PYRROLIDINE RING BIOSYNTHESIS IN TOBACCO

THE STEREOCHEMISTRY OF PYRROLIDINE RING BIOSYNTHESIS IN TOBACCO

by

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ABSTRACT

In four separate experiments, DL-[5-^{3}H]/DL-[5-^{14}C]ornithine, L-[5-^{3}H]/DL-[5-^{14}C]ornithine, D-[5-^{3}H]/DL-[5-^{14}C]ornithine and L-[2-^{3}H]/L-[5-^{14}C]ornithine were administered to intact tobacco plants (Nicotiana tabacum). Nicotine, ornithine and proline were isolated in each of these experiments. In another experiment, R-[1-^{2}H][1,4-^{14}C] putrescine was administered to intact tobacco plants and nicotine was isolated. The results of these experiments are consistent with the accepted mode of biosynthesis of nicotine from ornithine via putrescine (1,4-diaminobutane), N-methylputrescine, N-methyl-4-aminobutanal and N-methyl-1-pyrrolinium ion. The ^{3}H:^{14}C ratios of nicotine, the distribution of tritium within nicotine as established by chemical degradation and the distribution of deuterium within nicotine as established by ^{2}H NMR are interpreted as showing that L-ornithine is the preferred enantiomer for nicotine biosynthesis, that the decarboxylation of L-ornithine to yield putrescine proceeds with retention of configuration at the reaction site, and that the oxidation of N-methylputrescine to N-methyl-4-aminobutanal proceeds with loss of the 4(S)-hydrogen.

Contrary to earlier reports, ornithine isolated in the ^{3}H,^{14}C experiments had a changed ^{3}H:^{14}C ratio from the ornithine which was fed. These results are interpreted as showing that L-ornithine is

metabolised more rapidly than is D-ornithine in the tobacco plant.

In all $^{3}H,^{14}C$ experiments, proline was found to contain at least a small amount of tritium. In particular, when L-[2-^{3}H]/L-[5-^{14}C] ornithine served as substrate, proline was found to contain 40 ± 1% of the tritium, relative to ^{14}C, that had been present in the feeding material. This result is interpreted as showing that, contrary to earlier reports, L-ornithine can be converted into proline via either α-keto-δ-aminovaleric acid or glutamic semialdehyde. Together with the $^{3}H:^{14}C$ ratios of proline in the other experiments, the results of this work are interpreted as showing that, when DL-ornithine serves as the substrate for proline biosynthesis in tobacco, 88 ± 1% of the proline arises from D-ornithine via α-oxidation, 7 ± 1% of the proline comes from L-ornithine via α-oxidation and 5 ± 1% of the proline is produced from L-ornithine via δ-oxidation.

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I would like to thank Dr. Spenser for his help, his encouragement, his suggestions and insights, but most especially for his patience in guiding me through this work.

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INTRODUCTION

Design of Tracer Experiments

Biosynthetic tracer experiments are intended to provide investigators with improved understanding of the chemical reactions which convert precursors into the products of interest in a biological system. These experiments take several forms, but all are dependent upon a specific knowledge of the isotopic labelling pattern of the (putative) precursor which is administered, and upon determination of the presence and location of label in the product.[1,2] Both radioactive and stable isotopes have been put to effective use in tracer studies, and both have advantages and disadvantages associated with their use.

When radioactive labels are used, a high dilution of the tracer can be tolerated, often as high as one part in 10^7 or 10^8. This is due, in part, to the availability of high specific activity compounds - often virtually carrier-free at labelled sites - and in part to the high sensitivity and efficiency of the equipment available for the quantitative determination of radioactivity, such as modern scintillation counting equipment. Since a high dilution is acceptable, it is possible to administer amounts of compound which are chemically minute. This helps to minimize the perturbation of the many complex chemical equilibria present in a living organism, so that observations better approximate an accurate representation of what is happening in the normal organism.[2]

In order to determine in what manner the precursor was incorporated, however, it is necessary to perform an unambiguous degradation of the product, to establish where the activity of the product resides.[1] This requires chemical manipulation of small quantities of pure substance.

In the feeding of precursors labelled with stable isotopes, care must be taken to eliminate the possibility that incorporation of label into product is a consequence of the high concentrations of compound administered.[2] The need for larger amounts of precursor lies in the normal presence of detectable amounts of stable isotopes in unlabelled material. For example, ^{13}C has a natural abundance of about one per cent. If a labelled precursor were incorporated with a specific incorporation of 0.5% (more than sufficient if the label is ^{14}C), then unlabelled positions would contain 1% ^{13}C (natural abundance) and labelled positions would contain 1 + 0.5 = 1.5% ^{13}C. This is only 50% above background and it becomes exceedingly difficult, at this level, to garner any information as to the relative efficiencies of incorporation of label into various positions of the product .[3,4] At even lower levels of specific incorporation, which are not uncommon (e.g. 4), it becomes difficult to determine whether the precursor is incorporated at all unless special techniques are used (vide infra). The limit of detection for ^{13}C not bonded to another isotopic label which is magnetically active is generally agreed to be approximately 0.2% specific incorporation.[5,6]

Low concentration of label may also be a result of the method by which the product is isolated. If the product is present in only minute quantities in the organism, it is common practice, in experi-

ments where radioactive tracers are used, to add comparatively large amounts of unlabelled compound to act as a 'carrier' of the biosynthetic (i.e. labelled) molecules through the various isolation and purification steps (e.g. 5,7). While this carrier dilution technique is often well tolerated when radiotracers are used, the addition of large amounts of carrier to a stable isotope experiment essentially raises the 'background' of natural abundance material. For example, if only 2 μg of product is produced during an experiment, it will be necessary to add unlabelled carrier in order to isolate it. If 1 mg of carrier is added, itself a small amount to handle and isolate, even if the specific incorporation into the product were 100%, the five hundred-fold dilution with carrier reduces this to an effective 0.2%; labelled positions would contain 1 + 0.2 = 1.2% ^{13}C while unlabelled positions would contain 1% ^{13}C. If the incorporation is any lower, the amount of product any smaller, or the minimum amount of material (and therefore carrier) which can be practicably isolated any larger, this experiment may not be feasible.

This example is representative of the situation which might obtain in an experiment with microorganisms, where the great majority of the individual organisms, and therefore of the product, is produced in the course of the experiment.

The problem of isotopic dilution is compounded further in cases where a significant amount of the desired product is biosynthesised prior to the beginning of the experiment. An example of this situation is the study of biosynthesis in plants (e.g. 4,5). A comparatively large amount of product may already be present at the start of the experiment, and if the duration of the experiment is short, relative to

the age of the plant, the fraction of product isolated which is synthesised during the experiment will be small. This amounts to carrier dilution by endogenous material which, in turn, results in a lower effective specific incorporation of label.

To combat the problems caused by the natural abundance of stable isotopes, these experiments are often done on a larger scale, and the experiments are continued for a longer time to allow larger amounts of the products to accumulate (e.g. 8).

One of the great advantages of stable labelling techniques is that the sites of incorporation of label can often be determined by direct examination of the product, using NMR.[1] This can result in a large savings of time during analysis, although it may only offset an increased amount of time and effort required in the earlier stages of the experiment.

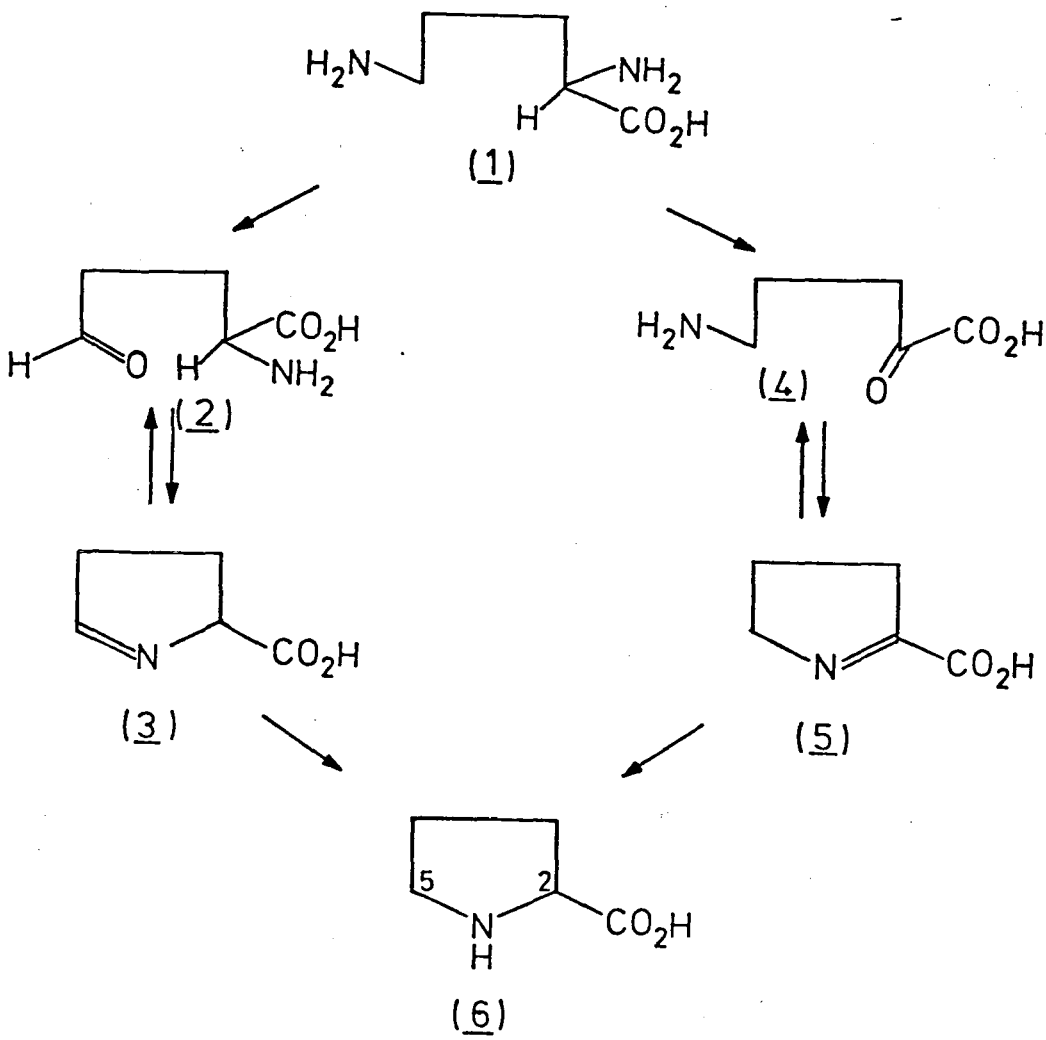
While it is true that mass spectral analysis (M.S.) is also a viable method for establishing isotopic enrichment, it has some disadvantages in biosynthetic investigations which it does not share with NMR. M.S. is a destructive technique whereas with NMR, many experiments can be performed with the same sample. It can also be very difficult to analyse the results from M.S. The fragmentations which occur in a mass spectrometer often involve loss of multi-atom fragments from the parent ion.[9] Therefore, although the incorporation of an isotopic label into a given fragment can generally be inferred, it may be difficult to establish the exact location of the label within that fragment, and therefore within the original molecule. This can be overcome, in part, by chemically synthesizing samples of the product which are isotopically

enriched in various positions, and comparing the mass spectra of these compounds with those of the biosynthetically derived compounds (e.g. 10). This approach entails a great deal of work which might be avoided with NMR as the chosen method of analysis.

Advances in recent years have made NMR a very useful and flexible tool for biosynthetic investigations. Consequently, examples in this introduction have been chosen which use NMR rather than M.S. to determine the mode of incorporation of stable isotopes in solving biosynthetic problems.

While the incorporation of activity into the product gives an indication of precursor-product relationships, there is certainly no assurance that the original precursor has not been degraded, and the product assembled from some metabolite not directly related to the precursor. This possibility can be minimized by administration of two or more different labels in the same compound.[1,11] If the distribution of label within the substrate and product is the same, then degradation of the precursor prior to incorporation is much less likely to have taken place.

The use of double labelling techniques also makes it possible to deduce what reactions may be involved in the biosynthesis without direct observation of the intermediates. This is possible by determining which atoms of the substrate molecule are lost during the conversion and which are retained. For instance, Mestichelli et al. inferred that oxidation of ornithine(1) during proline(6) biosynthesis occurs at carbon-2 rather than at carbon-5.[12] This conclusion was drawn from the observations that proline contained little tritium relative to ^{14}C



Scheme 1. The two possible routes from ornithine (1) into proline (6).

when DL-[2-^{3}H,5-^{14}C]ornithine was fed to higher plants, but retained all tritium relative to ^{14}C when DL-[5-^{3}H,5-^{14}C]ornithine was fed.

Leistner et al. made use of ^{3}H:^{14}C double labelling methods in determining the chirality of lysine(7) which served as the precursor of pipecolic acid(8) and of the piperidine alkaloids (e.g. N-methylpelletierine(9) and sedamine(10)) in Sedum sp.[11] Having established that when the substrate was DL-[4,5-$^{3}H_2$]/DL-[6-^{14}C]lysine, both pipecolic acid and the piperidine alkaloids contained the same amount of tritium relative to ^{14}C that had been in the lysine which was fed,[13,14] the authors then fed L-[4,5-$^{3}H_2$]/DL-[6-^{14}C]lysine to the plants. Any compounds derived from L-lysine would retain all tritium, but only one-half the ^{14}C; the ^{3}H:^{14}C ratio would double, relative to that of the feeding material. On the other hand, any compounds derived from D-lysine would retain none of the tritium (there were no D-tritiated molecules present) and half the ^{14}C; the ^{3}H:^{14}C ratio would be zero. In the event, all the alkaloids had a ^{3}H:^{14}C ratio which was twice that of the lysine that was fed, and the pipecolic acid contained virtually no tritium; the alkaloids were derived from L-lysine and pipecolic acid was derived from D-lysine.[11]

One of the primary assumptions of double-labelling experiments is that a mixture of singly labelled molecules will give rise to a product which is indistinguishable from one derived from a single, multiply labelled species.[1] While this is generally the case (but see references 8,15-17), there are occasions when it is helpful to be able to differentiate between the two possibilities. With the emergence of NMR as a new and powerful tool in biosynthetic studies, it is now

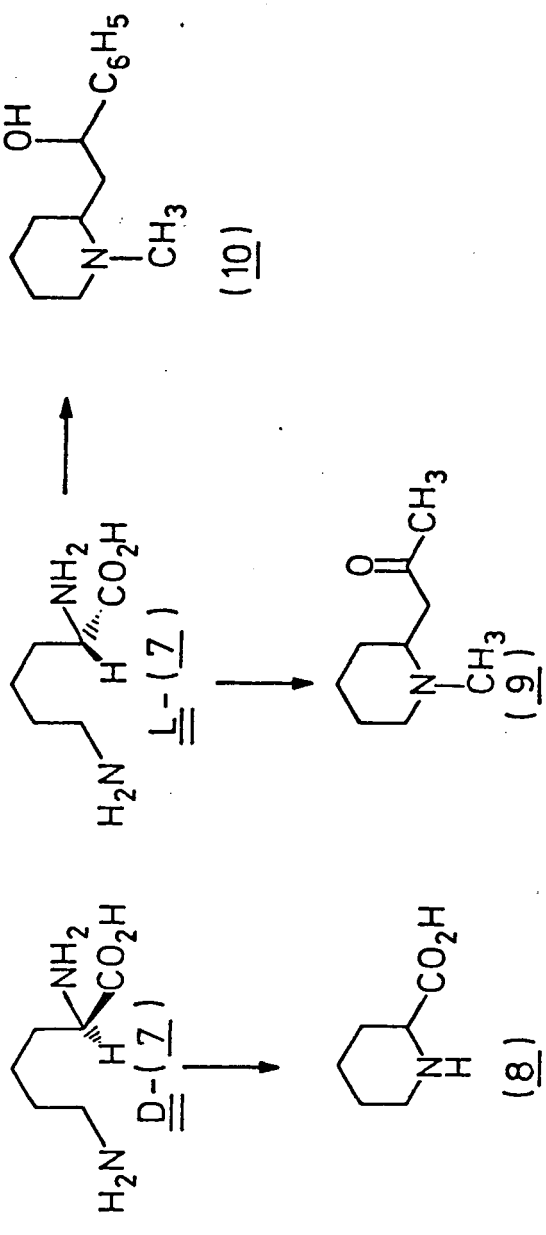
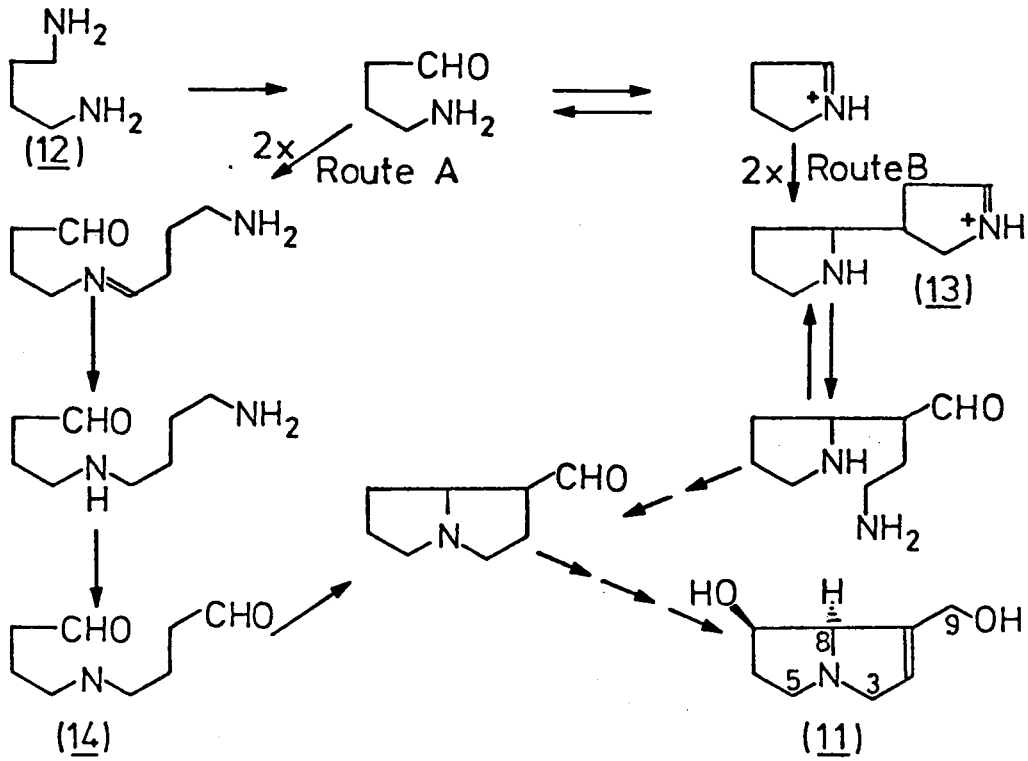


Figure 1. The biosynthetic fates of D- and L-lysine (7) in Sedum sp.

possible to distinguish between inter- and intramolecularly doubly labelled species, provided that at least one of the labels is magnetically active and therefore NMR-detectable.[1,4,18-22]

In their study of retronecine(11) biosynthesis, Grue-Sørensen and Spenser were unable to distinguish among several possible biosynthetic pathways on the basis of ^{3}H:^{14}C labelling experiments.[8,19] Retronecine is derived from two four-carbon-units related to putrescine(12) and it was important to establish whether, after the coupling of two C_4 units, the 'dimer' at any time developed C_{2v} symmetry. Two representative pathways were proposed by the authors in the cited work. One involved 2-(3'-(1'-pyrrolinyl))pyrrolidine (13) (not a symmetrical intermediate) and the other, N-(4'-oxobutyl)-4-aminobutanal(14) (a compound with C_{2v} symmetry).[19] To distinguish between these possibilities, [1-^{13}C,1-^{15}N] putrescine(12) was administered to Senecio vulgaris plants. If the doubly labelled putrescine were incorporated into retronecine(11) via a non-symmetrical intermediate, there would be only a single species wherein a carbon-nitrogen bond is ^{13}C-^{15}N labelled, the C-5-N-4 bond. If, on the other hand, an intermediate with C_{2v} symmetry were involved, there would be equal amounts of C-3-N-4 and C-5-N-4 bond-labelled species. The ^{13}C NMR spectrum of the former would have only a single signal showing coupling to ^{15}N, while the latter would have two. The ^{13}C NMR spectrum of retronecine had two signals which were coupled to ^{15}N, thus eliminating from consideration any pathway involving only non-symmetric intermediates.[8]

Leete and McDonell, among others, have also made use of contiguously ^{13}C-^{15}N-labelled substrates in biosynthetic investigations,[18]



Scheme 2. Two possible, representative routes from putrescine (12) into retronecine (11) (After Grue-Sørensen and Spenser[8]).

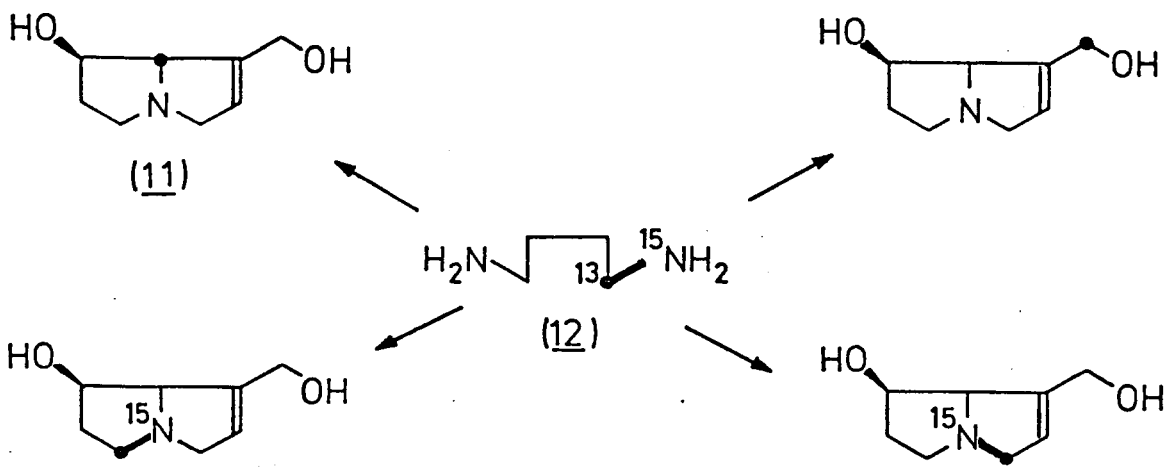


Figure 2. The four labelled species observed when [1-^{13}C,1-^{15}N] putrescine (12) is incorporated into retronecine (11).[8] For simplicity, species into which two molecules of labelled putrescine are incorporated have been omitted.

specifically to determine the mode of incorporation of N-methyl putrescine(15) into nicotine(16) and the tropane alkaloids. This work established that, when [1-^{13}C,1-^{15}N]-N-methylputrescine was fed to N. tabacum and Datura innoxia, ^{15}N was retained in the alkaloids and ^{13}C was incorporated specifically into the 5' position of nicotine(16) and the 5' position of scopolamine(17) respectively. These results served to confirm the validity of current views on the mode of biosynthesis of these alkaloids.[18]

One drawback of using ^{13}C-^{15}N-labelled substrates, as mentioned by Leete and McDonell,[18] is that ^{13}C-^{15}N coupling constants are usually much smaller than typical ^{13}C-^{13}C coupling constants, and consequently the observation of satellites in the ^{13}C NMR spectra is often difficult. Nonetheless this technique has been put to good use in the study of alkaloid biosynthesis.

