BIOSYNTHESIS OF THE PYRROLIDINE RING IN PLANTS

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By

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ABSTRACT

DL-[5-^{3}H,5-^{14}C]ornithine and DL-[2-^{3}H,5-^{14}C]ornithine were administered to intact tobacco plants (Nicotiana tabacum) in separate experiments. Proline, ornithine and nicotine were isolated. The ^{3}H:^{14}C ratio in proline derived from DL-[5-^{3}H,5-^{14}C]ornithine was the same as that in the reisolated ornithine, whereas proline derived from DL-[2-^{3}H,5-^{14}C]-ornithine contained ^{14}C but lost almost all tritium relative to ^{14}C. This evidence definitely excludes 5-oxo-2-aminopentanoic acid and Δ^{1}-pyrroline-5-carboxylic acid as intermediates on the route from ornithine to proline. These results together with earlier work are interpreted to show that proline is derived from ornithine via 2-oxo-5-aminopentanoic acid and Δ^{1}-pyrroline-2-carboxylic acid. The ^{3}H:^{14}C ratios found in nicotine samples isolated from experiments using DL-[5-^{3}H,5-^{14}C]ornithine and DL-[2-^{3}H,5-^{14}C]-ornithine are consistent with the accepted route from ornithine via 1,4-diaminobutane, mono-N-methyl-1,4-diaminobutane, 4-methylaminobutanal, and N-methylpyrrolinium ion. The ^{3}H:^{14}C ratio found in nicotine derived from DL-[5-^{3}H,5-^{14}C]ornithine is interpreted as showing that oxidation of mono-N-methyl-1,4-diaminobutane to 4-methylaminobutanal proceeds with loss of the 4(S)-hydrogen.

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I am grateful to Professor Ian Spenser for his patience and counsel, and for the suggestion of an interesting research topic. In our discussions he never failed to produce enthusiasm and new insights into biochemistry.

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I. THE DESIGN OF TRACER EXPERIMENTS

(Substanzen) die sich durch radioactive Merkmale leicht und sicher unterscheiden lassen, doch dasselbe chemische Verhalten zeigen. G. von Hevesy and L. Zechmeister (1920)[1].

The use of radionuclides as tracers of biochemical processes began in 1923 with Georg von Hevesy's studies of the absorption, circulation, storage and excretion of lead in plants of Vicia faba (horse bean), whose roots were allowed to soak in a solution of "Thorium B" nitrate ($^{212}Pb(NO_3)_2$).[2] The application of tracers to the study of more fundamental biochemical problems awaited the development of the cyclotron in the nineteen thirties and the subsequent generation of carbon-11 by bombardment of boron with high energy deuterons.[3] With the first biochemical experiment using radioactive carbon, in 1939, it became clear that radioactive tracers would greatly advance the study of life. Barley was exposed to $^{11}CO_2$ and, as expected, labelled chlorophyll was isolated after exposure to light. But, "$^*CO_2$ reduction and formation of some labelled carbohydrate in the absence of light"[3] was also reported. A fundamental controversy concerning the role of carbon dioxide in non-photochemical processes, and the reversibility of enzyme catalysed reactions was thus resolved.

Although the early experiments with carbon-11 were promising, the nuclide's short half-life (21.5 minutes) severely limited its biochemical utility. A continued search for biochemically useful radionuclides led to the discovery of tritium in 1939,[4] and carbon-14 in 1940.[5] World War II brought a temporary halt to research using the newly discovered nuclides but also provided impetus for the development of the Uranium pile reactor, which ultimately made the large scale synthesis of radionuclides possible.[6]

Thirty years ago metabolic pathways in green plants were little understood. Biogenetic theories based on hypothetical precursor-product relationships had been proposed at the turn of the century.[7,8] These speculative sequences of probable intermediates were based on the structural relationships of known metabolites, connected by plausible mechanisms analogous to chemical reactions in vitro.

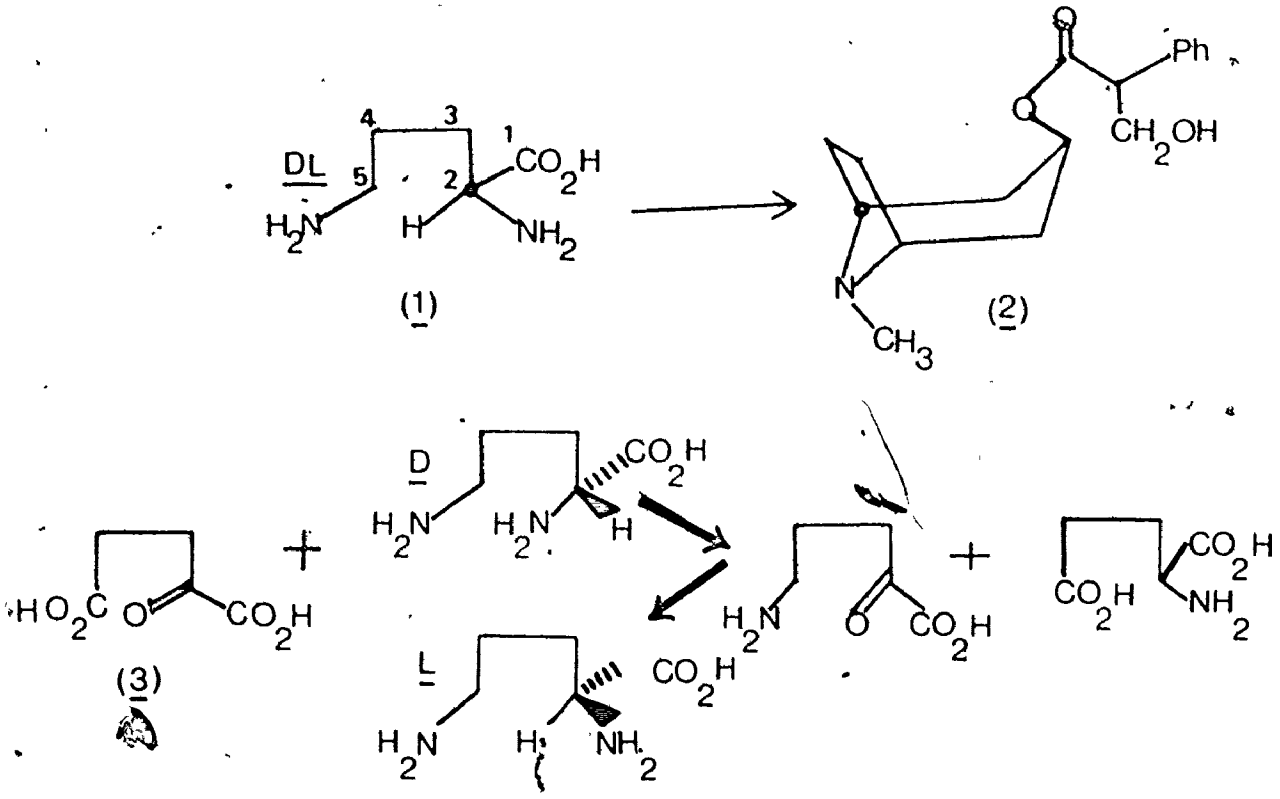
Before the discovery of carbon-14 and tritium there was no satisfactory way to test these hypotheses. The first attempts to establish precursor-product relationships with tracers used, as a criterion, a comparison of changes in specific activity with time, in the putative precursor and in the product.

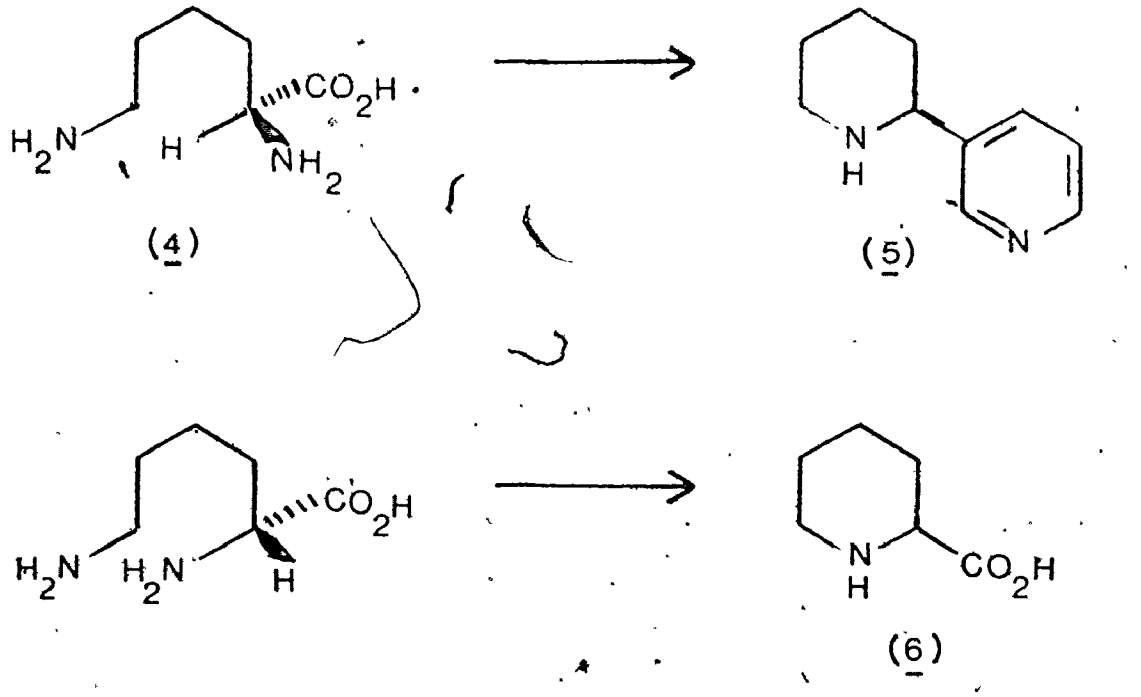
Currently accepted criteria for establishing precursor-product relationships have been reviewed by Spenser[10] and Brown.[11] A commonly used criterion is the specific incorporation of a substrate specifically labelled in one position, followed by unambiguous de-

gradation to known compounds. If the distribution of activity in the product is non-random, the substrate is considered to be a precursor of the product. However, it is not an obligatory precursor. The substrate may have undergone reversible degradation reactions before reaching the site of biosynthesis, leading to spurious limited randomisation or misleading specific incorporation of a degradation product. This experimental defect can be avoided by demonstrating the incorporation of a multiply labelled substrate into a product. If incorporation takes place without change in the isotopic distribution of the substrate, the likelihood of substrate degradation prior to incorporation is greatly reduced.

But such experiments provide no information concerning the stereochemistry of biosynthesis. Progress has recently been made in this respect by the publication of a general method for the determination of precursor-product relationships by Leistner, Gupta and Spenser.[12]

In the study of amino acid metabolism it was previously assumed that incorporation of label from a racemic substrate implicated the L enantiomer as the likely precursor, reflecting the influence of protein biochemistry. For example, Leete,[13] studying the stereospecific incorporation of DL-[2-^{14}C]ornithine (1) into hyoscyamine (2) in Datura stramonium, suggested that incorporation into only one of the bridgehead carbons was due to utilisation of only one ornithine isomer, "presumably the L-isomer" with possible inversion of the D isomer via α-ketoglutaric acid (3). Stratagems devised for the empirical determination of the chirality of the pre-


DL
4
3
1
5
2
CO_2H
H_2N
H
NH_2
(1)
Ph
O
CH_2OH
N
CH_3
(2)
HO_2C
O
CO_2H
(3)
D
L
H_2N
H
NH_2
CO_2H


CO_2H
H_2N
H
NH_2
(4)
N
H
N
(5)
CO_2H
H_2N
H_2N
H
N
H
CO_2H
(6)

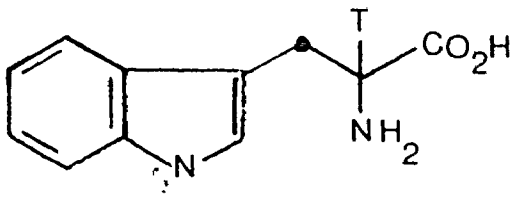
cursor were of three kinds:[12]

1) Comparison of incorporation efficiencies of labelled L and D isomers, individually and in racemic mixture. Using this approach, Gilbertson[14] fed D- and L-[2-^{14}C]lysine (4) to Nicotiana glauca in parallel experiments. He found that anabasine (5) was labelled in the L-lysine experiment and pipecolic acid (6) in the corresponding D-lysine experiment. A note of skepticism concerning the significance of this observation is evident in his remark, "that D-lysine is an extremely good precursor of pipecolic acid is not easy to rationalize". He speculated that it might be the result of an "aberrant metabolic pathway", and that pipecolic acid represents a detoxification product.

2) Comparison of incorporation efficiencies, of the labelled racemic mixture in the presence of an excess of unlabelled D or L enantiomer, or of one labelled enantiomer in the presence of an unlabelled sample of the other. For example, Figenshou et al[15] found that the efficiency of incorporation of DL-[5-^{14}C]ornithine into Gramicidin S, a macrolide antibiotic (cyclic polypeptide), in Bacillus brevis was ten times greater in the presence of unlabelled D-ornithine than in the presence of unlabelled L-ornithine. This was considered sufficient evidence to conclude that only L-ornithine is a precursor of Gramicidin S.

3) Comparison of the retention or loss, within the biosynthetic product, of α-tritium or α-^{15}N, relative to ^{14}C, observed in parallel experiments with multiply labelled samples of the L-enantiomer, of the D-enantiomer, and of the DL-racemate of an amino acid substrate. Using this technique, Floss et al[16] fed multiply labelled tryptophan (7) to Pseudomonas aureofaciens, and measured the retention of α-tritium relative to ^{14}C in pyrrolonitrin (8). The greatest retention of tritium relative to ^{14}C was found using L-tryptophan, almost no retention was observed with D-tryptophan, while DL-tryptophan gave an intermediate result. If criterion 3. were valid, this would mean that L-tryptophan is the precursor of pyrrolonitrin in Pseudomonas aureofaciens; but this conclusion is contradicted by the finding that D-tryptophan is incorporated more efficiently than L-tryptophan.

All of the experimental approaches described suffer from a failure to distinguish differences in incorporation due to biosynthetic stereospecificity and those due to differential absorption of enantiomers or racemisation. Further uncertainty results from the difficulty of obtaining sterically pure compounds, since incorporation efficiencies are frequently less than the percentage of enantiomeric impurity in reagents obtained by the resolution of racemic mixtures, so that activity incorporated into the product of interest may be preferentially or totally derived from the steric impurity.



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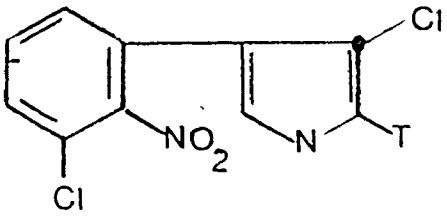

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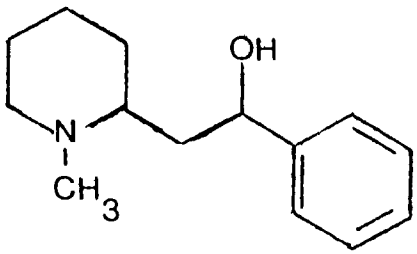

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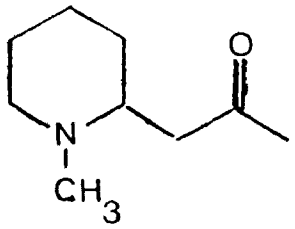

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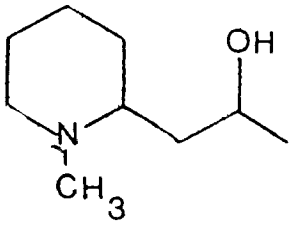


(11)

To avoid these pitfalls which are inherent in any experimental stratagem based on the comparison of parallel experiments, which by their nature involve many poorly controlled variables, it is necessary to obtain self-consistent and independently interpretable results from a single experiment. Leistner, Gupta and Spenser[12] devised such a method, in which the substrate is composed of a mixture of one enantiomer, singly labelled with either tritium or carbon-14, and the other enantiomer labelled with both radionuclides. One radionuclide acts as the internal standard of the other. The energy of beta particles emitted from tritium (0.018 Mev) being much less than the energy of beta particles emitted from carbon-14 (0.156 Mev), the two nuclides can be distinguished easily by Liquid Scintillation Counting. Provided that a precursor-product relationship has been established as described above*, the contribution of each enantiomer of the labelled substrate to the formation of the product may be deduced from a comparison of tritium/carbon-14 ratios.

