ - 0.52 ± 0.05°, $[\alpha]_{436}$ - 0.43 ± 0.06°, $[\alpha]_{546}$ - 0.21 ± 0.06° (*c* 1.84% in 0.1 *M* HCl, 26°C, uncorrected for incomplete deuteriation).

6.3.2.2 Conversion of (+)-(1-^2H)agmatine (106) sulfate into (+)-(1-^2H)putrescine (102) dihydrochloride (Experiment 10)

Hydrolysis of (+)-(1-^2H)agmatine sulfate (40 mg) gave (+)-(1-^2H)-putrescine dihydrochloride (22 mg) which was twice recrystallized from water: C.I. m.s., m/e 90 (M+H, 5%), 74 (5), 73 ($M-NH_3$, 100), 72 (10);

91.7 ± 0.8% ^2H, determined by C.I. m.s.; ORD, $[\alpha]_{313}$ + 0.85 ± 0.09°, $[\alpha]_{334}$ + 0.75 ± 0.09°, $[\alpha]_{365}$ + 0.56 ± 0.06°, $[\alpha]_{405}$ + 0.44 ± 0.05°, $[\alpha]_{436}$ + 0.41 ± 0.06°, $[\alpha]_{546}$ + 0.24 ± 0.06°, (*c* 1.61% in 0.1 *M* HCl, 26°C, uncorrected for incomplete deuteriation).

Paper chromatography (see Sections 6.2.3.2 and 6.3.1) of the samples of (-)-(1-^2H)- and (+)-(1-^2H)putrescine dihydrochloride obtained from Experiments 9 and 10 showed single ninhydrin positive and Sakaguchi[280] negative spots at R_f 0.27. The ^1H NMR spectra (^2H_2O) of both samples were identical with those of (1-^2H)putrescine dihydrochloride obtained from ornithine.

6.4 OXIDATION OF (1-^2H)AMINES TO ω-AMINOALDEHYDES

6.4.1 Chemical synthesis of solid derivatives of the ω-aminoaldehydes

6.4.1.1 5-Aminopentanal

6.4.1.1.1 3-(3'-Aminopropyl)quinoline (125) (*cf.* ref. 198)

α-Tripiperideine (127) (404 mg) was dissolved in hot citrate buffer (0.1 *M*, pH 4.7, 10 ml) and added to a hot solution of *o*-aminobenzaldehyde (Fluka A.G.) (610 mg) in the same buffer (190 ml). The mixture was heated on a steam bath for 6 h, cooled to 0°C and filtered. The filtrate was basified with aqueous sodium hydroxide (5%, w/v), saturated with sodium chloride and extracted with chloroform (4 x 100 ml). The combined chloroform extracts were dried over anhydrous magnesium sulfate, filtered and evaporated. 3-(3'-Aminopropyl)quinoline was obtained as a yellow oil (686 mg). ^1H 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 solution of the quinoline derivative (125) (113 mg) in ethanol (3 ml) was acidified with ethanolic hydrogen chloride. The dihydrochloride precipitated on addition of a small amount of ether, and was recrystallized from ethanol-ether. Yield 110 mg; m.p. 228-230°C (lit.[198] m.p. 225-230°C); ^1H 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°C (from water) (lit. m.p. 208-210°,[195] 216-217°[198]); ^1H 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).

6.4.1.1.2 3-(3'-Aminopropyl)-(2-^{2}H)quinoline dihydrochloride (*cf.* ref. 203)

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

When the solution had become colourless (45 min), the reaction mixture was basified with aqueous sodium hydroxide (5%, w/v), and was extracted with chloroform (4 x 5 ml). The chloroform extracts were dried over anhydrous magnesium sulfate, 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 (Section 6.4.1.1.1). 3-(3'-Aminopropyl)-(2-^{2}H)quinoline was obtained as its dihydrochloride and recrystallized from ethanol-ether. Yield 40 mg, 41%; m.p. 228-230°C. 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 of the 3-(3'-aminopropyl)quinoline derivative. Yield 26 mg (33%); m.p. 212-215°C. ^{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 ^2H in DMSO (2.6 ppm).

6.4.1.2 4-Aminobutanal

6.4.1.2.1 2,3-Trimethylene-1,2-dihydroquinazolinium (134) picrate

Oxidation of DL-ornithine monohydrochloride (333 mg) with *N*-bromosuccinimide (350 mg) in aqueous solution (20 ml) to give Δ^1-pyrroline (133), the cyclized form of 4-aminobutanal (132), was carried out by a published procedure,[199] the pH of the solution containing Δ^1-pyrroline (20 ml, pH 3.5) was adjusted to pH 4.5 with aqueous sodium hydroxide (5%, w/v). *o*-Aminobenzaldehyde (253 mg) in citrate buffer (0.1 *M*, pH 4.7, 40 ml) was added. The colour of the resulting solution changed from yellow to bright orange during stirring at room temperature. After 20 h the reaction mixture was filtered, and a solution of picric acid in methanol (10%, w/v, 5 ml) was added to the filtrate. The dihydroquinazolinium picrate (134), which precipitated at 0°C, was recrystallized from methanol-water. Yield 400 mg, 50%; m.p. 160-162°C (lit. m.p. 170-171°C,[202] 169-170°C,[199] 166-168°C[197]); ^1H 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*, 85,000 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 carbon atoms), 159.2 (C-4).

6.4.1.2.2 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 (Section 6.4.1.2.1). M.p. 154-155°C (from methanol-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 (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).

6.4.2 Enzymic synthesis of ω-aminoaldehydes

6.4.2.1 5-Aminopentanal

6.4.2.1.1 Incubation of cadaverine (121) with hog kidney diamine oxidase (E.C. 1.4.3.6) and isolation of the product as 3-(3'-aminopropyl)quinoline (125) dihydrochloride

(i) 40 h Incubation. A solution 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°C for 40 h. The mixture was then acidified (pH 4.5) with

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* 1 U will oxidise 1.0 μmole putrescine per hour at pH 7.2 at 37°C.

† 1 U will decompose 1.0 μmole hydrogen peroxide per minute at pH 7.0 at 25°C.

4 *M* hydrochloric acid, heated on a steam bath for 30 min and centrifuged. *o*-Aminobenzaldehyde (20 mg) was added, and the supernatant solution was refluxed for 6 h, cooled to room temperature and filtered through Celite. The pH of the filtrate was adjusted to 11-12 with 5% (w/v) sodium hydroxide, and the solution was extracted with diethyl ether (4 x 20 ml). The combined ether extracts were dried over anhydrous sodium sulfate, filtered and the solvent was evaporated to dryness. The residue was purified by preparative thin layer chromatography (silica gel plates, 0.5 mm, 20 x 20 cm; developed with 1-butanol:acetic acid: water (5:1:1)). The band corresponding to that of 3-(3'-aminopropyl)-quinoline, R_f 0.3, was eluted with ethanol. After removal of the solvent *in vacuo*, the residue was suspended in sodium hydroxide solution (2%, w/v, 1 ml) and the product extracted into ether (4 x 2 ml). The quinoline dihydrochloride was obtained by acidification with ethanolic hydrogen chloride, evaporation to dryness, and crystallization of the residue from ethanol-ether. Yield 5 mg; m.p. 229-231°C. The ^1H 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 (see Section 6.4.1.1.1).

(ii) 6 h 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 h. When picric acid in methanol was added, the products precipitated. The solid was extracted with boiling methanol (1 ml).

The yellow residue was recrystallized from water to give 3-(3'-aminopropyl)quinoline dipicrate (8 mg), m.p. 216-218°C. The ^1H NMR

spectrum (DMSO-$^2H_6$) was identical with that of an authentic sample of the quinoline dipicrate prepared from α-tripiperideine (see Section 6.4.1.1.1).

When water (0.2 ml) was added to the methanol extract the second product, 2,3-tetramethylene-1,2-dihydroquinazolinium (126) picrate (2 mg) precipitated. M.p. 165-167°C (from methanol-water) (lit.[198] m.p. 166-172°C); ^1H NMR (DMSO-$^2H_6$), δ 2.09 (6H, m, H-1',2'3'), 4.02 (*ca.* 2H, t (br), J *ca.* 10 Hz, H-4'), 5.75 (1H, d of d (br), J_1 *ca.* 9 Hz, J_2 *ca.* 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).

6.4.2.1.2 Enzymic conversion of (^2H)cadaverine into (^2H)-3-(3'-aminopropyl)quinoline

Samples (40 mg) of R-(1-^2H)-, S-(1-^2H)- and (1,1-$^2H_2$)cadaverine dihydrochloride (Experiments 1, 2 and 3a, respectively), obtained from the appropriate incubation with L-lysine decarboxylase, were incubated in separate experiments with diamine oxidase, as described in Section 6.4.2.1.1 (i). 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 deuterium at C-2 and/or C-3', with authentic unlabelled material, as revealed by ^1H and ^2H NMR spectroscopy.

Another sample of (1,1-$^2H_2$)cadaverine (30 mg) dihydrochloride (Experiment 3b) was incubated with diamine oxidase, as described in Section 6.4.2.1.1 (ii).

The major product, 3-(3'-aminopropyl)quinoline dipicrate (7 mg), m.p. 205-208°C gave a ^1H NMR spectrum (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 picrate (*ca.* 1 mg) showed a ^{1}H NMR spectrum (DMSO-$^{2}H_6$:$^{2}H_2O$, 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.

6.4.2.2 4-Aminobutanal

6.4.2.2.1 Incubation of putrescine (131) with hog kidney diamine oxidase and isolation of the product as 2,3-trimethylene-1,2-dihydroquinazolinium (134) picrate

A solution 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 temperature water bath at 36°C for 6 h. The pH of the reaction mixture was then adjusted to pH 4-4.5 with concentrated hydrochloric acid, and the solution concentrated (*ca.* 15 ml) by heating on a steam bath under a stream of nitrogen. The precipitated protein was removed by centrifugation and filtration of the supernatant solution through Celite. A saturated solution of picric acid in methanol (0.15 ml) was added, with swirling, to the clear yellow solution and the product, which precipitated when the mixture was cooled at 0°C overnight, was collected and recrystallized from methanol-water. Yield 14 mg. The m.p. (159-160°C) and ^{1}H NMR spectrum (DMSO-$^{2}H_6$) were identical

with those of the dihydroquinazolinium picrate prepared from ornithine (see Section 6.4.1.2.1).

6.4.2.2.2 Enzymic conversion of (^{2}H)putrescine into (^{2}H)-2,3-trimethylene-1,2-dihydroquinazolinium picrate

Samples (30 mg) of (-)-(1-^{2}H)-, (+)-(1-^{2}H)- and (1,1-$^2H_2$)-putrescine dihydrochloride (Experiments 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 Section 6.4.2.2.1), which were labelled with deuterium at C-2 and/or C-3', as determined by ^{2}H and ^{1}H NMR spectroscopy.

6.4.2.2.3 Incubation of agmatine (137) with hog kidney diamine oxidase and isolation of the product, 4-guanidinobutanal (138), as its dinitrophenylhydrazone (DNP) derivative

A potassium phosphate buffer solution (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°C in a constant temperature water bath. The course of the reaction was followed by thin layer chromatography (silica gel; 1-butanol:acetic acid:water (5:1:1)) which showed the formation of a single oxidation product (Sakaguchi^{280} positive) at R_f 0.35 (agmatine, R_f 0.06).

When oxidation was complete (*ca.* 23 h), the enzymic reaction mixture was acidified (pH 4) with 10% (w/v) hydrochloric acid, heated on a steam bath for 30 min, cooled to room temperature, and its pH adjusted to pH 6 with sodium hydroxide solution (2%, w/v). Ethanol (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 deuterium oxide (*ca.* 1 ml). ^{1}H NMR ($^{2}H_2O$), 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 ($^{2}H_2O$, 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).

After removal of the deuterium oxide *in vacuo*, the residue was redissolved in water (5 ml) and evaporated to dryness. It was then extracted with ethanol (3 x 2 ml). The combined ethanol extracts were evaporated to dryness and a solution of dinitrophenylhydrazine in ethanolic phosphoric acid (0.1 *M*, 5 ml) was added. The solution was heated at reflux for 30 min and after cooling at 0°C overnight the yellow-orange DNP-phosphate derivative precipitated (82 mg). ^{1}H NMR ($^{2}H_2O$), 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-$^{2}H_6$, 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 carbon 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-$^{2}H_6$ 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°C, the hydrochloride salt precipitated as yellow-orange needles which were recrystallized from ethanol. Yield 5 mg; m.p. 95-96°C. The ^{1}H NMR ($^{2}H_2O$) of the DNP-hydrochloride was identical with that of the phosphate.

6.4.2.2.4 Incubation of (1-^{2}H)agmatine with diamine oxidase

Incubation of (-)-(1-^{2}H)- and (+)-(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 in Section 6.4.2.2.3.

6.4.2.2.4.1 Conversion of (-)-(1-^{2}H)agmatine sulfate into 4-guanidino(1-^{2}H)butanal-DNP (Experiment 7)

The DNP-phosphate (25 mg) obtained from (-)-(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.

6.4.2.2.4.2 Conversion of (+)-(1-^{2}H)agmatine sulfate into 4-guanidinobutanal-DNP (Experiment 8)

The ^{1}H NMR spectrum (^2H_2O) of the DNP-phosphate (32 mg) obtained from (+)-(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, 14,612 transients) other than the one due to natural abundance deuterium in water.