Multiply, intramolecularly ^{13}C-labelled substrates have also found much use in biogenetic studies. First introduced by Seto and Tanabe and their co-workers,[21,22] doubly ^{13}C-labelled acetate has been widely used, particularly in the study of microbial polyketide biosynthesis. Since the administered precursor will be diluted by unlabelled acetate derived from other sources in the organism, it is unlikely that two labelled acetate units will be incorporated adjacent to one another in a given nascent polyketo acid. Therefore, in the average polyketide, though in few individual polyketides, only alternate carbon-carbon bonds will be doubly ^{13}C labelled; each representing the incorporation of an intact acetate unit. Those bonds which are not, in the average molecule, doubly ^{13}C labelled must have therefore been formed by

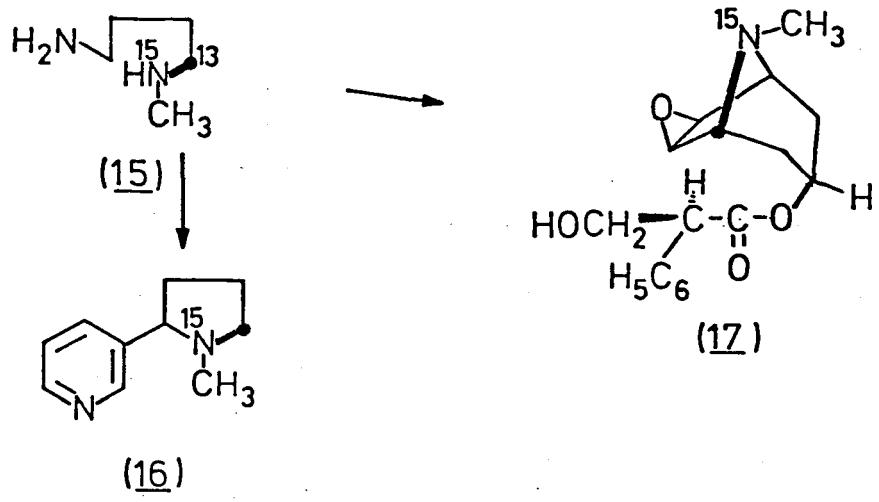


Figure 3. The mode of incorporation of [1-^{13}C,1-^{15}N]-N-methyl-putrescine (15) into nicotine (16) and scopolamine (17).[18]

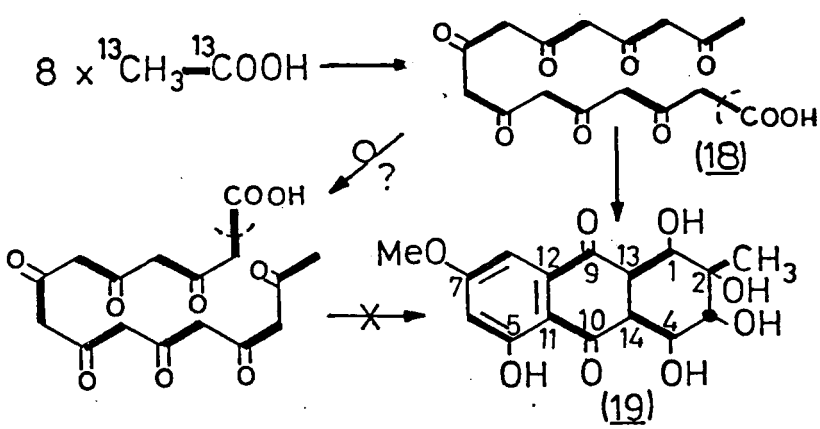


Figure 4. Predicted patterns of labelling of altersolanol A (19) by [1,2-$^{13}C_2$]acetate for two possible biosynthetic routes, along with the observed ^{13}C labelling pattern in altersolanol A.[23]

the organism in the course of biosynthesis.

Recently, Stoessl et al. have studied the biogenesis of some anthraquinones, octaketide metabolites of the fungus Alternaria solani, using among other precursors, [1,2-$^{13}C_2$]acetate as a substrate.[23] The anthraquinones in this study could be formed in one of two ways from the acetate-derived polyketo acid (18). These two routes would give rise to products, altersolanol A (19) for example, bond-labelled in either the C-6,C-7 bond or the C-5,C-6 and C-7,C-8 bonds. It was evident from the ^{13}C NMR spectrum that the octaketide is folded during biosynthesis in such a way as to label the C-5,C-6 and C-7,C-8 bonds of the anthraquinones.[23]

It was stated earlier that a detection limit of approximately 0.2% specific incorporation exists when ^{13}C incorporation is measured by ^{13}C NMR. By feeding precursors with two contiguous ^{13}C labels which remain together in the product, it is possible to extend this limit downward by at least an order of magnitude. This is possible because of the spin-spin coupling between the two ^{13}C atoms. The spin of ^{13}C is ½, therefore the signal arising from a ^{13}C atom which is coupled to another ^{13}C atom will appear as a doublet. These doublets will appear as satellite peaks on either side of the signals due to ^{13}C bonded only to ^{12}C. This effectively reduces the background due to natural abundance ^{13}C from $0.011 \times 100 = 1.1\%$ to $(0.011)^2 \times 100 = 0.000121 \times 100 = 0.012\%$, the natural occurrence of a ^{13}C-^{13}C bond. Using this approach, Leete and Yu were able to detect specific incorporations of [2,3-$^{13}C_2$] ornithine (1) into nicotine (16) and nornicotine (20) of as low as 0.020%.[4]

Other, recent work using multiply ^{13}C-labelled substrates includes a reinvestigation of nonactin (21) biosynthesis from acetate, propionate and succinate,[24] a study wherein glycolysis and succinic acid

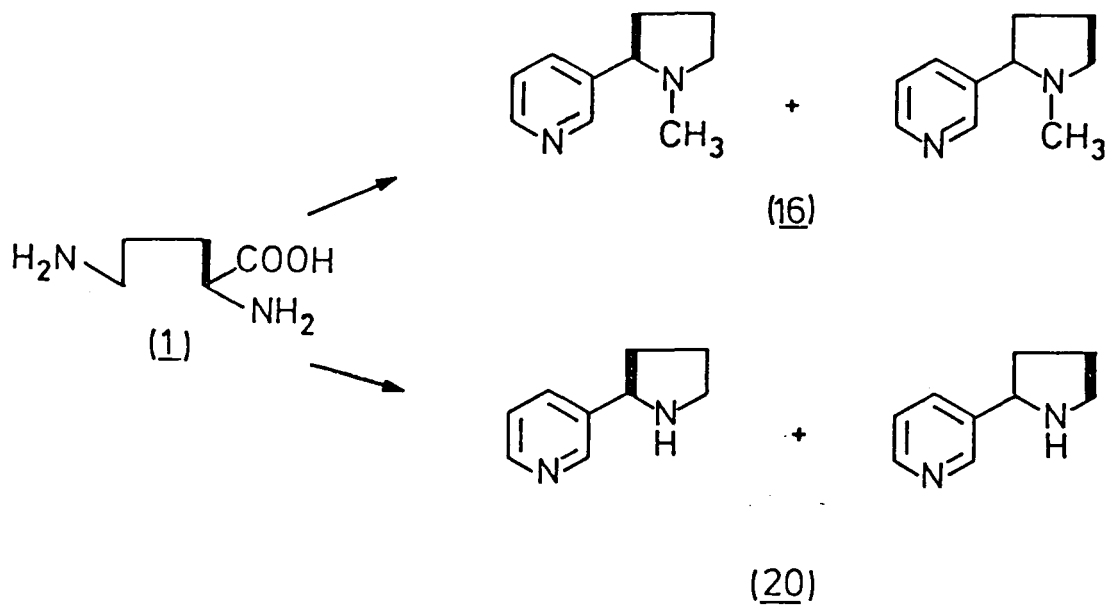
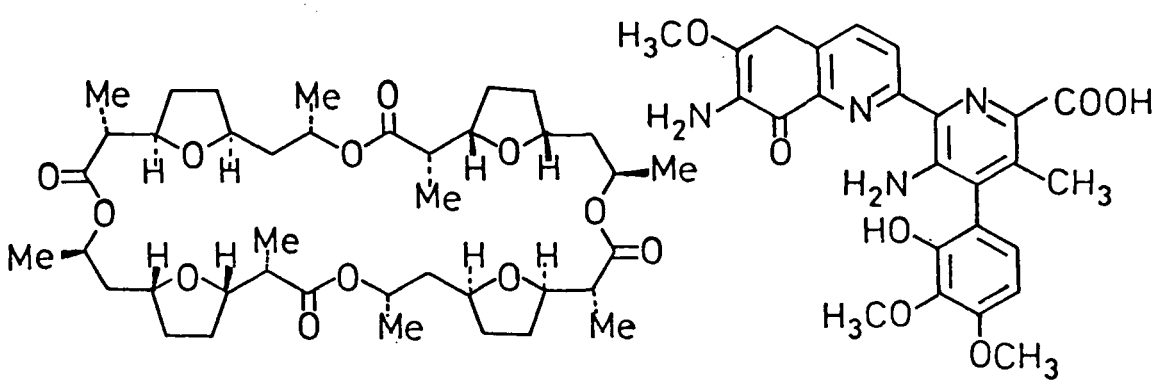


Figure 5. Observed patterns of incorporation of [2,3-$^{13}C_2$]ornithine (1) into nicotine (16) and nornicotine (20).[4]



(21) (22)

Figure 6. Nonactin (21)[24] and streptonigrin (22),[26] two bacterial metabolites whose biosynthesis has been studied with the aid of NMR and multiply ^{13}C-labelled substrates.

biosynthesis in Escherichia coli were monitored in vivo by ^{13}C NMR after the bacteria had been fed [U-$^{13}C_6$]-D-glucose,[25] and the elucidation of the mode of biosynthesis of the quinoline quinone moiety of streptonigrin (22), again using [U-$^{13}C_6$]-D-glucose.

This last work, in particular, serves to indicate the power of these multiple labelling techniques. Although extensive work had been done by Gould and his collaborators studying the 4-phenylpicolinic acid moiety of this metabolite, they had been unable to shed any light on the pathways involved in the quinoline moiety. A single experiment with multiply labelled glucose provided evidence for the identity of all the primary precursors to the antibiotic.[26]

Another characteristic of isotopic substitution which is particularly useful is the isotope shift in NMR. In general, heavy-atom substitution results in an upfield shift of the signal corresponding to the nucleus bearing the substituted atom.[27] This has been reported in studies using ^{13}C-^{15}N-labelled compounds[18,19] although in these instances, the visible coupling between ^{13}C and ^{15}N rendered the observation of an isotope shift redundant information. The isotope shift provides more useful information in cases where the substituted nucleus is not NMR-active (e.g. ^{18}O),[20,27] where the coupling constants are too small to be detected or the signals of interest are obliterated due to quadrupole relaxation (e.g. ^{13}C directly bonded to ^{2}H).[23]

In the investigations of Stoessl and co-workers mentioned earlier, samples of [1-^{13}C,2-$^{2}H_3$]acetate were administered to the fungus, and the ^{13}C NMR spectra of the anthraquinones were recorded. Additional, complementary evidence as to the mode of biosynthesis of these metabolites

was provided by the presence of small, upfield-shifted components of several of the NMR signals. These signified acetate units which had been incorporated without the complete loss of all hydrogen from their methyl groups.[23]

In his review, Vederas[20] describes several investigations, much of it his own work, where the isotope shift in ^{13}C NMR is used to detect the presence of ^{18}O directly bonded to carbon. In this way, it has been possible to establish the origins of oxygen atoms in natural products. In practice, organic acids (generally acetate, but also propionate and butyrate) labelled in the carboxyl group with both ^{13}C and ^{18}O are administered to the organism. Since many of these experiments have been done with microorganisms, wherein high specific incorporations are often found, unlabelled product is frequently added to that isolated from the experiment so that there is an internal chemical shift reference for the isotope-shifted peaks; signals corresponding to carbons bonded to labelled oxygen appear as doublets, the presence of the higher field line indicating the presence of labelled oxygen. Water and oxygen gas, which are also potential sources of oxygen atoms in natural products, have also been used as ^{18}O-labelled substrates.

In principle, ^{17}O, which is NMR-active, could also be used in these experiments, in a manner analogous to that in which ^{15}N is used. However, ^{17}O has a relatively high spin quantum number ($I = 5/2$), which would cause carbon resonances to appear as six lines of equal intensity if they could be seen. It is not likely, however, that these carbon resonances would be seen at all because ^{17}O has a relatively large quadrupole moment, and the carbon resonances would be completely

relaxed through quadrupole interactions.[20] Also, ^{18}O is more abundant than ^{17}O (0.204% vs. 0.037%), and is consequently less costly to use.[20,28]

Another use to which double labelling techniques can be put is the study of rearrangements in biosynthesis. One particular advantage that NMR investigations have in this regard is that it is possible to differentiate between intermolecular and intramolecular rearrangements. While it is possible to infer that a rearrangement has occurred from the results of a chemical degradation, it is not possible to say whether two labelled atoms were present in a single molecule or in separate molecules. With NMR-detectable isotopes, on the other hand, it is possible to label the precursor to be administered in such a way that if a rearrangement is intramolecular, the labelled sites within the product will interact differently than if the rearrangement were intermolecular. One of the earliest applications of this is Battersby's work on porphyrin biosynthesis.[29]

In the biosynthesis of uroporphyrinogen-III,(24) precursor of protoporphyrin-IX,(25) from porphobilinogen, (23) there is an apparent exchange of an acetate and a propionate side chain in one of the pyrrole rings. When [2,11-$^{13}C_2$]porphobilinogen(23) served as the substrate and the protoporphyrin-IX(25) which was produced was examined by ^{13}C NMR, the results indicated that an intramolecular rearrangement had occurred in such a way that the two ^{13}C atoms were now directly bonded to each other in the ring in question. In this way the majority of hypotheses which had been advanced to explain the mechanism of this conversion were eliminated.[29]

Another example of a rearrangement studied with ^{13}C NMR is

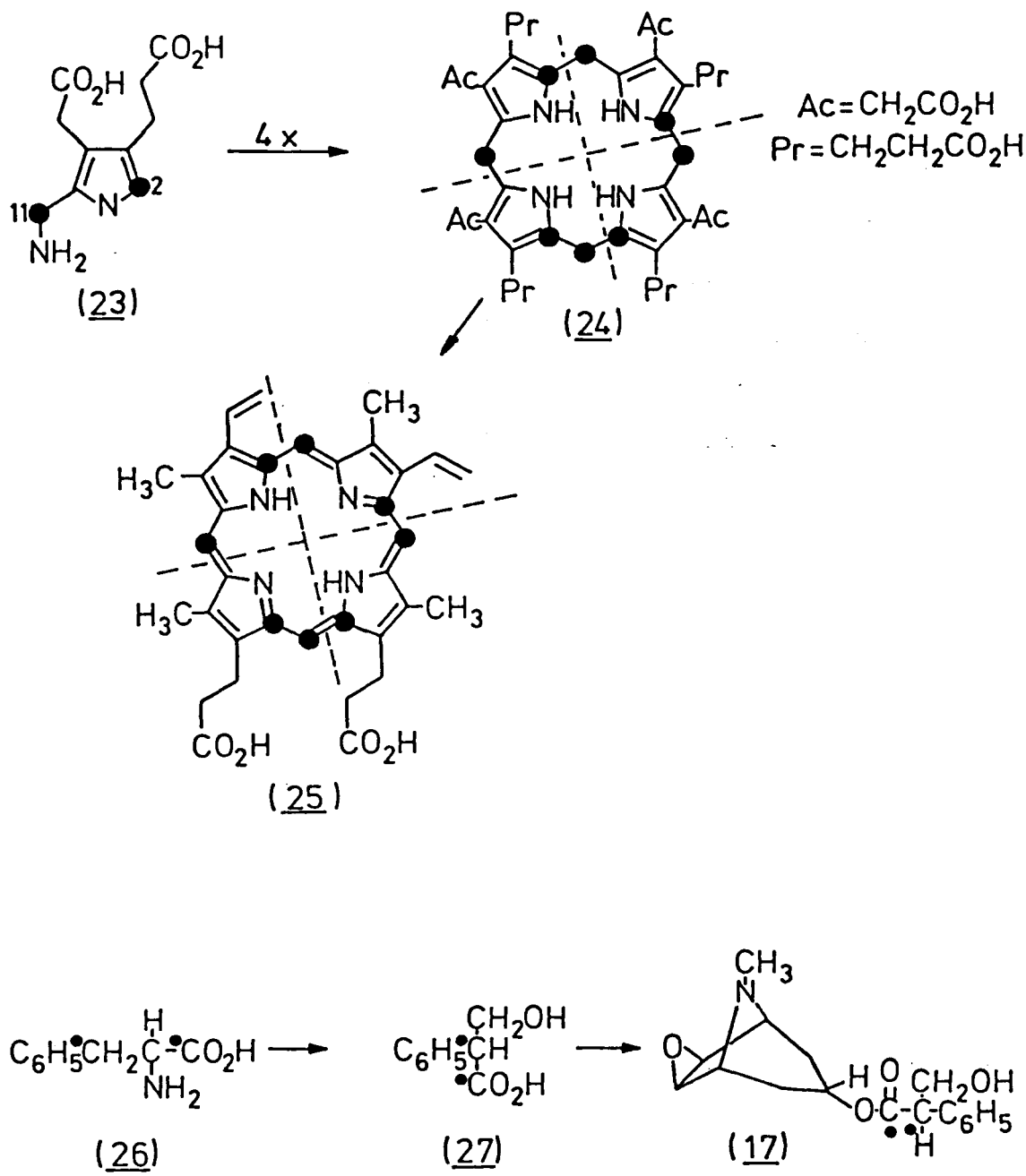


Figure 7. Use of intramolecular doubly ^{13}C-labelled precursors in the investigation of skeletal rearrangements in the biosynthesis of protoporphyrin IX (25) and tropic acid (27).

Leete's investigation of tropic acid(27) biosynthesis from phenylalanine(26).[5] The substrate in this case was [1,3-$^{13}C_2$]phenylalanine, and the product which was analysed was scopolamine(17). Although the specific incorporation was quite low (0.25%), the ^{13}C NMR spectrum clearly shows satellite peaks for C-1 and C-2 of scopolamine, indicating that two ^{13}C atoms were directly bonded and that therefore an intramolecular rearrangement had occurred. This result rendered untenable two hypotheses then being entertained.[5]

Through the use of heteronuclear double labelling, it is possible to detect not only skeletal rearrangements, but also more subtle rearrangements such as hydrogen migrations. This much is possible using radioisotopes and chemical degradation, but the use of NMR again allows the investigator to distinguish between inter- and intramolecular events. This technique has been applied to the study of hydrogen rearrangements in terpenoid biosynthesis by Hanson, Sadler, and co-workers.[30-32] The labels used are deuterium and ^{13}C, but rather than analysing the ^{13}C NMR spectrum for isotope-shifted peaks (cf 23), these workers recorded the ^{2}H NMR spectrum and looked for ^{2}H-^{13}C couplings. Although the signals in a deuterium spectrum are quite broad due to quadrupole relaxation, the deuterium signals are split into well-defined doublets due to coupling of ^{2}H to ^{13}C nuclei, and are not decreased in intensity since ^{13}C has no quadrupole moment. It would be difficult to do this experiment by analysing the ^{13}C spectrum for two reasons. Firstly, the spin of deuterium is 1 and therefore the area of the multiplet components would be one-third rather than one-half the area of the total multiplet, and secondly the quadrupole moment of the deuterium would act to decrease greatly the intensity of the signals.[30]

Biosynthesis of the Pyrrolidine Ring

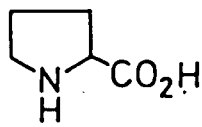
The pyrrolidine ring is a relatively common molecular grouping among natural products. It is found in simple compounds such as the amino acid proline(6) and the alkaloids hygrine(28) and nicotine(16), as well as in more complex, fused ring systems such as retronecine(11) and the tropane alkaloids (e.g. scopolamine(17) and cocaine(29)).

Early speculations on the biosynthesis of the pyrrolidine ring by Trier[33] and by Robinson[34] have proven to be surprisingly accurate in the light of modern tracer experiments. Trier surmised that nicotine(16) was derived from proline(6) and nicotinic acid(30);[33] it is now known that nicotinic acid is incorporated into nicotine,[35] as is proline,[36] although proline is much less efficiently incorporated than is ornithine(1).[36]

On the basis of Robinson's novel synthesis of tropinone(31) from methylamine, acetone and succindialdehyde,[37] Robinson proposed that the biosynthetic precursor of the pyrrolidine moiety of the tropane skeleton was ornithine(1), a supposed succindialdehyde-equivalent.[34] This hypothesis was confirmed nearly 40 years later in Marion's laboratory when activity from radioactive ornithine administered to intact Datura stramonium plants was isolated in the tropane alkaloid, hyoscyamine(32).[38]

Proline was also shown, in some of the earlier radiotracer studies, to be a pyrrolidine-related compound which can be derived from ornithine.[39]

Since these early beginnings, much work has been devoted to the study of both proline and nicotine biosynthesis. Excellent reviews



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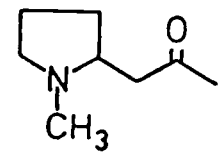

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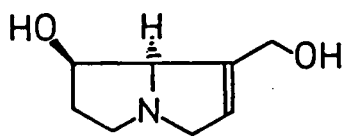

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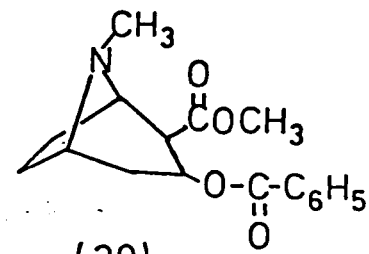
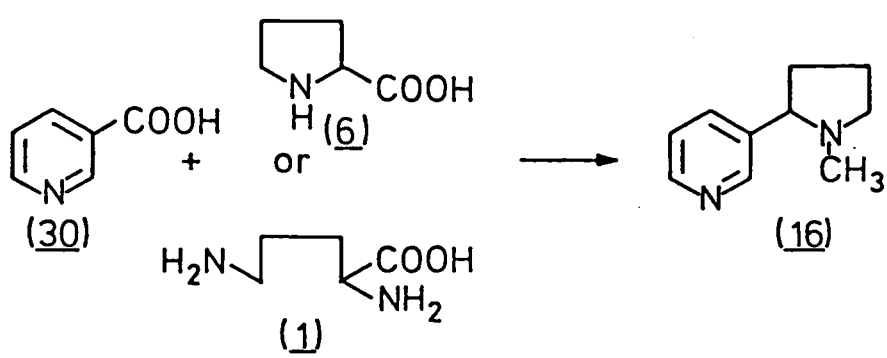


(29)

Figure 8. Some of the naturally occurring pyrrolidine compounds.



(30) (6) (1) (16)

Figure 9. Nicotinic acid (30), proline (6) and ornithine (1) were among the first predicted substrates of nicotine biosynthesis to be confirmed experimentally.

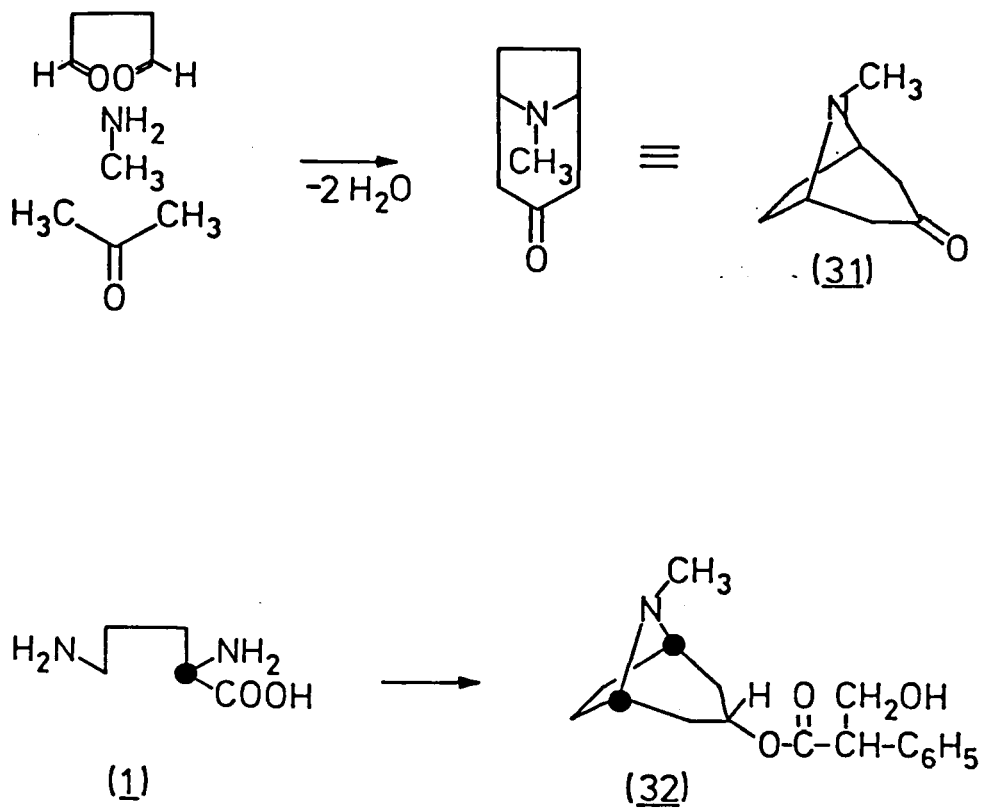


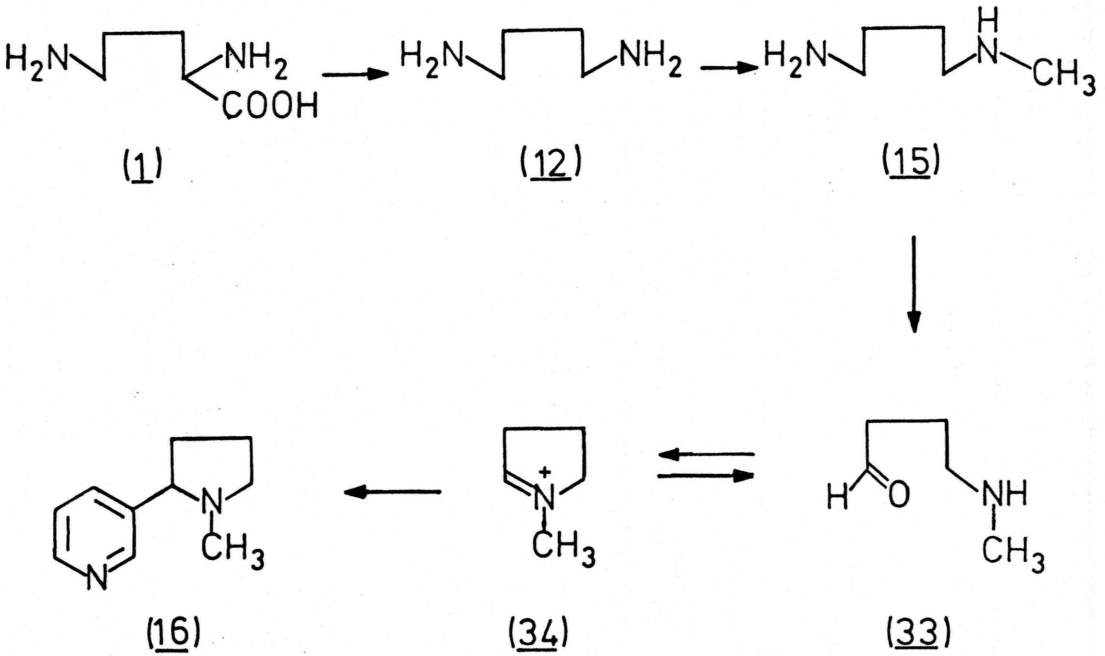
Figure 10. Robinson's novel synthesis of tropinone (31) which led to the prediction of ornithine (1) as a biosynthetic succindialdehyde-equivalent in tropane biosynthesis; a prediction ultimately confirmed experimentally.

and summaries dealing with the biosynthesis of both proline[12,40] and nicotine[2,41] have appeared in the literature, so only a brief overview will be presented here.