The utility of this approach was demonstrated by feeding L-[^{3}H]/DL-[^{14}C]lysine and DL-[^{3}H]/D-[^{14}C]lysine to Sedum and Nicotiana species in complementary experiments. When fed L-[4,5-$^{3}H_2$]/DL-[6-^{14}C]lysine, the ^{3}H/^{14}C ratio was doubled in sedamine (9), anabasine (5), N-methylpelletierine (10) and N-methylallosedridine (11), but approached zero for pipecolic acid (6). Conversely, the ^{3}H:^{14}C ratio was halved in pipecolic acid, but approached infinity in sedamine and anabasine when DL-[4,5-$^{3}H_2$]/D-[6-^{14}C]lysine was fed. These

* see page 2

results demonstrate that the alkaloids are derived from L-lysine, whereas pipecolic acid is derived from D-lysine.

However, precursor-product relationships determined by tracer experiments are not the last word in the study of biosynthesis. Ultimately, confirmation of proposed metabolic pathways must await demonstration of *in vitro* stereospecific transformations of precursor into product by appropriate well characterised enzymes and their co-factors or of *in vivo* suppression of biosynthesis using specific enzyme inhibitors, resulting in the accumulation of the obligatory precursor.

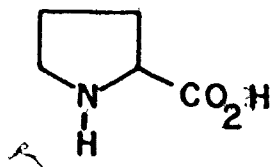
II. BIOSYNTHESIS OF THE PYRROLIDINE RING

"die Annahme von Fermenten nicht allein unnöthig, sondern geradezu sehr wenig plausibel erscheinen lassen." -- E. Pflüger (1878)[17]

The pyrrolidine ring is a frequently encountered molecular group in biochemistry. It is found in compounds as simple and ubiquitous as the amino acid proline (12), a protein constituent in microorganisms, plants and animals, and as a complex and limited in taxonomic distribution as the alkaloid α-truxillin (13), found only in the plant Erythroxylum coca of Peru and Java.

In his "Theory of the Mechanism of the Phytochemical Synthesis of Certain Alkaloids",[8] Robinson proposed ornithine (1) as the precursor of the pyrrolidine ring in the tropane alkaloids. This hypothesis arose from his observation of the facile chemical synthesis of tropinone (14) from acetone, methylamine, and the presumed ornithine equivalent succindialdehyde.[18] His hypothesis was confirmed four decades later when the first experiments on pyrrolidine ring biosynthesis using radioactive tracers were performed. [2-^{14}C]Ornithine was found to be incorporated into the pyrrolidine ring of hyoscyamine (2)[19] and of nicotine (15).[20,21]

In his "Theory of the Mechanism of the Phytochemical Synthesis of Certain Alkaloids", Robinson understated the importance of enzymes as catalysts for the transformations he proposed, speculating "that an equally important cause of the variety and complexity of syntheses in



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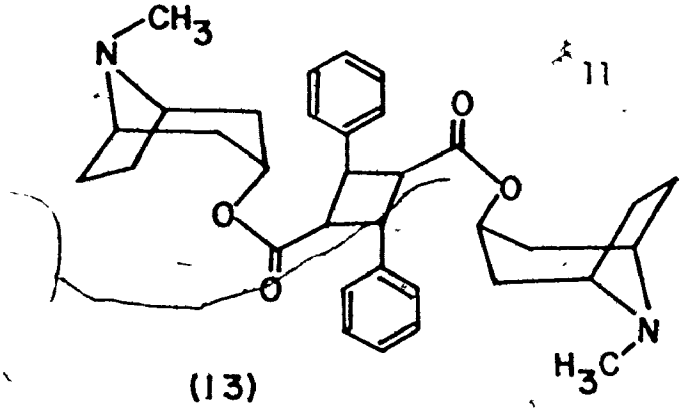

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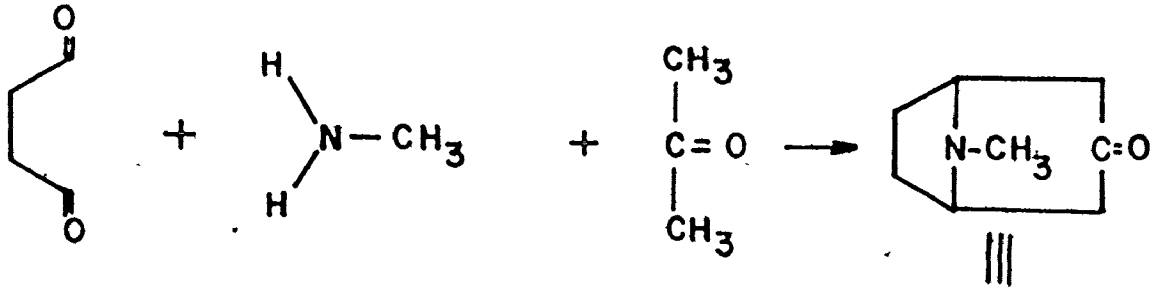

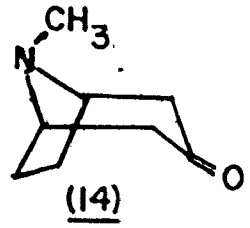

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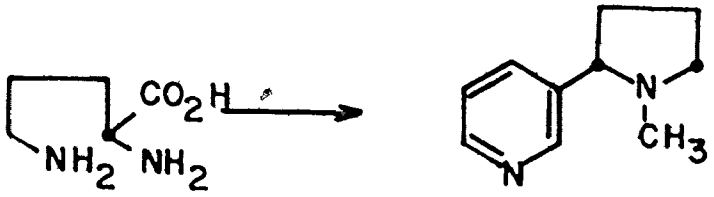

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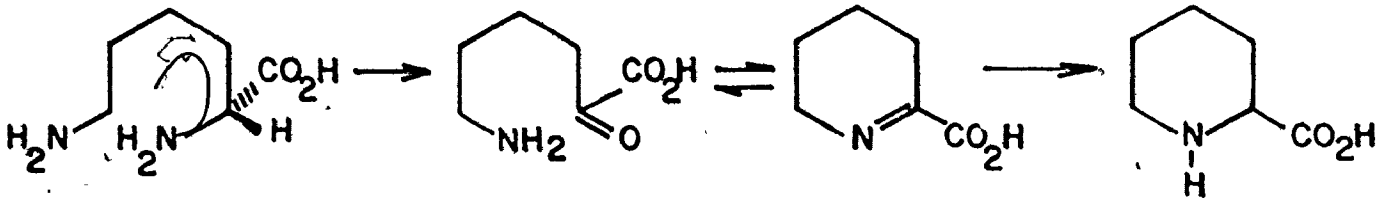


(4) (16) (17) (6)

plants resides in the highly reactive nature of the substances which function as intermediate products". In this classic thesis, Robinson also posited that the biogenetic relationship between ornithine and the pyrrolidine ring is paralleled in the relationship between their higher homologues lysine and the piperidine ring.

Using ^{3}H, ^{14}C double labelling techniques described in Chapter I, Gupta, Leistner and Spenser[22,12,23] elucidated the biosynthetic pathway from lysine to the piperidine ring. Separate pathways were found to exist from lysine (4) to pipecolic acid (6) and from lysine to the piperidine alkaloids. D-Lysine was found to be incorporated into pipecolic acid via ε-amino-α-ketocaproic acid (16) and Δ^{1}-piperideine-2-carboxylic acid (17) while L-lysine was incorporated into the alkaloids. It is of interest to investigate whether similar relationships exist between ornithine and its corresponding metabolites, proline and the alkaloids containing a pyrrolidine nucleus. The first step in such an investigation is the identification of intermediates on the metabolic pathway.

A. Ornithine and the Pyrrolidine Ring of Proline

Since homologues are often alternative substrates for the same enzymes, it is reasonable to expect that intermediates on pathways between homologous precursors and homologous products would also be homologues. It was surprising, therefore, to find that most texts and reviews on the subject of amino acid biosynthesis in plants show glutamic-γ-semialdehyde (5-oxo-2-aminopentanoic acid)(18) in equilibrium

with its cyclised form, Δ^1-pyrroline-5-carboxylic acid (19) as an intermediate on the pathway from ornithine to proline.[24,25,26,27]

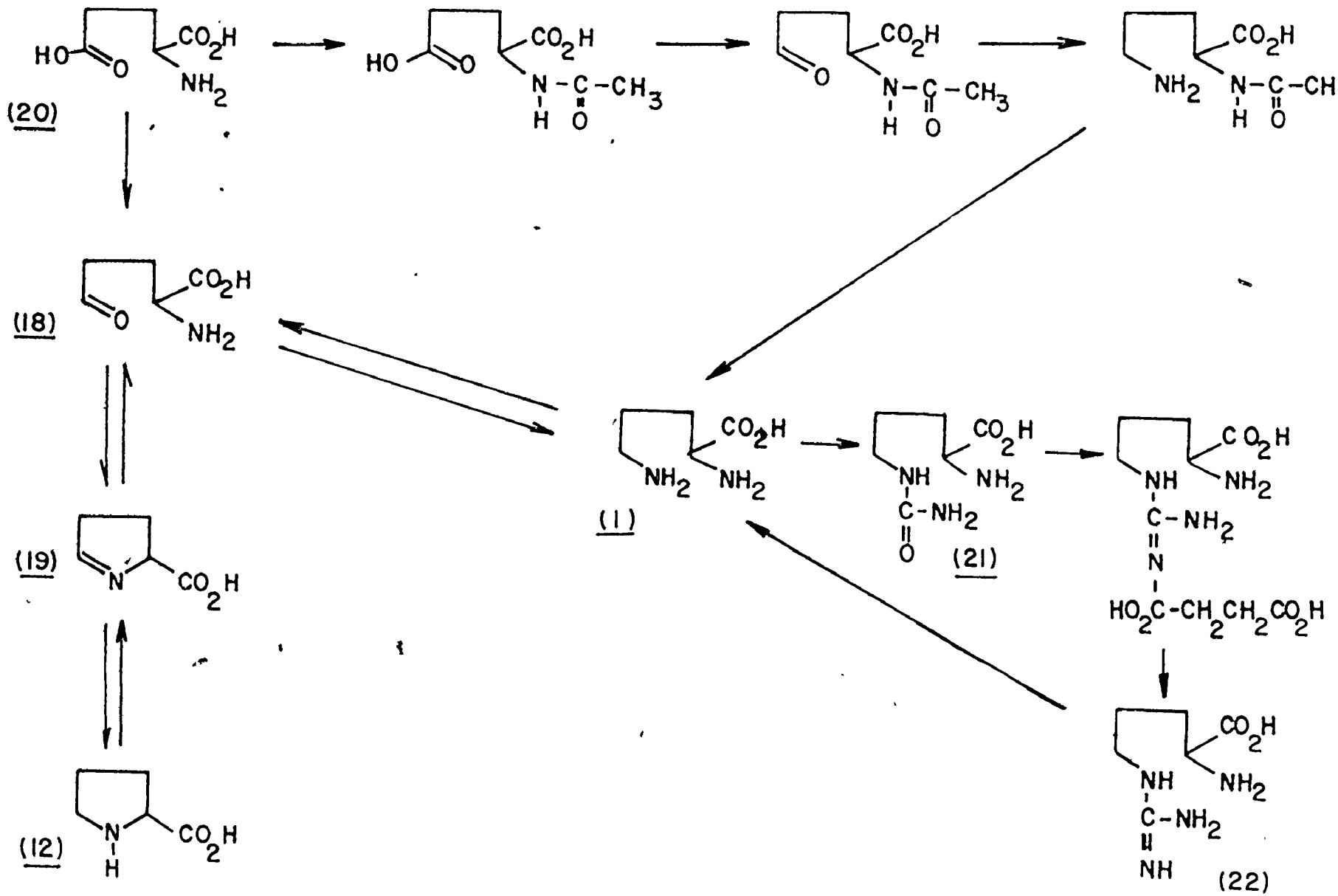
Only one review admits the possibility of an alternative pathway, but gives no supporting evidence.[28] Some authors completely ignore the pathway from ornithine to proline.[29,30] The topic is considered so uncontroversial that one recent review[27] of amino acid biosynthesis devoted only one sentence to proline biosynthesis. Fowden's review, "Aspects of Amino Acid Metabolism in Plants"[25] expresses the current consensus:

> The general unitary character of amino acid biogenesis, irrespective of the plant order involved, has gradually been confirmed as enzymic studies with higher plants have been made. Few major differences of enzymic mechanism have been revealed so far when observations made with angiosperm extracts are compared with those from bacterial or fungal systems."

Classical investigations of amino acid metabolism used bacteria and fungi as experimental organisms. The pathways deduced from these experiments have affected the design of later experiments with plants, where tests for alternative pathways often have not been applied. A thorough review of the classical experiments performed on microorganisms is beyond the scope of this introduction. Excellent reviews are presented in general treatises on metabolism.[31,32,33,34] The pathways connecting the structurally related amino acids, glutamic acid (20), ornithine (1), proline (12), citrulline (21), and arginine (22), as deduced from classical experiments, are summarised in Figure I.

Intermediates on these pathways were deduced from the nutritional

Figure I. Metabolic pathways in microorganisms for the amino acids related to glutamic acid.


(20)
(18)
(19)
(12)
(1)
(21)
(22)

requirements of mutant strains of intact organisms and from limited chemical analysis of compounds formed when cell free extracts or enzyme preparations from various organisms were incubated with various substrates. Tracer experiments, when performed, employed carbon-14 labelled substrates which usually were uniformly or generally labelled.

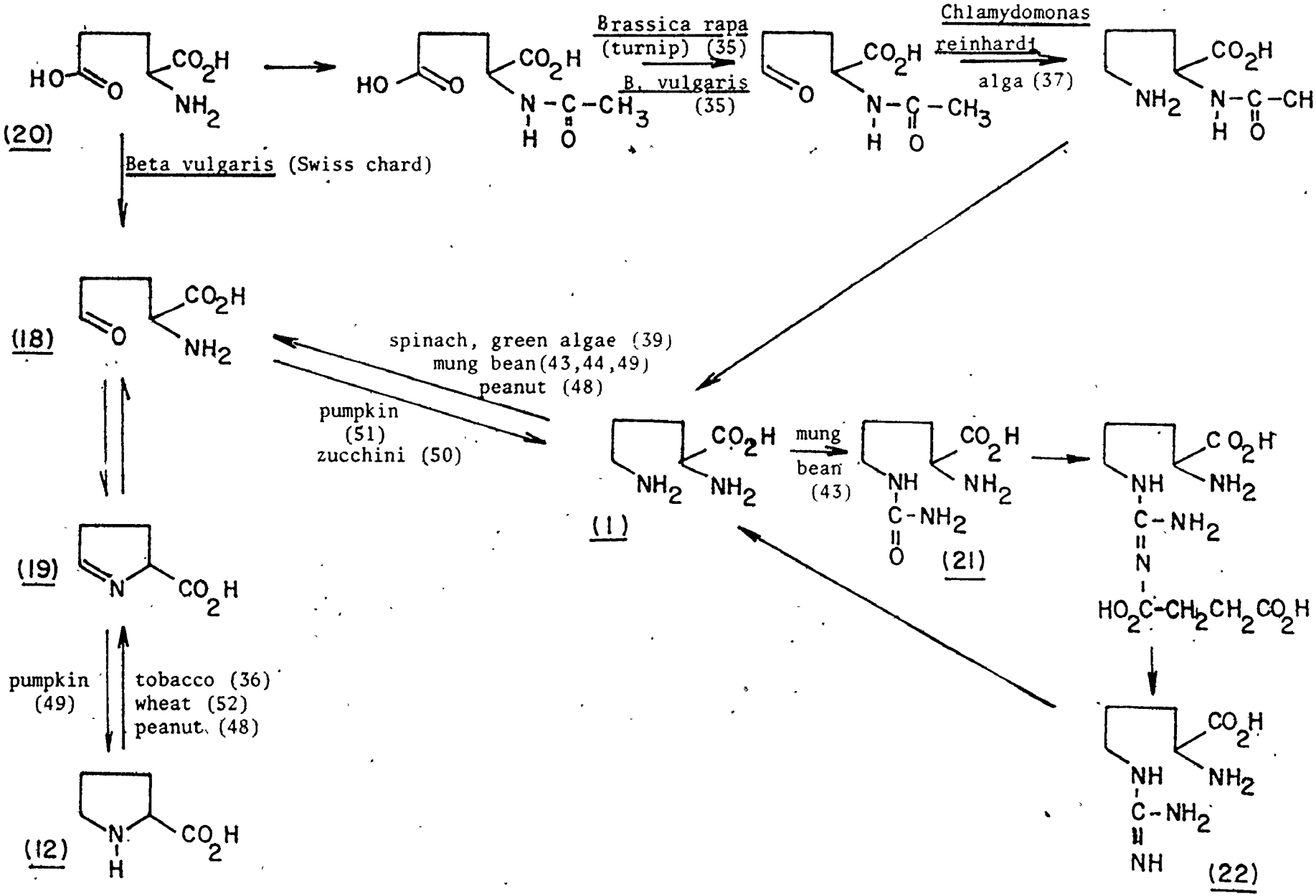
i. Evidence in favor of δ-deamination

The design of experiments intended to elucidate the pathways among members of the glutamic acid family in plants, even recently, has usually been copied from classical experiments, despite the availability of more definitive tracer methods. Conclusions drawn from these experiments are summarised in Figure II.