6.5 CONVERSION OF AGMATINE INTO PHTHALIMIDOBUTYRIC ACID

6.5.1 Preparation of 4-phthalimidobutyric acid

6.5.1.1 4-Phthalimidobutylguanidine (148) sulfate

To a stirred solution containing agmatine sulfate (228 mg) and sodium bicarbonate (168 mg) in water (3 ml) was added *N*-carbethoxyphthalimide (Aldrich) (232 mg) in small portions over 15 min at room temperature. After stirring for 1 h, the reaction mixture was cooled to 0°C and the product, which precipitated, was recrystallized from methanol. Yield 225 mg; m.p. 213-214°C; ^{1}H NMR ($C^2H_3CO_2{}^2H$), δ 1.70 (4H, m (br), H-2,3), 3.30 (2H, t (br), H-1), 3.84 (2H, t (br), H-4), 7.85 (4H, m, ArH). Anal. Calcd. for $(C_{13}H_{17}N_4O_2)_2 \cdot SO_4 \cdot 2H_2O$: C, 47.69; H, 5.85; N, 17.12; $SO_4^=$, 14.67. Found: C, 47.65; H, 5.98; N, 17.09; $SO_4^=$, 14.49%.

6.5.1.2 4-Phthalimidobutyric acid (149)

A solution of potassium permanganate (0.4 *M*) in 0.5 *M* sulfuric acid was added in small portions (*ca.* 0.1 ml) to a stirred suspension of 4-phthalimidobutylguanidine (27 mg) in 0.5 *M* sulfuric acid (1 ml) at room temperature. Additions of the oxidant were made until the colour of permanganate in the reaction mixture was no longer discharged (*ca.* 0.5 ml over 1 h). Stirring was continued for 1 h and the reaction mixture was then decolourized with sodium bisulfite. After cooling at 0°C, the product, which precipitated, was extracted into chloroform (3 x 2 ml). The combined extracts were dried over anhydrous sodium sulfate and filtered. Evaporation gave 4-phthalimidobutyric acid (13 mg) as a white solid which was recrystallized three times from water; m.p. 115-116°C

lit.[281] m.p. 115-117°C); ^{1}H NMR (C^2HCl_3), δ 2.01 (2H, quin., J 6.8 Hz, H-3), 2.32 (2H, t, J 6.8 Hz, H-2), 3.76 (2H, t, J 6.8 Hz, H-4), 7.75 (4H, m, ArH), 10.2 (1H, s (br), CO_2H); m.s., m/e 233 (M, 4%), 215 ($M-H_2O$, 19), 187 (15), 174 (70), 173 (42), 160 (100), 104 (19), 76 (24). The m.p. and spectral properties were the same as those of an authentic sample prepared from γ-aminobutyric acid and *N*-carbethoxyphthalimide.[121]

6.5.2 Conversion of (1-^{2}H)agmatine into 4-phthalimido-(4-^{2}H)butyric acid

6.5.2.1 (-)-4-Phthalimido(4-^{2}H)butyric acid (150) from (-)-(1-^{2}H)agmatine (104) sulfate (Experiment 11)

N-Carbethoxyphthalimide (82 mg) was added to a stirred solution of (-)-(1-^{2}H)agmatine sulfate (70 mg) in 0.5 *M* sodium bicarbonate (1.5 ml). The resulting mixture was stirred at room temperature for 20 min and then cooled to 0°C. After standing at 0°C for 30 min, the product was collected by filtration, washed with cold water (1 ml), cold ethanol (1 ml) and chloroform (1 ml), and dried *in vacuo* over sodium hydroxide. 4-phthalimido(4-^{2}H)butylguanidine (152) (66 mg), so obtained, was recrystallized from methanol:acetic acid (20:1); m.p. 210-212°C. The ^{1}H NMR spectrum ($C^2H_3CO_2{}^2H$) of the deuteriated sample was similar to that of unlabelled material (see Section 6.5.1.1) except that the signal at 3.84 ppm (H-4) corresponded to *ca.* 1.05 hydrogen atoms only.

Permanganate oxidation of a sample of the (^{2}H)guanidine derivative (49 mg) in aqueous sulfuric acid (0.5 *M*), as described for unlabelled material (Section 6.5.1.2), gave (-)-4-phthalimido(4-^{2}H)-butyric acid (150) (23 mg) which was recrystallized three times from water; m.p. 112-114°C; ^{1}H NMR (C^2HCl_3), δ 2.05 (2H, q, J 6.8 Hz), 2.39

(2H, t, J 6.8 Hz), 3.75 (*ca.* 1H, t, J 6.8 Hz), 7.80 (4H, m), 10.2 (1H, s (br)); ^{2}H NMR ($CHCl_3$, 30 *mM*, 2172 transients) showed a single signal at 3.74 ppm apart from that due to natural abundance ^{2}H in $CHCl_3$ (7.27 ppm); m.s., m/e 234 (M, 3%), 216 (M-H_2O, 7), 215 (M-H^2HO, 9), 188 (8), 187 (6), 175 (41), 174 (30), 173 (17), 161 (100), 160 (5), 104 (16), 76 (22); 97.2 ± 0.2% $^2H_1$, determined by m.s.; ORD, $[\alpha]_{365}$ - 5.39 ± 0.14°, $[\alpha]_{405}$ - 3.79 ± 0.22°, $[\alpha]_{436}$ - 3.07 ± 0.18°, $[\alpha]_{546}$ - 1.64 ± 0.13°, $[\alpha]_{577}$ - 1.37 ± 0.14°, $[\alpha]_{589}$ - 1.08 ± 0.22° (*c* 0.91% in methanol, 25°C, uncorrected for incomplete deuteriation) (lit.[121] $[\alpha]_{589}$ - 1.69°).

A sample of (-)-4-phthalimido(4-^{2}H)butyric acid (150) was dissolved in ether (1 ml), an excess of diazomethane in ether (1 ml)[282] was added and the mixture was allowed to stand overnight. The product, (-)-methyl 4-phthalimido(4-^{2}H)butyrate (63) was recrystallized from methanol. M.p. 87-88°C (lit. (nondeuteriated)[281] m.p. 89-90°C); m.s., m/e 248 (M, 9%), 217 (M-OCH_3, 18), 216 (8), 189 (10), 188 (10), 175 (100), 174 (20), 161 (95), 130 (15), 104 (17), 77 (15), 76 (12); 98.2 ± 0.3% $^2H_1$, determined by m.s.; ORD, $[\alpha]_{365}$ - 5.97 ± 0.60°, $[\alpha]_{405}$ - 4.84 ± 0.40°, $[\alpha]_{436}$ - 4.06 ± 0.30°, $[\alpha]_{546}$ - 2.02 ± 0.30°, $[\alpha]_{577}$ - 2.21 ± 0.30° (*c* 0.34% in methanol, 25°C, uncorrected for incomplete deuteriation). An unlabelled sample of methyl 4-phthalimidobutyrate was prepared analogously. M.p. 87-88°C (from methanol); m.s., m/e 247 (M, 13%), 216 (M-OCH_3, 19), 215 (17), 188 (9), 187 (11), 174 (100), 173 (23), 160 (79), 130 (11), 104 (14), 77 (12), 76 (6).

An authentic sample of (-)-methyl 4-phthalimido(4-^{2}H)butyrate (63) was supplied by Dr. M.H. O'Leary, University of Wisconsin. This sample had been prepared by methylation of (-)-4-phthalimido(4-^{2}H)-

butyric acid which had been obtained by decarboxylation in ^2H_2O of L-glutamic acid, catalysed by L-glutamate decarboxylase.[121] M.p. 88-89°C (from methanol); m.s., m/e 248 (M, 13%), 217 (M-OCH_3, 21), 216 (10), 189 (10), 188 (8), 175 (100), 174 (15), 161 (80), 130 (15), 104 (15), 77 (10), 76 (12); 92.5 ± 0.4% $^2H_1$, 4.7 ± 0.4% $^2H_2$, determined by m.s.; ORD, $[\alpha]_{365}$ - 4.45 ± 0.30°, $[\alpha]_{405}$ - 3.35 ± 0.20°, $[\alpha]_{436}$ - 2.76 ± 0.20°, $[\alpha]_{546}$ - 1.48 ± 0.30°, $[\alpha]_{577}$ - 1.43 ± 0.30° (*c* 0.39% in methanol, 25°C, uncorrected for incomplete deuteriation).

6.5.2.2 (+)-4-Phthalimido(4-^{2}H)butyric acid (151) from (+)-(1-^{2}H)agmatine (106) sulfate (Experiment 12)

A sample of 4-phthalimido(4-^{2}H)butylguanidine (153) (80 mg) obtained by the method described in Section 6.5.1 from (+)-(1-^{2}H)agmatine sulfate (72 mg) showed m.p. 213-214°C (from methanol:acetic acid (20:1)) and a ^{1}H NMR spectrum in which the signal at 3.84 ppm corresponded to *ca.* 1.15 hydrogen atoms. Oxidation of part of this sample (60 mg), as described in Section 6.5.1.2, gave (+)-4-phthalimido(4-^{2}H)butyric acid (151) (28 mg), m.p. 112-114°C (recrystallized 3 times from water). The ^{1}H (C^2HCl_3) and ^{2}H ($CHCl_3$, 30 *mM*, 1584 transients) NMR spectra of this material were similar to those obtained for the (-)-(4-^{2}H)phthalimide. M.s., m/e 234 (M, 3%), 216 (M-H_2O, 8), 215 (M-H^2HO, 10), 188 (9), 187 (7), 175 (44), 174 (39), 173 (19), 161 (100), 160 (16), 104 (17), 76 (25); 84.7 ± 0.3% $^2H_1$ determined by m.s.; ORD, $[\alpha]_{365}$ + 4.77 ± 0.27°, $[\alpha]_{405}$ + 3.26 ± 0.29°, $[\alpha]_{436}$ + 2.62 ± 0.17°, $[\alpha]_{546}$ + 1.32 ± 0.11°, $[\alpha]_{577}$ + 1.20 ± 0.13°, $[\alpha]_{589}$ + 0.96 ± 0.22° (*c* 0.97% in methanol, 25°C, uncorrected for incomplete deuteriation).

6.6 INSTRUMENTAL METHODS

6.6.1 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.

6.6.2 Nuclear magnetic resonance spectroscopy

6.6.2.1 ^{1}H-NMR

^{1}H-NMR spectra were recorded at ambient temperature 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. Chemical shifts are reported as ppm downfield (δ) from DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) for samples in $^{2}H_2O$ solution, from internal TMS (tetramethylsilane) for samples in DMSO-$^{2}H_6$ or $^{2}HCCl_3$ solutions, or from external TMS for samples in $C^{2}H_3CO_2{}^{2}H$ solution.

6.6.2.2 ^{2}H-NMR

^{2}H-NMR spectra were recorded on a Bruker WH90 pulsed Fourier transform spectrometer operating at 13.82 MHz at ambient temperature, with broad band proton noise decoupling. Spectra were obtained of samples in aqueous, dimethylsulfoxide or chloroform solution in 10 mm (o.d.) sample tubes, using $^{2}H_2O$ as external lock. The signal due to natural

abundance ^2H in ^2HOH, ^2H-DMSO or $^2HCCl_3$ served as internal standard. Chemical shifts are downfield from perdeuteriotetramethylsilane.

6.6.2.3 ^{13}C-NMR

^{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.

6.6.3 Mass Spectrometry

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.[283] Determinations were made from at least 10 m.s. traces of either the parent ion region or, with samples of (1-^2H)putrescine dihydrochloride,[284] the M-NH_3 region. Mean values and 95% confidence limits are reported.

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^{2}H NMR as a Probe of the Stereochemistry of Enzyme Reactions at Prochiral Centers. Deamination of Cadaverine, Catalyzed by Diamine Oxidase

Sir:

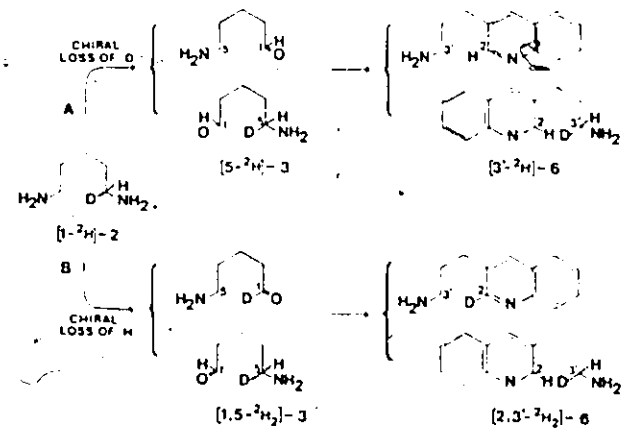
Diamine oxidase (E.C. 1.4.3.6, diamine:oxygen oxidoreductase (deaminating)) catalyzes the oxidative deamination of a wide range of primary amines, including diamines, histamine, and arylalkylamines.[1-3] The aliphatic diamines, cadaverine and putrescine, are the substrates which are most readily oxidized. Thus, cadaverine (**2**), by loss of one of the two equivalent amino groups, yields 5-aminopentanal (**3**), which is in equilibrium with its cyclized form, Δ^1-piperideine (**4**).[4-6] It is to be expected that the reaction takes place in a sterically controlled manner. The oxidation of histamine, catalyzed by hog kidney diamine oxidase,[7] and that of benzylamine, mediated by diamine oxidase from pea seedlings,[8] takes place with stereospecific release of one of the two enantiotopic hydrogen atoms from the carbon adjacent to nitrogen. In the latter case, the stereochemistry of the reaction was determined by means of tritium labeling and determination of $^3H/^{14}C$ ratios. The chirality of the analogous oxidation of tyramine, catalyzed by monoamine oxidase (E.C. 1.4.3.4) of rat liver, was determined by a kinetic method, using deuterium labeled substrates.[9]

The stereochemistry of the oxidative deamination of cadaverine has not been established. We have employed ^{2}H NMR spectrometry to demonstrate that, in the course of this reaction, catalyzed by diamine oxidase of hog kidney (E.C. 1.4.3.6), it is the *pro-S* hydrogen which is removed stereospecifically from the carbon atom adjacent to the reacting amino group.