Nicotine

The first evidence that ornithine(1) does indeed serve as a precursor of the pyrrolidine ring of nicotine(16) was provided by both Byerrum's group[42] and Leete.[43] In virtually identical experiments, DL-[2-^{14}C]ornithine was administered to the roots of intact plants, and nicotine was subsequently isolated from the plants. After isolation and purification, it was found that nicotine was radioactive, and that one-half of the radioactivity was present at C-2' of the pyrrolidine ring, and none was found in the pyridine ring.[42,43] Later, it was shown that the remainder of the activity was present at C-5'.[44,45]

Decarboxylation of ornithine(1) produces putrescine(12), which was shown by Leete to be an efficient precursor of nicotine.[36] The material fed was [1,4-^{14}C]putrescine and activity was evenly distributed between the 2' and 5' positions in nicotine.[36] The next step in the currently accepted pathway from ornithine to nicotine is the methylation of putrescine to give N-methylputrescine(15). Evidence for this comes from the work of Mizusaki et al.[46] who have isolated from tobacco roots an enzyme which catalyses this conversion. Further evidence is provided by the observed non-symmetrical incorporation of N-methylputrescine into nicotine.[47,18] This shows that while N-methylputrescine can act as a precursor, it must do so without regaining C_{2v} symmetry; N-methylputrescine is not converted back to putrescine prior to its incorporation into nicotine.



Scheme 3. The currently accepted biosynthetic route from ornithine (1) to nicotine (16).

(1) ORNITHINE → SERVES AS A PRECURSOR OF THE PYRROLIDINE RING OF NICOTINE (16)

(12) PUTRESCINE → PRODUCED BY DECARBOXYLATION OF ORNITHINE

(15) N-METHYLPUTRESCINE → BY METHYLATION OF PUTRESCINE

(33) N-METHYL-4-AMINOBUTANA → BY OXIDATION

(34) N-METHYL-1-PYRROLINIUM ION → CONDENSATION PRODUCT

The penultimate step in the biosynthesis is the oxidation of the diamine to give N-methyl-4-aminobutanal(33) which is in equilibrium with its internal condensation product, N-methyl-1-pyrrolinium ion(34). An enzyme capable of this conversion and highly specific for N-methyl-putrescine for its substrate has also been isolated from tobacco roots.[48] The product of this oxidation has been shown by Leete to be incorporated non-randomly.[49] When [2-^{14}C]-N-methyl-1-pyrrolinium ion was administered to intact plants, nicotine was found to contain radioactivity in only the 2' position of the pyrrolidine ring. This result provides evidence not only that N-methyl-1-pyrrolinium ion can act as a precursor to nicotine, but also that this ion does not tautomerize under biosynthetic conditions.[49]

The final step in the biosynthesis is a condensation between the pyrrolinium ion and a nicotinic acid derivative. Leete has found evidence that in Nicotiana spp., anatabine(35) is formed from two molecules of 2,5-dihydropyridine(36), which can be derived from nicotinic acid(30).[50] Leete suggests that nicotinic acid(30), which is also incorporated into nicotine(16) in Nicotiana spp.,[50] is initially reduced to yield 3,6-dihydronicotinic acid(37). This β-imino acid is expected to decarboxylate readily to give 2,5-dihydropyridine(36). This unsaturated imine could then participate in a nucleophilic attack upon N-methyl-1-pyrrolinium ion(34), 1-piperideine(38) or 2,5-dihydropyridinium ion(39) in the biosynthesis of nicotine(16), anabasine(40) or anatabine(35) respectively. These alkaloids all occur naturally in Nicotiana spp.[50] It is therefore possible that the nicotinic acid derivative which condenses with N-methyl-1-pyrrolinium ion in nicotine biosynthesis is

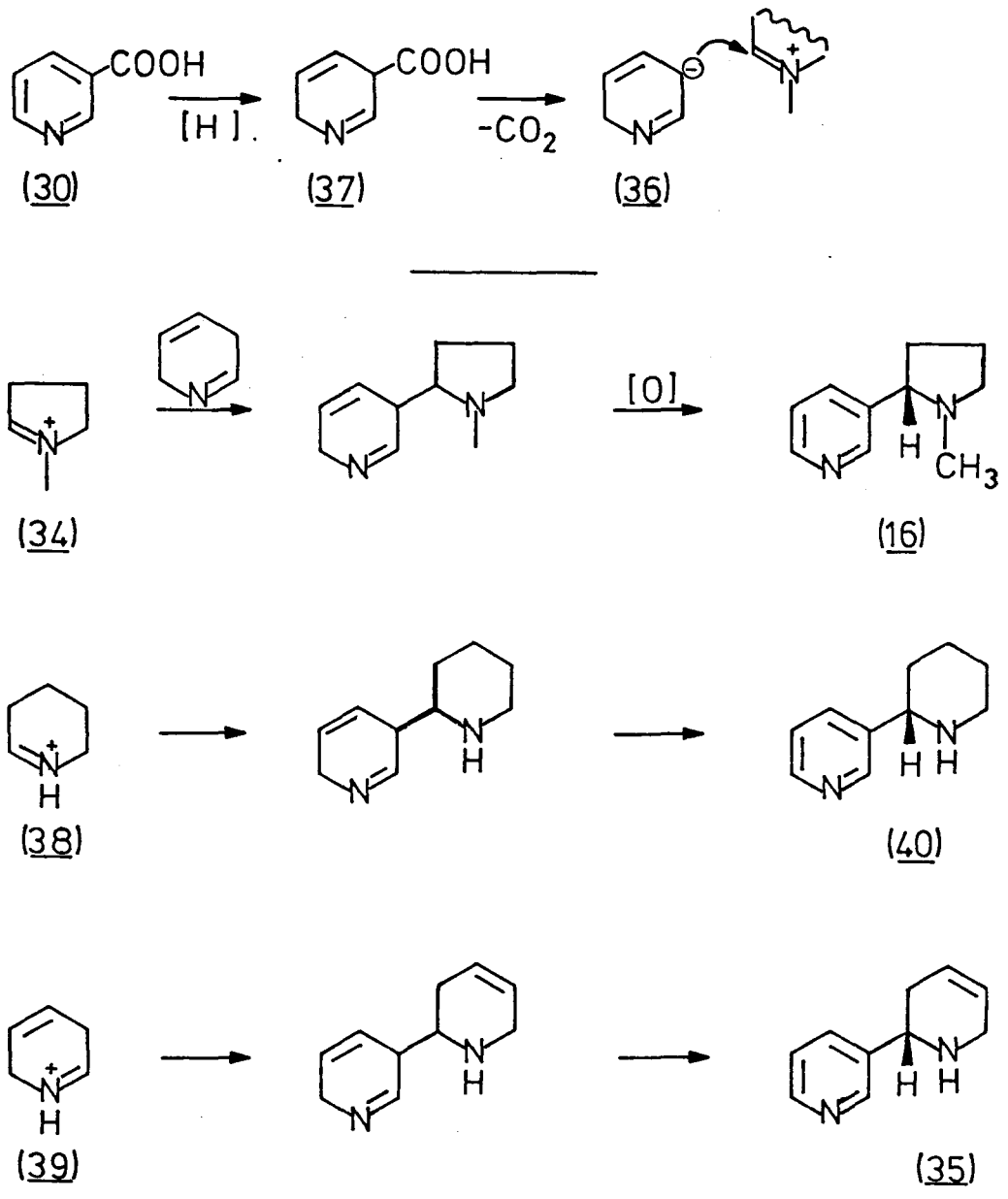


Figure 11. The nicotinic acid derivative, 2,5-dihydropyridine as a possible precursor to the pyridine moiety of tobacco alkaloids. (After Leete[50])

2,5-dihydropyridine, although there is as yet no direct experimental evidence that this is the case.

Stereochemical aspects of the biogenesis of the pyrrolidine ring of nicotine have yet to receive attention in the published literature. Mestichelli[51] has drawn the conclusion from his investigations that the oxidation of N-methylputrescine proceeds with loss of the 4-(S)-hydrogen. This conclusion, however, is based largely on the assumptions that, by analogy with lysine, it is L-ornithine which is the precursor of nicotine, and that ornithine is decarboxylated with retention of configuration.

The objective of the work was to answer three unsolved questions concerning the stereochemistry of nicotine biosynthesis.

1. Is it D-ornithine or L-ornithine which serves as the substrate for the decarboxylase which gives rise to putrescine?
2. Does the decarboxylase catalyse decarboxylation with retention or inversion of configuration at the reaction site?
3. Does the N-methylputrescine oxidase catalysed oxidation of N-methylputrescine proceed with loss of the 4-(R) or the 4-(S) hydrogen?

From evidence provided by the results of tracer experiments reported here, it was possible to infer the answers to these questions.

Proline

The first evidence of a radiochemical nature that proline(6) was a metabolite of ornithine(1) in plants was provided by Coleman and

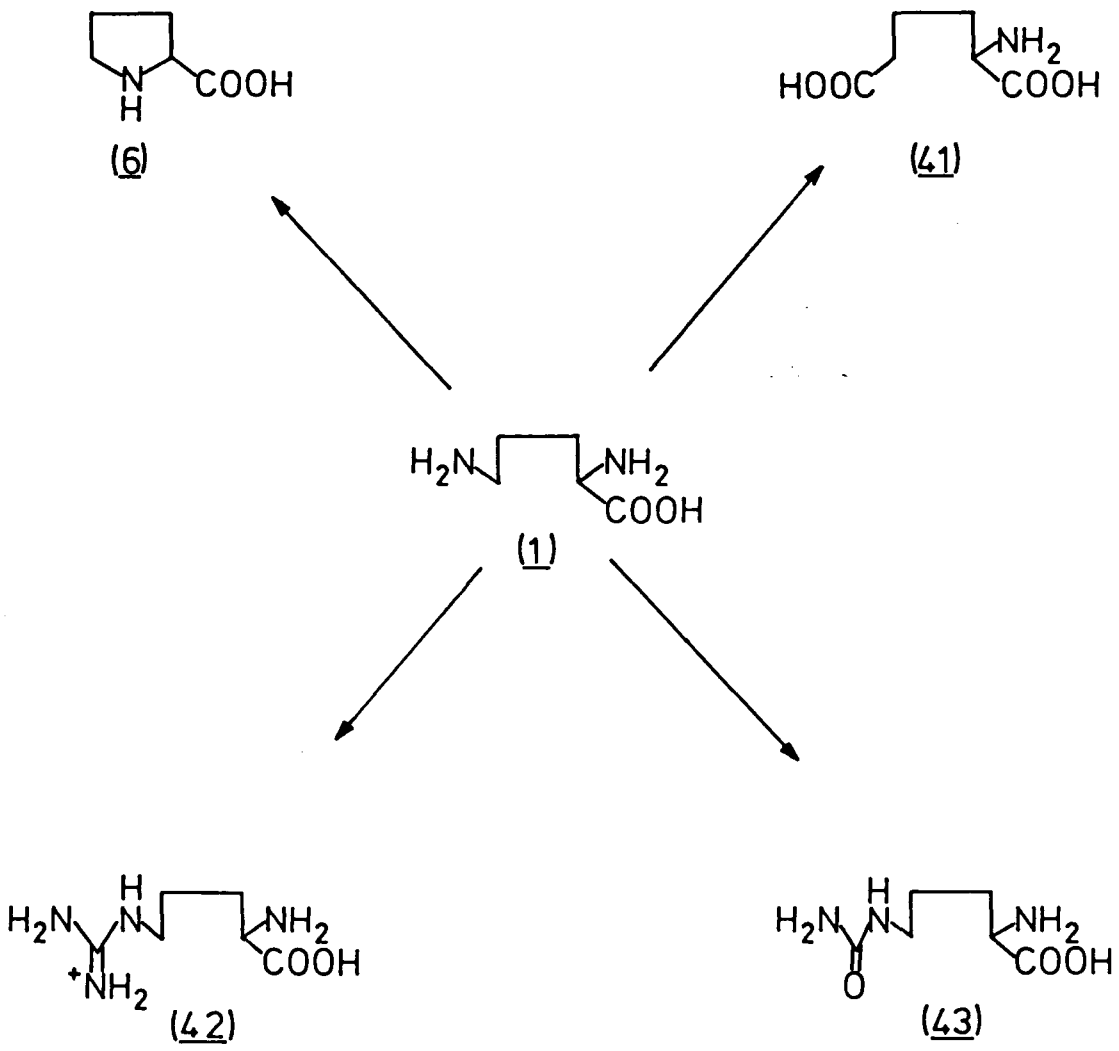


Figure 12. Proline (6), glutamic acid (41), arginine (42) and citrulline (43) can all be derived from ornithine in plant extracts.

Hegarty in 1957.[39] Labelled proline, glutamic acid(41), arginine(42) and citrulline(43) were detected by two-dimensional chromatography after extracts of barley and clover were incubated with DL-[2-^{14}C] ornithine. No evidence was presented in this work concerning the route of interconversion of ornithine and proline.[39]

As was pointed out by Mestichelli et al. in their investigation of proline(6) biosynthesis in 1979, most of the published evidence purporting to demonstrate that ornithine(1) is converted into proline(6) via glutamic semialdehyde(2) was equivocal at best.[12] The conclusion was based on a colour reaction between o-aminobenzaldehyde(44) and an oxo-amino acid, which was well established[52] to be non-specific; both possible intermediates in proline biosynthesis, α-keto-δ-aminovaleric acid(4) and glutamic semialdehyde(2), giving adducts with o-aminobenzaldehyde which are similar in colour.

There exist two plausible routes from ornithine(1) to proline(6). Both of these involve three steps: 1) The oxidation or transamination of an amino group; 2) Internal condensation between the resulting carbonyl and the remaining amino group; and 3) Reduction of the Schiff's base to yield proline. The route generally favoured to occur in micro-organisms and mammals[12,40] proceeds from ornithine to glutamic semialdehyde(2) by means of oxidation at the δ-amino group of ornithine, followed by condensation to give 1-pyrroline-5-carboxylic acid(3) and reduction to give proline.

There remains controversy as to which of the two pathways is the principal one in higher plants. Many feel that the experimental evidence for the pathway via glutamic semialdehyde(2) is sufficient

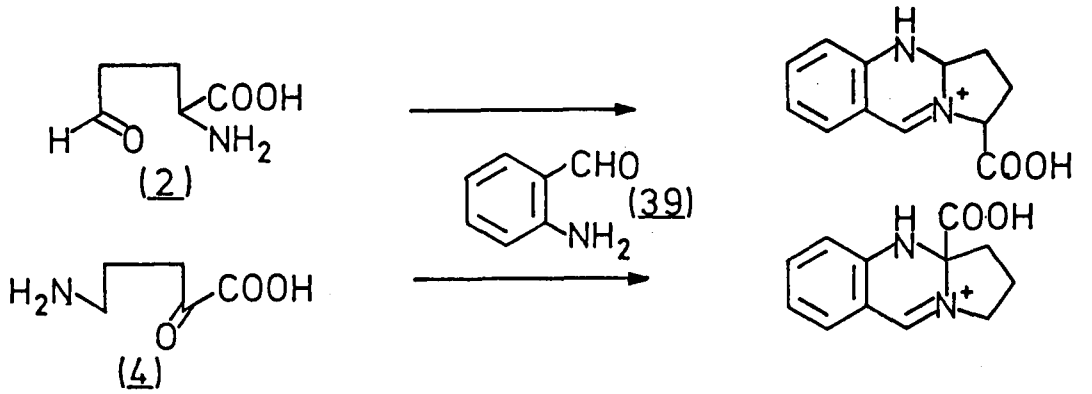
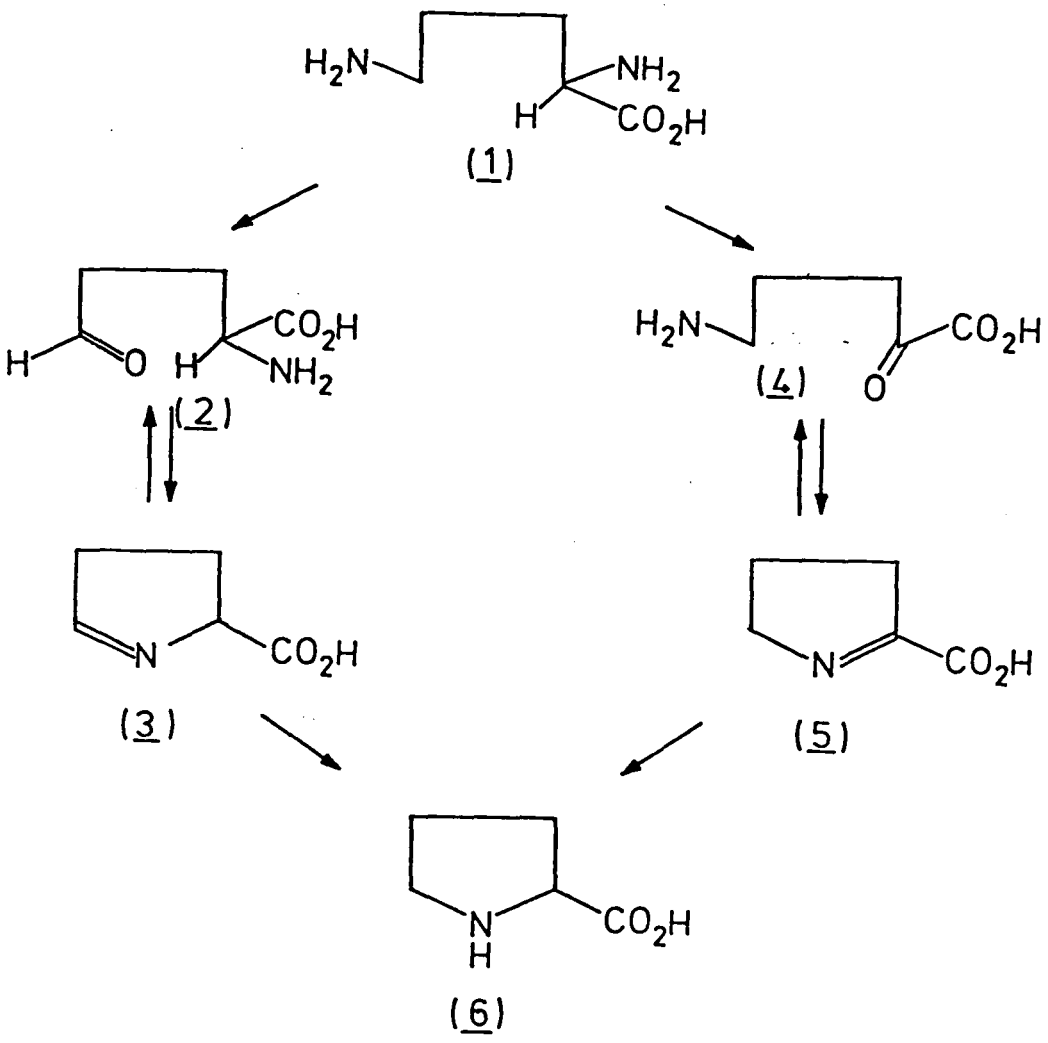


Figure 13. Both oxoamino acids derived from ornithine by oxidation give chemically similar adducts with o-aminobenzaldehyde.



Scheme 4. The two possible routes from ornithine (1) into proline (6).

and that further challenge of their beliefs is unnecessary.[40] Others, Spenser's group in particular, find the evidence unconvincing and have indeed found evidence to support the other pathway; α-oxidation of ornithine(1) to give α-keto-δ-aminovaleric acid(3), which is in equilibrium with 1-pyrroline-2-carboxylic acid(5), followed by reduction of the imine to yield proline.[12]

In their recent review, Adams and Frank have largely discounted the work of Spenser's group on the grounds that the roles of the two enantiomers of ornithine(1) were not fully accounted for, and that there was a possibility that the published data were the fortuitous result of kinetic tritium isotope effects.[40]

It was the aim of the work reported here to attempt to resolve some of these uncertainties, and to start a re-examination of proline biosynthesis, as advocated by Mestichelli et al.[12] Through the use of ^{3}H, ^{14}C double labelling techniques, insight was gained on the fates of the two enantiomers of ornithine in intact tobacco plants. On the basis of this insight, inferences may be drawn as to the modes of proline biosynthesis in Nicotiana tabacum.

EXPERIMENTAL

Labelled Compounds

The samples of labelled compounds were obtained from chemical suppliers (Table 1) and were used directly in the tracer experiments, except in the cases outlined below.

L-[2-^{3}H]Ornithine + L-[5-^{14}C]Ornithine (Experiment 4)

A solution of DL-[2-^{3}H]ornithine monohydrochloride (nominal total activity 1.6 mCi; nominal specific activity 24 Ci/mmol; CEA, France) in 0.01 M hydrochloric acid was mixed thoroughly with a solution of DL-[5-^{14}C]ornithine monohydrochloride (nominal total activity 0.1 mCi; nominal specific activity 11.5 mCi/mmol; CEA, France) in 0.01 M hydrochloric acid, and the resulting solution was evaporated to dryness *in vacuo*. The residue was re-dissolved in distilled water and evaporated to dryness *in vacuo* a further two times in order to remove any excess hydrochloric acid. To the residue was added a mixture of 0.1 M tris/HCl buffer (2.0 mL, pH 8.6), catalase (0.6 mg, from beef liver, 8250 units/mg*, Sigma) and D-amino acid oxidase (0.08 mL, from hog kidney, crystalline suspension in 3.2 M $(NH_4)_2SO_4$ solution, pH 6.5, 95 units/

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* One unit of catalase will decompose 1.0 μmole of hydrogen peroxide per minute at pH 7.0 at 25°C, while the hydrogen peroxide concentration falls from 10.3 to 9.2 μmoles per mL of reaction mixture.

Table 1

Administration of tracers to Nicotiana tabacum: Administered Compounds

Experiment No.	Precursor	Source	Nominal Total Activity (mCi)	Nominal Specific Activity (mCi/mmol)	$^{3}H/^{14}C$†
1	DL-[5-^{3}H]ornithine	R.C.C.	1.0	1.7×10^{4}	
	plus				7.2
	DL-[5-^{14}C]ornithine	CEA, France	0.1	11.5	
2	L-[5-^{3}H]ornithine	Schwarz/Mann Inc., New York	0.67	2.0×10^{4}	
	plus				2.6
	DL-[5-^{14}C]ornithine	CEA, France	0.1	11.5	
3	D-[5-^{3}H]ornithine	+	0.035	1.7×10^{4}	
	plus				5.5
	DL-[5-^{14}C]ornithine	CEA, France	0.005	55.0	
4	L-[2-^{3}H]ornithine	+	0.8	2.4×10^{4}	
	plus				13.3
	L-[5-^{14}C]ornithine	+	0.05	11.5	
5	R-(1-^{2}H)putrescine	+	-	96±3 atom % D	
	plus				-
	[1,4-^{14}C]putrescine	CEA, France	0.1	11.5	

+ See Experimental for Source and Preparation or Isolation.