That ornithine can serve as a precursor of the other structurally related amino acids in plants, as well as in microorganisms, was demonstrated almost twenty years ago.[38] Labelled glutamic acid, proline, citrulline, and arginine were detected on two-dimensional paper chromatograms of extracts of barley and of clover treated with DL-[2-^{14}C]-ornithine. The influence of classical microbial biochemistry was so strong that the authors felt compelled to speculate, without experimental justification, that:

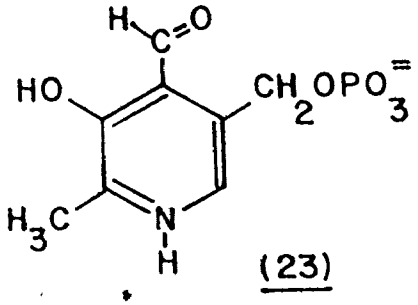
> "This close association of proline with ornithine metabolism points to a pathway of proline formation from ornithine in higher plants, presumably via glutamic-γ-semialdehyde and Δ^1-pyrroline-5-carboxylate, a metabolic process known to operate in Neurospora crassa."

Figure II. Currently accepted metabolic pathways in plants for the amino acids related to glutamic acid.


(20)
Beta vulgaris (Swiss chard)
Brassica rapa (turnip) (35)
B. vulgaris (35)
Chlamydomonas reinhardi
alga (37)
(18)
spinach, green algae (39)
mung bean (43,44,49)
peanut (48)
pumpkin (51)
zucchini (50)
(1)
mung bean (43)
(21)
(22)
(19)
pumpkin (49)
tobacco (36)
wheat (52)
peanut (48)
(12)

Scher and Vogel[39] attempted to establish glutamic-γ-semialdehyde as the product of ornithine deamination by incubating cell-free extracts of spinach leaf and green algae (Chlamydomonas reinhardi and Chlamydomonas moewusii) with L-ornithine, α-ketoglutaric acid and pyridoxal phosphate (23) at pH 7. They considered the presence of a substance (or substances) which gave a yellow color with o-aminobenzaldehyde (24)[40] (due to formation of 3a,4-(2H)dihydropyrrolidino[2,1-b]quinazolinium-1-carboxylic acid (25)) and which supported the growth of an E. coli mutant (55-25) which responds to glutamic-γ-semialdehyde or L-proline[41] (but not to α-keto-δ-aminovaleric acid (26), the product of ornithine α-deamination) as sufficient evidence to support their contention. Yet, α-keto-δ-aminovaleric acid also gives a yellow color with o-aminobenzaldehyde[41] due to 3a,4(2H)-dihydropyrrolidino [2.1-b]-quinazolinium-3a-carboxylic acid (27),[42] and only a small amount of glutamic-γ-semialdehyde, not necessarily the product of ornithine deamination, would have supported the growth of the E. coli mutants. Bone[43] also claimed to have demonstrated that glutamic-γ-semialdehyde is the product of ornithine transamination with glutamate serving as the amino acceptor, but omitted experimental details.

Nine years later, L. Fowden and his colleagues initiated a series of papers attempting to confirm the ornithine δ-transamination hypothesis. In the first of these[44] Seneviratne and Fowden studied the transamination of the homologous series of diaminoacids; α,β-diaminopropionic acid, α,γ-diaminobutyric acid, ornithine, and lysine, in mung bean seedling mitochondrial extracts from which endogenous amino acids and other small



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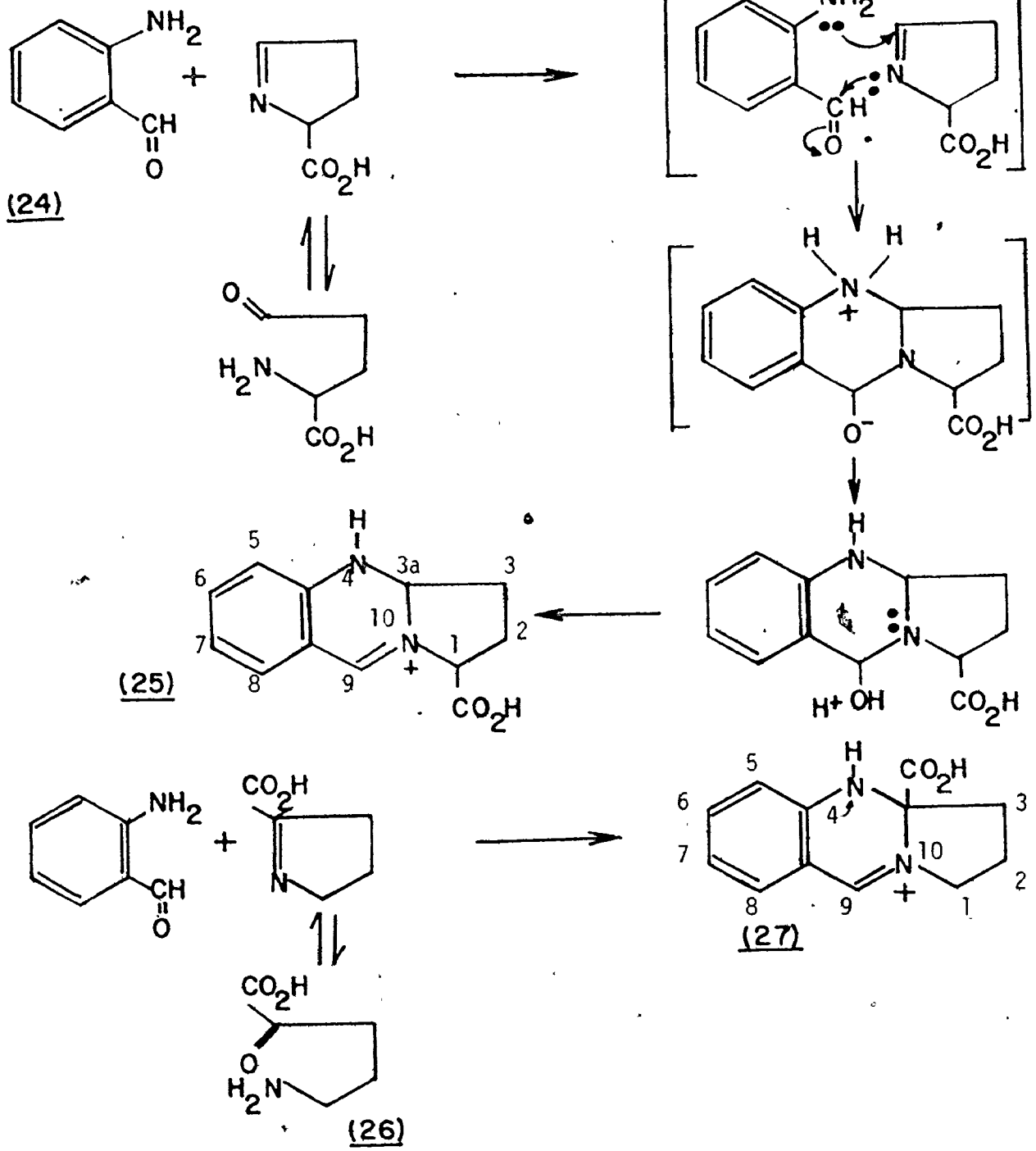

(24)

(25)

(26)

(27)

free molecules had been removed by dialysis or by gel-filtration. The extracts were incubated with α-ketoglutarate and each of the diamino acids, and the rate of formation of glutamic acid was measured. The rate of glutamic acid formation was found to decrease in the series: ornithine > α,β-diaminopropionic acid α,γ-diaminobutyric acid > lysine after gel-filtration of the mitochondrial extract. However after dialysis, instead of gel-filtration, the rate of glutamic acid produced from ornithine decreased by an order of magnitude.

To determine which amino group was involved in the transamination reaction, the products of diamino acid deamination were studied using L-[U-^{14}C]-2,3-diaminopropionic acid, DL-[4-^{14}C]diaminobutyric acid* and DL-[2-^{14}C]ornithine. After incubation with mung bean mitochondrial extracts, the reaction mixture was chromatographed on paper, and the radioactive spots were eluted. These labelled materials were identified by their reaction products on oxidation with hydrogen peroxide, or on reduction with sodium borohydride. If the α-amino group had been removed from the diamino acid in the course of transamination, an α-keto-ω-aminoacid would be formed which would yield, after peroxide oxidation, an ω-aminoacid with one less carbon atom, or, after borohydride oxidation, an α-hydroxy-ω-aminoacid (Scheme i). Alternatively, if the ω-amino group were removed during transamination, an α-amino-ω-aldehydo-acid would be formed yielding the corresponding aminodicarboxylic acid after peroxide oxidation and an α-amino-ω-hydroxyacid on borohydride reduction (Scheme ii).

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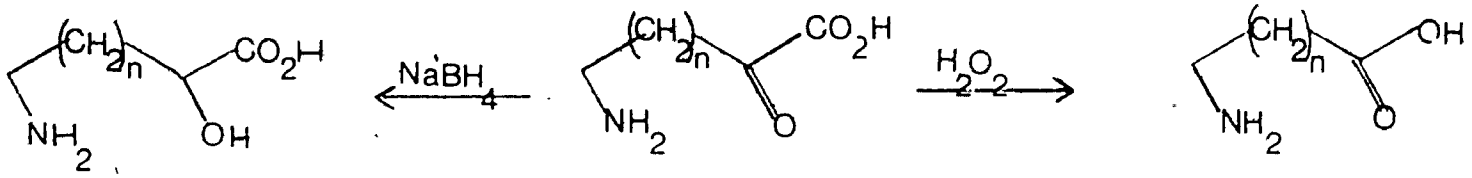
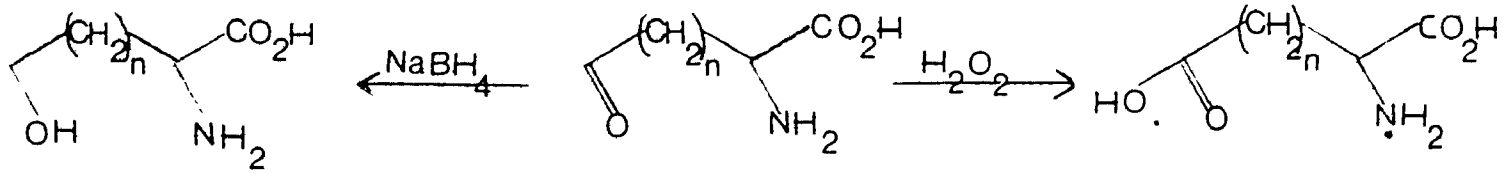
* presumably, DL-[4-^{14}C]-2,4-diaminobutyric acid



Scheme i.



Scheme ii.

When ornithine was studied, pyridoxal phosphate (23) was added to the reaction mixture before incubation. Using fresh, gel-filtered mung bean mitochondrial extract "labelled products formed from ^{14}C-ornithine included both α-keto-δ-aminovaleric acid ... and glutamic-γ-semialdehyde ... together with a compound tentatively identified as Δ^1-pyrroline-2-carboxylic acid (the product of spontaneous cyclization of α-keto-δ-aminovaleric acid). If, however, the mung bean mitochondrial extract was dialysed prior to incubation "glutamic-γ-semialdehyde formation was negligible".

Apparently unwilling to consider seriously the possibility of α-transamination in ornithine, Seneviratne and Fowden tried the experiment in the absence of mung bean mitochondrial extract, and found that a "facile chemical reaction"* occurred yielding pyridoxamine phosphate and a substance which was converted to γ-aminobutyric acid on oxidation with hydrogen peroxide. Remarkably, they concluded that "the enzyme therefore catalysed δ-transamination from ornithine", explaining that the observed α-transamination was a "chemical" reaction and therefore an artifact, and that "ornithine transaminase was quite labile under the experimental conditions used" (dialysis overnight at 0-4°). Yet Scher and Vogel[39] had reported that their extracts "could be stored at -15°C for several weeks without serious deterioration".

A number of derivative investigations based on the work of Seneviratne and Fowden appeared in ensuing years.[48-52] Lu and Mazelis studying ornithine transaminase in mung bean[49] and in zucchini (Cucurbita

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* A well-known reaction.[45,46,47]

pepo)[50] "using essentially the procedure of Seneviratne and Fowden with DL[2-^{14}C]ornithine as substrate, [showed] that the enzyme catalyzes the δ-transamination of ornithine to yield glutamic-γ-semialdehyde and its cyclic equilibrium form Δ^1-pyrroline-5-carboxylate". Splittstoesser and Fowden[51] in their study of the enzyme kinetics of ornithine transaminase from pumpkin (Cucurbita maxima) cotyledons simply assumed δ-transamination, quoting Seneviratne and Fowden as authority. Mazelis and Fowden[48] studying proline formation from ornithine in the peanut (Arachis hypogaea) likewise believed that further experimental evidence was unnecessary and assumed δ-deamination as the first step. In the last of this series of papers, Mazelis and Crevelling[52] in their description of experiments on the L-proline dehydrogenase of wheat (Triticum vulgare) begin by stating that:

> "The biosynthesis of proline in all organisms so far studied in any detail proceeds from either glutamic acid or ornithine via glutamic-γ-semialdehyde which is in equilibrium with its cyclic form P5C (Δ^1-pyrroline-5-carboxylate):"

But they observe that:

> "Strangely enough P5C, which is formed by transamination from ornithine, is reduced equally well to proline with either NADPH or NADH; however, chemically synthesized P5C uses NADH much more effectively than NADPH."

yet never question their opening statement.

ii. Evidence in Favour of α-deamination

In all the cited papers written by Fowden and his school on

ornithine deamination, only two experiments were mentioned which suggested loss of the α-amino group from ornithine. Seneviratne and Fowden[44] describe the work of Wickremasinghe and Swain[53] who found α-keto-δ-aminovaleric acid (26) on paper chromatograms of the products resulting from the incubation of ornithine and pyridoxal phosphate with an extract of bean callus tissue. Seneviratne and Fowden dismissed this finding as a chemical artifact. Mazelis and Fowden[48] with no elaboration or discussion cited the work of Hasse, Ratych and Salnikow[42] whose experiments paralleled, but whose results contradicted the work of Seneviratne and Fowden. Hasse et al. detected Δ^1-pyrroline-2-carboxylic acid or Δ^1-piperideine-2-carboxylic acid, respectively, after incubation of enzyme preparations from mung beans (Phaseolus aureus) and lupins (Lupinus angustifolius) with ornithine or with lysine. The identity of the products was determined by paper chromatography of the ortho-aminobenzaldehyde derivatives.

Using nitrogen-15 labelled ornithines, Duranton and Wurtz[54,55] reported loss of α-amino nitrogen from ornithine in the course of proline biosynthesis. Duranton and Wurtz found that when DL-ornithine, enriched 1.8 percent in nitrogen-15 at the α-position, was administered to tissue cultures of Jerusalem artichoke (Helianthus tuberosus) tubers, proline was not enriched in nitrogen-15. However, when DL-ornithine, enriched 2.75 percent in nitrogen-15 at the δ-position, was administered, 0.815 percent enrichment in nitrogen-15 was measured in proline.

Liebisch and Schütte[56] demonstrated the same relationship between ornithine and proline using DL-[2-^{14}C,α-^{15}N]ornithine and DL-[2-^{14}C,δ-^{15}N]-ornithine administered in parallel experiments to Datura species. When

DL-[2-^{14}C,δ-^{15}N]ornithine was the substrate, samples of proline which were isolated maintained the ^{14}C:^{15}N ratio of the substrate; while proline isolated from the other experiment lost all nitrogen-15 in excess of natural abundance, and remained rich in carbon-14. These experiments were not quoted in any of the papers written in English which were cited earlier.