The enantiomers of [1-^{2}H]cadaverine, required for the study, were prepared by decarboxylation of L-lysine (**1**), catalyzed by L-lysine decarboxylase (E.C. 4.1.1.18, L-lysine carboxylyase) from *Bacillus cadaveris*, a reaction which is known[10] to proceed with retention of configuration. (+)-(*S*)-[1-^{2}H]Cadaverine dihydrochloride (**2A**) (>95% deuterated at the *pro-S* position of one of the terminal carbon atoms)[11] was obtained from the L-component of DL-[2-^{2}H]-lysine.[12] (−)-(*R*)-[1-^{2}H]Cadaverine dihydrochloride (**2B**) (>95% deuterated at the *pro-R* position of one of the terminal carbon atoms)[11] was obtained by decarboxylation of L-lysine in deuterium oxide (99.7 atom %, Merck Sharp and Dohme). A third deuterated cadaverine, [1,1-$^2H_2$]cadaverine (**2C**) (>95% perdeuterated at one of the terminal C atoms),[11] was prepared by decarboxylation of DL-[2-^{2}H]lysine in deuterium oxide.

In separate experiments, each of the three deuterated samples of cadaverine (40 mg) was incubated (37 °C, 40 h) with hog kidney diamine oxidase (Sigma Grade II, 200 mg),

Scheme I



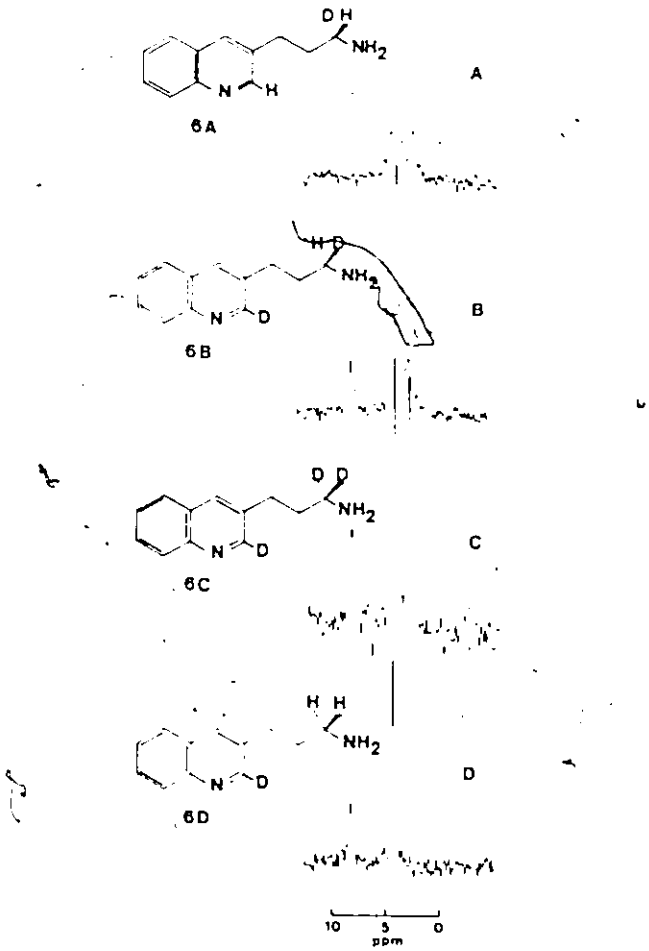


Figure 1. Proton decoupled ^2H NMR spectra of deuterium labeled samples of 3-(3′-aminopropyl)quinoline dihydrochloride (6): A (6A), derived from (*S*)-[1-^{2}H]cadaverine (2A) (via enzymic oxidative deamination) (5 mM, 56 628 transients); B (6B), derived from (*R*)-[1-^{2}H]cadaverine (2B) (5 mM, 57 000 transients); C (6C), derived from [1,1-$^2H_2$]cadaverine (2C) (13 mM, 3475 transients); D (6D), obtained from DL-[2-^{2}H]lysine by chemical oxidation[18] (8 mM, 4808 transients).

together with beef liver catalase[14] (E.C. 1.11.1.6, H_2O_2:H_2O_2 oxidoreductase) (Sigma, 0.5 mg) and *o*-aminobenzaldehyde (5, 32 mg). The latter traps the product of the enzymic reaction, 5-aminopentanal (3), to yield 3-(3′-aminopropyl)quinoline (6).[15]

The location of deuterium in the samples of 6 obtained from the three deuterated cadaverines 2A, 2B, and 2C was determined by ^{2}H NMR spectroscopy.[16]

Oxidation of a chirally deuterated [1-^{2}H]cadaverine with stereospecific loss of deuterium leads to a sample of 5-aminopentanal (3), enriched in deuterium exclusively at C-5, together with nondeuterated (3). The quinoline derivative (6) obtained from this 5-[5-^{2}H]aminopentanal will be deuterated exclusively at C-3′ of the side chain (Scheme I, A). Oxidation of a chirally deuterated [1-^{2}H]cadaverine, with stereospecific retention of deuterium but loss of protium from C-1, leads to a sample of 5-aminopentanal, intermolecularly doubly labeled at C-1 and C-5. This, in turn, yields an intermolecularly labeled quinoline derivative, deuterated at C-3′ of the side chain and at C-2 of the nucleus (Scheme I, B).

Assignment of NMR signals due to ^{1}H and ^{2}H at these two positions was made using compounds of known isotope distribution: The ^{1}H NMR[17] signals assigned to the protons at C-3′ and C-2 of undeuterated 6 appeared at δ 2.95 (t, J = 7.2 Hz) and 8.68 (s, br) ppm, respectively. The downfield signal (δ 8.68 ppm) was absent in the ^{1}H NMR spectrum of a sample of 3-[2-^{2}H](3′-aminopropyl)quinoline (6D).[18]

The ^{2}H NMR[16] spectrum of 6D showed a single peak at δ 8.8 ppm (Figure 1D) (apart from the signal at δ 4.5 ppm, due to the natural abundance of deuterium in water (^{1}H^{2}HO) present in all spectra in Figure 1). The ^{2}H NMR spectrum of [2,3′,3′-$^2H_3$]-6 (6C), derived from [1,1-$^2H_2$]cadaverine (2C) showed two signals at δ 2.9 and 8.8 ppm, of relative intensity 2:1, due to deuterium at C-3′ and C-2 (Figure 1C).

The spectra given by the quinoline derivatives obtained from the two enantiomeric [1-^{2}H]cadaverines are shown in Figures 1A and 1B. The quinoline derived from (*S*)-[1-^{2}H]cadaverine (2A) showed a single ^{2}H NMR signal at δ 2.9 ppm (Figure 1A), indicating that only C-3′ was labeled with deuterium. The quinoline from (*R*)-[1-^{2}H]cadaverine (2B), on the other hand, showed two signals at δ 2.9 and 8.8 ppm, in the ratio 1:1 (Figure 1B), due to the presence of deuterium at C-2 as well as at C-3′.

It follows that diamine oxidase (E.C. 1.4.3.6) from hog kidney mediates the stereospecific removal of the *pro-S* hydrogen from C-1 of cadaverine, and that the product of oxidative deamination, 5-aminopentanal (3), in equilibrium with Δ^1-piperideine (4), retains the *pro-R* hydrogen at the sp^2 carbon.

This stereospecificity corresponds to that of the oxidative deamination of benzylamine to benzaldehyde, catalyzed by the diamine oxidase (E.C. 1.4.3.6) of pea seedlings, which also involves loss of the *pro-S* hydrogen.[8] The analogous oxidation of tyramine, catalyzed by monoamine oxidase (E.C. 1.4.3.4) from rat liver, on the other hand, takes place with loss of the *pro-R*-hydrogen atom from the carbon adjacent to the amino group.[9]

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(12) DL-[2-^{2}H]Lysine (>98% deuterated at C-2, as determined by ^{1}H NMR) was prepared (cf. ref 13) by reaction of *N*-(4-bromobutyl)phthalimide with sodium diethyl acetamidomalonate in ethanol, followed by hydrolysis of the substitution product, diethyl 2-acetamido-2-(4-phthalimidobutyl)malonate, in deuterium chloride (38% w/w in D_2O) and isolated as its monohydrochloride.
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(16) ^{2}H NMR spectra were recorded on a Bruker WH90 pulsed Fourier transform spectrometer operating at 13.82 MHz at ambient temperature, with broad band proton noise decoupling. All spectra were obtained from aqueous solutions (1 mL) in 10-mm-o.d. sample tubes using ^2H_2O as external lock; repetition time, 1.024 s; pulse time, 15 μs. The natural abundance deuterium signal of ^{1}H^{2}HO served as internal standard. Chemical shifts are referenced to perdeuteriotetramethylsilane.

(17) ^{1}H NMR spectra at 90 MHz were of solutions of **6**, and **6D** dihydrochlorides in 0.2 M perdeuterioacetate buffer in deuterium oxide (pD ~5.2).
(18) 3-[2-^{2}H](3'-Aminopropyl)quinoline (**6D**) dihydrochloride was obtained in two steps from DL-[2-^{2}H]lysine. Oxidation with *N*-bromosuccinimide gave Δ^1-[2-^{2}H]piperideine,[19] in equilibrium with 5-[1-^{2}H]aminopentanal, which was condensed with *o*-aminobenzaldehyde.[13]
(19) R. N. Gupta and I. D. Spenser, *Can. J. Chem.*, **47**, 445 (1969).

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The stereochemistry of the enzymic decarboxylation of L-arginine and of L-ornithine[1]

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The decarboxylation of L-ornithine to yield putrescine, catalyzed by the L-ornithine decarboxylase (EC 4.1.1.17) of *E. coli*, and the decarboxylation of L-arginine to yield agmatine, catalyzed by the L-arginine decarboxylase (EC 4.1.1.19) of *E. coli*, take place with retention of configuration.

JAMES C. RICHARDS et IAN D. SPENSER. Can. J. Chem. 60, 2810 (1982).

La décarboxylation de la L-ornithine qui est catalysée par la décarboxylase de la L-ornithine (EC 4.1.1.17) de *E. coli* et qui conduit à la putrescine et la décarboxylation de la L-arginine, catalysée par la décarboxylase de la L-arginine (EC 4.1.1.19) de *E. coli* et qui conduit à la agmatine, se font avec retention de configuration.

[Traduit par le journal]

Introduction

Enzymes that catalyze the decarboxylation of L-α-amino acids are found in microorganisms, in mammalian tissues, and in plants. These enzymes show high substrate specificity for individual amino acids. The reaction that is catalyzed involves the conversion of a chiral centre within the L-α-amino acid, R—$CH(NH_2)CO_2H$, into a prochiral centre within the corresponding amine R—CH_2—NH_2. It was demonstrated (1) almost 30 years ago that the reaction mediated by three of these enzymes (EC 4.1.1.25 L-tyrosine decarboxylase, from *Streptococcus faecalis*; EC 4.1.1.18 L-lysine decarboxylase, from *Bacillus cadaveris*; EC 4.1.1.15 L-glutamate decarboxylase, from *Clostridium welchii* and from *Escherichia coli*) takes a stereochemically defined course, but it was not established at that time whether reaction takes place with net retention or with net inversion of configuration. Later, the actual stereochemical course of these reactions was determined.

The conversions of L-tyrosine into tyramine (2), of L-lysine into cadaverine (3), and of L-glutamate into γ-aminobutyric acid (4), each catalyzed by the appropriate enzyme, all take place with net retention of configuration, as shown by means of isotopic labelling with deuterium or tritium. Thus, decarboxylation of L-(i.e., *S*-)-tyrosine in ^2H_2O yields *R*-(1-^{2}H)tyramine (2), and of L-glutamate in ^2H_2O yields *R*-4-amino(4-^{2}H)butyric acid (4). Similarly, decarboxylation of L-[2-^{3}H]lysine in water generates *S*-[1-^{3}H]cadaverine, whereas L-lysine in tritiated water yields *R*-[1-^{3}H]cadaverine (3). The chirality of the products was determined by kinetic (2), enzymic (3), or chemical (4) correlation with compounds of known stereochemistry.

Net retention in the decarboxylation of L-tyrosine (5), of L-lysine (6, 7), and of L-glutamate (8–10), catalyzed by bacterial enzymes, was later confirmed by independent methods and was found also in reactions catalyzed by enzymes from mammalian sources (5, 8) and by plant systems (5, 6). Net retention has been demonstrated also in the decarboxylation of histidine, catalyzed by bacterial enzymes (EC 4.1.1.22 histidine decarboxylase, from *Lactobacillus* and from *Clostridium welchii*) (11, 12), and of tryptophan, catalyzed by a mammalian enzyme (EC 4.1.1.28 aromatic L-amino acid decarboxylase, from hog kidney) (13).

Very recently a D-amino acid decarboxylase (EC 4.1.1.20 *meso*-α,ε-diaminopimelate decarboxylase, from *Bacillus sphaericus*) has been shown to catalyze an analogous decarboxylation with inversion of configuration (14).

We now add two more L-α-amino acid decarboxylases of bacterial origin to the list of the enzymes that catalyze the decarboxylation of their substrates with net retention of configuration. The two enzymes are L-ornithine decarboxylase (EC 4.1.1.17) which catalyzes the decarboxylation of L-ornithine into putrescine, and L-arginine decarboxylase (EC 4.1.1.19), which mediates the conversion of L-arginine into agmatine (Scheme 4).