† $^{3}H/^{14}C$ determined by addition of ca 1% of the feeding solution to ca 200 mg of non-radioactive DL-Ornithinemonohydrochloride and recrystallization (water-ethanol) to constant $^{3}H:^{14}C$ ratio.

ml[†], Sigma). The solution was incubated at 27°C with gentle shaking. After 18 hours an additional 2.0 mL buffer, 0.6 mg catalase, 0.08 mL D-amino oxidase and 2.0 mg non-radioactive DL-ornithine monohydrochloride was added. After a further 4 hours, another portion (2.0 mg) of non-radioactive DL-ornithine monohydrochloride was added. The reaction was terminated after a further 20 h by addition of 1.0 mL concentrated hydrochloric acid. Paper chromatography (2-methylpropan-2-ol:butanone: 88% aqueous formic acid:water/40:30:15:15, Whatman #1, ascending, detection by radio-chromatoscanning) revealed that 2 radioactive compounds were present, at R_f 0.14 and R_f 0.41. In this system, ornithine migrates with R_f 0.14. The other compound (R_f 0.41) is presumably the expected product of oxidation at the α-carbon atom, α-keto-δ-aminovaleric acid, in equilibrium with its internal Schiff's base, 1-pyrroline-2-carboxylic acid. The yellow solution was filtered through cotton wool to remove precipitated protein and the filtrate was applied to an ion exchange column (Dowex 2-X8, OH^-, 35 ml). The column was washed with water (50 mL) and eluted with 1.0 M hydrochloric acid (50 mL). The acidic eluate was evaporated to dryness in vacuo, the residue was dissolved in water (2.0 mL) and was applied to an ion exchange column (Dowex 50W-X4, H^+, 10 mL). The column was washed with water (30 mL) and eluted, first with 1 M aqueous pyridine (25 mL), then with 1 M aqueous ammonia (25 mL). Paper chromatography showed that the pyridine eluate (expected to contain

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[†] One unit of D-amino acid oxidase will oxidatively deaminate 1.0 μmole of D-alanine to pyruvate per minute at pH 8.3 at 25°C, in the presence of catalase.

neutral compounds such as 1-pyrroline-2-carboxylic acid) gave a single radioactive spot at R_f 0.41 and that the ammoniacal eluate (expected to contain only basic compounds such as ornithine) gave a single radioactive spot at R_f 0.14. The ornithine-containing fractions were pooled and evaporated to dryness *in vacuo*, dissolved in water (*ca* 8 mL), the pH was adjusted to 6 with 0.01 M hydrochloric acid and the solution made up to 10 mL with distilled water. This was used as the feeding solution. A small sample of this solution (20 μL) was withdrawn and added to an aqueous solution of non-radioactive DL-ornithine monohydrochloride for subsequent determination of the $^{3}H/^{14}C$ ratio.

R-(1-^{2}H)Putrescine[53] (Experiment 5)

L-Arginine monohydrochloride (501 mg) was dissolved in $^{2}H_2O$ (5 ml) and the solution was evaporated to dryness *in vacuo* in order to remove all exchangeable ^{1}H from the starting material. To this residue was added sodium acetate buffer (100 mL $^{2}H_2O$, 1.22 g $CH_3CO_2{}^{2}H$, 837 mg Na_2CO_3; p^{2}H: 5.15) and L-arginine decarboxylase (27.1 mg, 6.6 units/ mg[††], Sigma). The resulting solution was gently shaken at 36°C. After 24 hours, concentrated deuterium chloride (1 mL) was added, along with 0.5 g activated charcoal, and the mixture was heated 30 minutes on a steam bath, and was then filtered through Celite. The filtrate was evaporated to dryness *in vacuo* and re-dissolved in a solution of 3.05 g sodium hydroxide in 30 mL of 70:30 ethanol-water (v/v). The solution was refluxed overnight under nitrogen. The pH of the solution was

[††] One unit of L-arginine decarboxylase will release 1.0 μmole of carbon dioxide from L-arginine per minute at pH 5.2 at 37°C.

adjusted to ca 4 with 10% hydrochloric acid and the solution was then evaporated to dryness in vacuo. The residue was dissolved in 50 mL 10% aqueous sodium hydroxide which had been saturated with sodium chloride. This solution was extracted with 1-butanol (4x40 mL), and the pooled butanol extracts were dried (anh. sodium sulfate), filtered, acidified to ca pH 3 with concentrated hydrochloric acid and evaporated to dryness in vacuo. The white residue was recrystallized twice from water-ethanol to yield R-(1-^{2}H)putrescine dihydrochloride (Yield: 132 mg, 35%) which was free from agmatine, as determined by paper chromatography (1-butanol: acetic acid: pyridine: water / 4:1:1:2, Whatman 3MM, ascending, detection with ninhydrin, agmatine Rf 0.38, putrescine Rf 0.26). Deuterium incorporation was 96 ± 3 atom %, as determined by mass spectral analysis of diacetylputrescine. The M-(CH_3CO-) peak (M/Z=129 → M/Z=130) was found to serve as the most reliable measure of deuterium incorporation.

Diacetylputrescine

Putrescine dihydrochloride (7 mg) and sodium carbonate (30 mg) were dissolved in water (0.3 mL) and acetic anhydride (0.1 mL) was added. The mixture was stirred until it had become homogeneous (ca. 15 m). The solution was concentrated to dryness in vacuo and the residue was extracted with hot chloroform (3x2 mL). The chloroform extract was filtered and concentrated to dryness in vacuo to give diacetylputrescine as a white residue (yield 4 mg, 54%) which was submitted for mass spectral analysis without further purification.

D-[5-^{3}H]Ornithine[53] (Experiment 3)

A solution of DL-[5-^{3}H]ornithine dihydrochloride (nominal

specific activity 17 Ci/mmol, 1.09 mCi/mL, Amersham, England; 1.0 mL) was evaporated to dryness *in vacuo* repeatedly from aqueous solution to remove excess hydrochloric acid. To the residue was added sodium acetate buffer (0.2 M, pH 5.5, 1.0 mL) and L-ornithine decarboxylase (type II from *E. coli*, 2.3 mg, 0.11 units/mg[§], Sigma) and the solution was shaken gently at 37°C. The progress of the reaction was followed by paper chromatography (2-propanol: water: concentrated ammonia / 5:1:1, Whatman #1, ascending, R_f ornithine: 0.1, R_f putrescine: 0.4, detection by radiochromatoscanning). After 20 hours, approximately 1/3 of the radioactivity was found at R_f 0.4 $\pm$ 0.1 and 2/3 at R_f 0.1 $\pm$ 0.1. Fresh enzyme (2 mg) was added and the incubation was continued for a further 24 hours, at which time there had been no further change in the relative amounts of radioactivity at R_f 0.1 and R_f 0.4, indicating the possibility of the presence of radioactive impurities. Analysis by tlc (ethanol: water: diethylamine / 77:22:1, silica, ascending, detection by radiochromatoscanning) showed significant amounts of activity spread between R_f 0 and R_f 0.4. Both ornithine and putrescine have R_f 0 in this chromatographic system.

Concentrated hydrochloric acid (1 mL) was added to the reaction mixture, which was then heated on a steam bath 30 minutes and centrifuged. The supernate was withdrawn and evaporated to dryness *in vacuo*. The residue was dissolved in water and applied to an ion exchange column (Dowex 50W-X4, H^+, 1 mL). The column was washed with water (5 mL) and eluted with 1 M aqueous pyridine (5 mL) and 1 M aqueous ammonia (5 mL).

[§] One unit of L-ornithine decarboxylase will release 1.0 μmole of carbon dioxide from L-ornithine per minute at pH 5.0 at 37°C.

Only the pyridine eluate contained radioactivity at R_f values other than R_f 0 in the ethanol/ water/ diethylamine tlc system. The ammoniacal eluate was neutralized with 1 M hydrochloric acid and applied to an ion exchange column (Dowex 2-X8, OH^-, 20 mL). The column was washed with water (25 mL) and eluted with 1 M hydrochloric acid (30 mL). The acidic eluate was evaporated to dryness *in vacuo*. The residue was dissolved in water (1 mL) and applied to a second Dowex 2-X8 column (OH^-, 5 mL), which was then washed with water (10 mL) and eluted with 1 M hydrochloric acid (10 mL). The acidic eluate contained *ca* 35 μCi tritium. This solution was repeatedly evaporated to dryness *in vacuo*, in order to remove excess hydrochloric acid. The residue was dissolved in a small volume of water, DL-[5-^{14}C]ornithine monohydrochloride (nominal total activity 5 μCi, nominal specific acitivity 11.5 mCi/mmol, CEA, France) was added and the solution was made up to 3 mL with water. This was used as the feeding solution. A small sample of this solution was removed and added to an aqueous solution of non-radioactive DL-ornithine monohydrochloride for subsequent determination of the $^3H/^{14}C$ ratio.

Administration of Labelled Compounds

Experiments 1-4. Tracers were typically made up to a volume of 10 mL with distilled water (3 mL in experiment 3) and after withdrawing a small portion for subsequent determination of the $^3H/^{14}C$ ratio of the ornithine fed, the feeding solutions were administered to the plants by means of cotton wicks which had been inserted into the stems. When all the tracer solution had been absorbed, the vials were refilled with distilled water. Experimental details appear in Table 2.

Table 2

Administration of Tracers to Nicotiana tabacum: Experimental Details

Experiment No.	Plants		Duration	Fresh Weight (g)	
	No.	Age		Tops	Roots
1	12	3 months	5 days	223	90
2	6	4 months	3 days	315	-
3	3	2 months	$1\frac{1}{2}$ days	18	9
4	5	3 months	2 days	214	-
5	12	3 months	17 days	468	150

Experiment 5. Tracers were dissolved in distilled water and made up to 50 mL. After extensive mixing, 4 mL was withdrawn for subsequent determination of the specific radioactivity of the administered putrescine dihydrochloride. In an attempt to maintain an approximately constant concentration of putrescine in the plants and to obtain a high degree of incorporation, the tracers were administered in equal portions daily for 15 days. After 2 further days during which the vials were refilled daily with distilled water, the plants were harvested.

Isolation of Nicotine

Immediately after harvesting and weighing, the plants were macerated in methanol and continuously extracted until the extracts were essentially colourless. After acidification to pH ca 3 with 2 M sulfuric acid to prevent loss of nicotine by steam distillation, the methanol extract was evaporated to small volume in vacuo. The residue was shaken with 2 M sulfuric acid (60 mL) and diethyl ether (50 mL), the layers were allowed to separate, and the ether layer was discarded. The aqueous layer was further extracted with ether (3x50 mL) and the ether was discarded. After cooling, the aqueous phase was basified with concentrated ammonium hydroxide to pH ca 10. The basic solution was then extracted extensively with ether (8x25 mL) and the ether extracts were pooled. Amino acids were isolated from the remaining aqueous solution (vide infra). The ether solution was extracted with 0.5 M hydrochloric acid (4x50 mL), the combined aqueous layers were basified with 10% aqueous sodium hydroxide to pH ca 11 and this solution was extracted with chloroform (7x50 mL). The combined chloroform extracts were dried (anh. sodium sulfate), filtered, and evaporated in vacuo to

yield a pale yellow-green oil. This crude alkaloid fraction was dissolved in water, a few granules of sodium carbonate were added and the solution was distilled. Nicotine is the only tobacco alkaloid that will steam distill.[54] After 40 mL of distillate had been collected, a further 50 mL of water was added to the remainder of the original solution and distillation was continued until a total of ca 90 mL distillate had been collected. The distillate was extracted with chloroform (5x10 mL), the extracts were combined and dried (anh. sodium sulfate), and chloroform was evaporated in vacuo to yield a pale yellow oil. In experiments 1-4, nicotine was converted to the diperchlorate by dissolving the oil in methanol (0.1 mL), adding 72% aqueous perchloric acid (ca 0.1 mL) and a few drops of ether and cooling the solution overnight. Solvent was decanted and the nicotine diperchlorate was recrystallized twice from absolute ethanol to give fine white crystals (130 mg, m.p. 208-209°C, lit. m.p. 208-209°C).[44] In experiment 5, a small sample of the nicotine was converted to the diperchlorate as described above. The remainder was dissolved in chloroform and chromatographed on a small (0.5x5 cm) column of alumina. This step was designed to remove any remaining high polarity impurities in order to ensure the absence of deuterioputrescine, the presence of which would have obscured the ^{2}H NMR spectrum of the desired product, ^{2}H-nicotine. The eluate was evaporated in vacuo to yield nicotine as a colourless oil (273 mg).

Isolation of Amino Acids

After extraction of alkaloids, the basic aqueous solution (vide supra) was evaporated to dryness in vacuo. The yellow-brown residue was extracted with boiling methanol (3x20 mL), and the extracts were

filtered and evaporated to dryness *in vacuo*. A solution of DL-ornithine monohydrochloride (29 mg) and DL-proline (30 mg) in water (5 mL) was added to the residue, and the solution was applied to an ion exchange column (Dowex 50W-X4, H^+, 15 mL). The column was washed with water (50 mL) and eluted first with 1 M aqueous pyridine (50 mL) to liberate proline, then with 1 M aqueous ammonia (50 mL) to liberate ornithine.

The proline-containing fractions were combined, the solution was evaporated to dryness *in vacuo*, and the residue was chromatographed (ethanol: water: diethylamine / 77:22:1, silica, 60-200 mesh, 50x1 cm). The proline fraction (isatin positive) was evaporated to dryness *in vacuo* and a solution of DL-proline (73 mg) dissolved in 95% aqueous ethanol (2 mL) was added to the residue. The resulting solution was slowly filtered through activated charcoal and celite to give a colourless solution. Evaporation to dryness *in vacuo* and recrystallization from absolute ethanol yielded DL-proline as white crystals (64 mg).

The ornithine-containing fractions were evaporated to dryness *in vacuo* and to the residue was added a solution of DL-ornithine monohydrochloride (87 mg) in water (2 mL). This solution was filtered through charcoal on celite, the pH of the filtrate was adjusted to pH 5 with dilute hydrochloric acid, and the solution was evaporated to dryness *in vacuo*. The residue was recyrstallized from water-ethanol, further carrier (50 mg) was added and further recrystallization yielded DL-ornithine monohydrochloride (77 mg, m.p. 232-233°C, lit. m.p. 225-232°C).[55]

DL-Proline Phenylthiohydantoin

Phenylisothiocyanate (23 mg) was added to a solution of DL-

proline (12.3 mg) in methanol (0.5 mL), and the mixture was kept at room temperature 24 h. Concentrated hydrochloric acid (0.1 mL) was then added and the mixture was kept at room temperature for 18 h and was then cooled to 0°C for 3 h. The white precipitate was recrystallized twice from methanol to yield DL-proline phenylthiohydantoin as white crystals (14.6 mg, 59%, m.p. 182°C, lit. m.p. 180-180.5°C).[12]

Cotinine Perchlorate

Cotinine was prepared from nicotine by a modification of a published procedure.[56]

Nicotine diperchlorate (13.7 mg) and non-radioactive nicotine diperchlorate (100 mg) were mixed and recrystallized from absolute ethanol, the mother liquor being reserved. After samples were taken for determination of specific activities (ca 3 mg in total), the mother liquor and 10 mL water were added to the crystals and the resultant solution was evaporated to dryness in vacuo. To the residue was added 80% aqueous acetic acid (0.3 mL) and a solution of bromine, acetic acid and water (2:4:1, V:V:V, 0.5 mL) and the red solution was stirred at room temperature overnight. Water (1.0 mL) was added and excess bromine was removed by heating the mixture on a steam bath until the solution was homogeneous. The orange-red solution was allowed to cool gradually to room temperature and then, when crystals had started to form, to 4°C. The mother liquor was decanted, the crystals were washed with a little cold 80% aqueous acetic acid, and recrystallized from the same solvent. To the crystals of 4',4'-dibromocotinine[©] hydrobromide perbromide was

[©] To obviate possible confusion in the discussion, the pyrrolidine ring of nicotine and the pyrrolidinone ring of cotinine will both be numbered in the same sense; in both molecules, the carbon bonded directly to the pyridyl ring will be C-2'. It is recognised by the author that in the case of cotinine, this is contrary to convention.

added 50% aqueous acetic acid (2 mL), concentrated hydrochloric acid (0.2 mL) and zinc dust (ca 100 mg) with stirring. After 3 hours the reaction mixture was cooled, basified to pH ca 10 with concentrated ammonia, filtered, and extracted with chloroform (5x2 mL). The extracts were dried (anh. sodium sulfate) and evaporated to dryness in vacuo to give a pale green oil. The oil was distilled at 0.02 torr and 65°C to yield a colourless, low-melting solid identified as cotinine by its infra-red[57,58] and mass[59] spectra, which were in good agreement with published data. ^{1}H NMR ($C^{2}HCl_3$) δ ($(CH_3)_4Si$): 8.8-8.5, m, 2H, 7.7-7.5, m, 1H, 7.5-7.3, m, 1H, (pyridyl ring protons); 4.61, d of d, 1H, (H-2'); 2.70, s, 3H, (CH_3); 2.8-1.7, m, 4H, (CH_2CH_2 of pyrrolidinone ring). The cotinine was dissolved in a few drops of methanol, the solution was cooled, and 72% aqueous perchloric acid (0.1 mL) was added. Fine white crystals formed rapidly and the mixture was kept at 4°C overnight. Solvent was decanted, the crystals were washed with a little cold methanol, and recrystallized from the same solvent to give cotinine perchlorate as fine white needles (35 mg, 65% from nicotine diperchlorate), m.p. 214.5-216°C with decomposition. Analysis: Calculated for $C_{10}H_{13}N_2O_5Cl$: C, 43.41%; H, 4.73%; N, 10.13%; Cl, 12.82%. Found: C, 43.34%; H, 4.91%; N, 10.10%; Cl, 12.76%.

(4',4'-$^{2}H_2$) Cotinine

A test of the lability of the 2'-hydrogen of nicotine under the above conditions was conducted. Nicotine diperchlorate was converted into cotinine as described above with the exception that all protic solvents were replaced by their deuterated analogues. The product was

not converted to its perchlorate salt. Mass spectral analysis revealed that the following deuterated species were present: d_3-0%; d_2-65%; d_1-15%; d_0-10%. ^{2}H-NMR (13.82 MHz) showed only one signal, at 2.50 ppm; there was no signal corresponding to the 2'-position at 4.61 ppm.

Instrumental Methods

Radioactivity was assayed by liquid scintillation counting (Beckman LS 9000 Liquid Scintillation System). All samples were re-crystallized to constant specific radioactivity or constant ^{3}H:^{14}C ratio, dissolved in Aquasol (New England Nuclear), and counted in triplicate, under comparable conditions of quenching.

Radioactivity in thin layer and paper chromatograms was assayed by radiochromatoscanning using a Model 7201, Radiochromatography Scanner, Packard Instrument Company.

The ^{2}H NMR spectrum was recorded at 61.42 MHz in the Fourier mode on a Bruker WM400 spectrometer at the Southwestern Ontario NMR Centre. The spectrum was determined in a 10 mm tube in chloroform and acquired in 16K data points, acquisition time 1.638 s, pulse width 30 μs. Natural abundance deuteriochloroform in the solvent served as an internal chemical shift reference. The chemical shift of chloroform was found to vary by as much as 0.5 ppm as a function of nicotine concentration. Consequently the chemical shift of chloroform relative to tetramethyl-silane in the sample was determined in a separate experiment on a Varian EM 390 spectrometer.

RESULTS

In order to gain a better understanding of the stereochemical aspects of pyrrolidine ring biosynthesis in the tobacco plant, Nicotiana tabacum, five tracer experiments were performed. In four of these experiments, samples of tritium and carbon-14 labelled ornithine of known stereochemistry were administered to intact tobacco plants and after a period of growth, nicotine and the amino acids ornithine and proline were isolated from the plants and assayed for radioactivity. In another experiment putrescine, stereospecifically labelled with deuterium, and also labelled with ^{14}C, was administered to intact plants and nicotine was isolated. The alkaloid was analysed for deuterium by ^{2}H NMR and for ^{14}C by liquid scintillation counting.

The $^{3}H/^{14}C$ ratios of the samples of ornithine administered in experiments 1-4, as well as those of the compounds isolated in these experiments are presented in Table 3. Table 4 and Figure 15 present the results of experiment 5, in which labelled putrescine served as precursor.

Nicotine isolated from plants which had been fed DL-[5-^{3}H]/DL-[5-^{14}C]ornithine (experiment 1) was found to retain 80 $\pm$ 1% of tritium relative to ^{14}C when compared with the starting ornithine. When L-[5-^{3}H]/DL-[5-^{14}C]ornithine served as precursor (experiment 2) the $^{3}H/^{14}C$ ratio of nicotine was greater (126 $\pm$ 3%) than that of the feeding material; whereas when D-[5-^{3}H]/DL-[5-^{14}C]ornithine was the

Table 3

Incorporation of Ornithine into Proline and Nicotine in Nicotiana tabacum

Experiment No.	Precursor	Products	$^{3}H:^{14}C$ Ratio (a)	% Retention of ^{3}H
1	DL-[5-^{3}H,5-^{14}C]ornithine		7.17 ± .09	(100)[b]
		Ornithine	8.4 ± .1	117 ± 2
		Proline	8.45 ± .06	118 ± 2
		Nicotine	5.75 ± .04	80 ± 1
		Cotinine	2.30 ± .03	32 ± 1
2	L-[5-^{3}H] + DL-[5-^{14}C]ornithine		2.60 ± .04	(100)[b]
		Ornithine	0.48 ± .01	18 ± 1
		Proline	0.46 ± .01	18 ± 1
		Nicotine	3.28 ± .04	126 ± 3
		Cotinine	1.44 ± .02	55 ± 2
3	D-[5-^{3}H] + DL-[5-^{14}C]ornithine		5.47 ± .09	(100)[b]
		Ornithine	9.43 ± .08	172 ± 3
		Proline	10.8 ± .1	197 ± 4
		Nicotine	0.48 ± .01	8 ± 0.2
4	L-[2-^{3}H,5-^{14}C]ornithine		13.3 ± .2	(100)[b]
		Ornithine	14.4 ± .2	108 ± 2
		Proline	5.37 ± .06	40 ± 1
		Nicotine	6.35 ± .08	48 ± 1
		Cotinine	0.18 ± .01	1.4 ± 0.1

[a] Standard deviation of the mean.

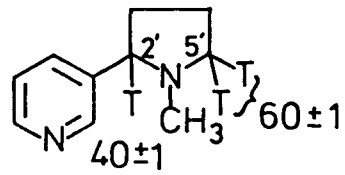
[b] Reference compound.

Figure 14

Distribution† of Tritium within Nicotine; Experiments 1, 2 and 4

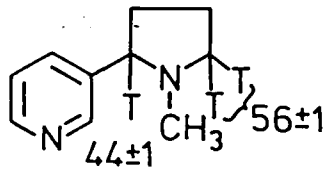
Experiment 1

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



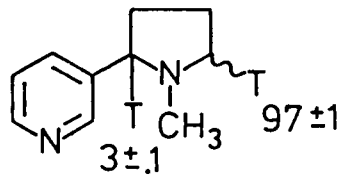
Experiment 2

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



Experiment 4

L-[2-^{3}H]/L-[5-^{14}C]Ornithine



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† Numbers represent percentage of tritium in nicotine present at Carbons 2' and 5' and are calculated as follows:

$$\%\ ^{3}H \text{ at } 2': \quad \frac{^{3}H/^{14}C \text{ Ratio of Cotinine}}{^{3}H/^{14}C \text{ Ratio of Nicotine}} \times 100$$

$$\%\ ^{3}H \text{ at } 5': \quad 100 - \%\ ^{3}H \text{ at } 2'$$

substrate (experiment 3), the nicotine was rich in ^{14}C but contained little tritium. A further experiment, in which L-[2-^{3}H]/L-[5-^{14}C] ornithine was administered to the plants (experiment 4), produced nicotine which had lost one-half of the tritium relative to ^{14}C. The distribution of tritium within nicotine isolated from experiments 1, 2 and 4, as determined by comparison of the $^{3}H/^{14}C$ ratios of nicotine and cotinine, is shown in Figure 14.

In experiment 5, where the precursor was R-(1-^{2}H) [1,4-^{14}C] putrescine, deuterium was present in nicotine at 40 times natural abundance in labelled positions, as inferred from the extent of ^{14}C incorporation. The ^{2}H NMR spectrum of this nicotine ($CHCl_3$) showed two deuterium resonances of approximately equal intensities at δ 2.33 ppm and δ 3.10 ppm downfield relative to tetramethylsilane.