However, these experiments suffer from the defect that the amino group is biochemically labile and the nitrogen pool is small. No amino-acid besides proline was isolated in any of the cited experiments performed either by Duranton and Wurtz or by Liebisch and Schütte, so that the degree of exchange between the amino groups of ornithine and the general nitrogen pool is unknown.

Thus much of the evidence concerning the relationship between ornithine and proline in plants is equivocal due to faults in experimental design and to the intricacies of interrelationships among the aminoacids and their metabolites. In the present study the two models of proline formation were tested experimentally using tritium and carbon-14. On the basis of the results obtained, the proposition that proline arises by δ-deamination of ornithine *via* glutamic-γ-semialdehyde becomes untenable; whereas the view that α-keto-δ-aminovaleric acid is an intermediate arising by α-deamination of ornithine is supported.

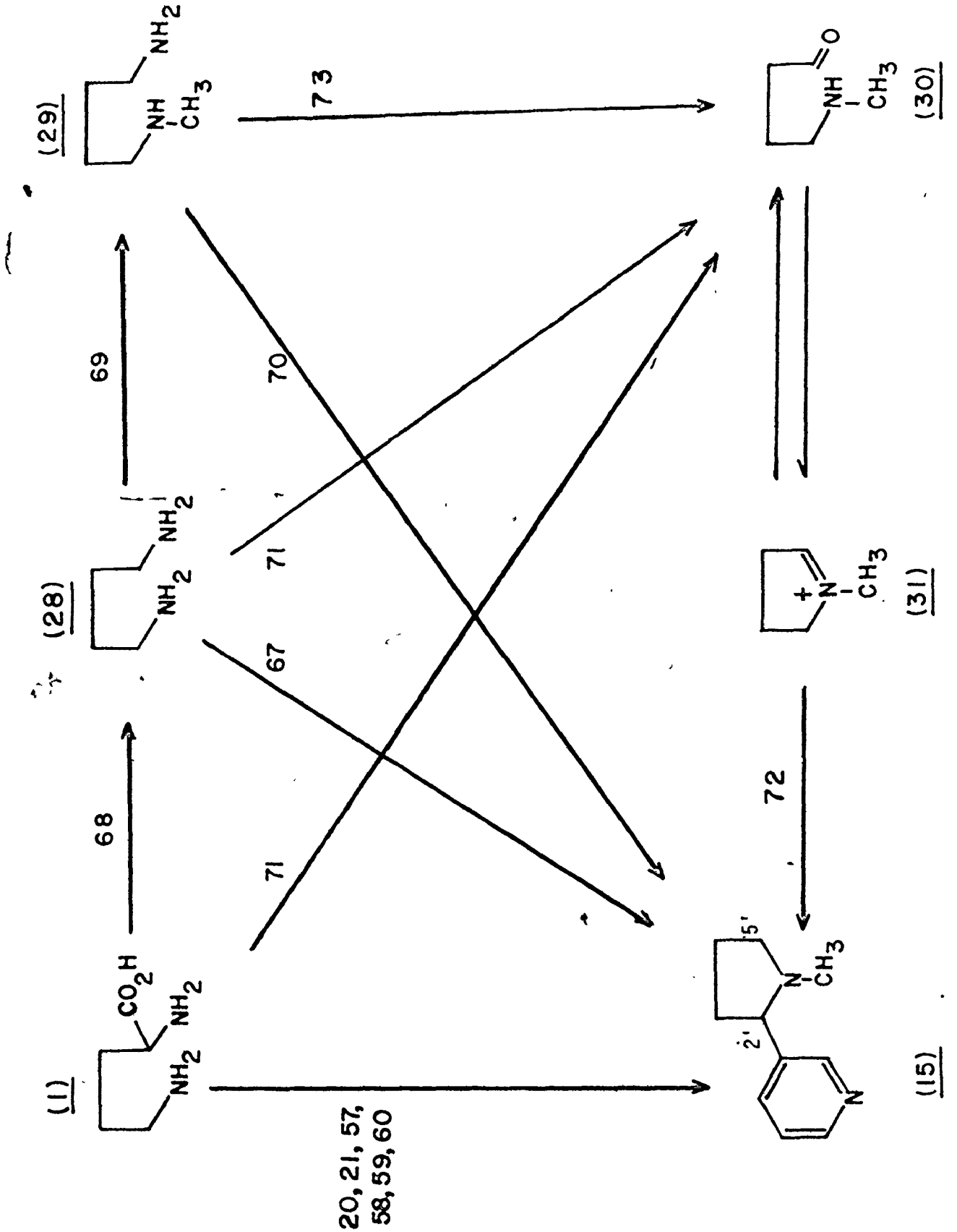
B. Ornithine and the Pyrrolidine Ring of Nicotine

Tracer studies of nicotine biosynthesis began twenty years ago when Leete[20] and Byerrum *et al.*[21] independently established that label from DL-[2-^{14}C]ornithine is incorporated into the pyrrolidine ring of

nicotine in such a manner that half of the radioactivity of the ring is located at C-2'. Further investigation revealed that the rest of the activity was located at C-5'.[57,58] The complementary experiment using [5-^{14}C]ornithine[59] gave the same result. When DL-[2-^{14}C,δ-^{15}N]ornithine was administered[60] to tobacco, the ^{15}N:^{14}C ratio in the nicotine isolated was half that of the administered ornithine. These results were interpreted as evidence for a symmetrical intermediate. Subsequently, intermediates on the pathway from ornithine to nicotine were discovered by several research groups. Excellent reviews have been written about nicotine biosynthesis[61-66] so only a brief summary, with emphasis on recent contributions, will be attempted here.

The currently accepted pathway[62,72] is presented in Figure III. The first step in this pathway is decarboxylation to give the established[67] nicotine precursor putrescine (1,4-diaminobutane) (28). This proposed decarboxylation was confirmed by Yoshida[68] who demonstrated incorporation of activity into putrescine when DL-[2-^{14}C]ornithine was injected into intact tobacco plants. The second step, N-methylation of putrescine to give N-methylputrescine (mono-N-methyl-1,4-diaminobutane) (29) was supported by Mizusaki, Tanabe, Noguchi and Tamaki's isolation of an enzyme which catalyses N-methyl transfer from S-adenosyl-L-methionine to putrescine (S-adenosyl-L-methionine:putrescine N-methyltransferase)[69] as well as by an experiment reported by Schütte, Meier and Mothes[70] in which non-random incorporation of label from [N-methyl-^{15}N,^{14}C]N-methyl-1,4-diaminobutane into nicotine was found to occur in plants of Nicotiana rustica with no change in ^{15}N:^{14}C ratio.

Figure III. Evidence in favour of Leete's hypothesis of nicotine biosynthesis from ornithine.


(1)
(28)
(29)
(30)
(31)
(15)
68
69
70
71
71
67
72
73
20, 21, 57, 58, 59, 60
5'
2'

4-Methylaminobutanal (30) in equilibrium with N-methylpyrrolinium ion (31), the next intermediate on the proposed pathway, was found to be labelled when DL-[2-^{14}C]ornithine, [1-^{14}C]putrescine, or DL-[methyl-^{14}C]-methionine were administered to tobacco plants.[71] Labelled 4-methylaminobutanal so obtained was itself an efficient precursor of nicotine when administered to tobacco root, in agreement with Leete's observation that the cyclised form, N-methylpyrrolinium ion, when labelled with carbon-14 in the 2-position yielded nicotine labelled at C-2'.[72] Further evidence was mustered in support of 4-methylaminobutanal as an intermediate on the pathway to nicotine by the isolation of a copper containing diamine oxidase from tobacco root which oxidized N-methylputrescine to 4-methylaminobutanal.[73]

All the evidence in its favour notwithstanding, this proposed pathway has been challenged by Rapoport and coworkers.[74] They have interpreted contradictory results of $^{14}CO_2$ feeding experiments in which unequal labelling at C-2' and C-5' of nicotine was sometimes found as evidence disproving the existence of a symmetrical intermediate on the normal biosynthetic route to nicotine. Yet Zielke, Byerrum and coworkers[76] found symmetrical labelling under similar conditions. Hutchinson's recently reported investigation[76] of ^{13}C nuclear magnetic resonance spectra of nicotine samples isolated from tobacco plants exposed to a mixture of $^{13}CO_2$ and $^{14}CO_2$ was equivocal in its answer to the problem of label distribution in the pyrrolidine ring of nicotine. Depending on the method used to measure relative ^{13}C enrichment, pathways involving either symmetrical or unsymmetrical intermediates could be rationalised. This uncertainty has been

resolved by Leete[77] who used an unambiguous degradative scheme to show that Hutchinson's nicotine samples contained symmetrically labelled pyrrolidine rings.

While the structural relationships between intermediates on the pathway from ornithine to nicotine have been discovered and the enzymes catalysing their transformations have been isolated, the stereochemistry of these enzymatic reactions has remained uninvestigated. The tritium, carbon-14 double labelling experiments presented in this study provide evidence that oxidative deamination of N-methylputrescine to 4-methylaminobutanal is stereospecific and proceeds with loss of the 4(S)-hydrogen.

EXPERIMENTAL

Labelled Compounds

DL-[5-^{3}H,5-^{14}C]Ornithine

Experiment 1. The intermolecularly doubly labelled ornithine used in this experiment was a mixture of DL-[5-^{14}C]ornithine (nominal total activity 0.1 mCi, nominal specific activity 9.3 mCi per mmole, Commissariat à l'Energie Atomique, France) and DL-[5-^{3}H]ornithine (nominal total activity 1.25 mCi, nominal specific activity 2.1 Ci per mmole, Amersham Searle). The ^{14}C labelled compound was prepared by reaction of K^{14}CN with α-benzamido-γ-butyrolactone, followed by Adams reduction and acid hydrolysis.[78] The tritium labelled compound was prepared by catalytic reduction of ethyl-α-acetamido-α-carbethoxy-γ-cyanobutyrate with tritium gas, followed by acid hydrolysis (cf. reference 79). The compound was purified by paper chromatography. The distribution of tritium within the material was established by chemical degradation.[80] DL-[5-^{3}H]Ornithine was oxidised with potassium permanganate; and γ-aminobutyric acid and succinic acid were isolated. The molar specific activity of the succinic acid was 10% of that of the ornithine. The molar specific activity of the γ-aminobutyric acid was 97% of that of the ornithine. This indicates that 87% of the tritium was present at C-5, 10% at one or both of C-3 and C-4, and no more than 3% of the label was present at C-2.

The two labelled samples of ornithine were dissolved in glass distilled water and the total volume adjusted to 5 ml. The mixture was stored at 5°C. Before feeding, the doubly labelled ornithine was puri-

fied on a (5 cm x 0.5 cm) column of Dowex 50W-X8-200 (H^+). After successive washings with water and 2M hydrochloric acid, ornithine was eluted with 4N hydrochloric acid. Excess hydrochloric acid was removed by repeated evaporation with water in vacuo. Paper chromatography (propanol:ammonium hydroxide, 7:3, Rf = 0.15) revealed only one spot on a scanning Geiger counter (Packard Radiochromatogram Scanner Model 7201) and on spraying with ninhydrin. The total activity of each of the radionuclides in the purified doubly labelled ornithine (0.165 mCi ^{3}H, 0.015 mCi ^{14}C) was determined by liquid scintillation counting (Nuclear Chicago Mark I liquid scintillation counter, New England Nuclear "Aquasol" cocktail) using the channels ratio method,[81] with a ^{133}Ba external standard.

Experiment 2. The intermolecularly doubly labelled ornithine used in this experiment was a mixture of DL-[5-^{14}C]ornithine (nominal total activity 0.033 mCi, nominal specific activity 6.6 mCi per mmole, New England Nuclear, plus nominal total activity 0.07 mCi, nominal specific activity 11.5 mCi per mmole, Commissariat à l'Énergie Atomique, France) and DL-[5-^{3}H]ornithine (nominal total activity 0.66 mCi, nominal specific activity 5.0 Ci per mmole, Radiochemical Centre, Amersham).

The labelled samples of ornithine were dissolved in glass distilled water and the total volume adjusted to 8 ml. The ^{3}H:^{14}C ratio of this stock solution was determined by liquid scintillation counting.

DL-[2-^{3}H,5-^{14}C]Ornithine

Experiment 3. This intermolecularly doubly labelled ornithine was a

mixture of DL-[5-^{14}C]ornithine (nominal total activity 0.066 mCi, nominal specific activity 11.5 mCi per mmole, Commissariat à l'Énergie Atomique, France) and DL-[2-^{3}H]ornithine (nominal total activity 0.66 mCi, nominal specific activity 146 mCi per mmole, Radiochemical Centre, Amersham). The tritium labelled compound was prepared by hydrolysis[79] of 3-acetamido-3-carbethoxypiperid-2-one with tritiated hydrochloric acid.

The two labelled samples of ornithine were dissolved in glass distilled water and the total volume adjusted to 8 ml. The ^{3}H:^{14}C ratio of this stock solution was determined by liquid scintillation counting.

Administration of Labelled Ornithine to Nicotiana tabacum

Experiment 1. DL-[5-^{3}H,5-^{14}C]Ornithine was administered to two twelve week old N. tabacum plants by infusion into the stems through cotton wicks. The plants were left to grow in contact with the tracer for sixty-seven hours and then were harvested. The tops and roots were collected separately (fresh weight: tops 250 g, roots 90 g). They were dried for two days at 50°C and stored in a polyethylene container.

Experiment 2. DL-[5-^{3}H,5-^{14}C]ornithine was administered to six eleven week old N. tabacum plants by infusion into the stems through cotton wicks. The plants were left to grow in contact with the tracer for fifty-two hours and then were harvested. The tops and roots were collected separately (fresh weight: tops 190 g, roots 45 g). They were dried for two days at 50°C and stored in a polyethylene container.

Experiment 3. DL-[2-^{3}H,5-^{14}C]ornithine was administered to seven eleven

week old N. tabacum plants as in Experiment 2. The fresh weight of the tops was 220 g and that of the roots 60 g.

Isolation of Proline and Ornithine

Experiment 1. The dried tops (7 g) were ground in an Osteriser blender. The coarse powder was extracted for three hours with 200 ml 4% acetic acid under reflux with occasional shaking. The mixture was allowed to cool to room temperature and to stand overnight, after which it was filtered through glass wool. The retained solids were again extracted for one hour with 100 ml boiling water, filtered and again extracted with 100 ml cold water overnight. The combined extracts were refrigerated for 24 hours. A few milliliters of benzene were added to prevent microbial growth in the extract. The cloudy chilled extract was then centrifuged at 2000 rpm for 15 minutes. The supernatant solution was decanted and the residue washed with two 30 ml portions of water. The washings were added to the decanted solution. The combined extracts were then evaporated in vacuo to ca. 25 ml.

The clear brown solution was extracted three times with 30 ml diethyl ether and made basic (pH = 12 approximately, using pHydrion paper) with 2M ammonium hydroxide solution. The solution darkened and became cloudy. It was centrifuged, the supernatant solution was decanted, and the residue washed twice with 10 ml portions of water. The combined supernatant solution and washings were extracted with four 50 ml portions of diethyl ether. The organic extract was saved for subsequent nicotine isolation (vide infra).

The aqueous phase was acidified with acetic acid, evaporated

to 50 ml *in vacuo* and dialyzed for 24 hours in a cellulose dialysis bag (nominal pore size 2.5 nm, Canlab), suspended in a glass cylinder containing 150 ml distilled water. The dialysate was removed and replaced with distilled water three times in succession. The combined dialysates were evaporated under reduced pressure to a volume of 20 ml and the solution was applied to a (42 cm x 2 cm) column of Dowex 50W-X8-200 (H^+). The column was washed with 50 ml water, then eluted in succession with hydrochloric acid as follows: 250 ml 1.5 N, 750 ml 2.5 N, and 250 ml 4N.[82] Fractions (approximately 15 ml) were collected and spotted on paper strips. Activity on the strips was monitored on a scanning Geiger counter. The strips were then sprayed with ninhydrin. It was found that proline emerged from the ion exchange column with 75 to 125 ml of 2.5 N hydrochloric acid, while ornithine emerged with 275 to 325 ml of 2.5 N hydrochloric acid.