Methods and results

DL-(2-^{2}H)Ornithine (4) and L-(2-^{2}H)arginine (9) were prepared by the reaction sequences shown in Schemes 1 and 2.

Decarboxylation of the L-component of DL-ornithine in deuterium oxide, and of the L-component of DL-(2-^{2}H)ornithine in water, catalyzed by L-ornithine decarboxylase (L-ornithine carboxylyase, EC 4.1.1.17) (ex *Escherichia coli*, Sigma-

[1]A preliminary account of part of this work was presented at the 62nd Canadian Chemical Conference of the Chemical Institute of Canada, Vancouver, B.C., June 1979.

[2]Author to whom correspondence may be addressed.

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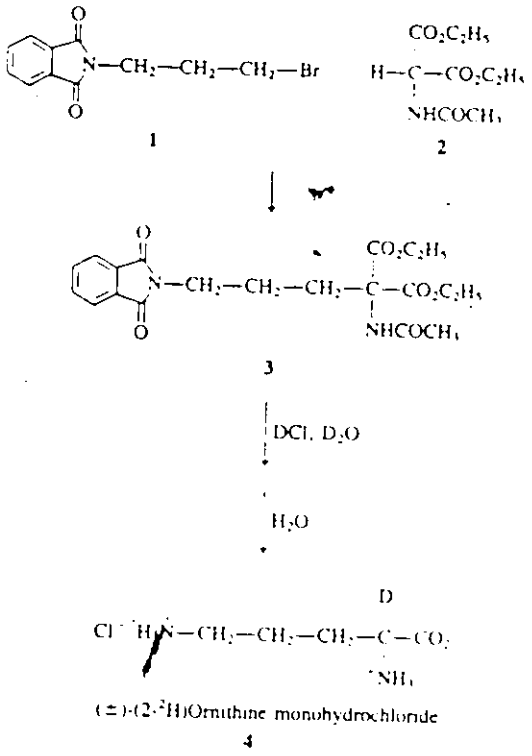


SCHEME 1. Synthesis of (±)-(2-²H)ornithine.

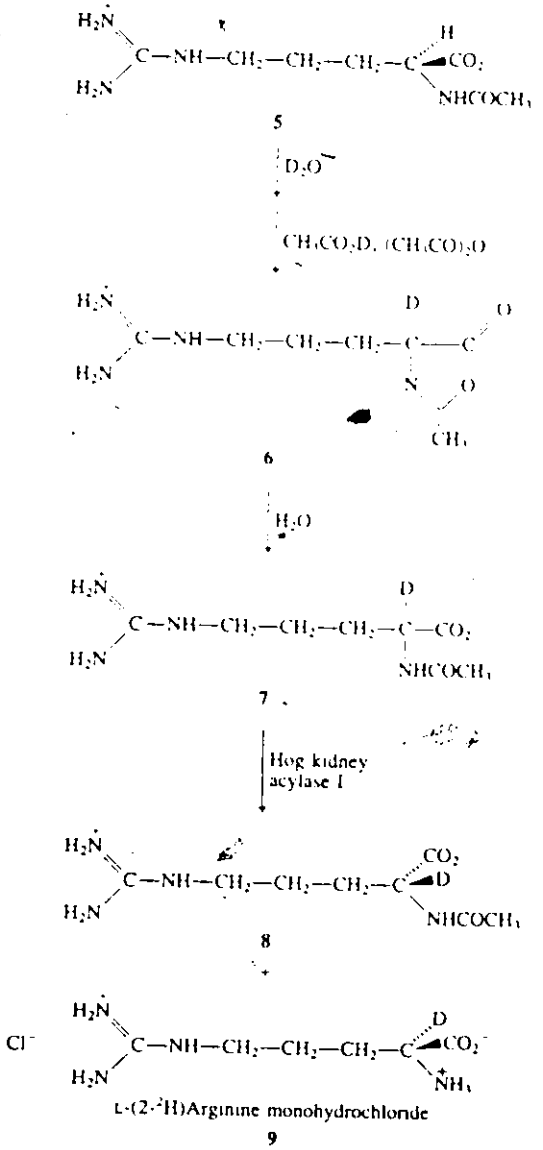
Chemical Co.) gave the enantiomers of (1-²H)-putrescine. L-Ornithine (**14**) in deuterium oxide gave (−)-(1-²H)putrescine dihydrochloride (**15**). L-(2-²H)ornithine in water gave (+)-(1-²H)putrescine dihydrochloride. The ORD curves of the two samples are shown in Fig. 1.

The enantiomers of (1-²H)agmatine were obtained similarly from L-arginine, catalyzed by L-arginine decarboxylase (L-argininine carboxylyase, EC 4.1.1.19) (ex *E. coli*, Sigma Chemical Co.). L-Arginine (**16**) in deuterium oxide gave (−)-(1-²H)agmatine sulfate (**10**). L-(2-²H)arginine in water gave (+)-(1-²H)agmatine sulfate. The ORD curves of the enantiomeric (1-²H)agmatine samples are shown in Fig. 2.

Alkaline hydrolysis with sodium hydroxide in ethanol led to quantitative conversion of agmatine into putrescine. (−)-(1-²H)Agmatine sulfate (**10**) yielded (−)-(1-²H)putrescine dihydrochloride (**15**). (+)-(1-²H)agmatine sulfate yielded (+)-(1-²H)-putrescine dihydrochloride. Within experimental error, the ORD curves of the (1-²H)putrescine samples, so obtained, were coincident with those of the samples obtained by decarboxylation (Fig. 1).

The samples of (1-²H)agmatine were converted in 2 steps (Scheme 3) into 4-phthalimidobutyric acid which was converted into the corresponding methyl ester.

The sample of (−)-(1-²H)agmatine sulfate (**10**) gave (−)-4-phthalimido(4-²H)butyric acid (**12**) which, in turn, gave (−)-methyl 4-phthalimido-(4-²H)butyrate (**13**). Conversely, (+)-(1-²H)agmatine sulfate gave (+)-4-phthalimido(4-²H)butyric acid. The ORD curves of the enantiomeric 4-phthalimido(4-²H)butyric acids are shown in Fig. 3.



SCHEME 2. Synthesis of L-(2-²H)arginine.

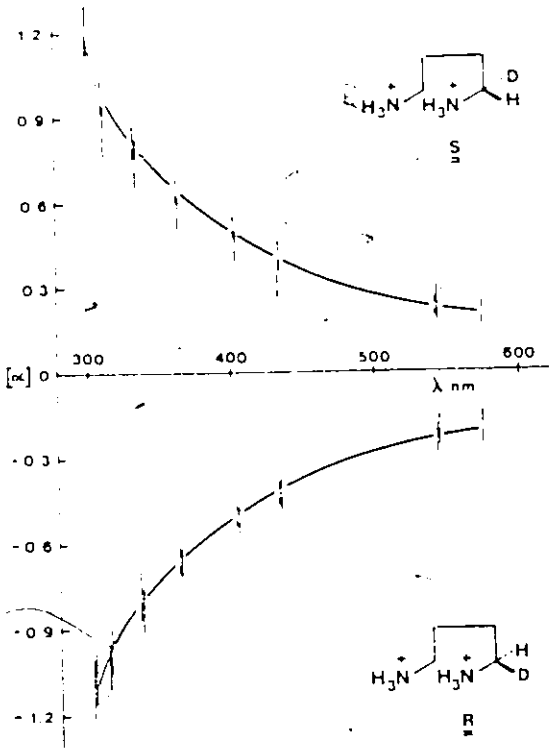


FIG. 1. Optical rotary dispersion (ORD) curves of (+)- and (−)-(1-²H)putrescine dihydrochloride. The samples were prepared by decarboxylation of ornithine (value plotted on the left, for each wave length) and by hydrolysis of agmatine (value on the right).

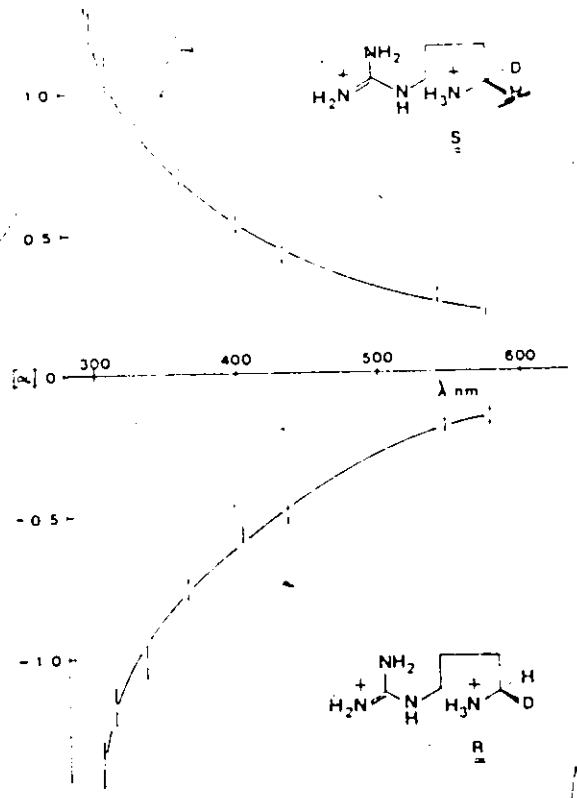


FIG. 2. ORD curves of (+)- and (−)-(1-²H)agmatine sulfate.

An authentic specimen of (−)-methyl *R*-4-phthalimido(4-²H)butyrate was obtained from M. H. O'Leary, University of Wisconsin. This sample had been prepared by methylation of (−)-4-phthalimido(4-²H)butyric acid which had been obtained by decarboxylation in ^2H_2O of L-glutamic acid, catalyzed by L-glutamate decarboxylase (L-glutamate carboxylyase, EC 4.1.1.15, *ex E. coli*), and had been shown to have *R*-chirality by direct correlation with an authentic specimen, prepared from chiral (2-²H)glycine by a series of reactions which did not affect the stereochemistry at the chiral centre (4).

The sample of (−)-methyl 4-phthalimido(4-²H)-butyrate (**13**) derived from (−)-(1-²H)agmatine sulfate (**10**) and the sample of (−)-methyl *R*-4-phthalimido(4-²H)butyrate supplied by M. H. O'Leary showed similar, but not coincident, plane negative ORD curves (Fig. 4), and identical mass spectra (Fig. 5).

Discussion

The stereochemistry of the replacement of the carboxyl group of an α-amino acid by a solvent proton, in the course of the reaction catalyzed by an L-α-amino acid decarboxylase (1, 15), can be determined if the prochiral centre that is generated in the course of the decarboxylation is rendered chiral by isotopic substitution. If the decarboxylation of the amino acid is carried out in the presence of isotopically labelled water (^3HOH or ^2H_2O), one of the enantiomers of the amine will be generated. The other enantiomer arises when a 2-labelled sample ([2-³H]- or (2-²H)-) of the L-α-amino acid undergoes decarboxylation in aqueous solution (3, 5–7, 13, 16, 17).

When tritium is employed as the tracer (3, 5–8, 10, 11, 13, 16) a very small fraction only of the molecules that comprise the labelled starting material ([2-³H]amino acid or ^3HOH) will carry ³H. It follows that only very few of the molecules comprising the product will be labelled, i.e., will be chiral and, in principle, optically active. The optical activity of the resulting product will thus be immeasurably small. Stereochemical analysis of the product is possible by means of enzymes of known stereospecificity.

When deuterium is employed as the marker (4, 9,

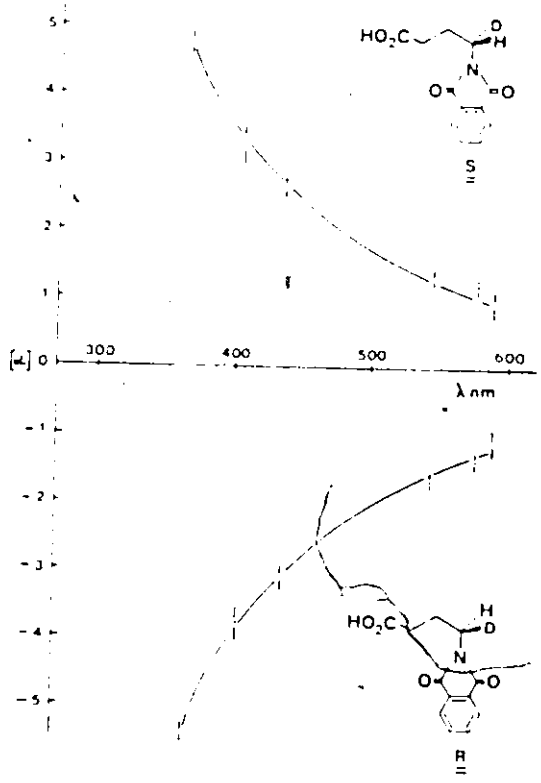


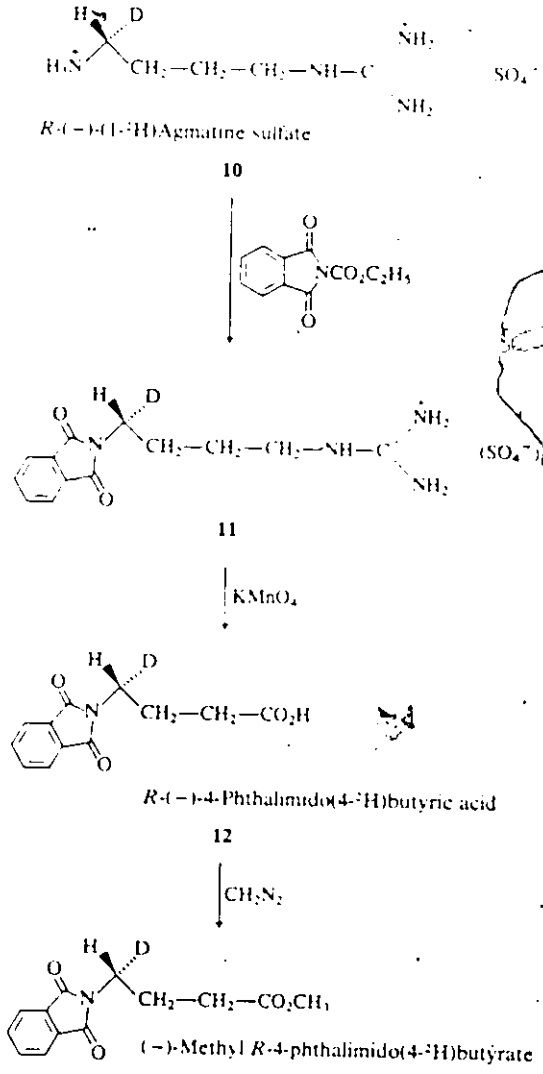
FIG. 3. ORD curves of (+)- and (−)-4-phthalimido(4-^{2}H)butyric acid.