Proline which was isolated from plants to which DL-[5-^{3}H]/DL-[5-^{14}C]ornithine had been administered contained more tritium relative to ^{14}C (118 $\pm$ 2) than did the ornithine which was fed. When L-[5-^{3}H]/DL-[5-^{14}C]ornithine served as precursor, proline retained little tritium relative to ^{14}C (18 $\pm$ 1% relative to feeding material), but when the substrate was D-[5-^{3}H]/DL-[5-^{14}C]ornithine, the $^{3}H/^{14}C$ ratio of the proline isolated was nearly twice that of the starting ornithine. When L-[2-^{3}H]/L-[5-^{14}C]ornithine was administered, proline was found to contain 40 $\pm$ 1% as much tritium relative to ^{14}C as the feeding material.

Contrary to earlier findings,[10] ornithine which was reisolated from the plants after the feedings was not found to have the same $^{3}H/^{14}C$ ratio as the ornithine which was fed. In experiments where DL-[5-^{3}H]/DL-[5-^{14}C] (experiment 1), D-[5-^{3}H]/DL-[5-^{14}C] (experiment 3)

Table 4

Incorporation of Putrescine into Nicotine (Experiment 5)

	Specific Activity ($dpm/mmol \times 10^{-7}$)	Specific Incorporation (^{14}C,%)	^{2}H Enrichment per deuterated position (Xnatural abundance)
Putrescine	18.8 $\pm$ 0.6	-	-
Nicotine	0.244 $\pm$ 0.002	1.3[†]	40[‡]

[†] $$\frac{\text{Molar specific activity of nicotine (dpm/mmol)}}{\text{Molar specific activity of putrescine (dpm/mmol)}} \times 100 = \frac{2.4 \times 10^{6} \times 10^{2}}{1.9 \times 10^{8}} = 1.3$$

[‡] $$\frac{\text{\% Specific incorporation} \times \text{Deuterium incorporation in putrescine} \times \frac{1}{2}}{\text{\% Natural abundance of deuterium}} = \frac{1.3 \times 0.96 \times \frac{1}{2}}{0.0156} =$$

40 times natural abundance ^{2}H per enriched position.

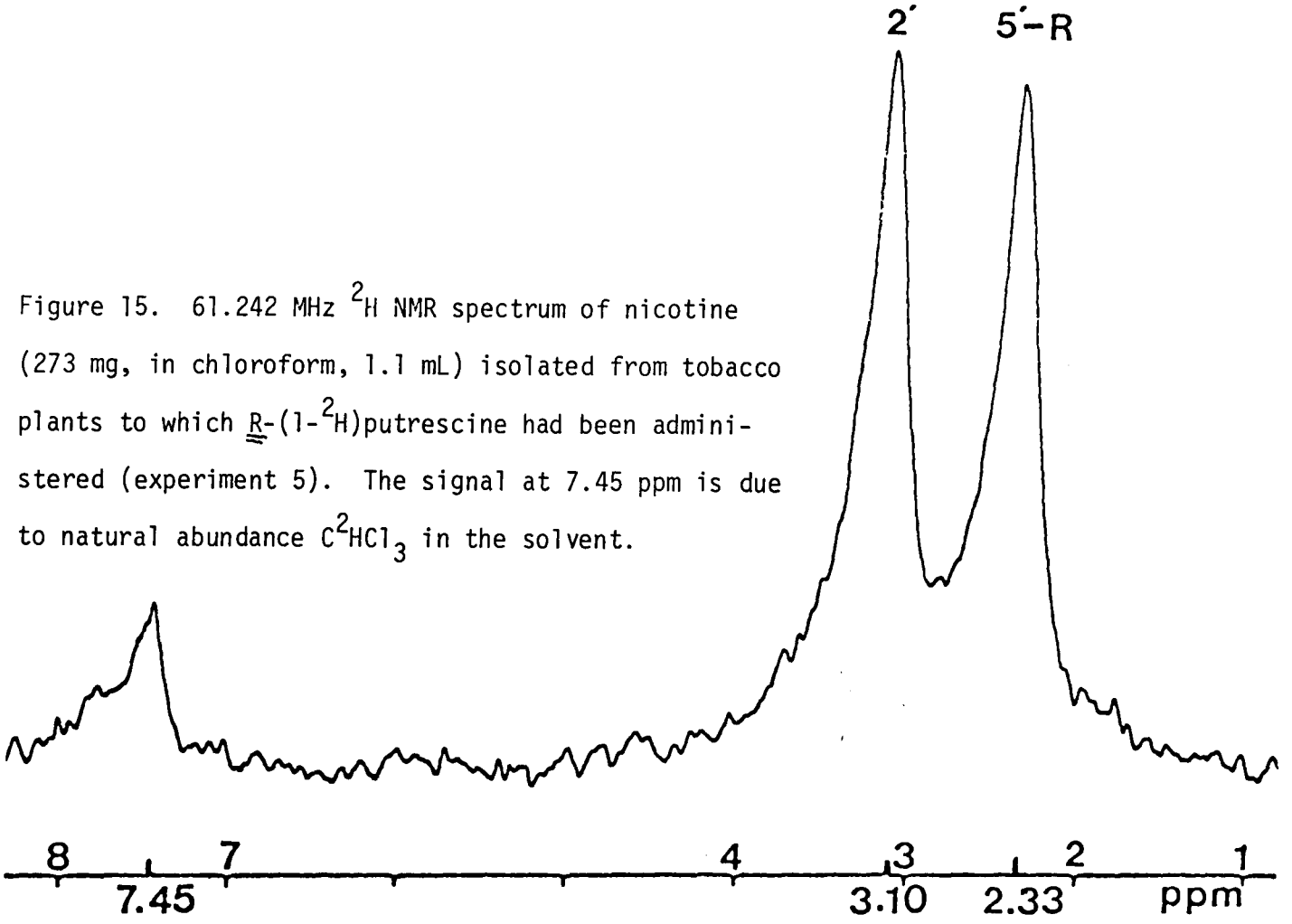

Figure 15. 61.242 MHz ^{2}H NMR spectrum of nicotine (273 mg, in chloroform, 1.1 mL) isolated from tobacco plants to which R-(1-^{2}H)putrescine had been administered (experiment 5). The signal at 7.45 ppm is due to natural abundance $C^{2}HCl_3$ in the solvent.

and L-[2-^{3}H]/L-[5-^{14}C] (experiment 4) ornithines were fed, the ^{3}H/^{14}C ratios in reisolated ornithine were found to have increased by 17 ± 2%, 8 ± 2% and 72 ± 3% respectively. In experiment 2, where the substrate was L-[5-^{3}H]/DL-[5-^{14}C]ornithine, the ^{3}H/^{14}C ratio of reisolated ornithine had dropped to 18 ± 1% of its initial value.

DISCUSSION

For nearly 30 years, it has been known that the pyrrolidine ring of nicotine (16) is derived from ornithine (1) in the intact tobacco plant, Nicotiana tabacum.[42,43] Since then further work has refined our understanding of this biosynthetic pathway to the point where there is now a general concensus as to the identity of all the intermediates which are involved.[41] However the stereochemistry of the reactions has yet to be investigated.

Likewise, it has been known for some time that the amino acid proline (6), another metabolite containing a pyrrolidine ring, is derived from ornithine (1),[39] but the stereochemistry of the conversion has been either assumed or ignored (e.g. 40).

There has been speculation about stereochemical aspects of these conversions,[51] based on the work of Spenser and co-workers[11,60] and upon assumed homologies between the origin of lysine-related compounds whose stereochemistry had been studied by Spenser's group, and that of the ornithine-related metabolites. These speculations have not been tested experimentally, however.

It was the aim of the present work to elucidate the stereochemical course of the steps in the conversion of ornithine (1) into nicotine (16) and proline (6) in tobacco.

Nicotine

In order to determine the stereochemistry of any reaction by the analysis of its products, it is necessary first to establish the chirality of the starting material. The $^{3}H:^{14}C$ labelling techniques of Leistner et al.[11] were well suited to the task of determining the chirality of the ornithine which serves as precursor to nicotine (16). It was necessary first, however, to establish a basis for the interpretation of results. In part, this entailed finding a suitable chemical degradation for the analysis of the biosynthetic nicotine.

The 2' carbon of nicotine has only one hydrogen bonded to it. Since the carbon atom of putrescine which enters this position bears two hydrogens, one of these hydrogens must be lost, presumably stereospecifically, during the conversion to nicotine. Therefore it was necessary to have a method whereby the extent to which tritium entered this position could be established. Oxidation of nicotine (16) to cotinine (46) was chosen to meet this need as the conversion is relatively straightforward and it was possible to prepare a solid, colourless derivative of cotinine (46) which was suitable for scintillation counting.

In the conversion of nicotine into cotinine via 4',4'-dibromocotinine (45), any tritium which might be present at the 4' or 5' positions of nicotine is lost. Also, since deuterium did not enter into the 2' position of cotinine when deuteriated solvents were used in the degradation, it is unlikely that any tritium is lost from the 2' position due to exchange processes during the degradation of tritiated nicotine. Therefore the $^{3}H:^{14}C$ ratio of cotinine is likely

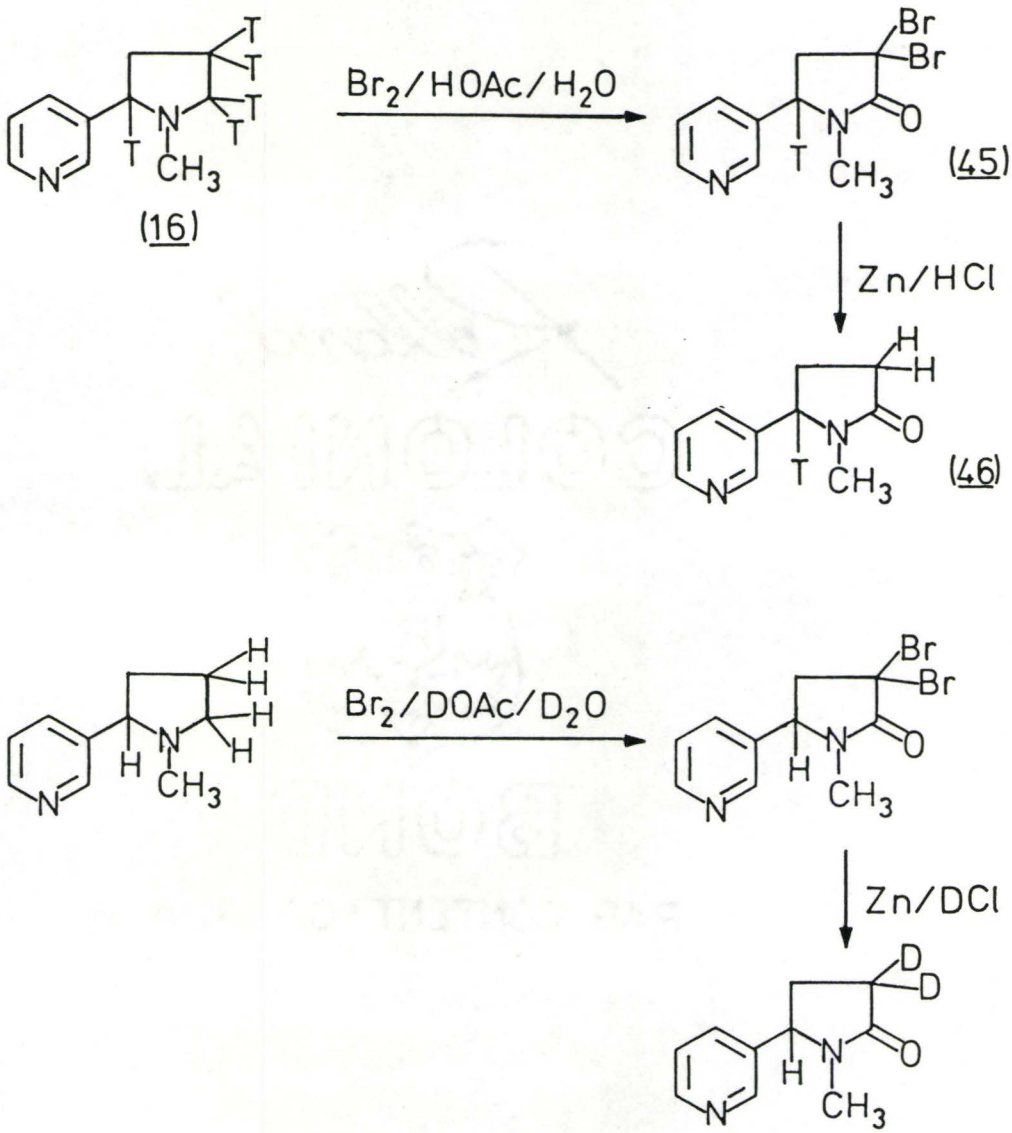

Figure 16. Bromine oxidation of nicotine, followed by zinc reduction will remove tritium from the 4' and 5' positions of nicotine, but not the 2' position.

to provide a good measure of the extent to which tritium enters the 2' position of nicotine in these experiments.†

In an experiment in which DL-[5-^{3}H]/DL-[5-^{14}C]ornithine was administered to tobacco (experiment 1), the nicotine which was isolated retained 80 ± 1% of tritium relative to ^{14}C of the ornithine that was fed. Following bromine oxidation of the nicotine and reduction of the resultant dibromo lactam, cotinine (46) was found to contain 40 ± 1% of the tritium activity that had been in nicotine.

If DL-[5-^{3}H]/DL-[5-^{14}C]ornithine were converted into nicotine by the currently accepted route, then no carbon-14 is expected to be lost during the conversion. Decarboxylation will proceed with no loss of tritium to give (RS)-[1-^{3}H]putrescine (12). Methylation is expected to proceed with equal facility at either nitrogen[51] to provide an equimolar mixture of (RS)-[1-^{3}H]-N-methylputrescine (15) and (RS)-[4-^{3}H]-N-methylputrescine; however it is important to note, in this context, that the pK_a of amines is sensitive to hydrogen isotope substitution at the α-carbon atom.[15] The nucleophilicity of the nitrogen lone pair may therefore exhibit a similar sensitivity, resulting in a non-equimolar mixture of [1-^{3}H]-N-methylputrescine (15) and [4-^{3}H]-N-methylputrescine. Oxidation of N-methylputrescine (15) will result in the loss of one of

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† Without complete degradation, it cannot be stated with absolute certainty that tritium is present only at the 2' and 5' positions of nicotine and only at the 2' position of cotinine; however it is well established[2] that activity from [2-^{14}C]ornithine and from [5-^{14}C]ornithine enters only and equally into the 2' and 5' positions of the pyrrolidine ring of nicotine. Furthermore, the biosynthesis of nicotine, as it is currently understood,[41] does not involve intermediates which would lend themselves to hydrogen rearrangements resulting in the incorporation of tritium into the 3' or 4' positions of nicotine from the substrates used in these experiments.

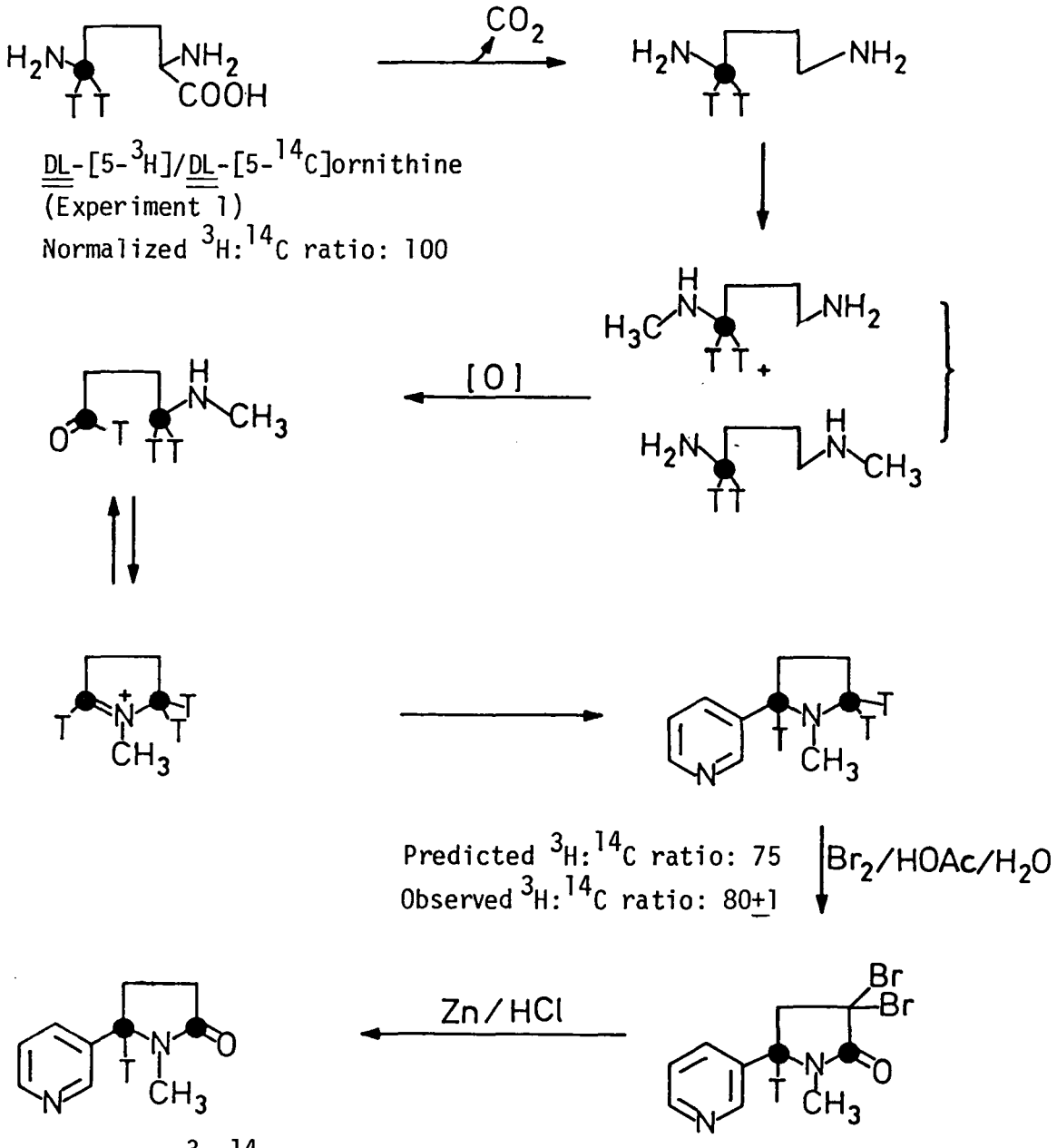


Figure 17. Predicted and observed $^{3}H:^{14}C$ ratios of nicotine into which DL-[5-^{3}H]/DL-[5-^{14}C]ornithine has been incorporated.

the hydrogens from C-4 in the conversion to (RS)-[1,4-^{3}H]-N-methyl-4-aminobutanal (33); 25% of the tritium will thus be lost, if isotope effects can be ignored. This amino aldehyde can then condense to form (RS)-[2,5-^{3}H]-N-methyl-1-pyrrolinium ion (34), which will in turn give rise to 5'-(RS)-[2',5'-^{3}H]nicotine (16). Retention of tritium relative to ^{14}C is predicted to be 75%, and of the tritium within nicotine, one-third is expected to be found in each of the 5'-R, 5'-S and 2' positions.

The experimental values for retention of tritium relative to ^{14}C (80 ± 1%) by, and distribution of tritium (40 ± 1% at C-2', 60 ± 1% at C-5') within nicotine are not, within experimental error, the same as the predicted values (75% retention of tritium, 1/3 at C-2', 2/3 at C-5'). They are however sufficiently close to confirm current thinking and to establish a basis for further work. The small differences between these values are probably due to isotope effects in ornithine metabolism (vide infra) and putrescine metabolism.

Experiment 2 was intended to establish the chirality of the ornithine which served as substrate for the ornithine decarboxylase which produces putrescine. When L-[5-^{3}H]/DL-[5-^{14}C]ornithine was fed to tobacco the biosynthetic nicotine had a ^{3}H:^{14}C ratio which was much greater (126 ± 3%) than that of the precursor. Distribution of tritium within nicotine was much the same as when DL-[5-^{3}H]/DL-[5-^{14}C]ornithine was fed (experiment 1) (44 ± 1% ^{3}H at C-2', 56 ± 1% ^{3}H at C-5').

This experiment uses the technique of Leistner et al.[11] in that one of the radiolabels is present in the feeding material in both enantiomers of ornithine, while the other is present in only one. Thus, when L-[5-^{3}H]/DL-[5-^{14}C]ornithine is administered, products derived

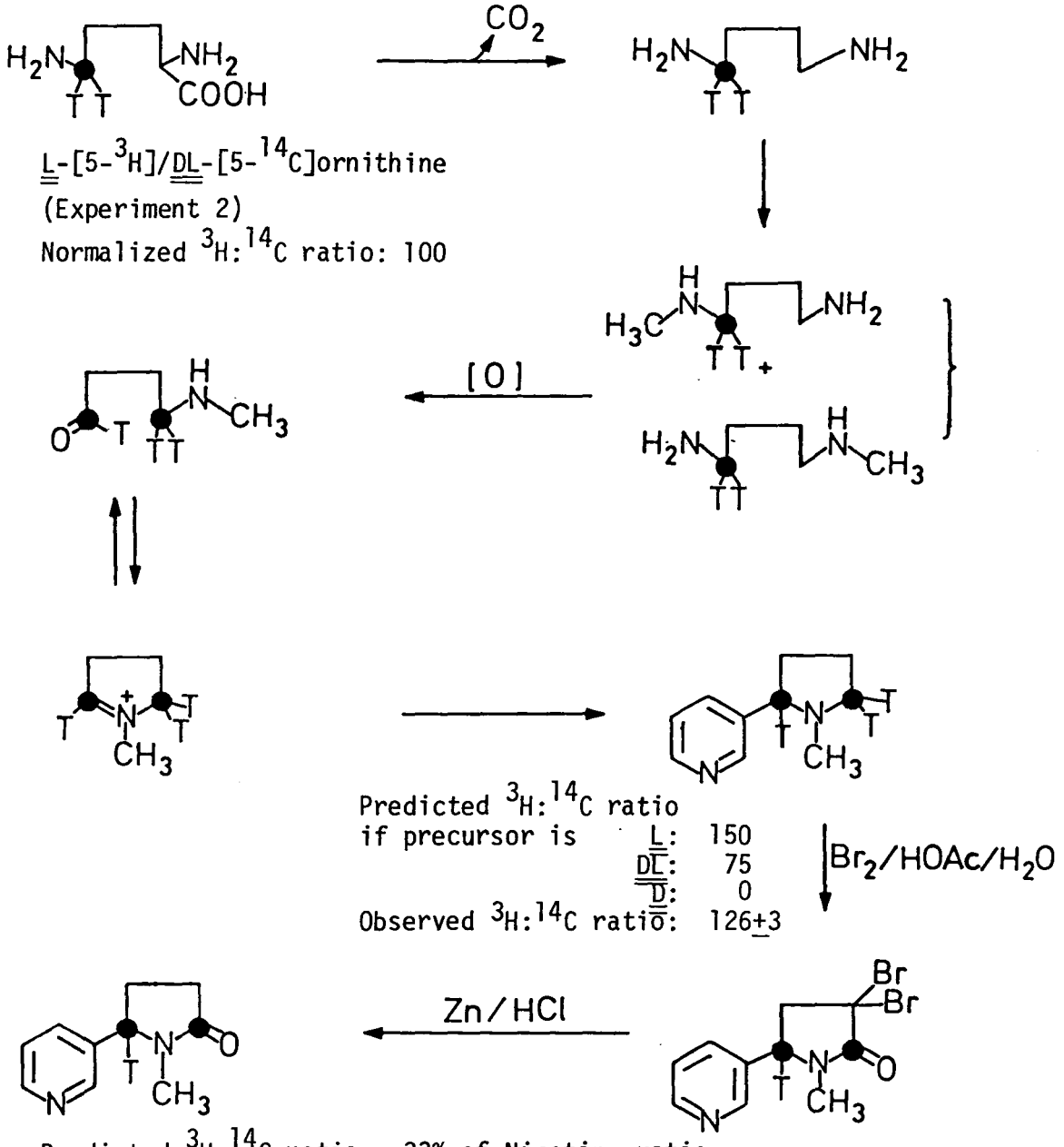


Figure 18. Predicted and observed $^{3}H:^{14}C$ ratios of nicotine into which L-[5-^{3}H]/DL-[5-^{14}C]ornithine has been incorporated.

from D-ornithine will contain no tritium, as no tritium is available in the form of D-ornithine, and those derived from L-ornithine will retain as much tritium as in the analogous DL/DL feeding, but only half the carbon-14; effectively the $^{3}H:^{14}C$ ratio will be doubled. Hence the predicted retention of tritium relative to ^{14}C by nicotine is 75% x 2 = 150%. The distribution of tritium within nicotine should be the same as predicted before, regardless of which enantiomer of ornithine serves as the precursor of nicotine; 1/3 at C-2' and 2/3 at C-5'.