Experiments 2 and 3. The dried roots were ground and extracted as in Experiment 1. Unlabelled L-proline (5 mg) was added as carrier. Instead of cellulose dialysis, membrane ultra-filtration was employed to separate the amino acids from high molecular weight compounds.[83,84] The concentrated plant extract (10 ml) was made basic (pH = 8.5) with ammonium hydroxide, and was placed in a mechanically stirred ultra-filtration cell (Amicon model UMM) fitted with a membrane having a nominal pore size of 1.1 nm (Amicon UM-2). This membrane allows passage to molecules with molecular weights less than approximately 1000 daltons. The cell was pressurized with nitrogen to 35 psi. When the volume of solution in the cell was reduced to 2 ml, 8 ml water was added to the cell, three times in succession.

The combined ultrafiltrates were applied to the same Dowex column used in Experiment 1; but, the column was eluted in succession with 250 ml each of 1.5 N, 2N, 2.5 N, 3 N, and 4 N hydrochloric acid. The activity of the eluted fractions was monitored by liquid scintillation counting. Radioactive fractions whose elution volumes corresponded to those of proline and ornithine were spotted on paper. The presence of proline was confirmed by spraying with ninhydrin, which gives a characteristic yellow color, and with isatin, which gives a characteristic blue color. The presence of ornithine was confirmed by spraying with ninhydrin, giving a purple color, and with vanillin, giving a green-yellow fluorescence in ultraviolet light.[85]

Purification of Labelled Ornithine

The column fraction containing the greatest amount of ornithine was repeatedly evaporated with water in vacuo to remove excess hydrochloric acid. Ornithine hydrochloride was then recrystallized from water-ethanol.[86] Recrystallization was repeated until paper chromatography (1% diethylamine, 77% ethanol/water (specific gravity 0.872), Rf = 0.39) showed a single radioactive ninhydrin-positive spot.

Purification of Labelled Proline

The column fraction containing the greatest amount of proline was repeatedly evaporated with water in vacuo to remove excess hydrochloric acid. The phenylthiohydantoin derivative was prepared, and was crystallised repeatedly from methanol until no change in $^{3}H:^{14}C$ ratio was observed by liquid scintillation counting.

Preparation of the Phenylthiohydantoin of Proline, 2-phenyl-1-oxo-pyrrolo[1,2-c]imidazolidine-3-thione

The procedure described by W. D. Marshall et al.[87] for the preparation of the phenylthiohydantoin of pipecolic acid was used. Proline hydrochloride (23 mg) was dissolved in aqueous pyridine (50% v/v, 3 ml) and the pH of the solution adjusted to pH 8.6 with sodium carbonate. Phenylisothiocyanate (4 drops) was added and the heterogeneous mixture heated with agitation for 2 hours at 38°C. The mixture was acidified with dilute hydrochloric acid and extracted with chloroform (2 x 2 ml). The extract was concentrated under reduced pressure, the oily residue dissolved in methanolic hydrochloric acid (1 M, 5 ml) and the solution refluxed for one hour. Solvent was evaporated and proline phenylthiohydantoin (20 mg) recrystallized four times from methanol; m.p. 180-180.5°C (uncorrected), literature m.p. 179°C[87a] mass spectrum, molecular ion m/e 232, M - 29, M - 97 (base peak).

Isolation and Purification of Labelled Nicotine

The diethyl ether extract of the basified tobacco extract which had been set aside during the isolation of amino acids (vide supra) was washed with 0.1 M ammonium hydroxide solution and acidified with hydrochloric acid. The resulting mixture of hydrochloride salts was extracted into water and made basic with sodium hydroxide. The free bases were extracted into chloroform. The chloroform solution was dried ($MgSO_4$) to prevent subsequent chloroform-steam distillation of nicotine, and evaporated under reduced pressure. Water (30 ml) and a few crystals of Na_2CO_3 were added and the mixture was steam distilled. Nicotine forms

an azeotropic mixture with water at the boiling temperature, while the other organic bases of tobacco do not.[88] Nicotine was then extracted from the distillate into chloroform, the solution was dried ($MgSO_4$) and evaporated *in vacuo*. The diperchlorate salt was prepared, and was recrystallised repeatedly from ethanol-diethyl ether, with the aid of nicotine diperchlorate seed crystals, until no change in the $^{3}H:^{14}C$ ratio was observed by liquid scintillation counting.

Preparation of Nicotine Diperchlorate

Nicotine (63 mg) was dissolved in a minimal volume of methanol. Perchloric acid (71% aqueous solution, 0.2 ml) was added and the solution cooled. A few drops of diethyl ether was added. The solution was covered lightly and allowed to stand undisturbed for several days, after which time crystals of nicotine diperchlorate appeared. Subsequent recrystallizations from ethanol were accomplished readily with the use of these crystals as seeds; m.p. 208-209°C (uncorrected) (literature m.p. 208-209°C)[20]. Analysis: calculated for $C_{10}H_{16}N_2O_8Cl_2$: C, 33.1%; H, 4.4%; N, 7.7%. Found: C, 33.2%; H, 4.4%; N, 7.7% (Galbraith Laboratories).

A perchlorate analysis was performed by weighing potassium perchlorate precipitated from a cold solution of the perchlorate salt of nicotine to which excess potassium hydroxide was added. Analysis: calculated for $C_{10}H_{16}N_2O_8Cl_2$: ClO_4, 54.7%. Found: ClO_4, 55.4% ± 1.4%.

RESULTS

The $^{3}H:^{14}C$ ratios of the samples of ornithine administered in each of the tracer experiments, and those of the products isolated from these experiments, are shown in Table 1. The percent retention of tritium, relative to carbon-14, within each of the products, using ornithine reisolated from the plant as a reference, is also shown for each experiment. Little difference between the $^{3}H:^{14}C$ ratios of ornithine feeding solutions and the reisolated ornithine was observed. Samples of proline isolated from experiments using DL-[5-^{3}H,5-^{14}C]ornithine as the substrate maintained the $^{3}H:^{14}C$ ratio of the reisolated ornithine. On the other hand, proline isolated from plants utilising DL-[2-^{3}H,5-^{14}C]-ornithine as the substrate lost almost all tritium, while remaining rich in carbon-14. The $^{3}H:^{14}C$ ratios of nicotine samples isolated from experiments using DL-[5-^{3}H,5-^{14}C]ornithine and DL-[2-^{3}H,5-^{14}C]ornithine were respectively, sixty-three percent and forty-six percent of the $^{3}H:^{14}C$ ratios of corresponding reisolated ornithine samples.

Table 1. Incorporation of DL-ornithine into proline and nicotine in Nicotiana tabacum

Expt. no.	Precursor	Products	$^{3}H:^{14}C$ ratio	% retention of ^{3}H
1	DL-[5-^{3}H,5-^{14}C]ornithine		11.9	
		ornithine	12.0 ±0.8 (a)	(100)[c]
		proline	11.4 ±0.1 (b)	95 ±6
		nicotine	7.5 ±0.1 (b)	63 ±4
2	DL-[5-^{3}H,5-^{14}C]ornithine		6.6 ±0.1 (b)	
		ornithine	6.39±0.04 (b)	(100)[c]
		proline	6.13±0.04 (b)	95 ±1
3	DL-[2-^{3}H,5-^{14}C]ornithine		9.6 ±0.6 (a)	
		ornithine	9.99±0.06 (b)	(100)[c]
		proline	0.77±0.06 (b)	7.8±0.6
		nicotine	4.6 ±0.1 (b)	46 ±1

(a) standard deviation of the mean

(b) standard error (95% confidence)

[c] reference compound

Table 2. Incorporation of DL-ornithine into proline in Datura stramonium and Lupinus angustifolius [a]

Plant	Ornithine Fed		Proline	
	Labelling Pattern	^{3}H:^{14}C ratio	^{3}H:^{14}C ratio	% retention of ^{3}H
Datura stramonium	5-^{3}H,5-^{14}C	9.4	10.6	113
Lupinus angustifolius		14.5	14.6	101
Datura stramonium	2-^{3}H,5-^{14}C	8.4	0.55	6.5
Lupinus angustifolius		6.2	0.96	15

[a] R.N. Gupta. personal communication.

DISCUSSION

Ornithine

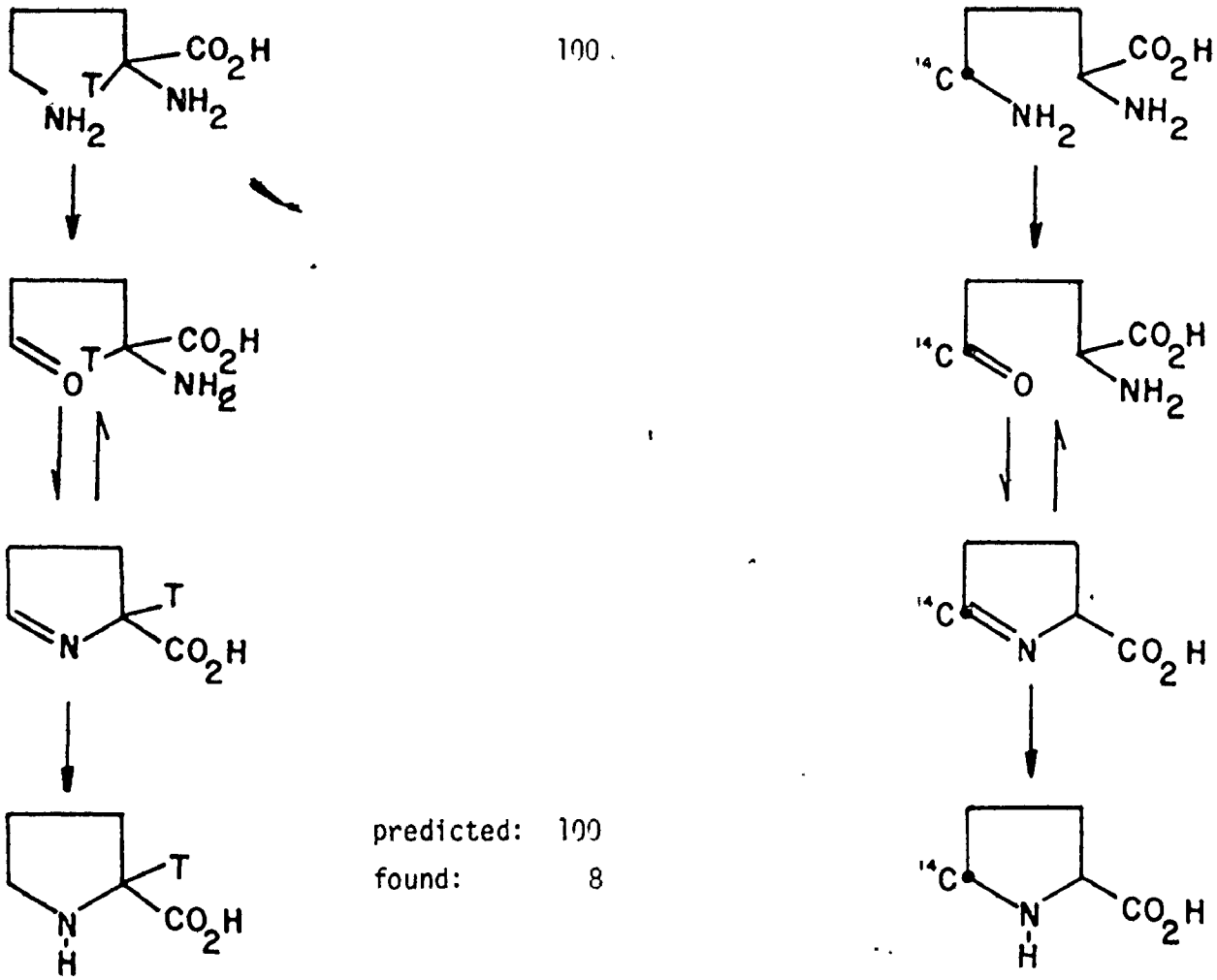
The virtually complete retention of tritium relative to carbon-14 in ornithine recovered from the plant extracts in all three experiments demonstrates that ornithine was absorbed by the plant with little or no isotope effect and that deamination and subsequent reamination of ornithine did not occur to an appreciable extent during the course of the two day feeding experiments. Deamination of the α- or δ-amino group would lead to loss of tritium relative to carbon-14, in [2-^{3}H,5-^{14}C]ornithine or [5-^{3}H,5-^{14}C]ornithine respectively, while an isotope effect in absorption would lead to tritium loss in both.

Proline

The currently accepted model[24,25,27] for the route from ornithine to proline, involving glutamic-γ-semialdehyde (5-oxo-2-aminopentanoic acid) (18) and Δ^{1}-pyrroline-5-carboxylic acid (19), would predict maintenance of the ^{3}H:^{14}C ratio in proline derived from DL-[2-^{3}H,5-^{14}C]ornithine (Figure IV) regardless of stereochemistry, since the carbon bearing tritium is remote from the site of reaction in each step. However, experiment showed only 7.8 per cent retention of tritium relative to carbon-14.

In an experiment using DL-[5-^{3}H,5-^{14}C]ornithine the model would predict reduction of the ^{3}H:^{14}C ratio by one-half in comparison with reisolated ornithine, ignoring secondary isotope effects, regardless of the stereochemical course of the reactions or of the chirality of the

Figure IV. Predicted retention of tritium relative to carbon-14 in proline derived from DL-[2-^{3}H,5-^{14}C]ornithine by stereo-specific or non-stereospecific deamination of ornithine at the δ-position (now disproved).


3H/14C ratio
100
predicted: 100
found: 8

precursor (Figure V). Assuming equal numbers of pro-R and pro-S tritium atoms, loss of either pro-R or pro-S hydrogen nuclides from ornithine in the course of stereospecific oxidative deamination would lead to loss of all tritium from either pro-R or pro-S sites; while non-stereospecific oxidative deamination would lead to loss of one-half of the tritium from both the pro-R and the pro-S sites. Only five per cent loss of tritium relative to carbon-14 was observed experimentally. Thus, the results of this investigation are clearly in disagreement with predictions based on this model.

An alternative model involving α-keto-δ-aminovaleric acid (2-oxo-5-aminopentanoic acid) (26) and Δ^1-pyrroline-2-carboxylic acid, on the other hand, would predict loss of all tritium relative to carbon-14 in the course of oxidative deamination of DL-[2-^{3}H,5-^{14}C]ornithine, producing proline labelled with carbon-14 only (Figure VI). In the complementary experiment, proline derived from DL-[5-^{3}H,5-^{14}C]ornithine would maintain the ^{3}H:^{14}C ratio of the precursor (Figure VII) since the carbon bearing tritium is remote from the site of reaction in each step of the proposed pathway. The data presented in Table 1 are consistent with this model, as are the unpublished results of R. N. Gupta, who performed similar experiments with Datura stramonium and Lupinus angustifolius (Table 2). The results of experiments described in the Introduction using α-^{15}N ornithine and δ-^{15}N ornithine with the Jerusalem artichoke (Helianthus tuberosus)[54,55] also fit the model well.

Further support for this pathway is provided by Meister, Radhakrishnan and Buckley[89] who have shown that separate enzymes exist in the rat for

Figure V. Predicted retention of tritium relative to carbon-14 in proline derived from DL-[5-^{3}H,5-^{14}C]ornithine by stereospecific or non-stereospecific deamination of ornithine at the δ-position (now disproved).