12, 14, 17) starting material can, in principle, be fully deuterated (^2H_2O), or fully deuterated at the appropriate position ((2-^{2}H)amino acid). The product will then consist entirely of molecules that are correspondingly deuterated, and is therefore not only chiral but its optical activity will, in all likelihood, be measurable (subject to the sensitivity of the detection instruments). The chirality of the product can then be determined by the classical method of measuring optical activity followed by assignment of configuration by comparison of this activity with that of standards of known configuration (4, 12, 14). Alternatively, nmr methods can be applied (9, 17) in the configurational assignment.

We have determined the stereochemistry of the enzymic decarboxylations of ornithine and arginine by deuterium labelling, followed by adaptation of the classical approach to configurational assignment, measurement of optical activity, and comparison with standards of known stereochemistry.

Samples of the two enantiomeric (1-^{2}H)putrescines were isolated as dihydrochlorides. They were obtained, respectively, by the action of L-ornithine decarboxylase (from *E. coli*) on L-ornithine (**14**) in the presence of ^2H_2O (ca. 98 at.% ^{2}H), and on the L-component of DL-(2-^{2}H)ornithine. The DL-(2-^{2}H)ornithine (**4**) was prepared by adaptation of a conventional synthetic route (18) (Scheme 1). The ORD curves of the enantiomeric (1-^{2}H)putrescines are shown in Fig. 1. The sample derived from L-(2-^{2}H)ornithine was (+)-(1-^{2}H)putrescine dihydrochloride, that derived from L-ornithine (**14**) in ^2H_2O (Scheme 4) was (−)-(1-^{2}H)putrescine dihydrochloride (**15**).

Similarly, samples of the two enantiomeric (1-^{2}H)agmatines, isolated as sulfates, were obtained by action of L-arginine decarboxylase (from *E. coli*) on L-arginine in the presence of ^2H_2O, and on L-(2-^{2}H)arginine, respectively. The L-(2-^{2}H)arginine (**9**) was prepared from α-*N*-acetyl-L-arginine



SCHEME 3. Conversion of (1-^{2}H)agmatine into methyl 4-phthalimido(4-^{2}H)butyrate.

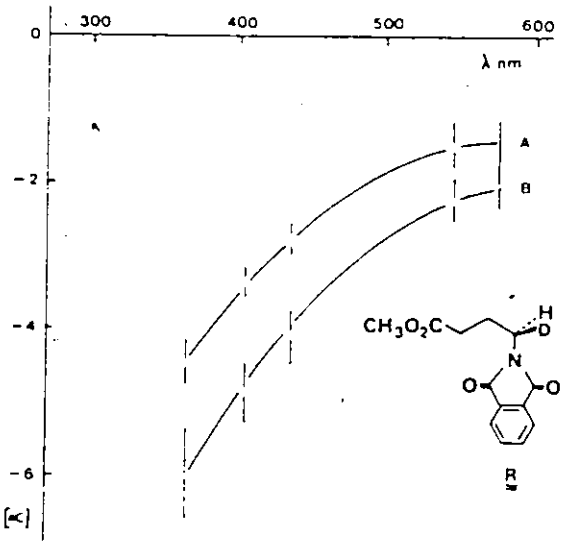


FIG. 4. ORD curves of (−)-methyl 4-phthalimido(4-^{2}H)-butyrate (13). Curve A: Sample supplied by M. H. O'Leary. Curve B: Sample obtained from (−)-(1-^{2}H)agmatine sulfate (10) (Scheme 3).

(5) by exchange (via the oxazolone (6)) and stereospecific hydrolysis employing acylase I (19) (Scheme 2). The sample of agmatine sulfate derived from L-(2-^{2}H)arginine was (+)-(1-^{2}H)agmatine sulfate, that derived from L-arginine (16) in ^2H_2O (Scheme 4) was (−)-(1-^{2}H)agmatine sulfate (10).

It was a simple matter to establish the relative configuration of the samples of (1-^{2}H)putrescine and (1-^{2}H)agmatine. Hydrolysis of (−)-(1-^{2}H)-agmatine sulfate (10) with ethanolic sodium hydroxide (10% w/v),[3] which maintained the chiral integrity at C-1 but cleaved the guanidino group, gave (−)-(1-^{2}H)putrescine dihydrochloride (15) (Scheme 4). Similarly, (+)-(1-^{2}H)agmatine sulfate gave (+)-(1-^{2}H)putrescine dihydrochloride.

It follows that the levorotary samples of (1-^{2}H)-putrescine dihydrochloride and of (1-^{2}H)agmatine sulfate have the same configuration at C-1. Since these two samples were derived from L-ornithine, and from L-arginine, respectively, by decarboxylation in ^2H_2O, it follows further that the steric course of the decarboxylation of L-ornithine, catalyzed by L-ornithine decarboxylase, is identical with that of the decarboxylation of L-arginine, catalyzed by L-arginine decarboxylase.

[3]Hydrolysis with barium hydroxide solution (20, 21) or with aqueous sodium hydroxide (22), conditions which have been employed to convert L-arginine into L-ornithine (20, 21) and DL-ornithine (22), respectively, did not lead to quantitative conversion of chiral deuterio-agmatine into chiral deuterio-putrescine.

Congruently, the dextrorotary samples of (1-^{2}H)-putrescine dihydrochloride and of (1-^{2}H)agmatine sulfate correspond in configuration at C-1. Each was derived from the corresponding L-(2-^{2}H)amino acid. Thus, the decarboxylations catalyzed by L-ornithine decarboxylase and by L-arginine decarboxylase take the same stereochemical course. Either both reactions proceed with net retention of configuration or they both proceed with net inversion.

This question was settled by a chemical conversion of (−)-(1-^{2}H)agmatine into a product of known chirality by a reaction sequence which did not affect the chiral centre at C-1 (Scheme 3).

(1-^{2}H)Agmatine rather than (1-^{2}H)putrescine was chosen for chemical correlation with a known standard in order to avoid possible complications due to the C_{2v} symmetry of putrescine.

(1-^{2}H)Agmatine was converted into the 1-*N*-phthaloyl derivative (11). The primary amino group was thus afforded protection from oxidative attack. The guanidino group was then removed by oxidation with potassium permanganate, yielding 4-phthalimido(4-^{2}H)butyric acid (12) whose stereochemical integrity had been maintained. By this reaction sequence (−)-(1-^{2}H)agmatine sulphate (10) yielded (−)-4-phthalimido(4-^{2}H)butyric acid (12), and (+)-(1-^{2}H)agmatine sulfate gave (+)-4-phthalimido(4-^{2}H)butyric acid.

A sample of (−)-4-phthalimido(4-^{2}H)butyric acid (12) was converted into the corresponding methyl ester, (−)-methyl 4-phthalimido(4-^{2}H)butyrate (13) (isotopic enrichment (ms) 98.2 ± 0.3 at.% $^2H_1$). This derivative was compared directly with an authentic specimen of (−)-methyl 4-phthalimido-*R*-(4-^{2}H)butyrate (isotopic enrichment 92.5 ± 0.4 at.% $^2H_1$, 4.7 ± 0.4% $^2H_2$), which had been prepared in the laboratory of M. H. O'Leary, University of Wisconsin (4). The absolute configuration of this sample ultimately rests on chemical correlation with that of *R*-(2-^{2}H)glycine (17) (4).

The two samples of (−)-methyl 4-phthalimido-(4-^{2}H)butyrate (13), that from (−)-(1-^{2}H)agmatine sulfate (10) and that supplied by O'Leary, both showed plane negative ORD curves (Fig. 4). They gave identical mass spectra (Fig. 5).

Since (−)-methyl 4-phthalimido(4-^{2}H)butyrate has the *R*-configuration, the (−)-(1-^{2}H)agmatine sulfate from which it was obtained, by a reaction sequence which did not affect the chiral centre, also has the *R*-configuration (see Scheme 3, no change in ligand priorities around the chiral centre).

Since this *R*-(−)-(1-^{2}H)agmatine sulfate had been obtained from L-arginine by decarboxylation in ^2H_2O, catalyzed by L-arginine decarboxylase

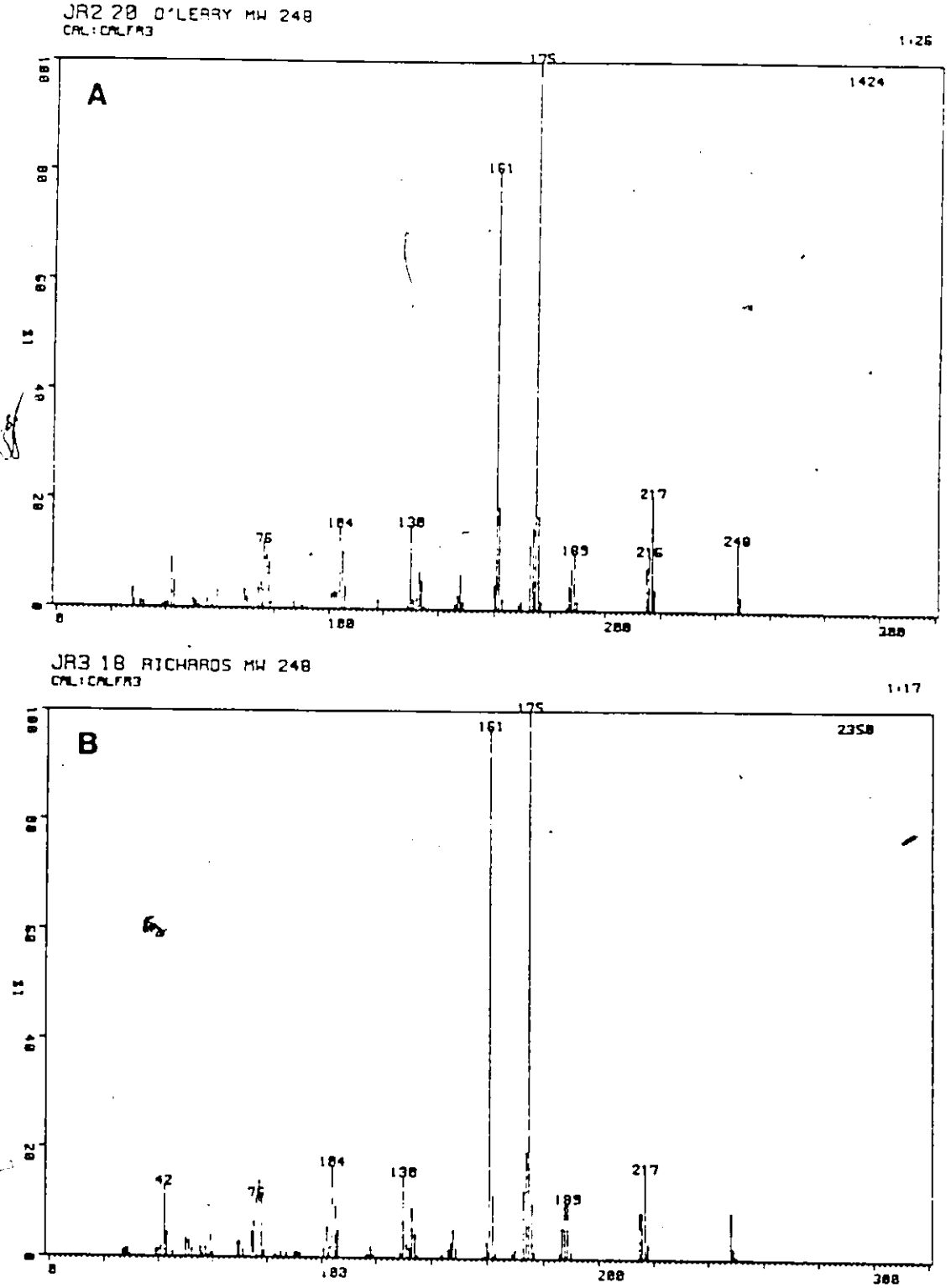
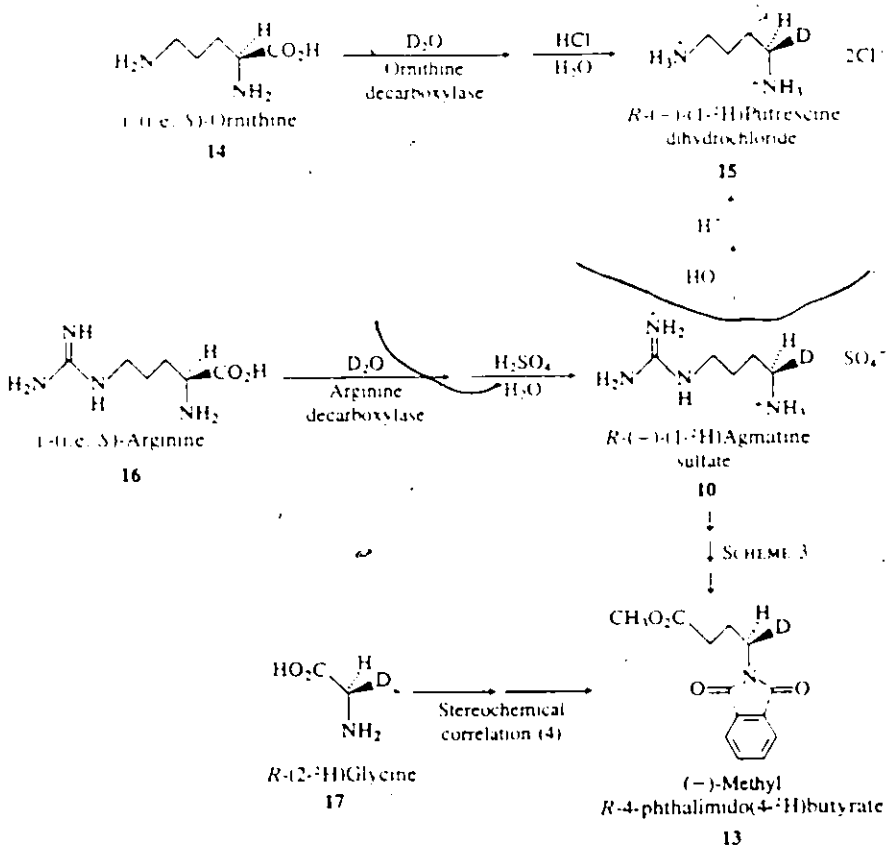


FIG. 5. Mass spectra of (−)-methyl 4-phthalimido(4-^{2}H)butyrate (13). Spectrum A: Sample supplied by M. H. O'Leary. Spectrum B: Sample obtained from (−)-(1-^{2}H)agmatine sulfate (10) (Scheme 3).