Again the results do not agree completely with prediction, but the inference may clearly be drawn that a majority of the nicotine produced during the experiment was derived from L- and not D-ornithine. In order to confirm this result, the answer to a complementary question was required: Does D-ornithine enter nicotine? To answer this question, a mixture of D-[5-^{3}H]-ornithine and DL-[5-^{14}C]ornithine (experiment 3) was fed to tobacco plants. The nicotine which was isolated contained much ^{14}C activity, but little tritium activity.

With D-[5-^{3}H]/DL-[5-^{14}C]ornithine serving as precursor, nicotine is expected to contain no tritium if L-ornithine is converted into nicotine. If it were D-ornithine which is the true precursor however, the nicotine should be rich in tritium. The conclusion to be drawn is clear, and confirms the earlier finding that it is L-ornithine, and not D-ornithine which is decarboxylated to form putrescine during nicotine biosynthesis in tobacco. This result is qualitatively similar to the finding[11] that the homolog of L-ornithine, L-lysine, and not D-lysine is the precursor of anabasine, the piperidine alkaloid of Nicotiana glauca. This apparent homology in the reactions of structurally and chemically similar systems is perhaps not surprising and indeed was predicted to

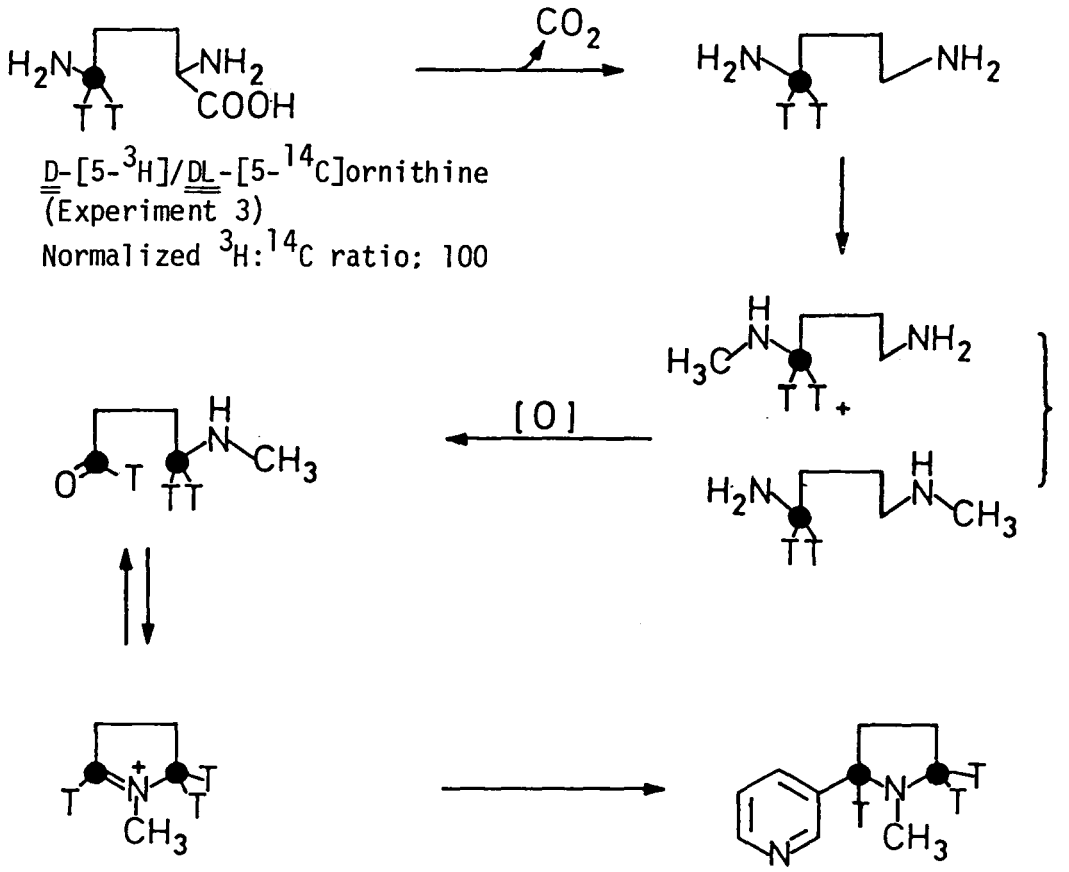


Figure 19. Predicted and observed $^{3}H:^{14}C$ ratios of nicotine into which D-[5-^{3}H]/DL-[5-^{14}C]ornithine has been incorporated.

occur in biochemical systems by Robinson some years ago.[34]

Having established that L-ornithine is the substrate of the decarboxylase which produces putrescine, there remain two questions of a stereochemical nature: 1) Does the decarboxylation of L-ornithine proceed with retention or with inversion of configuration at the reaction site? and 2) Does the oxidation of N-methylputrescine involve loss of the 4-(R) or the 4-(S) hydrogen? There are four possible combinations of answers to these questions, and therefore four possible routes from L-ornithine to nicotine. In conducting further investigations, each experiment should be designed in such a way that the results will clearly eliminate as many of these routes as possible.

Experiment 4 was designed to eliminate two of the four possible routes from L-ornithine to nicotine. With L-[2-^{3}H]/L-[5-^{14}C]ornithine serving as the substrate for nicotine biosynthesis, the nicotine which was isolated was found to contain one-half the amount of tritium relative to ^{14}C that had been present in the administered ornithine. Degradation of this biosynthetic nicotine yielded cotinine which contained virtually no tritium. Clearly there was no tritium at the 2' position of nicotine; it had all been located at the 5' position. There are only two of the four possible routes which could give this result.

Decarboxylation of L-[2-^{3}H]ornithine may proceed with either retention or inversion of configuration. Decarboxylation with retention of configuration would produce (S)-[1-^{3}H]putrescine, while decarboxylation with inversion would result in the enantiomeric (R)-[1-^{3}H]putrescine being produced. Once methylated, these would each give rise to a mixture of two different N-methylputrescines (15). Methylation of

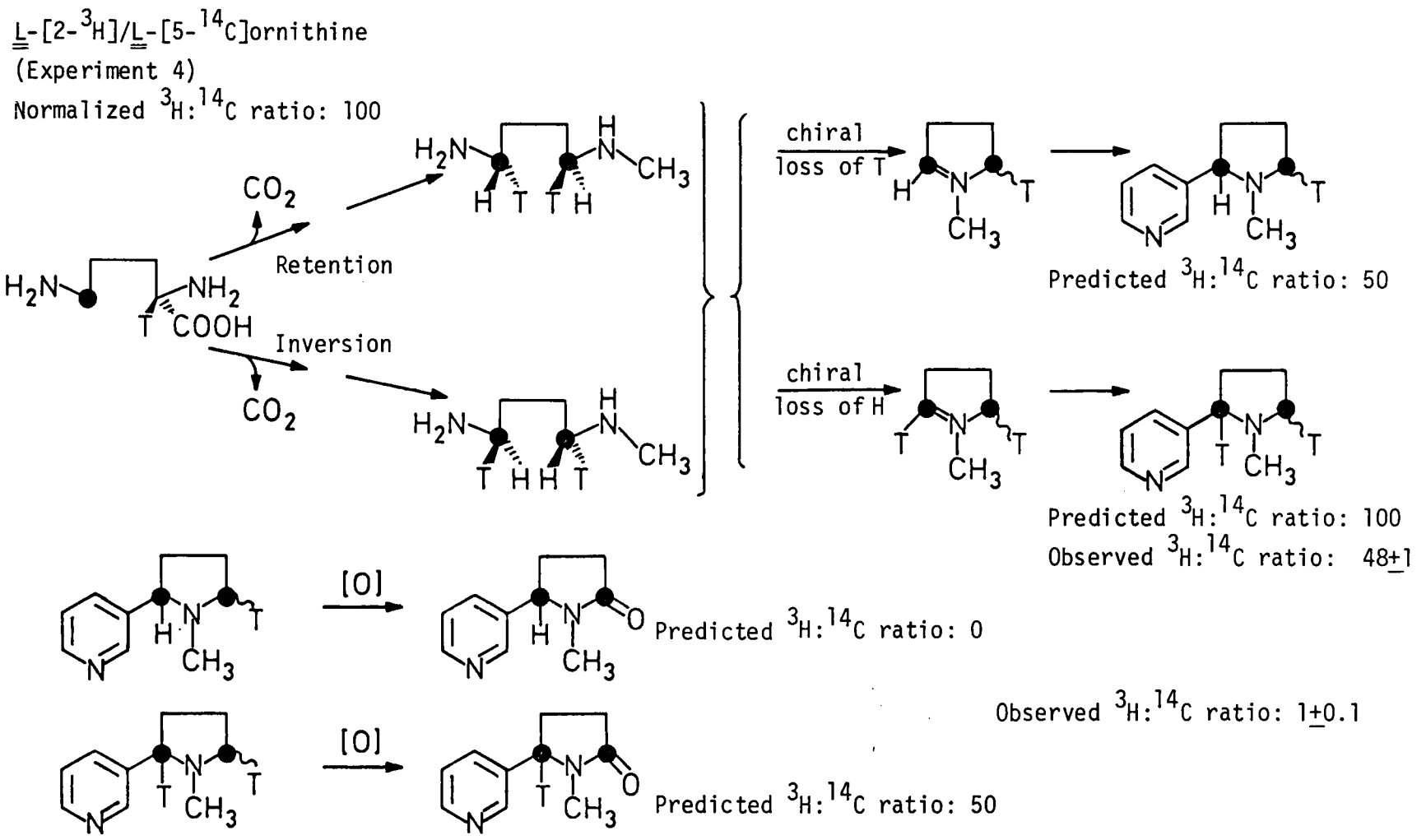


Figure 20: Predicted and observed $^{3}H:^{14}C$ ratios of nicotine into which L-[2-^{3}H]/L-[5-^{14}C] ornithine has been incorporated.

(S)-[1-^{3}H]putrescine will result in an equimolar mixture of N-methyl-(S)-[1-^{3}H]putrescine and N-methyl-(S)-[4-^{3}H]putrescine, whereas methylation of (R)-[1-^{3}H]putrescine will give an equimolar mixture of N-methyl-(R)-[1-^{3}H]putrescine and N-methyl-(R)-[4-^{3}H]putrescine.

Oxidation occurs only at C-4 of N-methylputrescine,[48,18] and the condensation product of the resultant N-methyl-4-aminobutanal (33), N-methyl-1-pyrrolinium ion (34), does not tautomerize under biosynthetic conditions.[49] Therefore, since C-1 of N-methylputrescine becomes C-5 of N-methyl-1-pyrrolinium ion, which in turn becomes C-5' of nicotine, all activity from any 1-tritio-N-methylputrescine must be incorporated into the 5' position of nicotine. Furthermore, since half of the tritiated N-methylputrescine is labelled at C-1, at least 50% of the tritium from L-[2-^{3}H]ornithine must enter the C-5' position of nicotine, regardless of which of the four possible routes is being considered.

Now, the stereospecificity of decarboxylation eventually results in the formation from L-[2-^{3}H]ornithine of only one of two possible enantiomers of 4-tritio-N-methylputrescine. Oxidation of this enantiomer of N-methylputrescine must proceed stereospecifically,[51] i.e., oxidation must occur with either chiral loss of tritium from C-4 of [4-^{3}H]-N-methylputrescine, or chiral retention of tritium. In the former case, 1-protio-N-methyl-4-aminobutanal and hence 2-protio-N-methyl-1-pyrrolinium ion and 2'-protio-nicotine, will be produced. Chiral retention of tritium, on the other hand, will result in [1-^{3}H]-N-methyl-4-aminobutanal, [2-^{3}H]-N-methyl-1-pyrrolinium ion, and ultimately, [2'-^{3}H]nicotine being produced.

We have already seen that tritium from L-[2-^{3}H]ornithine will be incorporated into the 5' position of nicotine in any event. If decarbox-

ylation and oxidation combine to result in chiral retention of tritium (either by decarboxylation with retention of configuration followed by loss of the pro-R hydrogen on oxidation or by decarboxylation with inversion and loss of the pro-S hydrogen), 50% of the tritium from L-[2-^{3}H]ornithine will be incorporated into the 2' position of nicotine and 50% into the 5' position, i.e., nicotine will retain 50% + 50% = 100% of the tritium relative to ^{14}C from the administered ornithine. If on the other hand decarboxylation and oxidation combine to result in a chiral loss of tritium (either due to decarboxylation with retention of configuration followed by oxidation with loss of the pro-S hydrogen, or decarboxylation with inversion followed by loss of pro-R hydrogen), there will be no tritium in the 2' position of nicotine and nicotine will retain only 50% of tritium, relative to ^{14}C, from L-[2-^{3}H]/L-[5-^{14}C] ornithine.

The finding that nicotine had, in fact, retained half of the tritium only, and that none of the tritium was present at C-2', allows the inference to be drawn that the concatenation of decarboxylation and oxidation results in the loss, during oxidation, of that hydrogen atom which was originally bonded to the α-carbon of L-ornithine. Therefore, either L-ornithine is decarboxylated with retention of configuration and N-methylputrescine is oxidised with loss of the 4-(S) hydrogen, or L-ornithine is decarboxylated with inversion of configuration and N-methylputrescine is oxidised with loss of the 4-(R) hydrogen, in the course of nicotine biosynthesis.

To solve the problem of nicotine biosynthesis it is necessary to eliminate one of the remaining two possible stereochemical routes.

These remaining possibilities differ in the stereochemical course of each enzymic step. It is therefore sufficient to establish the stereochemistry of one of these steps in order to differentiate between the two pathways.

In experiment 5, the substrate that was administered to the plants was (R)-(1-^{2}H)[1,4-^{14}C]putrescine. The ^{2}H NMR spectrum of the nicotine which was isolated in this experiment showed two resonances of approximately equal intensities at 2.33 ppm and 3.10 ppm. These signals correspond to the 5'-pro-R and the 2' hydrogen atoms of nicotine, respectively. These assignments were made on the basis of work published by Pitner et al., who report chemical shifts in ^{2}H NMR of 2.31 ± 0.02 ppm for the 5'-pro-R deuteron and 3.07 ± 0.02 ppm for the 2' deuteron.[61] The 5'-pro-S deuteron is reported to give a resonance at 3.25 ± 0.02 ppm;[61] there was no indication of any incorporation of deuterium into the 5'-pro-S position of the nicotine isolated from experiment 5.

Methylation of (R)-(1-^{2}H)putrescine will give rise to an equimolar mixture of N-methyl-(R)-(1-^{2}H)putrescine and N-methyl-(R)-(4-^{2}H)putrescine. Since oxidation occurs only at C-4 of N-methylputrescine, N-methyl-(R)-(1-^{2}H)putrescine will be oxidised to N-methyl-4-amino-(R)-(4-^{2}H)butanal, in equilibrium with N-methyl-(R)-(5-^{2}H)-1-pyrrolinium ion. This in turn will give rise to (5'-R)-(5'-^{2}H)nicotine.

Oxidation of N-methyl-(R)-(4-^{2}H)putrescine will lead to N-methyl-4-amino(1-^{2}H)butanal or unlabelled N-methyl-4-aminobutanal, depending upon whether it is the 4-pro-S or the 4-pro-R hydrogen, respectively, which is lost upon oxidation. These two species will be in equilibrium with N-methyl-(2-^{2}H)-1-pyrrolinium ion or unlabelled

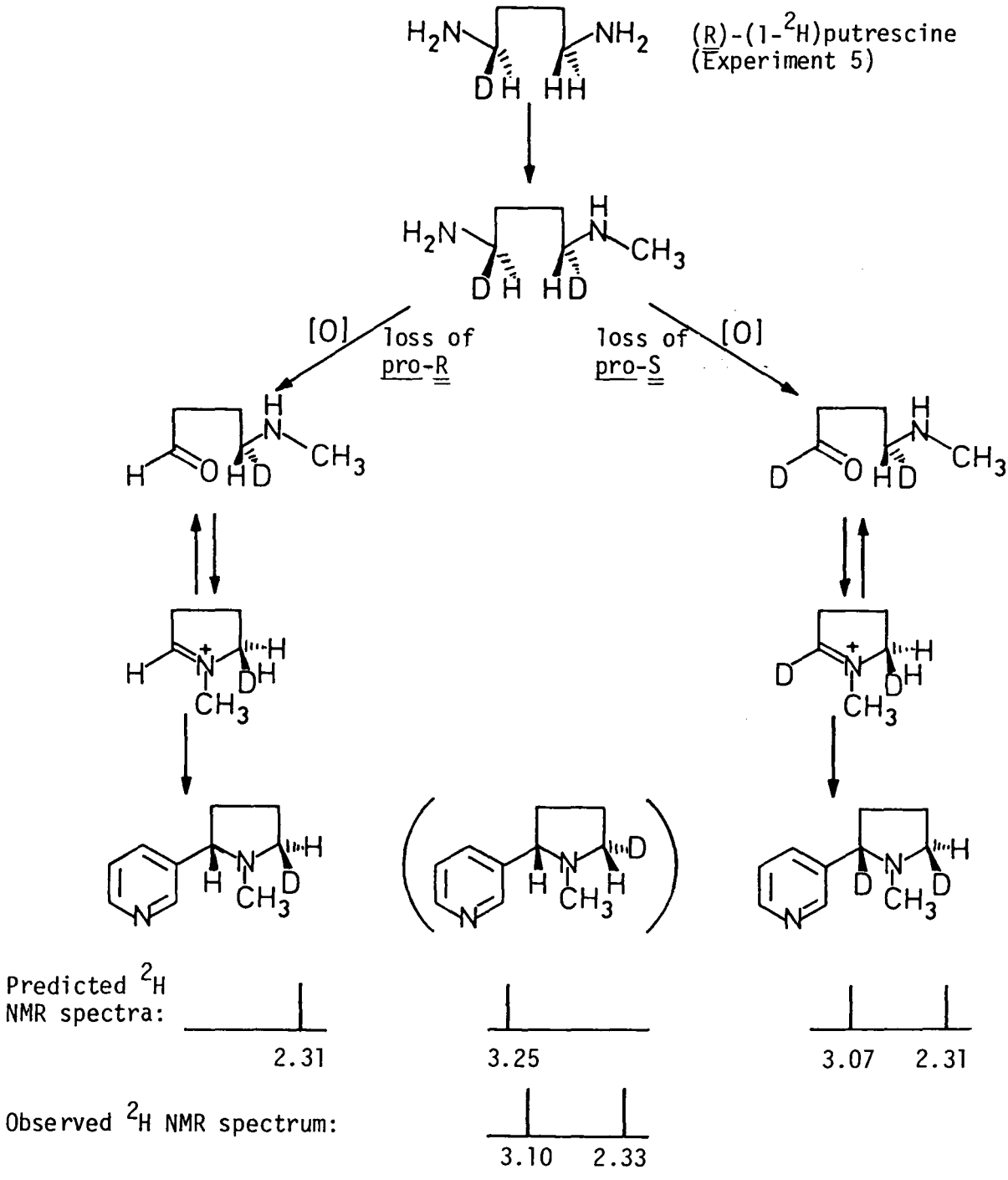


Figure 21. Predicted and observed ^{2}H NMR spectra of nicotine into which (R)-(1-^{2}H)putrescine has been incorporated.

N-methyl-1-pyrrolinium ion, respectively, which would, in turn, give rise to (2'-^{2}H)nicotine or unlabelled nicotine, respectively.

The precursor in this experiment, (R)-(1-^{2}H)putrescine, was labelled in such a way that there way only one deuterium atom in each molecule. Therefore there can be at most one deuterium atom in each nicotine molecule. Since there were two signals in the ^{2}H NMR spectrum, corresponding to the 2' and 5'-pro-R positions of nicotine, the sample must have contained two deuteriated species: (2'-^{2}H)nicotine and (5'-R)-(5'-^{2}H)nicotine.

The presence of (5'-R)-(5'-^{2}H)nicotine in the sample is expected, regardless of the stereochemical course of the oxidation step. The presence of (2'-^{2}H)nicotine, however, is a clear indication that the 4-pro-R hydrogen of N-methylputrescine is the same one which eventually becomes the 2' hydrogen of nicotine. Therefore, it must be the 4-pro-S hydrogen which is lost when N-methylputrescine is oxidixed to 4-methylaminobutanal.

This result allows the elimination of one of the two remaining pathways: Since the pro-R hydrogen is retained in the oxidation step, it follows, on the basis of the argument presented earlier, that the decarboxylation of L-ornithine proceeds with retention of configuration at the reaction site.

Summary

The three stereochemical ambiguities of nicotine biosynthesis have thus been resolved. The substrate for the initial decarboxylation is L-ornithine. This is decarboxylated with retention of configuration

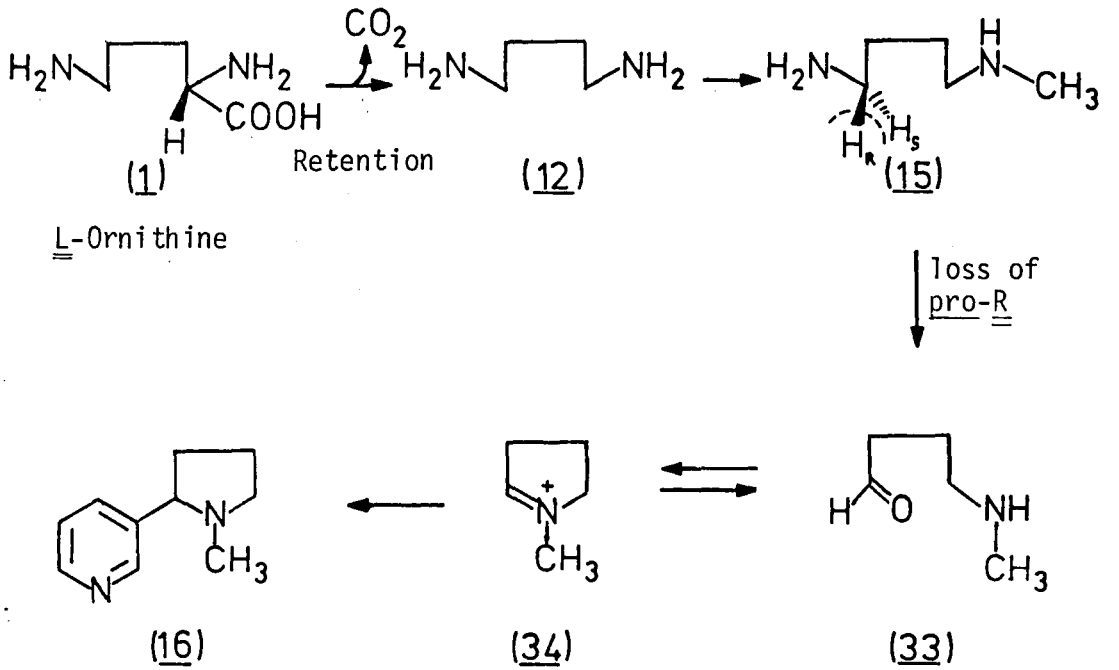


Figure 22. The stereochemical course of nicotine biosynthesis from ornithine.

at the reaction centre to give putrescine. After methylation, the N-methylputrescine is oxidatively deaminated with loss of the 4-pro-S hydrogen.

These findings are consistent with the known stereospecificities of bacterial L-ornithine decarboxylase[53] and of various diamine oxidases.[62-64] Furthermore, in testament to the intuition of Robinson,[34] these findings are consistent with the established stereospecificity of L-lysine decarboxylase of bacterial origin,[60,64,65] and they establish that a similarity exists between the biosynthetic systems responsible for the ornithine-related alkaloids and the lysine-related alkaloids in plants.[9,57]

Further consideration of the results of experiment 5 provides insight into the stereochemistry of enzymic decarboxylation of L-arginine (42). The (1-^{2}H)putrescine which was used as the feeding material in experiment 5 was derived from L-arginine by a published procedure.[53] L-Arginine was converted into (4-^{2}H)agmatine (47) by the action of bacterial L-arginine decarboxylase in ^2H_2O solution, and the (4-^{2}H)agmatine was then converted into (1-^{2}H)putrescine (12) by alkaline hydrolysis. Richards and Spenser deduced that the decarboxylation of L-arginine by bacterial L-arginine decarboxylase proceeds with retention of configuration. This conclusion was arrived at using "... the classical approach to configurational assignment, measurement of optical activity, and comparison with standards of known stereochemistry".[53] The ^{2}H NMR results of experiment 5 provide independent evidence to the same effect.