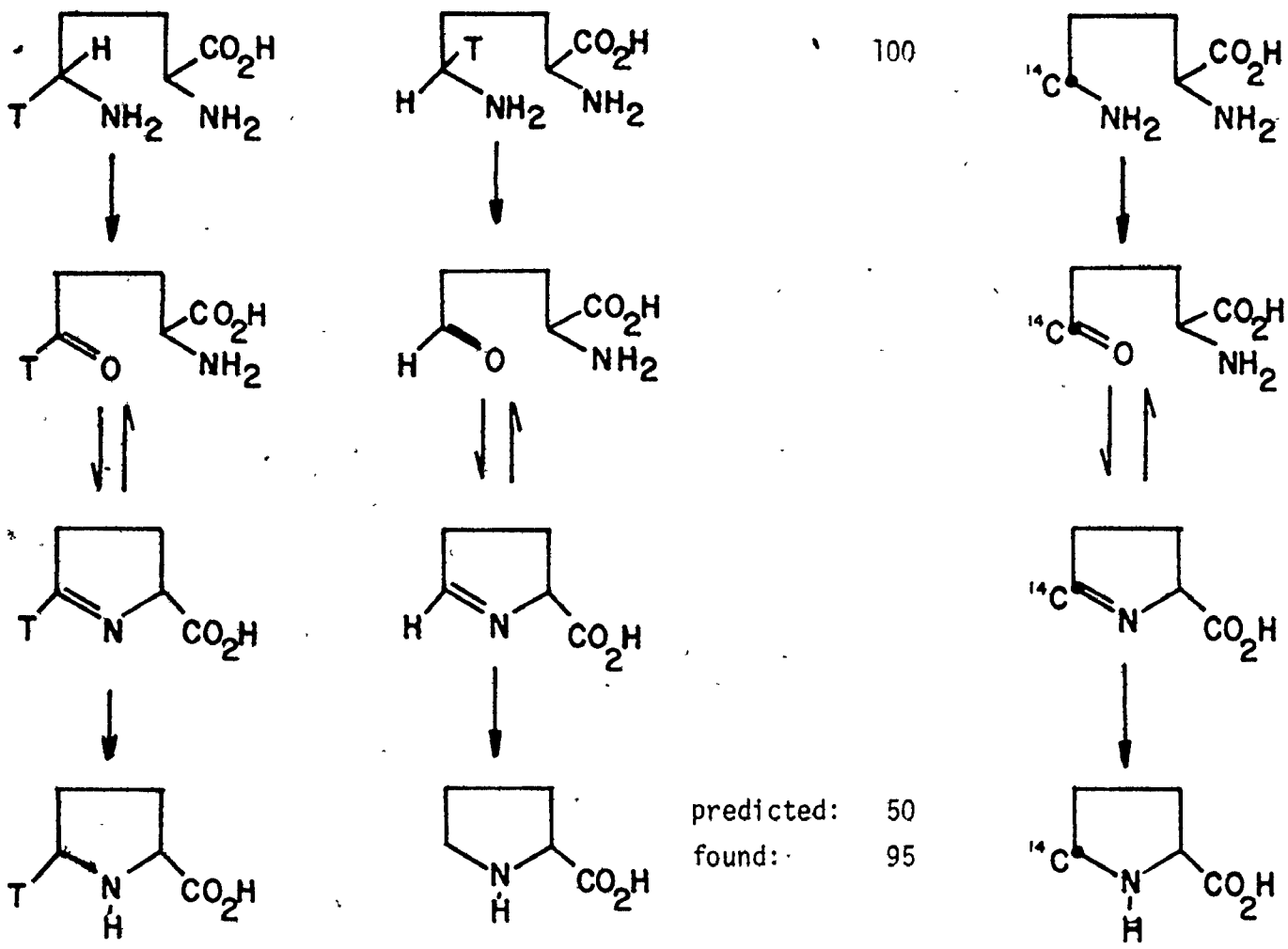
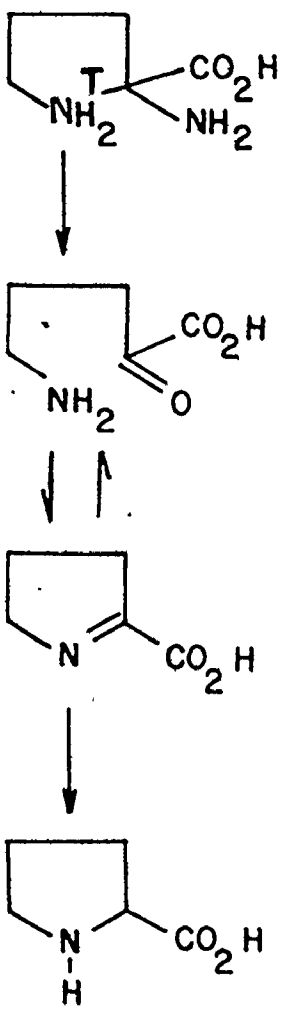

$^3H/^{14}C$ ratio
100
predicted: 50
found: 95

Figure VI. Predicted retention of tritium relative to carbon-14 in proline derived from DL-[2-^{3}H,5-^{14}C]ornithine assuming deamination of ornithine at the α-position.



$^{3}H/^{14}C$ ratio

100

predicted: 0

found: 8

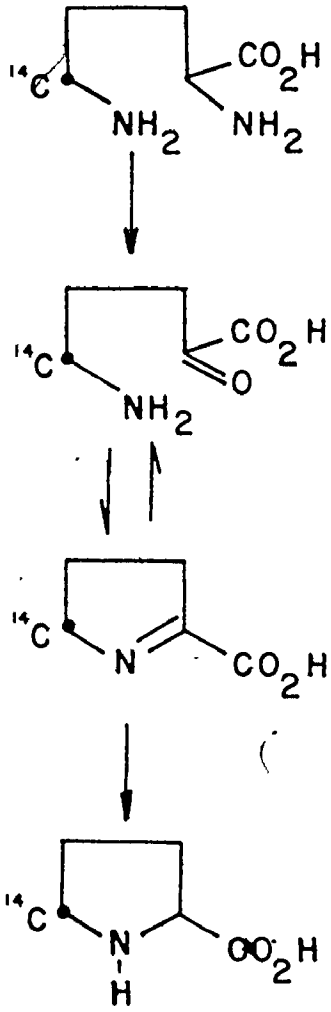
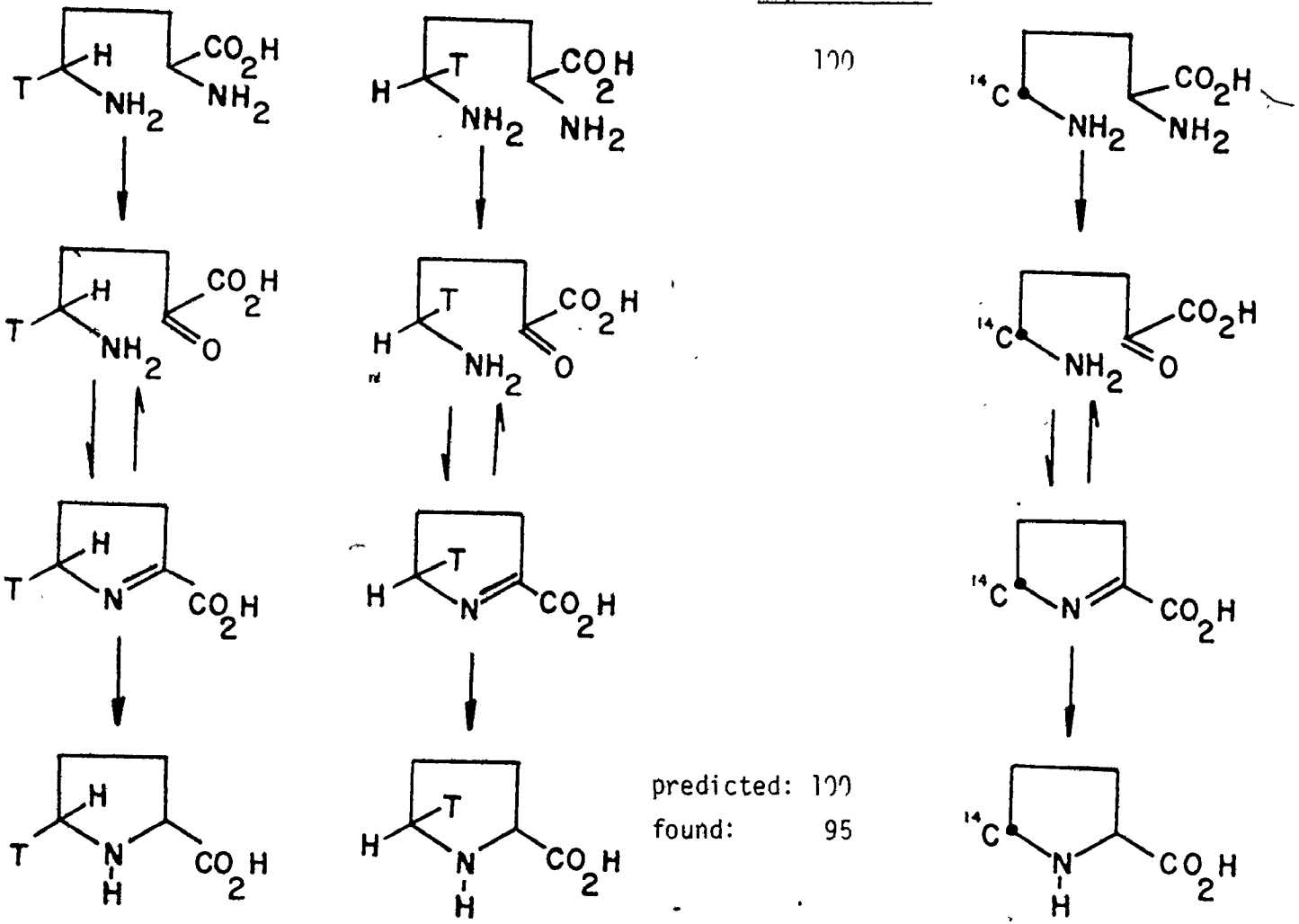


Figure VII. Predicted retention of tritium relative to carbon-14 in proline derived from DL-[5-^{3}H,5-^{14}C]ornithine assuming deamination of ornithine at the α-position.

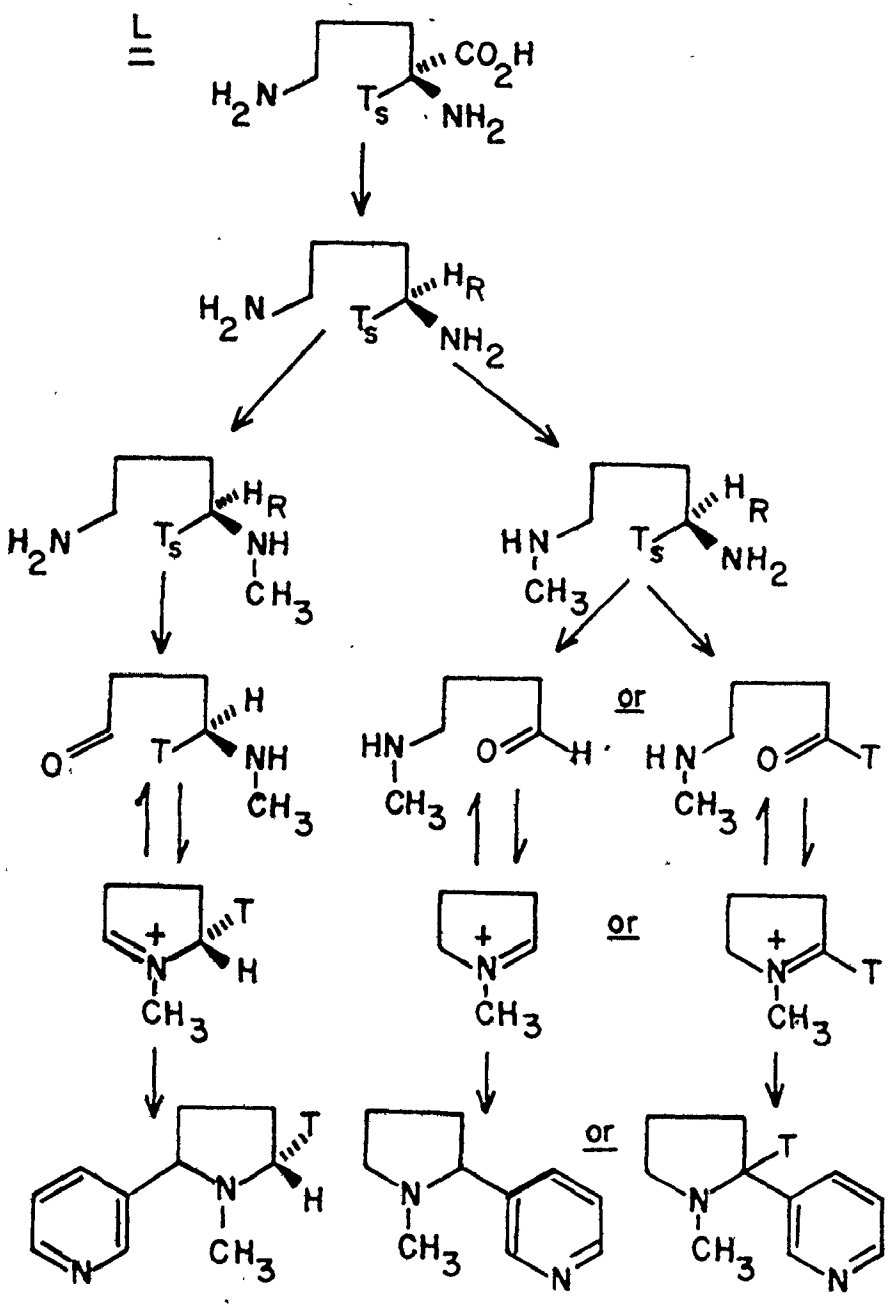

$^3H/^{14}C$ ratio
100
predicted: 100
found: 95

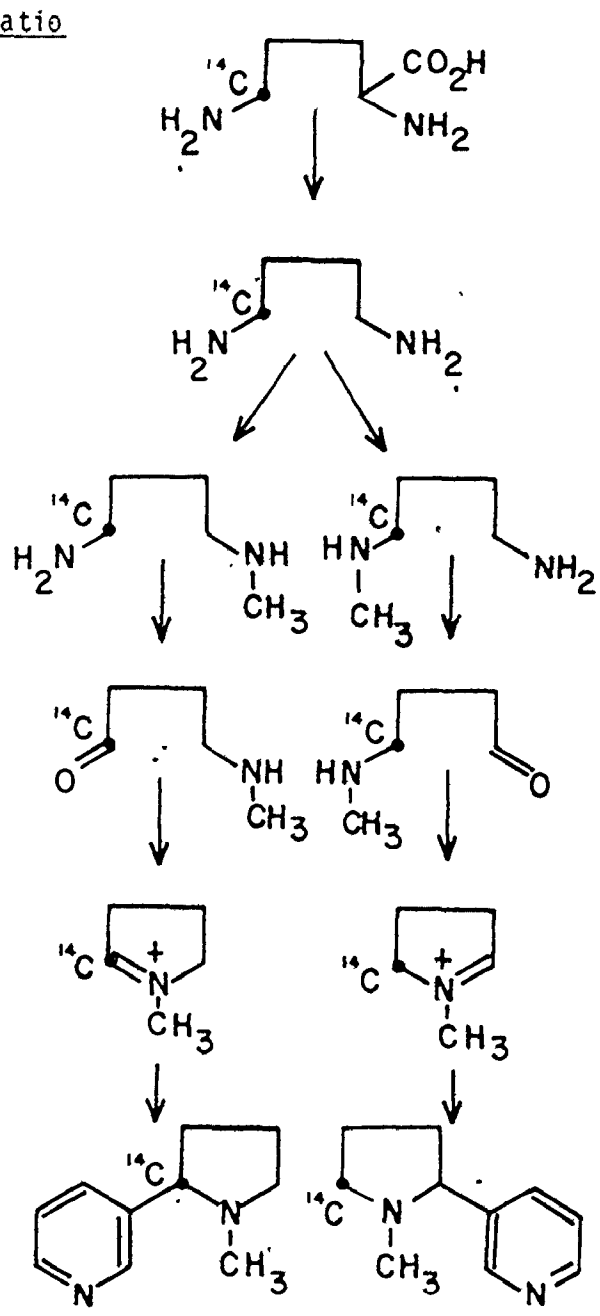
the reduction of Δ^1-pyrroline-2-carboxylic acid and Δ^1-pyrroline-5-carboxylic acid and that the enzyme which catalyses the reduction of Δ^1-piperideine-2-carboxylic acid to pipecolic acid in mammals (rat), fungi (Neurospora crassa), and higher plants (Phaseolus radiatus and Pisum sativum) also catalyses the reduction of Δ^1-pyrroline-2-carboxylic acid in these organisms. The results of this investigation together with Gupta and Spenser's experiments[22] inferring 2-oxo-6-aminohexanoic acid and Δ^1-piperidine-2-carboxylic acid as intermediates in the conversion of lysine to pipecolic acid and Meister's observations indicate the generality of the proposed metabolic pathway in taxonomically diverse organisms and in homologous series of amino acids as predicted by Robinson in 1917.[8]

Nicotine

The currently accepted model[62,72] for the route from ornithine to nicotine supposes decarboxylation of ornithine to putrescine, transmethylation from S-adenosyl-L-methionine to produce N-methylputrescine, followed by oxidative deamination to 4-methylaminobutanal in equilibrium with its cyclised form, N-methyl-Δ^1-pyrrolinium ion, and condensation with nicotinic acid. Assuming, by analogy with results obtained in experiments using lysine,[12] that L-ornithine is the precursor of the pyrroline ring of nicotine and that decarboxylation occurs with retention of configuration, as it does with lysine,[90] the stereospecificity of tobacco diamine oxidase towards N-methylputrescine can be tested in an experiment using DL-[2-^{3}H,5-^{14}C]ornithine as substrate, by comparing the ^{3}H:^{14}C ratios in isolated nicotine and ornithine. Thus, as illutrated in Figure (VIII) decarboxylation of L-[2-^{3}H]ornithine would lead to formation of 1(S)-^{3}H putrescine. Methylation would occur with equal facility at

Figure VIII. Predicted retention of tritium relative to carbon-14 in nicotine derived from L-[2-^{3}H,5-^{14}C]ornithine using Leete's proposed pathway, showing the alternative consequences of stereospecific loss of 4(S)-tritium or 4(R)-hydrogen during deamination of [4(S)-^{3}H]-1-methylamino-4-aminobutane.