L-(1-^{2}H)Ornithine
14

R-(−)-(1-^{2}H)Putrescine dihydrochloride
15

L-(1-^{2}H)Arginine
16

R-(−)-(1-^{2}H)Agmatine sulfate
10

R-(2-^{2}H)Glycine
17

(−)-Methyl *R*-4-phthalimido(4-^{2}H)butyrate
13

SCHEME 4. Stereochemical interrelationship of L-ornithine, L-arginine, and their conversion and degradation products

(EC 4.1.1.19, from *E. coli*) (Scheme 4), the reaction catalyzed by this enzyme proceeds with net retention of configuration.

Since *R*-(−)-(1-^{2}H)agmatine sulfate yielded (−)-(1-^{2}H)putrescine dihydrochloride in a reaction which does not affect the chiral centre, this compound is *R*-(−)-(1-^{2}H)putrescine dihydrochloride.

Since the *R*-(−)-(1-^{2}H)putrescine dihydrochloride had been obtained from L-ornithine by decarboxylation in ^2H_2O, catalyzed by L-ornithine decarboxylase (EC 4.1.1.17, from *E. coli*), the reaction catalyzed by this enzyme proceeds with net retention of configuration.

These configurational relationships are summarized in Scheme 4.

Experimental

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

1. DL-*(2-^{2}H)Ornithine (18)*

Diethyl 2-acetamido-2-(3-phthalimidopropyl)malonate (3) (23)

Diethyl acetamidomalonate (2) (Aldrich Chemical Co.) (2.16 g) was dissolved in hot dry ethanol[4] in a two-necked round bottom flask, fitted with a reflux condenser, calcium sulfate drying tube, and a pressure equalizing dropping funnel. A solution of sodium ethoxide (250 mg of sodium in 14 mL ethanol) was added and the resulting solution was heated at reflux while *N*-(3-bromopropyl)phthalimide (1) (Aldrich Chemical Co.) (2.69 g) in hot ethanol (14 mL) was added dropwise over 10 min. The reaction mixture was refluxed 20 h, and was then cooled to 0°C. Water (35 mL) was added to give the substitution product, Yield 2.85 g, 71%; mp 116–116.5°C (from 95% ethanol) (lit. (20) mp 115–116°C; (25) 111–112°C); ^{1}H nmr (^{2}HCCl$_3$), δ: 1.20 (6H, t, *J* 7.2 Hz), 1.53 (2H, m), 2.00 (3H, s), 2.40 (2H, m), 3.67 (2H, t, *J* 7.2 Hz), 4.23 (4H, q, *J* 7.2 Hz), 6.80 (1H, s), 7.77 (4H, m).

DL-*(2-^{2}H)Ornithine monohydrochloride (4)*

Diethyl 2-acetamido-2-(3-phthalimidopropyl)malonate (3) (784 mg) was suspended in a solution of deuterium oxide containing deuterium chloride (38%, w/w in ^2H_2O, 10 mL) (Merck, Sharp, and Dohme, 99.7 at.% ^{2}H) in a small flask fitted with a reflux condenser and calcium sulfate drying tube. The mixture was heated at reflux for 18 h, cooled to 0°C, 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 deuterium. The residue, crude ornithine dihydrochloride, was dissolved in hot 95% ethanol (8 mL), the solution was cooled to room temperature, filtered, and pyridine (ca. 600 mg) was added. DL-(2-^{2}H)-Ornithine monohydrochloride (398 mg) which precipitated was recrystallized from aqueous ethanol, mp 235–237°C (dec.) (lit. (nondeuteriated) (22) mp 232°C (dec.); (25) 222–224°C (dec.);

[4]Absolute ethanol purified by the Lund–Bjerrum method (24) was used throughout.

(216 °C(dec.)). The ^{1}H nmr spectrum (^2H_2O) was similar to that of authentic unlabelled material and indicated that this sample was ca. 98% deuterium labelled at C-2, δ 1.90 (4H, m, H-3, 4), 3.05 (2H, t, *J* 6.8 Hz, H-5), 3.80 (ca. 0.02 H, H-2). The ^{2}H nmr spectrum (H_2O, 107 MHz, 200 transients) showed one signal at 3.7 ppm (relative to ^2HCO at 4.7 ppm).

In another experiment, hydrolysis of 3 (900 mg) gave a sample of DL-(2-^{2}H)ornithine monohydrochloride (4) (330 mg) which was ca. 92% ^{2}H-labelled in the 2-position as determined by ^{1}H nmr.

2. L-(2-^{2}H)Arginine

α-N-Acetyl-L-arginine dihydrate (5)

L-Arginine monohydrochloride (4.24 g) was acetylated with acetic anhydride in aqueous sodium bicarbonate by a published procedure (26), yielding α-acetyl-L-arginine dihydrate (4.10 g, 81%), mp 267–268 °C (from water) (lit. (27) (DL) mp 256 °C); ^{1}H nmr (^2H_2O), δ 1.73 (4H, m, H-3, 4), 2.05 (3H, s, $CO(^2H_3)$), 3.23 (2H, t, *J* 6.8 Hz, H-5), 4.20 (1H, d of d, *J* ~ 7.8 Hz, *J* ~ 4.8 Hz, H-2). ORD, $[\alpha]_{546}$ +10.1 ± 0.3°, $[\alpha]_{500}$ +12.3 ± 0.3°, $[\alpha]_{502}$ +14.1 ± 0.3°, $[\alpha]_{436}$ +15.8 ± 0.3°, $[\alpha]_{414}$ +16.6 ± 0.3°, $[\alpha]_{365}$ +16.0 ± 0.3°, $[\alpha]_{365}$ +13.6 ± 0.3°, $[\alpha]_{436}$ +11.9 ± 0.3°, $[\alpha]_{546}$ +7.6 ± 0.2°, $[\alpha]_{589}$ +6.7 ± 0.1°, $[\alpha]_{589}$ +6.3 ± 0.2° (*c* 2% in 1 N HCl, 25 °C) (lit. (26) $[\alpha]_{589}$ +7.0° (*c* 2% in 1 N HCl)).

α-N-Acetyl-DL-(2-^{2}H)arginine dihydrate (7) (cf. ref. 28)

α-N-Acetyl-L-arginine dihydrate (3.9 g) was dissolved in deuterium oxide (5 mL) and the solvent was evaporated *in vacuo*. This process was repeated three times to effect replacement of the exchangeable protons by deuterium. The residue was then recrystallized from deuterium oxide (3 mL) and deuterium oxide of crystallization was removed by drying over phosphorus pentoxide at 75 °C for 24 h. The product so obtained, α-acetylarginine, in which all exchangeable protons had been replaced by ^{2}H, mp 253–255 °C (lit. (nondeuterated) (28) mp 250 °C), was dissolved in deuterioacetic acid (25 mL) which had been prepared in the following way (cf. ref. 29).

A mixture of freshly distilled acetic anhydride (Fisher) (bp 137–138 °C, 40 mL) and deuterium oxide (Merck, Sharp and Dohme, 99.7 at.% ^{2}H) (8 g) was stirred at room temperature under a stream of dry nitrogen until the solution was homogeneous (ca. 3 h). Distillation under anhydrous conditions gave deuterioacetic acid, $CH_3CO_2^2H$, bp 112–114 °C. The ^{1}H nmr spectrum of a sample of the product indicated that it was ≥ 98% deuterium labelled and contained a trace of acetic anhydride (ca. 2%).

Acetic anhydride (bp 137–138 °C, 1.7 mL) was added to the solution of α-N-acetylarginine in deuterioacetic acid, and the mixture was heated at reflux under dry nitrogen. After 2 h deuterium oxide (0.4 mL) was added and heating at reflux was continued for 30 min. The solution was cooled to room temperature, acetic acid removed *in vacuo*, and the residue, a yellow oil, was dissolved in water (5 mL). The solution was evaporated, the residue redissolved in water, and the solution again evaporated. The oil was then redissolved in water (5 mL), the pH of the solution was adjusted to pH 8 with concentrated ammonium hydroxide, acetone (1 mL) was added, and the mixture cooled to 0 °C. The crystals which formed were filtered off, washed with cold water, and dried *in vacuo* over phosphorus pentoxide. The sample of α-N-acetyl-DL-(2-^{2}H)arginine dihydrate (3.4 g) so obtained (optically inactive, mp 268–270 °C (from water)), was 85–90% deuterium labelled at C-2 (^{1}H nmr). Deuterium was also present in the acetyl group.

L-(2-^{2}H)Arginine (9) monohydrochloride and α-acetyl-D-(2-^{2}H)arginine (8) dihydrate (cf. refs. 19, 26)

The pH of a solution of α-N-acetyl-DL-(2-^{2}H)arginine dihydrate (3.2 g) in water (140 mL) was adjusted to pH 6.8 with hydrochloric acid (10% w/v). Hog kidney acylase I (Sigma, 6300 U/mg,[1] 8.6 mg) in water (20 mL) was added. The resulting solution was incubated at 36 °C in a constant temperature water bath for 23 h. The pH of the enzyme reaction mixture was then adjusted to pH 4.8 with glacial acetic acid. Norite was added, and the mixture filtered through Celite. The clear filtrate was concentrated *in vacuo* (~ 7 mL), and after cooling in a refrigerator for several days, α-N-acetyl-D-(2-^{2}H)arginine (8) dihydrate, which crystallized, was filtered off and washed with water. Yield 1.4 g, 89%; mp 268–269 °C (from water); ORD, $[\alpha]_{546}$ +14.0 ± 0.3°, $[\alpha]_{500}$ +16.9 ± 0.4°, $[\alpha]_{502}$ +18.0 ± 0.4°, $[\alpha]_{412}$ +19.4 ± 0.4°, $[\alpha]_{414}$ +19.6 ± 0.4°, $[\alpha]_{365}$ +17.9 ± 0.4°, $[\alpha]_{365}$ +15.3 ± 0.3°, $[\alpha]_{436}$ +13.2 ± 0.3°, $[\alpha]_{436}$ +8.3 ± 0.2°, $[\alpha]_{546}$ +7.2 ± 0.2°, $[\alpha]_{589}$ +6.8 ± 0.2° (*c* 2.06% in 1 N HCl, 25 °C) (lit. (26) (nondeuterated) $[\alpha]_{589}$ +7.0° (*c* 2% in 1 N HCl)).

The combined filtrate and washings containing L-(2-^{2}H)arginine (9) (10 mL) were diluted with water (25 mL), sodium hydroxide solution (5% w/v) was added to pH 11, and the solution was cooled in an ice bath. Benzaldehyde (1.3 mL) was added and the mixture stirred at 0 °C for 2 h. The benzylidine derivative which separated was filtered off and was washed with ethanol and ether. Yield, 1.54 g, 93%; mp 198–199 °C (dec.) (lit. (31) mp 206–207 °C (dec.)).