It was shown, based upon the ^{2}H NMR data in experiment 5, that the 5'-pro-R and not the 5'-pro-S position of nicotine was deuteriated. It follows, from arguments advanced earlier, that the (1-^{2}H)putrescine

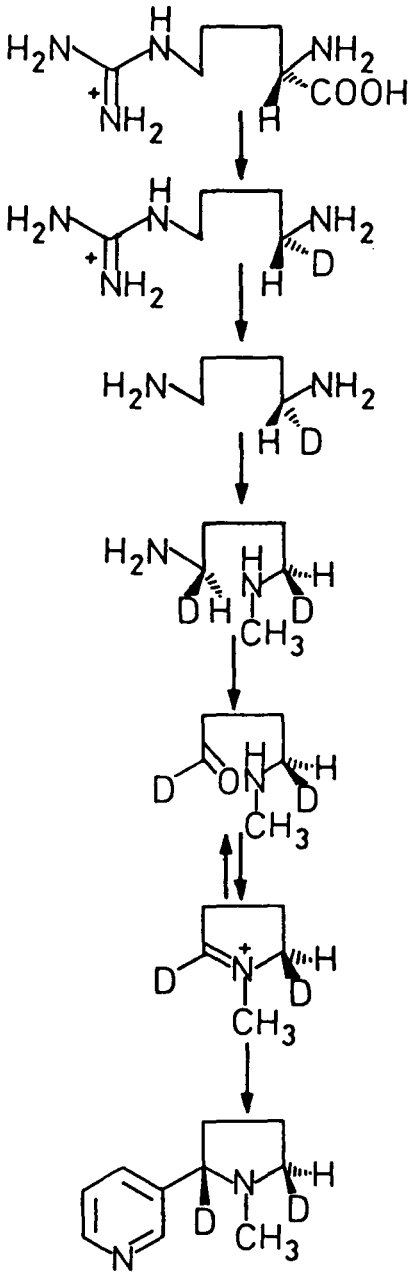


Figure 23. Correlation between the stereochemical course of L-arginine decarboxylase and the configuration at C-5' of nicotine isolated from experiment 5.

from which (5'-$\underline{\underline{R}}$)-(5'-^{2}H)nicotine was derived was ($\underline{\underline{R}}$)-(1-^{2}H)putrescine; that the (4-^{2}H)agmatine from which the ($\underline{\underline{R}}$)-(1-^{2}H)putrescine was obtained was ($\underline{\underline{R}}$)-(4-^{2}H)agmatine and that therefore the reaction, catalysed by bacterial $\underline{\underline{L}}$-arginine decarboxylase, by which the agmatine was obtained, proceeds with retention of configuration.

Proline

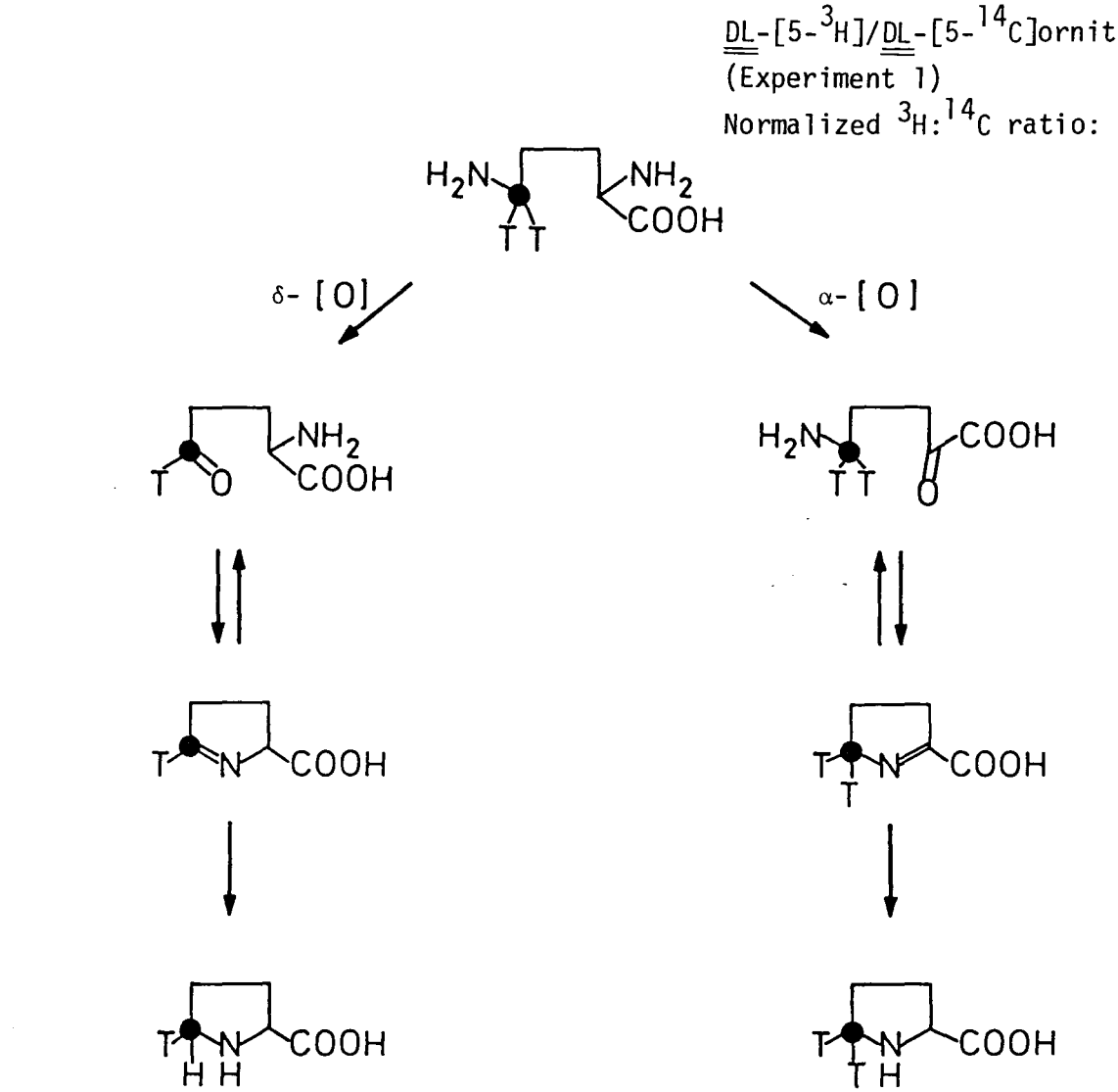
It is well known that ornithine can serve as a substrate in proline biosynthesis.[12,39,40] Although the experiments described here were designed primarily to elucidate the stereochemical aspects of nicotine biosynthesis, the substrate administered in all cases but one was ornithine. It should therefore also be possible to garner useful information about proline biosynthesis from these experiments.

When DL-[5-^{3}H]/DL-[5-^{14}C]ornithine served as substrate (experiment 1), proline was found to be rich in tritium. In fact the $^{3}H:^{14}C$ ratio of the proline was 118 $\pm$ 2% relative to the ornithine fed.

If [5-^{3}H]ornithine were converted to proline via oxidation or transamination at the α-carbon, i.e., via α-keto-δ-aminovaleric acid (4) and 1-pyrroline-2-carboxylic acid (5), no tritium would be lost in the oxidation or in the reduction step. If, on the other hand, oxidation or transamination were to occur at the δ-carbon, one of the two hydrogens, and therefore 50% of the tritium, would be lost in the conversion to glutamic semialdehyde (2). No further loss of tritium is expected during equilibration with 1-pyrroline-5-carboxylic acid (3) or reduction to proline.

It would seem to be clear that the route from DL-ornithine to proline is via α-oxidation.

An alternative explanation for the complete retention of tritium by proline, although improbable, is not ruled out by this experiment. The terminal CH_2NH_2 group of ornithine is prochiral. Ornithine which is non-stereospecifically labelled with tritium in the 5 position will have equal amounts of tritium in each of the 5-pro-R



Observed $^{3}H:^{14}C$ ratio: 118±2

Figure 24. Predicted and observed $^{3}H:^{14}C$ ratios of proline into which DL-[5-^{3}H]/DL-[5-^{14}C]ornithine has been incorporated.

and 5-pro-S positions. If, as is likely to be the case (cf 62-64,66), oxidation at this site proceeds stereospecifically, all of the tritium in one of the two positions will be lost. If, on the other hand, this reaction is non-stereospecific and there is also an infinite tritium isotope effect, only protium will be lost in the oxidation and all the tritium will be retained in glutamic semialdehyde and therefore in proline. An infinite tritium isotope effect in this reaction, however, is in poor agreement with reported deuterium isotope effects in similar enzymic reactions (monoamine oxidase,[66,67] $k_H/k_D = 2.3$, $k_H/k_T = 3.3$ (calculated from $k_H/k_T = (k_H/k_D)^{1.442}$),[68] pig diamine oxidase,[69] $k_H/k_D = 2.0$, calculated[68] $k_H/k_T = 2.7$). This is therefore an unlikely explanation of the observed retention of tritium by proline from ornithine.

The observed increase in the $^3H:^{14}C$ ratio of proline over that of ornithine can, in principle, be explained in one of two ways. There may be an isotope effect acting either to decrease the amount of ^{14}C (an improbable situation[15]), or to increase the amount of tritium in proline. A chemical reaction in which the rate is slower for a tritiated species than for a protiated species, if not allowed to go to completion, will tend to enrich the starting material in tritium.[15] It may therefore be the case that once [5-^{3}H]proline has been formed via α-oxidation of [5-^{3}H]ornithine, oxidation at C-5 of proline results in enrichment of tritium within proline by virtue of discrimination against tritium. Oxidation of proline in this manner has been shown to occur readily,[70] although this pathway would seem to be strongly inhibited under conditions of stress such as may be experienced by the plant during a feeding experiment.[71] Also, tritium isotope effects have been observed in the

oxidation of proline to 1-pyrroline-5-carboxylic acid (3).[40] Alternatively, a similar mechanism (δ-oxidation of ornithine) may be responsible for an increase in the amount of tritium in ornithine. This increase in the $^{3}H:^{14}C$ ratio would be passed on to proline, since α-oxidation would not affect tritium in the 5-position. The observed increase in $^{3}H:^{14}C$ ratio of ornithine during the experiment tends to support the latter explanation.

In the two experiments designed to determine the chirality of ornithine which serves as precursor to nicotine, it was possible also to establish which of the two enantiomers of ornithine is oxidised preferentially to proline.

When L-[5-^{3}H]/DL-[5-^{14}C]ornithine was fed (experiment 2), proline was isolated which had retained 18 ± 1% of the tritium, relative to ^{14}C, present in the feeding material. Had L-ornithine been the only precursor of proline, the $^{3}H:^{14}C$ ratio would have been twice that of the ornithine which was fed. On the other hand, if D-ornithine were the sole precursor, proline would have contained no tritium. It follows[11] that the D isomer of ornithine is a far more efficient precursor of proline than is the enantiomeric L-ornithine.

At this stage, it is not possible to assess precisely the relative importance of L-ornithine as a precursor of proline in these experiments. If it is assumed that L-ornithine is converted into proline via α-oxidation, none of the tritium would be lost from 5-tritio-ornithine during the conversion and it can be calculated[11] that 9% of the proline produced arises from L-ornithine. If, on the other hand, the conversion proceeds via δ-oxidation of L-ornithine, only half the tritium from 5-tritio-ornithine would be retained in proline, and twice as much of the

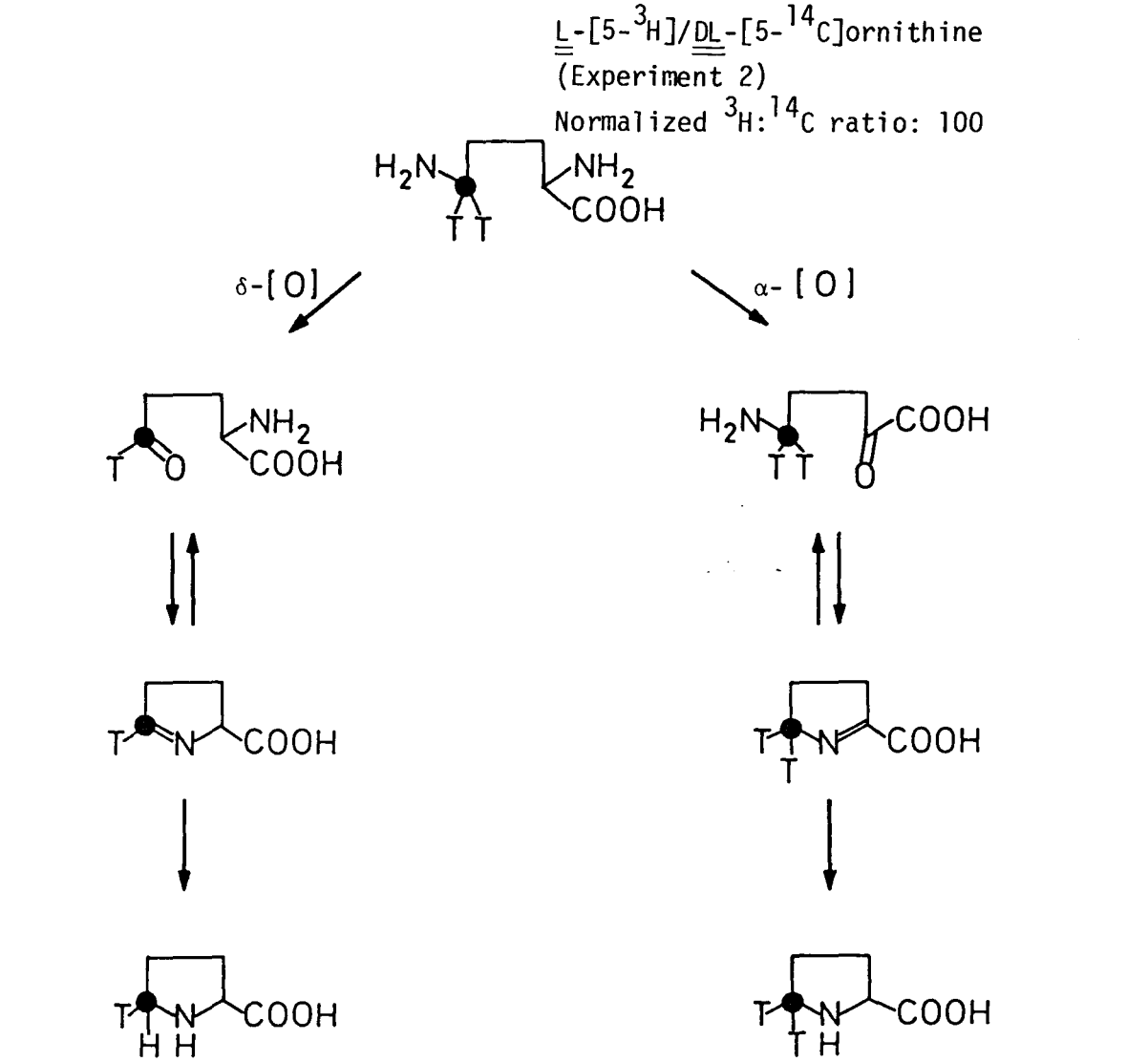


Figure 25. Predicted and observed $^{3}H:^{14}C$ ratios of proline into which L-[5-^{3}H]/DL-[5-^{14}C]ornithine has been incorporated.

proline (18%) must have arisen from L-ornithine; i.e., since only half the tritium is retained, twice the amount of L-ornithine must be converted to arrive at the same amount of tritium in proline. Therefore it can only be said that between 9% and 18% of the proline produced in this experiment was derived from L-ornithine.

It is interesting to note that the $^{3}H:^{14}C$ ratio of ornithine decreased drastically during this experiment; reisolated ornithine contained only one-fifth of the tritium relative to ^{14}C of the ornithine that was fed. Since tritium was present only in the L isomer of ornithine, one possible explanation for this observation is that L-ornithine is metabolised at a faster overall rate than D-ornithine, leaving less tritium and therefore a lower $^{3}H:^{14}C$ ratio in the remaining ornithine.

In experiment 3, in which D-[5-^{3}H]/DL-[5-^{14}C]ornithine served as substrate, there was twice as much tritium relative to ^{14}C in proline as there was in the ornithine which was fed. It follows from arguments similar to those used above that had L-ornithine been the precursor of proline, proline would have been poor in tritium and rich in ^{14}C. If D-ornithine were the precursor of proline, however, the $^{3}H:^{14}C$ ratio would be twice that of the administered ornithine. Clearly, this result provides support for the conclusion that D-ornithine is the preferred precursor of proline in the tobacco plant.

This experiment also provides support for the conclusion that L-ornithine is metabolised more rapidly than is its enantiomer. Ornithine reisolated in this experiment was greatly enriched in tritium relative to the ornithine that was fed, or alternatively, ornithine was

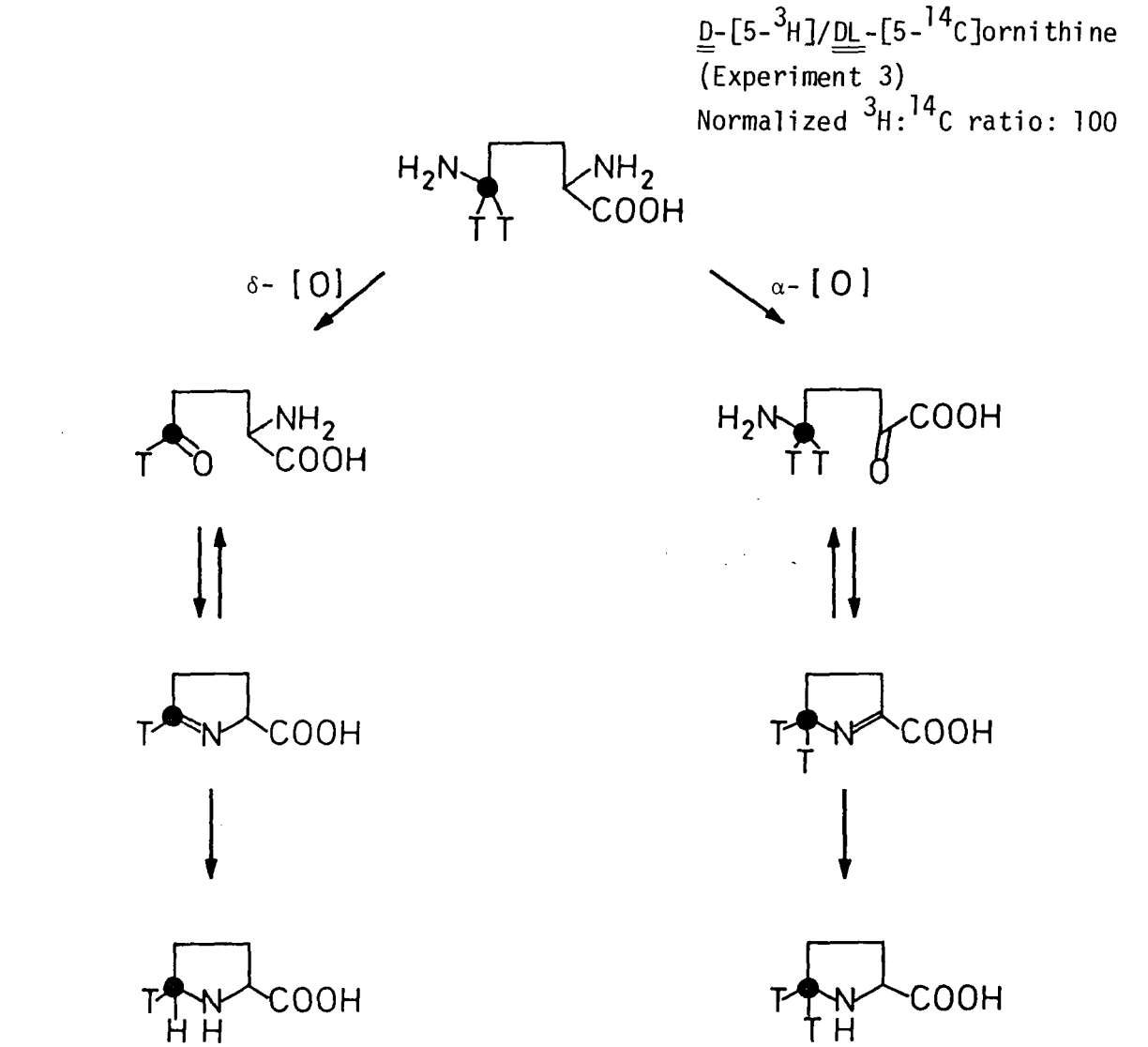


Figure 26. Predicted and observed $^{3}H:^{14}C$ ratios of proline into which D-[5-^{3}H]/DL-[5-^{14}C]ornithine has been incorporated.

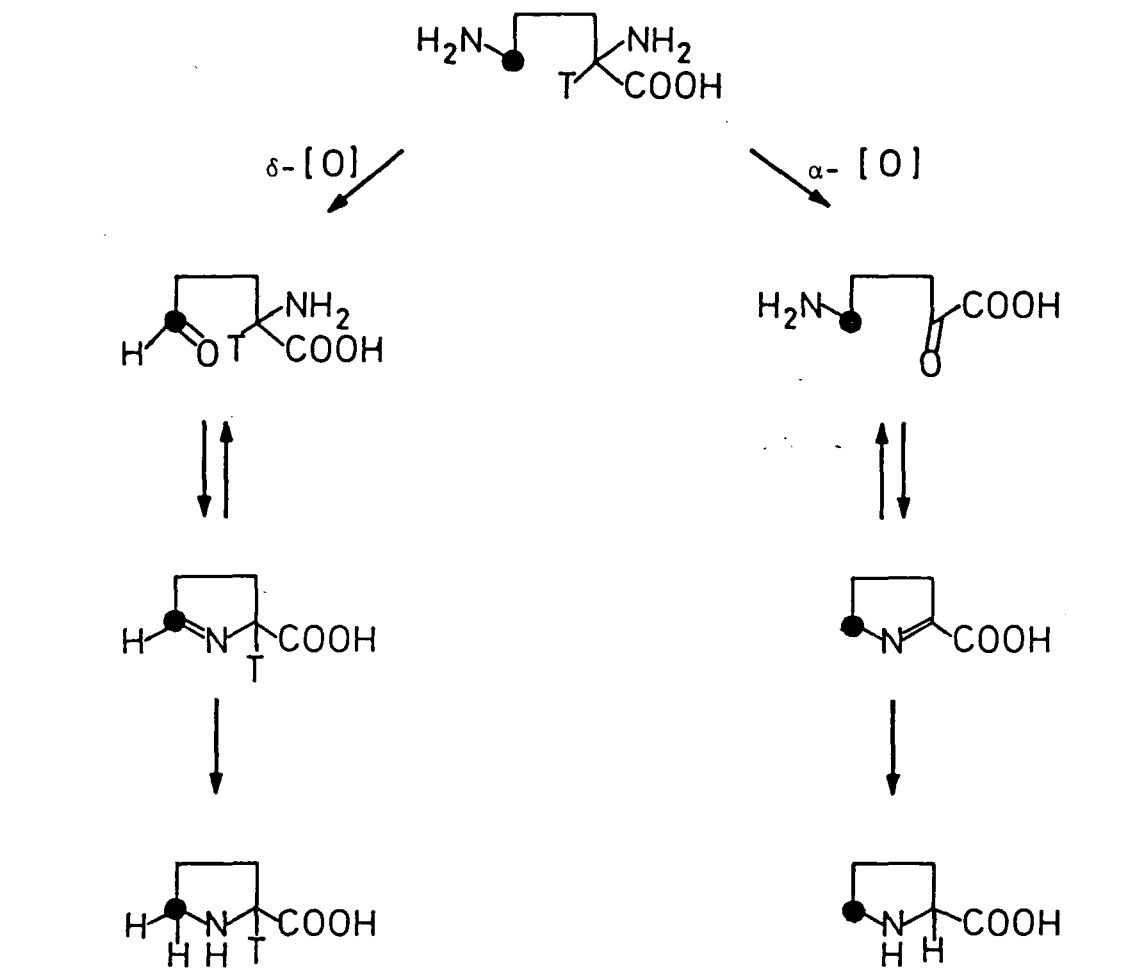
much depleted in the amount of ^{14}C relative to tritium, as compared with starting material. A relative increase in the amount of singly, ^{14}C-labelled L-ornithine would effectively decrease the ^{3}H:^{14}C ratio of ornithine in the plant, whereas a decrease in the amount of L-ornithine would result in an increase in the ^{3}H:^{14}C ratio. In this experiment, there was an apparent decrease in the amount of L-ornithine relative to the amount of D-ornithine; such a decrease as would be occasioned by the more rapid metabolism of L-ornithine.