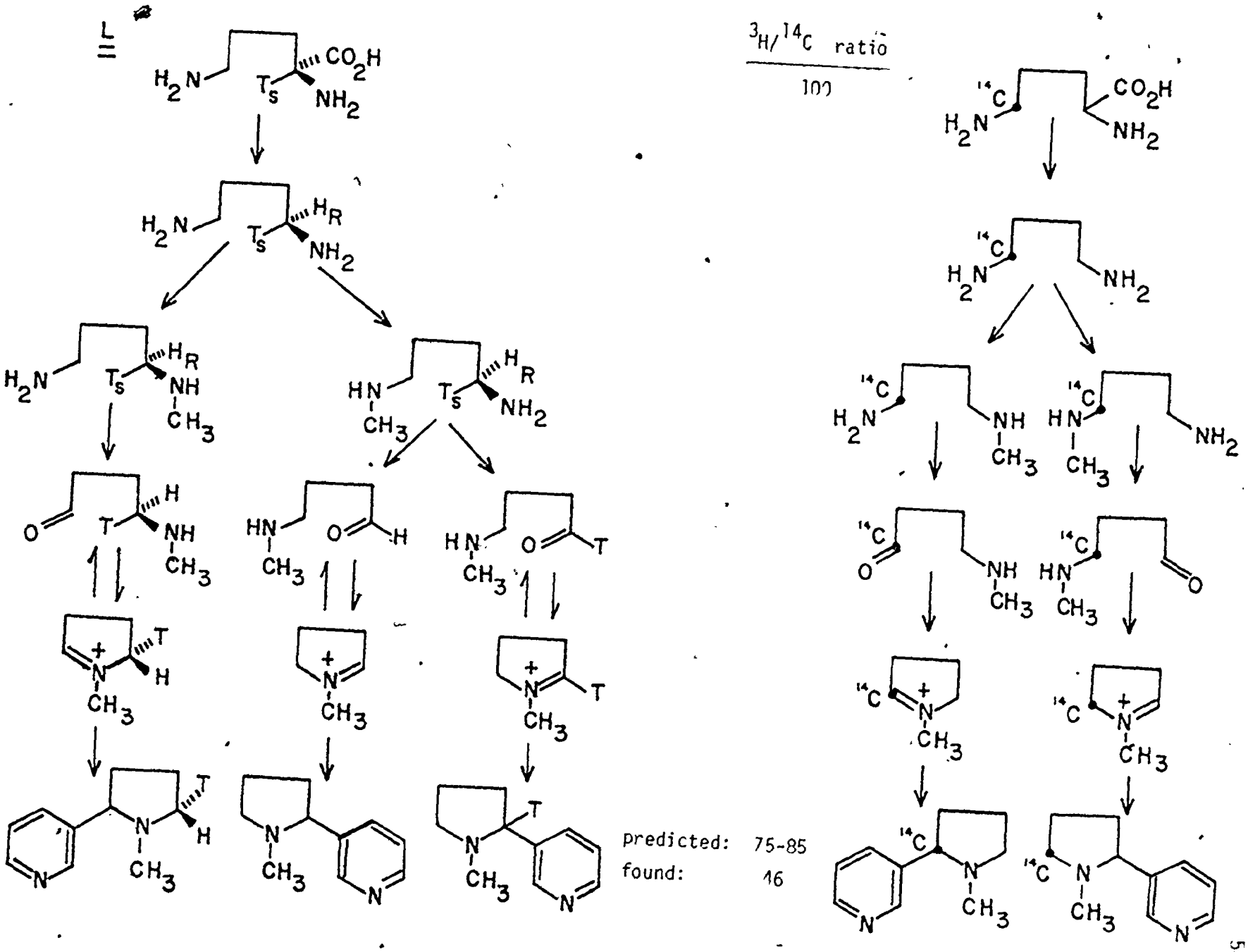


$^3H/^{14}C$ ratio	
100	
predicted: loss of H_R	82-100
loss of T_S	50
found:	46

either nitrogen, yielding equal amounts of [1(S)-^{3}H]-1-methylamino-4-aminobutane and [4(S)-^{3}H]-1-methylamino-4-aminobutane. The former compound would be converted to nicotine with retention of tritium regardless of enzyme stereospecificity or lack of it; while the latter would lose tritium if oxidation were stereospecific with loss of 4(S)-hydrogen or would retain tritium with stereospecific loss of 4(R)-hydrogen. The net retention of tritium would be 100 per cent for stereospecific loss of 4(R)-tritium and 50 per cent for stereospecific loss of 1(S)-tritium, neglecting isotope effects. If the secondary kinetic hydrogen isotope effect k_T/k_H = 0.63 - 0.90 for oxidation of [4-^{3}H]-1-methylamino-4-aminobutane (discussed on page 63) were considered, the expected retention of tritium would be between 82% (50% derived from [1-^{3}H]-1-methylamino-4-aminobutane and 0.63 x 50% = 32% derived from [1-^{3}H]-1-methylamino-4-aminobutane) and 95% relative to carbon-14, for stereospecific loss of 4(R)-hydrogen. No isotope effect would be expected for loss of 4-(S)-hydrogen.

Non-stereospecific oxidation of N-methylputrescine (Figure IX) would produce nicotine with 75% retention of tritium relative to carbon-14 in the absence of isotope effects. Fifty per cent would be derived from oxidation of [1-^{3}H]-1-methylamino-4-aminobutane and twenty-five per cent from oxidation of [4-^{3}H]-1-methylamino-4-aminobutane. However, by analogy with pig diamine oxidase, oxidation of [4-^{3}H]-1-methylamino-4-aminobutane would occur with a primary isotope effect k_H/k_T = 2.7 (from an observed k_H/k_D = 2.0 [91] and Swain's equation[92] $k_H/k_T = (k_H/k_D)^{1.442}$) for tritium loss, while a secondary isotope effect $(k_H/k_T)'$ = 1.11 - 1.59, $((k_T/k_H)'$ = 0.63 - 0.90) would accompany loss of protium. If substrate-enzyme binding occurs without an isotope effect as it does for pig plasma benzylamine oxidase,[93] it follows that the retention of tritium relative to carbon-14 would be:

Figure IX. Predicted retention of tritium relative to carbon-14 in nicotine derived from L-[2-^{3}H,5-^{14}C]ornithine using Leete's proposed pathway, showing the consequences of non-stereospecific loss of hydrogen isotopes during deamination of [4(S)-^{3}H]-1-methylamino-4-aminobutane.

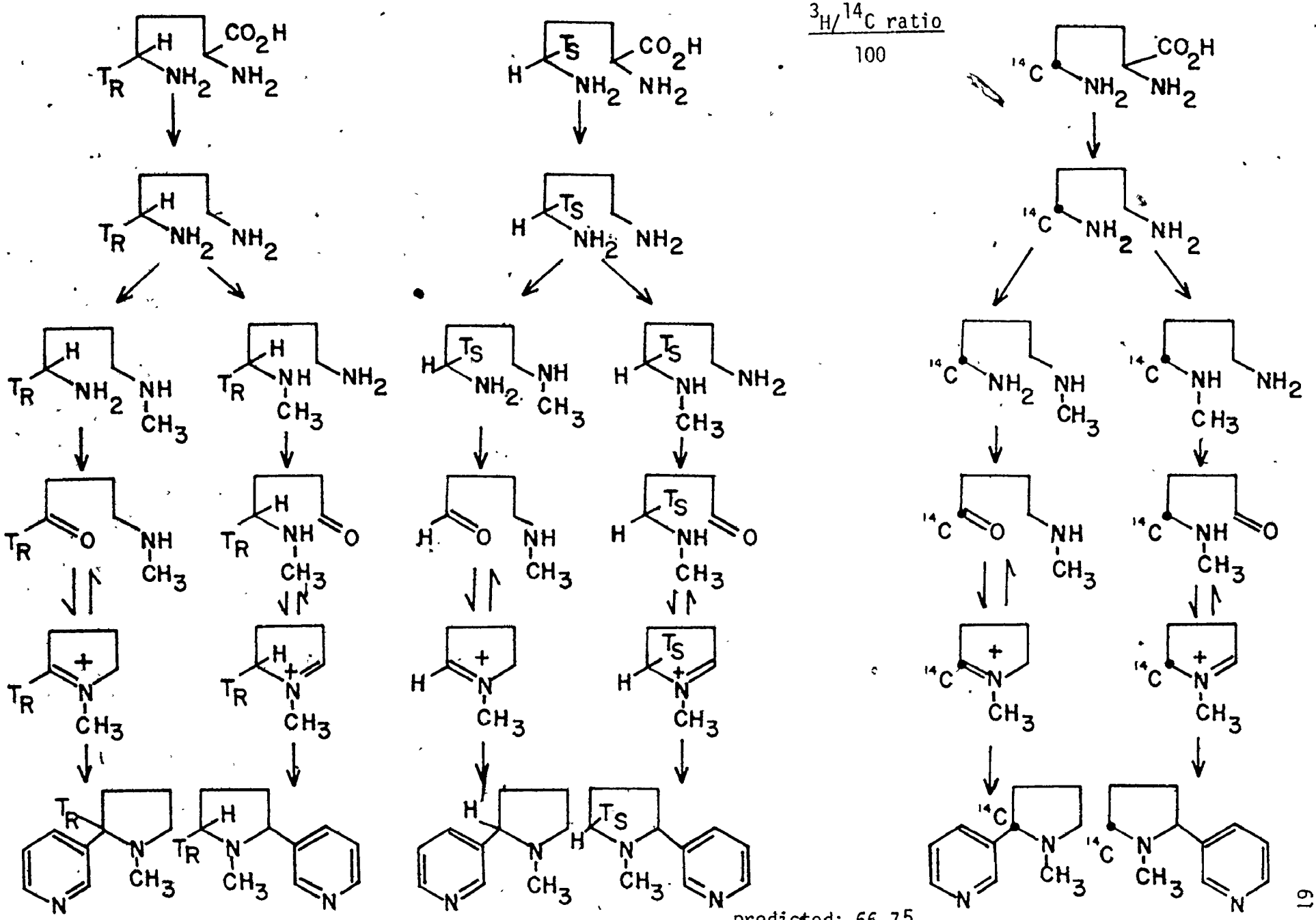

L
$^3H/^{14}C$ ratio
100
predicted: 75-85
found: 16

$$50\% + \left[\frac{(k_T/k_H)'}{(k_T/k_H)' + (k_T/k_H)}\right]50\%$$

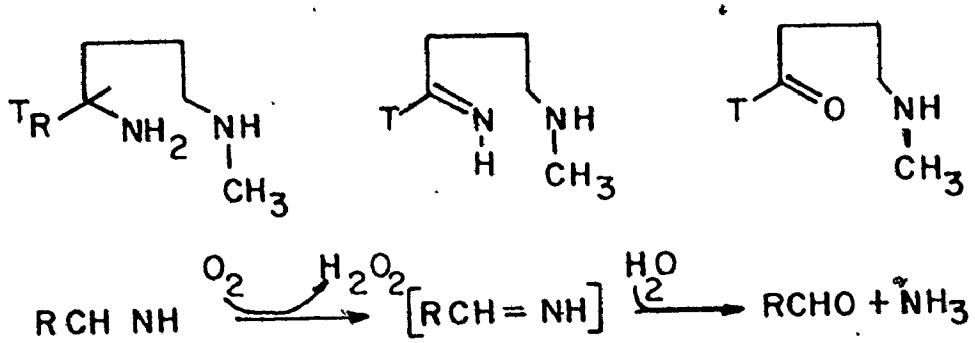
or, between 81% ($50\% + \left[\frac{0.63}{0.63 + 1/2.7}\right]50\%$) and 85% ($50\% + \left[\frac{0.90}{0.90 + 1/2.7}\right]50\%$). The observed retention of 46% tritium relative to carbon-14 in nicotine derived from DL-[2-^{3}H,5-^{14}C]ornithine therefore leads to the inference that oxidation of N-methylputrescine occurs with stereospecific loss of 4(S)-hydrogen.

Using DL-[5-^{3}H,5-^{14}C]ornithine as substrate, Leete's model would predict (Figure X) the formation of [1(R)-^{3}H]putrescine and [1(S)-^{3}H]-putrescine in equal amounts in the course of ornithine decarboxylation. Methylation would yield equal quantities of four isomeric tritiated N-methylputrescines. However, oxidation of one of these isomers, [4(R)-^{3}H]-1-methylamino-4-aminobutane, would be retarded relative to [1(R)-^{3}H]-1-methylamino-4-aminobutane, [1(S)-^{3}H]-1-methylamino-4-aminobutane, and the two isomeric carbon-14 labelled mono-N-methyldiaminobutanes due to a secondary kinetic isotope effect on the rate limiting step, which involves breaking the C-H bond of the amine[91] (Scheme iii). Such a change in hybridisation from sp^3 to sp^2 is usually associated with a secondary isotope effect k_H/k_D between 1.12 and 1.15 [95] which corresponds[92] to k_H/k_T between 1.18 and 1.22. Similarly, cyclisation of the resulting [1-^{3}H]-4-methylaminobutanal to [2-^{3}H]-Δ^1-N-methylpyrrolinium ion would also be retarded due to a secondary kinetic hydrogen isotope effect in the course of hydroxyl elimination to form the Schiff's base (Scheme iv). The secondary kinetic hydrogen isotope effect associated with OH elimination from malic acid by fumarate hydratase was found to be $k_H/k_T = 1.13 \pm 0.02$ [96] (or between 1.11 and 1.15); although secondary kinetic isotope effects for solvolytic

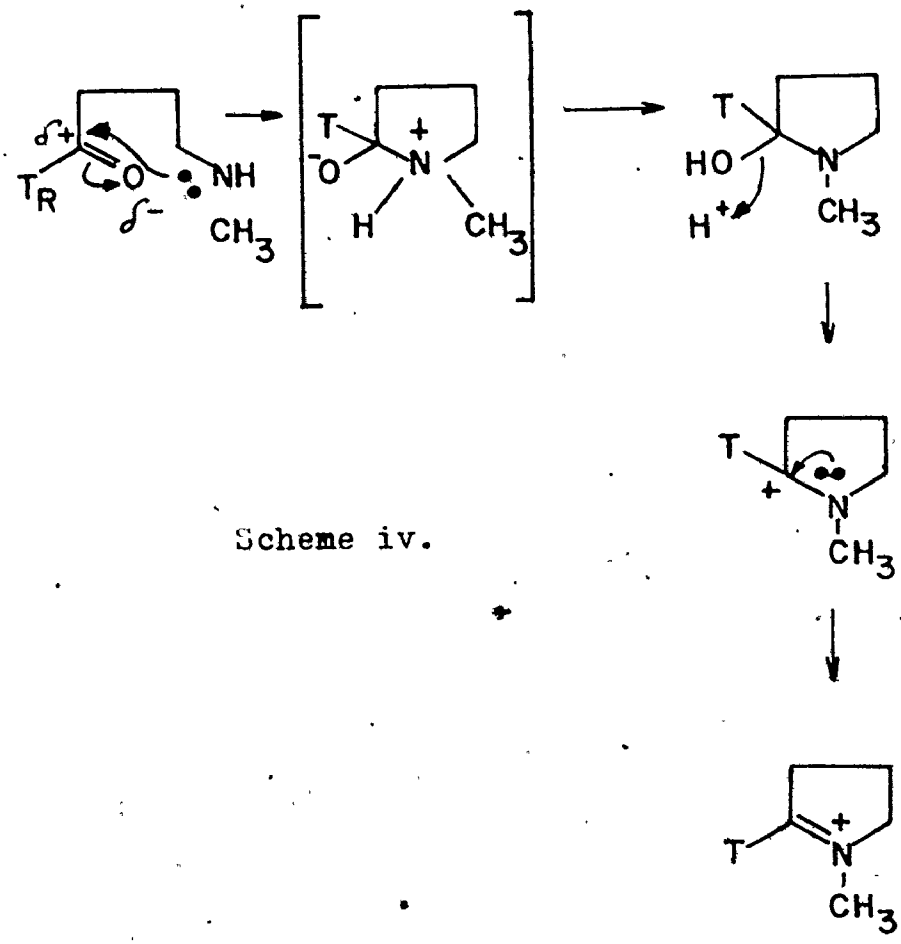
Figure X. Predicted retention of tritium relative to carbon-14 in nicotine derived from DL-[5-^{3}H,5-^{14}C]ornithine using Leete's proposed pathway.