The solution of the benzylidine derivative (1.53 g) in aqueous hydrochloric acid (2 N, 12 mL) was heated (95 °C) 20 min with stirring. After cooling to room temperature, the mixture was extracted with ether (2 × 5 mL) and the ether layers were discarded. The aqueous phase, containing the dihydrochloride of L-(2-^{2}H)arginine, was evaporated *in vacuo*. The residue was dissolved in hot 95% ethanol (5 mL), the solution cooled to room temperature, and filtered. L-(2-^{2}H)Arginine monohydrochloride was precipitated from the filtrate by addition of pyridine (1.2 mL), and was recrystallized from aqueous ethanol. Yield 1.1 g, 91% from the benzylidine derivative; mp 222–223 °C (lit. (nondeuterated) (31) mp 220 °C); ^{1}H nmr (^2H_2O), δ: 1.74 (4H, m, H-3, 4), 3.17 (2H, t, *J* 6.8 Hz, H-5), 3.72 (ca. 0.15 H, H-2, i.e., ca. 85% of ^{2}H); ORD, $[\alpha]_{546}$ +94.5 ± 1.9°, $[\alpha]_{502}$ +87.7 ± 1.9°, $[\alpha]_{412}$ +75.6 ± 1.5°, $[\alpha]_{414}$ +58.6 ± 1.2°, $[\alpha]_{365}$ +43.3 ± 0.9°, $[\alpha]_{365}$ +31.4 ± 0.6°, $[\alpha]_{436}$ +25.3 ± 0.5°, $[\alpha]_{436}$ +14.1 ± 0.3°, $[\alpha]_{546}$ +12.4 ± 0.3°, $[\alpha]_{589}$ +11.7 ± 0.2° (*c* 2.06% in water, 25 °C). An authentic sample of unlabelled L-arginine monohydrochloride showed a plane positive ORD spectrum: $[\alpha]_{546}$ +94.0 ± 1.9°, $[\alpha]_{502}$ +87.2 ± 1.7°, $[\alpha]_{412}$ +75.4 ± 1.5°, $[\alpha]_{414}$ +58.6 ± 1.2°, $[\alpha]_{365}$ +43.3 ± 0.9°, $[\alpha]_{365}$ +31.5 ±0.6°, $[\alpha]_{436}$ +25.4 ± 0.5°, $[\alpha]_{436}$ +14.0 ± 0.3°, $[\alpha]_{546}$ +12.4 ± 0.3°, $[\alpha]_{589}$ +11.7° ± 0.2° (*c* 2.07% in water, 25 °C) (lit. (28) $[\alpha]_{589}$ +11.30° (*c* 5.0% in water, 25 °C), (31) $[\alpha]_{589}$ +12.2°, +12.3° (*c* 5.0% in water, 25 °C)).

A sample of L-(2-^{2}H)arginine monohydrochloride (67 mg) which was ≥ 92% ^{2}H-labelled (by ^{1}H nmr) was obtained from α-N-acetyl-L-arginine under a similar set of reaction conditions (see above) except that the ^{2}H for ^{1}H exchange reaction was optimized by following the course of the reaction in perdeuterioacetic acid – acetic anhydride solution by ^{1}H nmr.

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

1. *Ornithine*

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

(i) (−)-(1-^{2}H)Putrescine dihydrochloride (15) by decarboxylation of the L-component of unlabelled DL-ornithine in deuterium oxide solution

Anhydrous sodium carbonate (Analar) (111 mg) was added to a stirred solution of perdeuterioacetic acid (Merck, Sharp, and Dohme, 99.7 at.% ^{2}H) in deuterium oxide (Stohler Isotopes, 99.8 at.% ^{2}H) (0.2 M, 15 mL) under an atmosphere of dry

[1] 1 U will hydrolyse 1 μmol of *N*-acetyl-L-methionine/h at pH 7.0 at 25 °C. Hydrolysis of α-*N*-acetyl-L-arginine is ca. 60 times slower (30).

nitrogen. After stirring for 1 h, DL-ornithine monohydrochloride (Aldrich) (98 mg), which had been stripped of exchangeable protons by repeated solution 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 solution (ca. p-H 5). The enzyme reaction mixture, in a flask fitted with a calcium sulfate drying tube, was agitated at 36°C for 40 h. The enzyme reaction mixture was then acidified with 4 *N* hydrochloric acid, heated on a steam bath for 30 min, and cooled to room temperature. The precipitated enzyme was removed by centrifugation, followed by filtration of the supernatant solution through Celite. The filtrate was lyophilyzed, the residue dissolved in 10% sodium hydroxide (3 mL), and the alkaline solution saturated with sodium chloride. The product was extracted into 1-butanol (4 × 5 mL), the butanol extract was acidified by addition of a solution of hydrogen chloride in butanol, and the solution was then concentrated *in vacuo* (5 mL). (+)-(1-^{2}H)Putrescine dihydrochloride (46 mg) was recrystallized from 95% ethanol; ^{1}H nmr (^2H_2O), δ: 1.77 (4H, m, H-2,3), 3.06 (ca. 3H, m, H-1,4); CI ms 90 (M + H, 5%), 74 (5), 73 (M − NH_3, 100), 72 (2). (CI ms of authentic unlabelled material, 89 (M + H, 5%), 73 (5), 72 (M − NH_3, 100), 71 (2)); 99.7 ± 0.3% ^{2}H, determined by CI 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 CI ms, and gave ORD, $[\alpha]_{592}$ +1.42 ± 0.11°, $[\alpha]_{578}$ +1.03 ± 0.08°, $[\alpha]_{546}$ +0.78 ± 0.09°, $[\alpha]_{436}$ +0.66 ± 0.05°, $[\alpha]_{405}$ +0.50 ± 0.04°, $[\alpha]_{436}$ +0.43 ± 0.04°, $[\alpha]_{446}$ +0.24 ± 0.05°, $[\alpha]_{577}$ +0.20 ± 0.06° (*c* 2.26% in 0.1 *N* HCl, 26°C, uncorrected for incomplete deuteration).

(ii) (+)-(1-^{2}H)Putrescine dihydrochloride by decarboxylation of the L-component of DL-(2-^{2}H)ornithine

A solution of DL-(2-^{2}H)ornithine monohydrochloride (~ 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°C for 20 h. After work-up, (+)-(1-^{2}H)putrescine dihydrochloride was obtained and recrystallized from 95% ethanol (yield 45 mg). The ^{1}H nmr spectrum (^2H_2O) was identical with that of (+)-(1-^{2}H)-putrescine and the sample showed a plane positive ORD curve: CI ms, 90 (M + H, 3%), 74 (5), 73 (M − NH_3, 100), 72 (5); 97.2 ± 0.3% ^{2}H, determined by CI ms.

Another sample of (+)-(1-^{2}H)putrescine dihydrochloride obtained by enzyme decarboxylation of DL-(2-^{2}H)ornithine (ca. 92% ^{2}H) was 93.1 ± 0.9% mono-^{2}H labelled at C-1 (determined by CI ms); ORD, $[\alpha]_{592}$ +1.21 ± 0.09°, $[\alpha]_{578}$ +0.98 ± 0.06°, $[\alpha]_{546}$ +0.81 ± 0.06°, $[\alpha]_{436}$ +0.64 ± 0.04°, $[\alpha]_{405}$ +0.51 ± 0.04°, $[\alpha]_{436}$ +0.30 ± 0.03°, $[\alpha]_{446}$ +0.23 ± 0.03°, $[\alpha]_{577}$ +0.20 ± 0.04° (*c* 2.86% in 0.1 *N* HCl, 26°C, uncorrected for incomplete deuteration).

Unreacted D-(2-^{2}H)ornithine was reisolated from the incubation mixture. The alkaline solution remaining after extraction of the (1-^{2}H)putrescine into butanol was neutralized with concentrated hydrochloric acid, and was then applied to a column (25 × 1 cm) of Dowex 50-X8 (H^+ form). The column was washed with water (25 mL), hydrochloric acid (2 *N*, 25 mL), and water (25 mL). D-(2-^{2}H)ornithine was eluted with ammonia (1 *M*, 25 mL), and was isolated as the monohydrochloride, as described earlier.

The ^{1}H and ^{2}H nmr spectra of the sample of D-(2-^{2}H)ornithine monohydrochloride (ca. 92% ^{2}H) were identical with those of the DL-(2-^{2}H)ornithine from which it was derived. ORD, $[\alpha]_{365}$ −157.8 ± 7.9°, $[\alpha]_{350}$ −114.8 ± 5.7°, $[\alpha]_{330}$ −98.0 ± 4.9°, $[\alpha]_{298}$ −87.3 ± 4.4°, $[\alpha]_{592}$ −80.7 ± 4.0°, $[\alpha]_{578}$ −69.2 ± 3.5°, $[\alpha]_{546}$ −52.8 ± 2.6°, $[\alpha]_{436}$ −39.3 ± 2.0°, $[\alpha]_{405}$ −29.0 ± 1.5°, $[\alpha]_{436}$ −23.4 ± 1.2°, $[\alpha]_{446}$ −14.4 ± 0.8°, $[\alpha]_{577}$ −12.9 ± 0.7°, $[\alpha]_{644}$ −12.1 ± 0.8° (*c* 1.03% in water, 25°C).

An authentic sample of unlabelled L-ornithine (10) monohydrochloride gave the following ORD: $[\alpha]_{365}$ +166.3 ± 3.3°, $[\alpha]_{350}$ +124.9 ± 2.5°, $[\alpha]_{330}$ +107.6 ± 2.2°, $[\alpha]_{298}$ +96.1 ± 1.9°, $[\alpha]_{592}$ +88.9 ± 1.8°, $[\alpha]_{578}$ +78.1 ± 1.6°, $[\alpha]_{546}$ +61.7 ± 1.2°, $[\alpha]_{436}$ +45.1 ± 0.9°, $[\alpha]_{405}$ +32.3 ± 0.7°, $[\alpha]_{436}$ +26.1 ± 0.5°, $[\alpha]_{446}$ +17.0 ± 0.4°, $[\alpha]_{577}$ +11.5 ± 0.3°, $[\alpha]_{644}$ +11.8 ± 0.5° (*c* 0.49% in water, 25°C); (lit. (32) $[\alpha]_{589}$ +11.0° (*c* 5.5% in water, 23°C)).

2. *Arginine*

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

(i) (−)-(1-^{2}H)Agmatine sulfate (10) by decarboxylation of L-arginine (16) in deuterium oxide solution

L-Arginine monohydrochloride (Eastman) (90 mg) was twice dissolved in deuterium oxide and evaporated to dryness to effect replacement of exchangeable protons with deuterium. The amino acid was then dissolved in perdeuterioacetate–^2H_2O buffer (ca. p-H 5.2, 20 mL) to yield a 0.02 *M* solution. The buffer had been prepared from perdeuterioacetic acid (0.2 *M* in ^2H_2O) and anhydrous sodium carbonate (8.36 mg/mL) (see under Decarboxylation of ornithine). L-Arginine decarboxylase (Sigma; 0.7 U/mg* (43 mg) was added and the resulting solution incubated at 36°C in a flask equipped with a calcium sulfate drying tube. After 30 h the incubation mixture was acidified (ca. pH 2) with concentrated 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 sodium hydroxide and then dissolved in 5% sodium hydroxide solution (1–2 mL). The alkaline solution was saturated with sodium chloride and extracted with 1-butanol (4 × 5 mL). Evaporation of the butanol gave an oil which was redissolved in aqueous sulfuric acid (0.1 *M*, ca. 1 mL). After addition of methanol and cooling at 0°C overnight the crystalline sulfate of (−)-(1-^{2}H)agmatine separated and was recrystallized from water–methanol. Yield 22 mg; mp 238–240°C (lit. (nondeuterated) (33) mp 236–239°C); ^{1}H nmr (^2H_2O, relative to ^{1}H-HO at 4.63 ppm), δ: 1.66 (4H, m, H-2,3), 2.95 (ca. 1H, t (br), J ~ 6.8 Hz, H-1), 3.18 (2H, m, H-4); ORD, $[\alpha]_{592}$ −1.37 ± 0.08°, $[\alpha]_{578}$ −1.17 ± 0.07°, $[\alpha]_{546}$ −1.01 ± 0.06°, $[\alpha]_{436}$ −0.76 ± 0.04°, $[\alpha]_{405}$ −0.57 ± 0.03°, $[\alpha]_{436}$ −0.50 ± 0.03°, $[\alpha]_{446}$ −0.19 ± 0.02°, $[\alpha]_{577}$ −0.16 ± 0.03° (*c*, 6.52% in water, 25°C, uncorrected for incomplete deuteration); ^{2}H nmr (H_2O, 180 *mM*, 100 transients) showed one signal at 3.0 ppm apart from that due to natural abundance ^{2}H in water (4.7 ppm).

(ii) (+)-(1-^{2}H)Agmatine sulfate by decarboxylation of L-(2-^{2}H)arginine

A solution of L-(2-^{2}H)arginine monohydrochloride (ca. 92% ^{2}H) (0.02 *M*) and L-arginine decarboxylase (0.7 U/mg)* (75 mg) in acetate buffer (0.2 *M*, pH 5.2, 35 mL) was incubated at 36°C for 40 h. The reaction mixture was worked up, as described under (i), to give (+)-(1-^{2}H)agmatine sulfate (100 mg). The mp and nmr spectra (^{1}H and ^{2}H) were similar to those obtained for (−)-(1-^{2}H)agmatine sulfate. The sample showed a plane positive ORD curve: $[\alpha]_{597}$ +1.42 ± 0.11°, $[\alpha]_{592}$ +1.21 ± 0.08°, $[\alpha]_{578}$ +1.07 ± 0.07°, $[\alpha]_{546}$ +0.84 ± 0.06°, $[\alpha]_{436}$ +0.70 ± 0.04°, $[\alpha]_{405}$ +0.53 ± 0.03°, $[\alpha]_{436}$ +0.42 ± 0.03°, $[\alpha]_{446}$ +0.27 ± 0.03°, $[\alpha]_{577}$ +0.23 ± 0.04° (*c*, 4.18% in water, 25°C, uncorrected for incomplete deuteration).

C. *Conversion of agmatine into putrescine*

1. *Hydrolysis of agmatine to putrescine*

A solution of agmatine sulfate (55 mg) in ethanol–water

*1 U will release 1.0 μmol carbon dioxide from L-ornithine per min at pH 5.0 at 37°C.

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

(70:30, v/v, 3 mL) containing sodium hydroxide (10% w/v) was heated at reflux under a nitrogen atmosphere. When the reaction was complete (ca. 20 h) as revealed by paper chromatography (Whatman 3 MM, 1-butanol – acetic acid – pyridine – water 4:1:1:2; visualized with ninhydrin (0.3% w/v, in 1-butanol – acetic acid, 97:3, v/v); agmatine, R_f 0.36, putrescine, R_f 0.27), the reaction mixture was cooled to room temperature and acidified to pH 4 with 10% hydrochloric acid. After evaporation of the solvent the residue was dissolved in sodium hydroxide solution (10% w/v, 3 mL), the solution saturated with sodium chloride and extracted with 1-butanol (3 × 3 mL). The butanol extract was acidified with HCl–butanol and concentrated *in vacuo* (~4 mL). Putrescine dihydrochloride which separated was collected and recrystallized from aqueous ethanol. Yield, 33 mg, 87%. The ^{1}H nmr spectrum was identical with that of authentic material.

(i) *Conversion of (−)-(1-^{2}H)agmatine sulfate (10) into (−)-(1-^{2}H)putrescine dihydrochloride (15)*

Hydrolysis of (−)-(1-^{2}H)agmatine sulfate (65 mg), as described for unlabelled material, gave (−)-(1-^{2}H)putrescine dihydrochloride (41 mg) which was twice recrystallized from aqueous ethanol: CI ms, 90 (M + H, 3%), 74 (5), 73 (M − NH_3, 100), 72 (2); 99.4 ± 0.4% ^{2}H, determined by CI ms; ORD, $[\alpha]_{365}$ −1.07 ± 0.09°, $[\alpha]_{313}$ −0.98 ± 0.08°, $[\alpha]_{334}$ −0.84 ± 0.08°, $[\alpha]_{365}$ −0.67 ± 0.05°, $[\alpha]_{405}$ −0.52 ± 0.05°, $[\alpha]_{436}$ −0.43 ± 0.06°, $[\alpha]_{546}$ −0.21 ± 0.06° (*c*, 1.84% in 0.1 *N* HCl, 26°C, uncorrected for incomplete deuteration).

(ii) *Conversion of (+)-(1-^{2}H)agmatine sulfate into (+)-(1-^{2}H)putrescine dihydrochloride*

Hydrolysis of (+)-(1-^{2}H)agmatine sulfate (40 mg) gave (+)-(1-^{2}H)putrescine dihydrochloride (22 mg) which was twice recrystallized from water: CI ms, 90 (M + H, 5%), 74 (5), 73 (M − NH_3, 100) 72 (10); 91.7 ± 0.8% ^{2}H, determined by CI ms; ORD, $[\alpha]_{313}$ +0.85 ± 0.09°, $[\alpha]_{334}$ +0.75 ± 0.09°, $[\alpha]_{365}$ +0.56 ± 0.06°, $[\alpha]_{405}$ +0.44 ± 0.05°, $[\alpha]_{436}$ +0.41 ± 0.06°, $[\alpha]_{546}$ +0.24 ± 0.06° (*c*, 1.61% in 0.1 *N* HCl, 26°C, uncorrected for incomplete deuteration).

Paper chromatography (see above) of the samples of (−)- and (+)-(1-^{2}H)putrescine dihydrochloride obtained from Experiments (i) and (ii), above, showed single ninhydrin positive and Sakaguchi (34) negative spots at R_f 0.27. The ^{1}H nmr spectra (^2H_2O) of both samples were identical with those of (1-^{2}H)-putrescine dihydrochloride obtained from ornithine.

D. *Conversion of agmatine into phthalimidobutyric acid*

1. *4-Phthalimidobutylguanidine sulfate* (cf. 11)

To a stirred solution containing agmatine sulfate (228 mg, 1 mmol) and sodium bicarbonate (168 mg, 2 mmol) in water (3 mL) was added *N*-carbethoxyphthalimide (Aldrich) (232 mg, 1 mmol) in small portions over 15 min at room temperature. After stirring for 1 h, the reaction mixture was cooled to 0°C and the product, which precipitated, was recrystallized from methanol. Yield 225 mg; mp 213–214°C; ^{1}H nmr ($C^2H_3CO_2^2H$), δ: 1.70 (4H, m (br), H-2,3), 3.30 (2H, t (br), H-1), 3.84 (2H, t (br), H-4), 7.85 (4H, m, ArH). *Anal.* calcd. for $(C_{13}H_{19}N_4O_2)_2SO_4.2H_2O$: C 47.69, H 5.85, N 17.12, SO_4^{2-} 14.67; found: C 47.65, H 5.98, N 17.09, SO_4^{2-} 14.49%.

2. *4-Phthalimidobutyric acid* (cf. 12)

A solution of potassium permanganate (0.4 *M*) in 1 *N* sulfuric acid was added in small portions (ca. 0.1 mL) to a stirred suspension of 4-phthalimidobutylguanidine (27 mg) in 1 *N* sulfuric acid (1 mL) at room temperature. Additions of the oxidant were made until the colour of permanganate in the reaction mixture was no longer discharged (ca. 0.5 mL over 1 h). Stirring was continued for 1 h and the reaction mixture was then decolourized with sodium bisulfite. After cooling at 0°C, the product, which precipitated, was extracted into chloroform (3 × 2 mL). The combined extracts were dried over anhydrous sodium sulfate and filtered. Evaporation gave 4-phthalimidobutyric acid (13 mg) as a white solid which was recrystallized three times from water; mp 115–116°C (lit. (35) mp 115–118°C); ^{1}H nmr (C^2HCl_3), δ: 2.01 (2H, quin., *J* 6.8 Hz, H-3), 2.32 (2H, t, *J* 6.8 Hz, H-2), 3.76 (2H, t, *J* 6.8 Hz, H-4), 7.75 (4H, m, ArH), 10.2 (1H, s (br), CO_2H); ms, *m/e*: 233 (M, 4%), 215 (M − H_2O, 19), 187 (15), 174 (70), 173 (42), 160 (100), 104 (19), 76 (24). The mp and spectral properties were the same as those of an authentic sample prepared from γ-aminobutyric acid and *N*-carbethoxyphthalimide (4).

3. *Conversion of (1-^{2}H)agmatine into 4-phthalimido(4-^{2}H)-butyric acid*

(i) *N*-Carbethoxyphthalimide (82 mg) was added to a stirred solution of (−)-(1-^{2}H)agmatine sulfate (10) (70 mg) in 0.5 *M* sodium bicarbonate (1.5 mL). The resulting mixture was stirred at room temperature for 20 min and then cooled to 0°C. After standing at 0°C for 30 min, the product was collected by filtration, washed with cold water (1 mL), cold ethanol (1 mL), and chloroform (1 mL), and dried *in vacuo* over sodium hydroxide. 4-Phthalimido(4-^{2}H)butylguanidine (11) (66 mg), so obtained, was recrystallized from methanol – acetic acid (20:1); mp 210–212°C. The ^{1}H nmr spectrum ($C^2H_3CO_2^2H$) of the deuteriated sample was similar to that of unlabelled material (see above) except that the signal at 3.84 ppm (H-4) corresponded to ca. 1.05 hydrogen atoms only.

Permanganate oxidation of a sample of the (4-^{2}H)guanidine derivative (49 mg) in aqueous sulfuric acid (1 *N*), as described for unlabelled material, gave (−)-4-phthalimido(4-^{2}H)butyric acid (12) (23 mg) which was recrystallized three times from water; mp 112–114°C; ^{1}H nmr (C^2HCl_3), δ: 2.05 (2H, q, *J* 6.8 Hz), 2.39 (2H, t, *J* 6.8 Hz), 3.75 (ca. 1H, t, *J* 6.8 Hz), 7.80 (4H, m), 10.2 (1H, s (br)); ^{2}H nmr ($CHCl_3$, 30 *mM*, 2172 transients) showed a single signal at 3.74 ppm apart from that due to natural abundance ^{2}H in $CHCl_3$ (7.27 ppm); ms, *m/e*: 234 (M, 3%), 216 (M − H_2O, 7), 215 (M − H^2HO, 9), 188 (8), 187 (6), 175 (41), 174 (30), 173 (17), 161 (100), 160 (5), 104 (16), 76 (22); 97.2 ± 0.2% $^2H_1$, determined by ms; ORD, $[\alpha]_{365}$ −5.39 ± 0.14°, $[\alpha]_{405}$ −3.79 ± 0.22°, $[\alpha]_{436}$ −3.07 ± 0.18°, $[\alpha]_{546}$ −1.64 ± 0.13°, $[\alpha]_{578}$ −1.37 ± 0.14°, $[\alpha]_{589}$ −1.08 ± 0.22° (*c*, 0.91% in methanol, 25°C, uncorrected for incomplete deuteration) (lit. (4) $[\alpha]_{589}$ −1.69°).

A sample of (−)-4-phthalimido(4-^{2}H)butyric acid (12) (5 mg) was dissolved in ether (1 mL), a solution of diazomethane in ether (1 mL) (36) was added, and the mixture was allowed to stand overnight. The product, (−)-methyl 4-phthalimido(4-^{2}H)butyrate (13) was recrystallized from methanol, mp 87–88°C (lit. (nondeuterated) (35) mp 89–90°C); ms, *m/e*: 248 (M, 9%), 217 (M − OCH_3, 18), 216 (8), 189 (10), 188 (10), 175 (100), 174 (20), 161 (95), 130 (15), 104 (17), 77 (15), 76 (12); 98.2 ± 0.3% $^2H_1$, determined by ms; ORD, $[\alpha]_{365}$ −5.97 ± 0.60°, $[\alpha]_{405}$ −4.84 ± 0.40°, $[\alpha]_{436}$ −4.06 ± 0.30°, $[\alpha]_{546}$ −2.21 ± 0.30°, $[\alpha]_{589}$ −2.02 ± 0.30° (*c*, 0.34% in methanol, 25°C, uncorrected for incomplete deuteration).

An unlabelled sample of methyl 4-phthalimidobutyrate was prepared analogously; mp 87–88°C (from methanol); ms, *m/e*: 247 (M, 13%), 216 (M − OCH_3, 19), 215 (17), 188 (9), 187 (11), 174 (100), 173 (23), 160 (79), 130 (11), 104 (14), 77 (12), 76 (6).

An authentic sample of (−)-methyl 4-phthalimido(4-^{2}H)-butyrate (13) was supplied by Dr. M. H. O'Leary, University of Wisconsin. This sample had been prepared (4) by methylation of (−)-4-phthalimido(4-^{2}H)butyric acid which had been obtained by decarboxylation in ^2H_2O of L-glutamic acid, catalyzed by L-glutamic decarboxylase; mp 88–89°C (from methanol); ms, *m/e*: 248 (M, 13%), 217 (M − OCH_3, 21), 216 (10), 189 (10), 188 (8), 175 (100), 174 (15), 161 (80), 130 (15), 104 (15), 77 (10), 76 (12); 92.5 ± 0.4% $^2H_1$, 4.7 ± 0.4% $^2H_2$, determined by ms; ORD,

$[\alpha]_{365}$ +4.45 ± 0.30°, $[\alpha]_{405}$ +3.35 ± 0.20°, $[\alpha]_{436}$ +2.76 ± 0.20°, $[\alpha]_{546}$ +1.48 ± 0.30°, $[\alpha]_{578}$ +1.43 ± 0.30°C, (0.39% in methanol, 25°C, uncorrected for incomplete deuteration).

(iii) A sample of 4-phthalimido(4-^{2}H)butylguanidine (80 mg) obtained by the method described above from (+)-(1-^{2}H)-agmatine sulfate (72 mg) showed mp 213–214 °C (from methanol – acetic acid (20:1)) and a ^{1}H nmr spectrum in which the signal at 3.84 ppm corresponded to ca. 1.15 hydrogen atoms. Oxidation of part of this sample (60 mg), as described above, gave (+)-4-phthalimido(4-^{2}H)butyric acid (28 mg), mp 112–114 °C (3 times from water). The ^{1}H (HCCl$_3$) and ^{2}H (CHCl$_3$, 30 *mM*, 1584 transients) nmr spectra of this material were similar to those obtained for the (+)-(4-^{2}H)phthalimide; ms, *m/e*: 234 (M, 3%), 216 (M – H$_2$O, 8), 215 (M – HDO, 10), 188 (9), 187 (7), 175 (44), 174 (39), 173 (19), 161 (100), 160 (16), 104 (17), 76 (25); 84.7 ± 0.3% ^{2}H, determined by ms; ORD, $[\alpha]_{365}$ +4.77 ± 0.27°, $[\alpha]_{405}$ +3.26 ± 0.29°, $[\alpha]_{436}$ +2.62 ± 0.17°, $[\alpha]_{546}$ +1.32 ± 0.11°, $[\alpha]_{578}$ +1.20 ± 0.13°, $[\alpha]_{644}$ +0.96 ± 0.22°C, (0.97% in methanol, 25 °C, uncorrected for incomplete deuteration).

E. *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.

The ^{1}H nmr spectra at 90 MHz were recorded on a Varian EM 390 spectrometer at ambient temperature. Chemical shifts are reported as ppm downfield from DSS or TMS as internal standards, respectively, for samples in ^{2}H$_2$O or ^{2}HCCl$_3$ solution.

The ^{2}H nmr spectra were recorded on a Bruker WH 90 pulsed Fourier transform spectrometer operating at 13.82 MHz at ambient temperature, with broad band proton noise decoupling. Spectra were obtained of samples in aqueous or chloroform solution in 10 mm (od) sample tubes, using ^{2}H$_2$O as external lock. The signal due to natural abundance ^{2}H in ^{2}HOH or ^{2}HCCl$_3$ served as internal standard. Chemical shifts are downfield from perdeuteriotetramethylsilane.

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 (37).

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