$^3H/^{14}C$ ratio
100
predicted: 66-75
found: 63±4

Scheme iii.



Scheme iv.



reactions involving similar carbonium ion intermediates commonly have values as high as $k_H/k_D = 1.20$ [96] which is equivalent to $k_H/k_T = 1.20$. Therefore a reasonable secondary isotope effect for Schiff's base formation, assuming rate-limiting E_1 elimination would be k_H/k_T between 1.11 and 1.30.

Oxidation of [4(S)-^{3}H]-1-methylamino-4-aminobutane leads to inactive nicotine and loss of 25% of the tritium, relative to carbon-14, contained in the ornithine precursor. If isotope effects were ignored, the ^{3}H:^{14}C ratio in nicotine would be 75% of that in the DL-[5-^{3}H,5-^{14}C]-ornithine precursor, in poor agreement with experiment. But, if the proposed isotope effects for N-methyldiaminobutane oxidation and for N-methylpyrrolinium ion formation are considered, the range of the combined isotope effect k_T/k_H is between $\frac{1}{1.11}$ and $(\frac{1}{1.22} \times \frac{1}{1.30})$ or between 0.90 and 0.63. The lower limit would apply if Schiff's base formation is rate limiting and the carbonium ion is not well-developed in the transition state; while the upper limit would apply if oxidation and Schiff's base formation have similar rate constants and if the carbonium ion is well-developed in the transition state leading to N-methylpyrrolinium ion.

Since [4(R)-^{3}H]-1-methylamino-4-aminobutane accounts for 25% of the tritium relative to carbon-14 in the ornithine substrate, [2'-^{3}H]-nicotine, to which it is metabolised, would retain between (0.63 x 25% = 16%) and (0.90 x 25% = 22.5%) of tritium relative to carbon-14. The two isomers of [5'-^{3}H]nicotine would each retain 25% tritium relative to carbon-14. A total of between 66% and 72.5% retention of tritium is therefore expected. This is in good agreement with the experimentally observed

63% ± 4% retention of tritium relative to carbon-14 in nicotine derived from DL-[5-^{3}H,5-^{14}C]ornithine.

SUMMARY

It was noted in the Introduction that the primary metabolism of amino acids is considered by some to be a closed subject in biochemical research. This investigation shows the folly of such opinions. Interpretations of the results of inconclusive experiments, no matter how plausible, must be tested when more discriminating experimental techniques become available. The results of experiments described herein illustrate the utility of tritium, carbon-14 double labelling techniques in the elucidation of metabolic pathways. δ-Deamination of ornithine, the classical microbiological pathway for proline biosynthesis, was found to be an untenable hypothesis for proline biosynthesis in plants; while experiment supported a scheme based on α-deamination of ornithine. It would be interesting to study other metabolic relationships outlined in Figure II using tritium, carbon-14 double labelling techniques in plants and in other organisms.

There is an unexpressed controversy about the pathway involved in proline formation from ornithine in birds, paralleling that which existed for plants. The current opinion among those writing in English language journals[97] is that δ-deamination of ornithine is involved, while contradictory results of nitrogen-15 experiments, performed by Boulanger and coworkers[98] supporting α-deamination are ignored. Tritium, carbon-14 double labelling experiments similar to those described in this thesis would resolve the controversy.

The next step in the investigation of pyrrolidine ring biosynthesis in plants is to establish which ornithine enantiomer is involved in each of the two pathways leading to proline and nicotine. The double labelling technique of Leistner, Gupta and Spenser[12] described earlier is ideally suited to the problem. Using this approach, L-ornithine labelled with tritium on carbon-3 or on carbon-4, remote from reactive sites, would be administered together with DL-ornithine labelled with carbon-14 at any site other than carbon-1. Products derived from L-ornithine would have $^{3}H:^{14}C$ ratios equal to twice the $^{3}H:^{14}C$ ratio of the administered ornithine; while products derived from D-ornithine would lose all tritium relative to carbon-14.

REFERENCES

1. G. V. Hevesy and L. Zechmeister, Ber. Dtsch. Chem. Ges. 53, 410 (1920).
2. G. V. Hevesy, Biochem. J. 17, 439 (1923).
3. S. Ruben, W. Z. Hassid and M. D. Kamen, J. Am. Chem. Soc. 61, 661 (1939).
4. L. W. Alvarez and R. Cornog, Phys. Rev. 56, 613 (1939).
5. S. Ruben and M. D. Kamen, Phys. Rev. 57, 549 (1940).
6. M. D. Kamen. A Tracer Experiment. Holt, Rinehart and Winston, New York. 1964.
7. E. Winterstein and G. Trier, Die Alkaloide, Bornträger, Berlin, 1910.
8. R. Robinson, J. Chem. Soc. 111, 876 (1917).
9. D. P. Zilversmit, C. Entenman and M. C. Fishler, J. Gen. Physiol. 26, 325 (1943).
10. I. D. Spenser, In Chemistry of the Alkaloids, Edited by S. W. Pelletier, Van Nostrand Reinhold, New York, 1970.
11. S. A. Brown, In Biosynthesis, Vol. I, Edited by T. A. Geissman, The Chemical Society, Specialist Periodical Reports, London, 1972.
12. E. Leistner, R. N. Gupta and I. D. Spenser, J. Am. Chem. Soc. 95, 4040 (1973).
13. E. Leete, J. Am. Chem. Soc. 84, 55 (1962).
14. T. J. Gilbertson, Phytochemistry 11, 1737 (1972).
15. K. J. Figenshou, L. O. Frøholm and S. G. Laland, Biochem. J. 105, 451 (1967).
16. H. G. Floss, P. E. Manni, R. L. Hamill and J. A. Mabe, Biochem. Biophys. Res. Commun. 45, 781 (1971).
17. E. Pflüger, Pflüger's Arch. Gesammte Physiol. Menschen Thiere, 18, 249 (1878).

18. R. Robinson, J. Chem. Soc. 111, 762 (1917).

19. E. Leete, L. Marion and I. D. Spenser, Nature, 174, 650 (1954).

20. E. Leete, Chem. Ind. (London), 537 (1955).

21. L. J. Dewey, R. U. Byerrum and C. D. Ball, Biochim. Biophys. Acta, 18, 141 (1955).

22. R. N. Gupta and I. D. Spenser, Phytochemistry, 9, 2329 (1970).

23. E. Leistner and I. D. Spenser, J. Am. Chem. Soc. 95, 4715 (1973).

24. L. Fowden, in Plant Biochemistry, Edited by J. Bonner and J. E. Varner, Academic Press, New York, 1965.

25. L. Fowden, Annu. Rev. Plant. Physiol. 18, 85 (1967).

26. L. Fowden, in Phytochemistry, Vol. II, Edited by L. P. Miller, Van Nostrand Reinhold, New York, 1973.

27. B. J. Mifflin, in Biosynthesis and Its Control in Plants, Edited by B. V. Milborrow, Academic Press, London, 1973.

28. I. W. Goodwin and E. I. Mercer, Introduction to Plant Biochemistry, Pergamon Press, London, 1973.

29. D. D. Davies, J. Giovanelli and T. ApRees, Plant Biochemistry, Blackwell Scientific Publications, Oxford, 1964.

30. T. Robinson, The Organic Constituents of Higher Plants, Second Edition, Burgess Publishing, Minneapolis, 1967.

31. A. Meister, Biochemistry of the Amino Acids, Vol. II, Second Edition, Academic Press, New York, 1965.

32. J. R. Matoon, in Biogenesis of Natural Compounds, Second Edition, Edited by P. Bernfeld, Pergamon Press, Oxford, 1967.

33. D. M. Greenberg, Metabolic Pathways, Third Edition, Vol. III, Academic Press, New York, 1969.

34. S. Dagley and D. E. Nicholson, An Introduction to Metabolic Pathways, Blackwell Scientific Publications, Oxford, 1970.

35. C. J. Morris, J. F. Thompson and C. M. Johnson, Plant Physiol. 44, 1023 (1969).

36. M. Noguchi, A. Koiwai and E. Tamaki, Agric. Biol. Chem. 30, 452 (1966).

37. M. Staub and G. Denes, Biochim. Biophys. Acta, 128, 82 (1966).

38. R. G. Coleman and M. P. Hegarty, Nature (London), 179, 376 (1957).

39. W. I. Scher and H. J. Vogel, Proc. Nat. Acad. Sci. U.S.A. 43, 796 (1957).

40. H. J. Vogel and B. D. Davis, J. Am. Chem. Soc. 74, 109 (1952).

41. A. Meister, J. Biol. Chem. 206, 577 (1954).

42. K. Hasse, O. T. Ratych and J. Salnikow, Hoppe-Seyler's Z. Physiol. Chem. 348, 843 (1967).

43. D. H. Bone, Plant Physiol. 34, 171 (1959).

44. A. S. Seneviratne and L. Fowden, Phytochemistry, 7, 1047 (1968).

45. G. C. Webster, Nitrogen Metabolism in Plants, Ron Peterson and Co., Evanston, 1959.

46. E. E. Snell, J. Am. Chem. Soc. 67, 194 (1945).

47. D. E. Metzler and E. E. Snell, J. Am. Chem. Soc. 74, 979 (1952).

48. M. Mazelis and L. Fowden, Phytochemistry, 8, 801 (1969).

49. M. Mazelis and T. S. Lu, Plant Physiol. 47, 17 (1971).

50. T. S. Lu and M. Mazelis, Plant Physiol. 55, 502 (1975).

51. W. E. Splittstoesser and L. Fowden, Phytochemistry, 12, 785 (1973).

52. M. Mazelis and R. K. Crevelling, Phytochemistry, 13, 559 (1974).

53. R. L. Wickremasinghe and T. Swain, Phytochemistry, 4, 687 (1965).

54. H. Duranton and R. Wurtz, C. R. Hebd. Seances, Acad. Sci. 259, 2506 (1964).

55. H. Duranton and R. Wurtz, Physiol. Veg. 3, 7 (1965).

56. H. W. Liebisch and H. R. Schütte, Z. Pflanzenphysiol. 57, 434 (1967).

57. E. Leete and K. J. Siegfried, J. Am. Chem. Soc. 79, 4529 (1957).

58. B. L. Lamberts and R. V. Byerrum, J. Biol. Chem. 233, 939 (1958).

59. A. A. Liebman, B. P. Mundy and H. J. Rapoport, J. Am. Chem. Soc. 89, 664 (1967).

60. E. Leete, E. G. Gros and T. J. Gilbertson, Tetrahedron Letters, 587 (1964).

61. I. D. Spenser, in Comprehensive Biochemistry, Vol. XX, Edited by M. Florkin and E. H. Stotz, Elsevier, Amsterdam, 1968.

62. E. Leete, Adv. Enzymol. Rel. Areas Mol. Biol. 32, 373 (1969).

63. R. B. Herbert, in The Alkaloids, Vol. III, Edited by J. E. Saxton, The Chemical Society, Specialist Periodical Reports, London, 1973.

64. R. B. Herbert, in The Alkaloids, Vol. V, Edited by J. E. Saxton, The Chemical Society, Specialist Periodical Reports, London, 1975.

65. E. Leete, in Biosynthesis, Vol. II, Edited by T. A. Geissman, The Chemical Society, Specialist Periodical Reports, London, 1973.

66. E. Leete, in Biosynthesis, Vol. IV, Edited by T. A. Geissman, The Chemical Society, Specialist Periodical Reports, London, 1976.

67. E. Leete, J. Am. Chem. Soc. 80, 2162 (1958).

68. D. Yoshida, Plant Cell Physiol. 10, 393 (1969).

69. S. Mizusaki, Y. Tanabe, M. Noguchi and F. Tamaki, Plant. Cell Physiol. 12, 633 (1971).

70. H. R. Schütte, W. Meier and K. Mothes, Acta. Biochim. Polon. 13, 401 (1966); reported in (62) (E. Leete, Adv. Enzymol. Rel. Areas Mol. Biol. 32, 373 (1969).)

71. S. Mizusaki, T. Kisaki and E. Tamaki, Plant Physiol. 43, 93 (1968).

72. E. Leete, J. Am. Chem. Soc. 89, 7081 (1967).

73. S. Mizusaki, Y. Tanabe, M. Noguchi and E. Tamaki, Phytochemistry 11, 2757 (1972).

74. M. L. Rueppel, B. P. Mundy and H. Rapoport, Phytochemistry 13, 141 (1974).

75. H. R. Zielke, R. V. Byerrum, R. M. O'Neal, L. C. Burns and R. E. Koeppe, J. Biol. Chem. 243, 4757 (1968).

76. C. R. Hutchinson, M-T. S. Hsia and R. A. Carver, J. Amer. Chem. Soc. 98, 6006 (1976).

77. E. Leete, J. Org. Chem. 41, 3438 (1976).

78. L. Pichat, J. Mizon and M. Herbert, Bull. Soc. Chim. Fr. 1787 (1963).

79. N. F. Albertson and S. Archer, J. Am. Chem. Soc. 67, 2043 (1945).

80. R. N. Gupta, personal communication.

81. R. S. Hendler, Anal. Biochem. 7, 110 (1964).

82. W. H. Stein and S. Moore, Cold Spring Harbour Symp. Quant. Biol. 14, 179 (1949).

83. W. F. Blatt, B. G. Hudson, S. M. Robinson and E. M. Zipilivan, Nature (London), 216, 511 (1967).

84. C. Dennison, Anal. Biochem. 58, 646 (1974).

85. G. Curzon and J. Giltrow, Nature (London), 172, 356 (1953).

86. J. P. Greenstein and M. Winitz, Chemistry of the Amino Acids, Vol. III, John Wiley and Sons, New York, 1961, p. 2478.

87. W. D. Marshall, T. T. Nguyen, D. B. MacLean and I. D. Spenser, Can. J. Chem. 53, 41 (1975).

87a. R. M. C. Dawson, D. C. Elliott, W. H. Elliott and K. M. Jones, Data for Biochemical Research, Second Edition, Oxford University Press, New York, 1969, p. 53.

88. C. R. Smith, Ind. Eng. Chem., 34, 251 (1942). Quoted in L. Marion, in The Alkaloids, Vol. I, Edited by R. H. F. Manske and H. L. Holmes, Academic Press, New York, 1950, p. 231.

89. A. Meister, A. N. Radhakrishnan and S. D. Buckley, J. Biol. Chem., 229, 789 (1957).

90. E. Leistner and I. D. Spenser, J. Chem. Soc. Chem. Commun. 378 (1975).

91. W. G. Bardsley, M. J. C. Crabbe and J. S. Shindler, Biochem. J. 131, 459 (1973).

92. C. G. Swain, E. C. Stivers, J. F. Reuwer and L. J. Schaad, J. Am. Chem. Soc. 80, 5885 (1958).

93. B. Olsson, J. Olsson and G. Petterson, Eur. J. Biochem. 64, 327 (1976).

94. A. R. Battersby, J. Staunton and M. C. Summers, J. Chem. Soc. Perkin Trans. 1, 1052 (1976).

95. H. Simon and D. Palm, Angew. Chem. Internat. Ed. 5, 920 (1966).

96. D. E. Schmidt, Jr., W. G. Nigh, C. Tanzer and J. H. Richards, J. Am. Chem. Soc. 91, 5849 (1969).

97. R. E. Austic, Fed. Proc., Fed. Am. Soc. Exp. Biol. 35, 1914 (1976).

98. P. Boulanger, J. Corsaget, J. Bertrand and R. Osteux, C. R. Hebd. Seances Acad. Sci. 244, 2255 (1957).