The evidence presented thus far is in agreement with the conclusions of Mestichelli[12,51] that the major metabolic pathway from DL-ornithine to proline is via α-oxidation of ornithine. No support can be found, however, for the assertion by Mestichelli *et al.* that "... it must be concluded that the conversion of L-ornithine into L-proline ... takes place via α-keto-δ-aminovaleric acid ...".[12] Rather, the work presented here would indicate that it is D-ornithine which is converted into proline via α-keto-δ-aminovaleric acid. These findings are qualitatively similar to those of Leistner *et al.*[11] who showed that D-lysine, the higher homolog of D-ornithine, is converted via α-oxidation into pipecolic acid, the higher homolog of proline.

No instance has been found where D-ornithine is reported to occur as a natural product in plants. It was of some interest, therefore, to investigate the mode of incorporation of L-ornithine into proline, in the absence of D-ornithine.

In an experiment where L-[2-^{3}H]/L-[5-^{14}C]ornithine was fed to tobacco, proline was isolated which retained 40 ± 1% of the tritium, relative to ^{14}C, of the substrate ornithine. In view of the other

L-[2-^{3}H]/L-[5-^{14}C]ornithine
(Experiment 4)
Normalized ^{3}H:^{14}C ratio: 100



Predicted ^{3}H:^{14}C ratio: 100

Predicted ^{3}H:^{14}C ratio: 0

Observed ^{3}H:^{14}C ratio: 40±1

Figure 27. Predicted and observed ^{3}H:^{14}C ratios of proline into which L-[2-^{3}H]/L-[5-^{14}C]ornithine has been incorporated.

experiments reported here, this result is surprising.

If L-[2-^{3}H]ornithine is converted to proline via α-oxidation, the resultant proline should contain no tritium, the tritium being lost in the oxidation step. If, on the other hand, the conversion into proline is effected via δ-oxidation, the carbon bearing the tritium would not be affected, and there would be no loss of tritium; proline would have the same $^{3}H:^{14}C$ ratio as the ornithine which was fed.

Since both tritium and ^{14}C were present only in L-ornithine, the incorporation of either radiolabel implies that L-ornithine can serve as a precursor to proline in tobacco plants. Furthermore, since proline retained 40% of tritium from the ornithine substrate, at least 40% of the proline produced during the experiment must have arisen from ornithine via some pathway which does not involve loss of hydrogen from C-2 of ornithine. Biosynthesis of proline from L-ornithine via δ-oxidation satisfies this requirement.

The remaining 60% of the proline isolated in this experiment which was not labelled with tritium may have arisen from L-ornithine in one of two ways. Either L-ornithine was oxidatively deaminated at the α-carbon and converted to proline by reduction of 1-pyrroline-2-carboxylic acid, or L-ornithine underwent reversible α-transamination before being converted to proline via δ-oxidation.

The latter of these possibilities may be eliminated on the basis of the distribution of tritium within nicotine isolated from this experiment.

Argument was marshalled in the previous section of this discussion to show that in all events, nicotine isolated from plants to which L-

[2-^{3}H]/L-[5-^{14}C]ornithine had been administered, must retain at least 50% of the tritium relative to ^{14}C of the ornithine fed. This argument made no allowance for the possibility that a reversible α-transamination might have occurred prior to decarboxylation of L-ornithine to produce putrescine. Had reversible α-transamination occurred, tritium would have been lost into the environment and the predicted 50% retention of tritium relative to ^{14}C by nicotine would have been reduced to the same extent that tritium was lost from the α-carbon of ornithine.

In the present case, a 60% decrease in the amount of tritium in L-ornithine would be required in order to explain the 'missing' 60% of tritium from proline on the basis of reversible α-transamination. This in turn would result in the predicted 50% retention of tritium by nicotine being reduced by 60%, down to 20%. This was not the case. Nicotine was found to have retained fully one half of the tritium in its 5' position. Therefore, reversible α-transamination of L-ornithine is not an acceptable explanation of the observation that 60% of the proline contained no tritium.

This conclusion is supported by the observed ^{3}H:^{14}C ratio of reisolated ornithine. Ornithine was enriched in tritium relative to ^{14}C during the experiment by 8%. This is indicative, not of a loss of tritium by reversible α-transamination, but of an isotope effect discriminating against tritium in an irreversible reaction occurring at the α-carbon of ornithine.[13] Such a reaction is oxidative deamination.

These observations are consistent with the alternative explanation, advanced earlier, that the 60% of proline which was non-tritiated arose from L-ornithine via α-oxidation and subsequent reduction of 1-

pyrroline-2-carboxylic acid.

The apparently contradictory observations that the route from DL-ornithine to proline is via α-oxidation (12, and this work), that the route from D-ornithine into proline is via α-oxidation, but that the route from L-ornithine into proline is to a large extent via δ-oxidation, can be reconciled.

This work has shown that D-ornithine, and apparently DL-ornithine are converted into proline via α-oxidation. If a comparatively small amount of DL-ornithine were converted into proline via δ-oxidation, this numerically smaller result might well go unnoticed. Since D-ornithine goes into proline via α-oxidation, the small component being converted via δ-oxidation would need to be L-ornithine. Evidence to support this hypothesis, direct and indirect, exists in this work and in the literature.[12,51]

The finding by Mestichelli et al.[12,51] that proline, isolated from plants to which DL-[2-^{3}H]/DL-[5-^{14}C]ornithine had been fed, contained small but measurable amounts of tritium (6-14% retention of tritium relative to ^{14}C of the feeding material) indicates that there is some mechanism for the conversion of ornithine into proline which leaves the α-hydrogen in place, i.e., δ-oxidation, a possibility which the authors imply.[12]

In experiment 2, where L-[5-^{3}H]/DL-[5-^{14}C]ornithine was fed to tobacco, proline was isolated which showed 18% retention of tritium relative to ^{14}C. This indicates, since there was no tritiated D-ornithine present, that L-ornithine is converted into proline, albeit at a slower rate than is D-ornithine. Furthermore, it is now possible to calculate what proportion of the L-ornithine which was incorporated into proline

underwent the conversion via α-oxidation, what proportion proceeded via δ-oxidation, and what proportion of the proline was formed from L-ornithine, rather than from D-ornithine.

If we assume that when L-[5-^{3}H]/DL-[5-^{14}C]ornithine was fed (experiment 2), the ^{3}H:^{14}C ratio was 100, then the ^{3}H:^{14}C ratio of the L-ornithine was 200. Proline derived via α-oxidation of L-ornithine would therefore have a ^{3}H:^{14}C ratio of 200 and that derived via δ-oxidation, where half the tritium is lost, would have a ^{3}H:^{14}C ratio of 100. Since tritium in proline is derived only from L-ornithine, we may express the relative contributions of α- and δ-oxidation of L-ornithine thus:

$$40\% \times n \times 100 + 60\% \times n \times 200 = 18,$$

where n represents the proportion of proline derived from L-ornithine via both routes and 18 is the observed ^{3}H:^{14}C ratio of the proline isolated. Rearranging this expression, we may find the value of n:

$$40n + 120n = 18\%$$

$$n = \frac{18}{160}\%$$

$$= 11.25\%.$$

Of this 11.25% of proline derived from L-ornithine, 4.5% (i.e. 40 X 11.25%) was derived via δ-oxidation and the remainder (6.75% or 60 X 11.25%) was derived via α-oxidation. Therefore, when L-[5-^{3}H]/DL-[5-^{14}C]ornithine served as the substrate for proline biosynthesis (experiment 2), 88 ± 1%

of the proline was derived from D-ornithine via α-oxidation, 7 ± 1% of the proline was derived from L-ornithine via α-oxidation and 5 ± 1% was derived via δ-oxidation of L-ornithine.

Further evidence for the existence of an ornithine-δ-transaminase, an enzyme which could catalyse the oxidative δ-deamination of ornithine, is available in the form of an apparent isotope effect, observed indirectly in experiment 1. When ornithine was reisolated from the plants after DL-[5-^{3}H]/DL-[5-^{14}C]ornithine had been administered, it was found that ornithine had been enriched in tritium relative to ^{14}C in the course of the experiment. The only plausible explanation for this increase of tritium is an isotope effect in the δ-oxidation of ornithine which discriminates against tritiated ornithine (vide supra).

Therefore no contradiction exists in these results. D-ornithine is converted into proline at a faster rate than is L-ornithine. Also, D-ornithine is converted into proline only via α-oxidation, whereas L-ornithine can be oxidised at either the α-carbon, or the δ-carbon in the course of its conversion into proline.

Summary

We have seen that proline can be produced in two different ways from L-ornithine. It is possible that the existence of two pathways is an evolutionary response to two different needs for proline: proline produced for protein synthesis, and that produced in response to water stress, a condition under which large amounts of proline accumulate in many plants.[71,72]

Under water stress, protein begins to break down. This alone does not account for the amount of free proline which accumulates, since

there is more proline and less of the other amino acids than would result from simple proteolysis.[71] Therefore there must be an increase in the de novo synthesis of proline.

The function of this increased amount of proline is poorly understood, although several theories have been advanced to explain this phenomenon. One hypothesis is that as the concentration of other amino acids and ammonia increases during proteolysis, a problem of toxicity arises and the conversion of these compounds into the presumably less harmful proline acts as a detoxification mechanism.[71] An alternative explanation is that proline serves as an efficient source of reduced carbon and nitrogen during the plant's recovery from drought, and that therefore proline is 'stockpiled' in the initial stages of drought.[72] Regardless of the cause, proline accumulates in plants during water stress.

Glutamic acid (41) has been shown to be a more efficient precursor of proline under drought conditions than under normal conditions.[73] The conversion of glutamic acid (41) into proline proceeds via glutamic semialdehyde (2). It is conceivable then, that the conversion of L-ornithine into proline via δ-oxidation and glutamic semialdehyde might occur more readily under drought conditions, conditions under which the conversion of glutamic acid into proline via glutamic semialdehyde is more facile.

This leads to the speculation that normally, the major route from L-ornithine into proline is via α-oxidation and α-keto-δ-aminovaleric acid, and that under stress, such as might be encountered in the course of a feeding experiment, the biogenesis of proline via δ-oxidation of L-ornithine may become a contributing or major pathway.

Useful insight into the source(s) of stress-induced proline might be gained through the use of $^{3}H,^{14}C$ double labelling techniques such as have been employed in the present work. By varying the conditions under which the plants are maintained, from arid to humid, and by feeding α- or δ-tritiated L-ornithine, the extent to which tritium was retained by proline would provide an indication of the relative contribution of the two pathways.

As was mentioned earlier, D-ornithine has not been reported as a natural product in higher plants. It is clear, however, that D-ornithine is incorporated efficiently into proline in tobacco. This apparent inconsistency may be rationalized in one of two ways. On the one hand, the incorporation of D-ornithine may not represent a normal biosynthetic pathway; rather, it may be the fortuitous result of a general D-amino acid oxidase acting on D-ornithine to produce α-keto-δ-aminovaleric acid. The same product results when L-ornithine is oxidised at the α-position. This work has shown that L-ornithine can be converted into proline via α-keto-δ-aminovaleric acid. Therefore the presence of a D-amino acid oxidase in tobacco could result in the conversion of D-ornithine into proline.

On the other hand, D-ornithine may be a naturally occurring compound in tobacco which has yet to be identified. If this were the case, the presence of enzymes for which D-ornithine is a specific substrate is to be expected. Likewise, there would need to be an enzyme capable of producing D-ornithine, such as an ornithine racemase. While it is true that an amino acid racemase has not yet been reported to occur in plants, such enzymes have been identified in bacterial systems.

A reversible D-amino acid transaminase could also result in the production of D-ornithine if a suitable substrate, i.e., α-keto-δ-aminovaleric acid, the product of the α-transamination or α-oxidation of ornithine were present. It is noteworthy that a D-amino acid transaminase has been isolated from pea seedlings,[74] and that therefore, there may be similar enzymes present in other higher plants.

Racemisation of ornithine has been found to occur in one plant system, although the result may be suspect. When L-ornithine which was tritiated at other than the α-position was administered to Senecio vulgaris plants, approximately 10% of the tritium in reisolated ornithine was in D-ornithine.[75] This result indicates that racemisation of ornithine did occur in the course of the experiment. However, the workup included reducing conditions at such time as α-imino-δ-aminovaleric acid may have been present in the mixture being worked up. Non-enzymic reduction of α-imino-δ-aminovaleric acid, a prochiral molecule, will give rise to an equimolar mixture of D-ornithine and L-ornithine. The presence of D-ornithine may therefore have been an artifact of the isolation procedure.

Whether D-ornithine which is formed by racemisation of L-ornithine is an obligatory precursor to proline biosynthesis via α-oxidation, or whether the incorporation of D-ornithine is simply the fortuitous result of the action of a D-amino acid oxidase cannot be resolved on the basis of the results presented here. I tend to favour the latter alternative since the former seems an inefficient process to have evolved for proline biosynthesis, and the dictates of Occam's Razor require that the simpler (i.e. the latter, in this instance) of two hypotheses be accepted as the

more probable.

Further, unambiguous experimentation is required if these uncertainties are to be resolved. For example, to establish whether or not racemisation of ornithine occurs, and in which of the two senses, samples of radio-labelled L-ornithine and D-ornithine, in separate experiments, could be administered to tobacco, ornithine reisolated after a time, and the reisolated ornithine be analysed for the amount of radioisotopes present in each of the D and L isomers.

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^{2}H N.M.R. Spectroscopy as a Probe of the Stereochemistry of Biosynthetic Reactions. The Biosynthesis of Nicotine

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The pyrrolidine ring of nicotine is derived from L-ornithine which, in intact *Nicotiana tabacum*, is decarboxylated with retention of configuration; the resulting putrescine is *N*-methylated and then oxidised, with loss of the *pro-S* proton on C-4, to yield *N*-methyl-1-pyrrolinium ion, which is attacked by the pyridine ring precursor at the 1-*si*,2-*re* face to yield (*S*)-nicotine.

The accepted route from ornithine[1,2] (1) into the pyrrolidine ring of nicotine (7)[3,4] *via* putrescine (2),[5] *N*-methylputrescine (3),[6] *N*-methyl-4-aminobutanal (4)[7] and *N*-methyl-1-pyrrolinium ion (5),[3] which is based on the results of tracer experiments

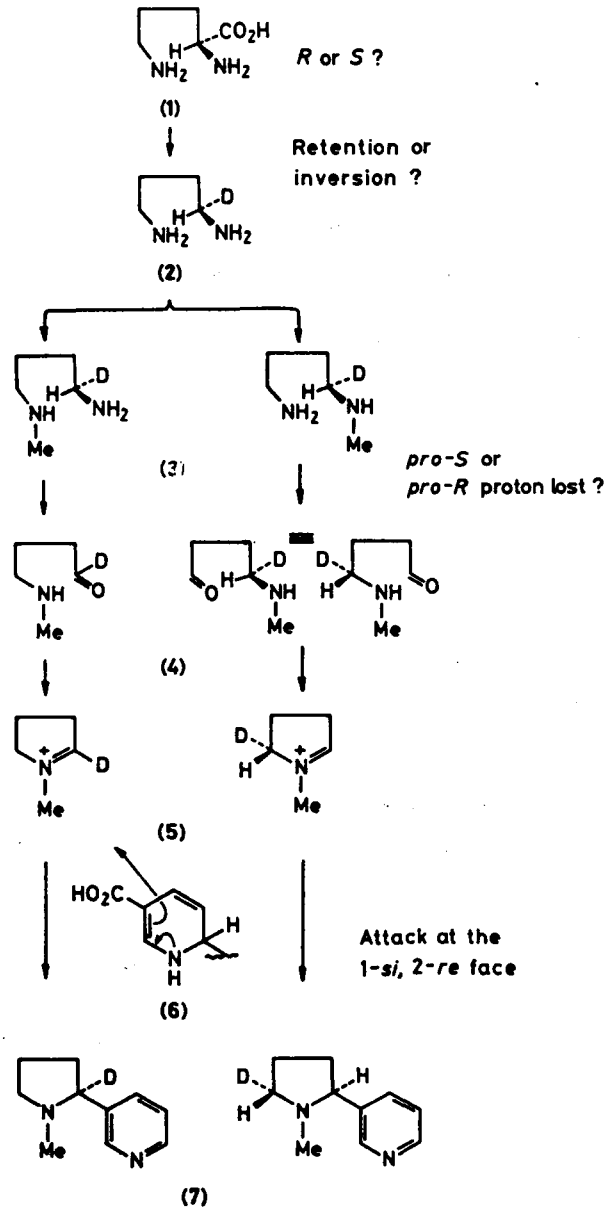
with intact plants[1-3,5,6] and tissue cultures[7] of *Nicotiana tabacum*, and on enzyme studies,[8,9] fully accounts for the origin of the pyrrolidine nucleus, in chemically rational terms. Stereochemical aspects of the biosynthetic sequence have so far not received attention.

We have employed ^{2}H n.m.r. spectroscopy to resolve one of the stereochemical ambiguities of the pathway, the prochirality of the conversion of *N*-methylputrescine (3) into *N*-methyl-4-aminobutanal (4).

(*R*)-[1-^{2}H]Putrescine dihydrochloride (2) (125 mg) was obtained by hydrolysis of (*R*)-[1-^{2}H]agmatine which had been prepared[10] by enzymic decarboxylation of L-arginine in ^2H_2O, a reaction which proceeds with retention of configuration.[10] A solution containing the (*R*)-[1-^{2}H]putrescine, together with [1,4-^{14}C]putrescine (68 μCi) (New England Nuclear), was administered to 12 3-month-old tobacco plants over a period of 15 days, by the wick method. Nicotine was isolated and purified and a small sample was converted into the diperchlorate for determination of radioactivity.† From the observed specific incorporation of ^{14}C (1.3%)‡ a deuterium enrichment corresponding to *ca.* 40 times natural abundance at deuteriated position(s) was inferred.‡

The ^{2}H n.m.r. spectrum§ (Figure 1) showed that deuterium was present at two positions of the pyrrolidine ring of nicotine, to an approximately equal extent. The chemical shifts¶ of the two ^{2}H n.m.r. signals corresponded to those of the 5′-*pro*-*R*-proton (δ 2.33 ± 0.02 p.p.m.)** and of the 2′-proton (δ 3.10 ± 0.02 p.p.m.) of nicotine, respectively. (Literature[11] values: 5′-*pro*-*R*, δ 2.31 ± 0.02; 2′-, δ 3.07 ± 0.02; 5′-*pro*-*S*, δ 3.25 ± 0.02 p.p.m.)

From this result the stereochemical course of the incorpora-



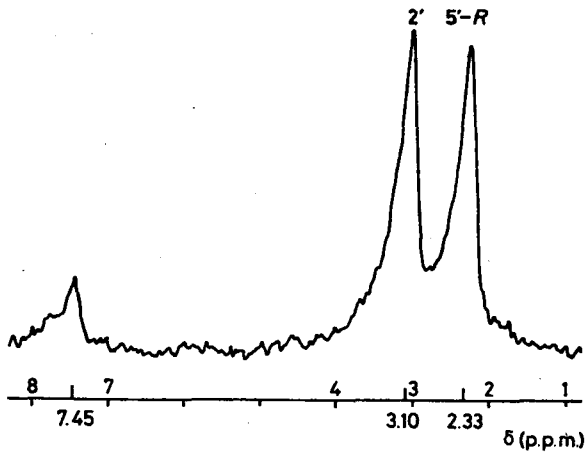


Figure 1. 61.42-MHz ^{2}H N.m.r. spectrum of nicotine (273 mg, in chloroform, 1.1 ml) isolated from tobacco plants to which *R*-[1-^{2}H]putrescine had been administered. The signal at δ 7.45 p.p.m. is due to natural abundance C[^{2}H]Cl$_3$ in the solvent.

† Radioactivity was assayed by liquid scintillation counting (Beckman LS 9000 Liquid Scintillation System). All samples were recrystallized to constant specific radioactivity, dissolved in Aquasol (New England Nuclear), and counted in triplicate, under comparable conditions of quenching.

‡ $$\frac{\text{Molar specific activity of nicotine (dpm/mmol)}}{\text{Molar specific activity of putrescine (dpm/mmol)}} \times 100 = \frac{2.4 \times 10^6}{1.9 \times 10^8} \times 10^2 = 1.3$$

$$\frac{\%\ \text{Specific incorporation} \times \frac{1}{2}}{\%\ \text{Natural abundance of deuterium}} = \frac{1.3 \times \frac{1}{2}}{0.0156} = 42 \text{ times natural}$$

abundance ^{2}H per enriched position.

§ The ^{2}H n.m.r. spectrum was recorded at 61.42 MHz in the Fourier mode on a Bruker-WM400 spectrometer (Southwestern Ontario NMR Centre, funded by a Major Installation grant from the Natural Sciences and Engineering Research Council of Canada, and located at the University of Guelph, Guelph, Ontario). The spectrum was determined in $CHCl_3$ with external ^2H_2O serving as frequency lock: acquisition time, 1.638 s; pulse width 30 μs. Natural abundance C[^{2}H]Cl$_3$ served as an internal standard.

¶ The chemical shift of $CHCl_3$ was found to change by as much as 0.5 p.p.m. as a function of nicotine concentration. Consequently the chemical shift of $CHCl_3$ relative to Me_4Si in the sample was determined in a separate experiment on a Varian EM 390 spectrometer.

** The observation that the 5′-*pro*-*R*-proton but not the 5′-*pro*-*S*-proton of nicotine was replaced by deuterium serves as independent evidence that the starting [1-^{2}H]putrescine was (*R*)-[1-^{2}H]-putrescine, that the [1-^{2}H]agmatine from which it was obtained was (*R*)-[1-^{2}H]agmatine, and that the reaction, catalysed by bacterial L-arginine decarboxylase, by which the agmatine was obtained, proceeds with retention of configuration.

tion of putrescine into nicotine may be inferred. Putrescine, a non-dissymmetric (C_{2v}) molecule, will be methylated at either nitrogen atom to give *N*-methylputrescine. Methylation of (*R*)-[1-^{2}H]putrescine will then give rise to an equimolar mixture of *N*-methyl-(*R*)-[1-^{2}H]putrescine and *N*-methyl-(*R*)-[4-^{2}H]putrescine.

Oxidative deamination of *N*-methyl-(*R*)-[1-^{2}H]putrescine, followed by cyclization, generates *N*-methyl-(*R*)-[5-^{2}H]-1-pyrrolinium ion as the sole product. This structure is known not to tautomerize under biosynthetic conditions.[3] *N*-Methyl-(*R*)-[5-^{2}H]-1-pyrrolinium ion in turn leads to nicotine labelled with deuterium in the 5′-*pro*-*R*-position.

Oxidative deamination of *N*-methyl-(*R*)-[4-^{2}H]putrescine, followed by cyclization, can lead either to *N*-methyl-1-pyrrolinium ion or to *N*-methyl-[2-^{2}H]-1-pyrrolinium ion, depending upon whether the *pro*-*R* or *pro*-*S* hydrogen, respectively, is stereospecifically lost during the oxidation. These two species would, in turn, give rise to unlabelled nicotine or to [2′-^{2}H]nicotine, respectively.

The nicotine generated in the course of the feeding experiment with (*R*)-[1-^{2}H]putrescine will be a mixture of several molecular species. The observed deuterium distribution of the isolated sample of nicotine permits inferences to be drawn about the mode of derivation of these species. Thus, if oxidation of *N*-methylputrescine proceeds with stereospecific loss of the *pro*-*R* hydrogen atom, deuterium will be incorporated into only one position in nicotine (5′-*pro*-*R*). Stereospecific loss of the *pro*-*S* hydrogen, on the other hand, will result in nicotine labelled with deuterium in two positions (5′-*pro*-*R* and 2′).

The results (Figure 1) show that two positions in nicotine, 5′-*pro*-*R* and 2′, are enriched in deuterium. It follows that it is the *pro*-*S* proton which is lost from the -CH_2NH_2 group of putrescine in the course of its conversion into nicotine. This is consistent with the known stereospecificity of reactions catalysed by diamine oxidase.[12–14]

Further experiments, with radioactive tracers, resolved the remaining stereochemical ambiguities of the route from ornithine into nicotine. Whereas the nicotine, isolated from a feeding experiment with a mixture of DL-[(*RS*)-5-^{3}H]ornithine and DL-[5-^{14}C]ornithine, showed a ^{3}H/^{14}C ratio which was approximately 0.7 times that of the doubly labelled ornithine that served as the substrate (calculated value, 0.75 times), the nicotine, isolated from an experiment with a mixture of L-[(*RS*)-5-^{3}H]ornithine and DL-[5-^{14}C]ornithine, showed a ^{3}H/^{14}C ratio which was 1.4 times that of the administered tracer. This demonstrates[15] that L-ornithine (predicted ^{3}H/^{14}C ratio of nicotine, 1.5 times that of substrate) rather than D-ornithine (predicted ^{3}H/^{14}C ratio of nicotine, zero) is the precursor of the pyrrolidine ring of nicotine.

Furthermore, a feeding experiment with intermolecularly doubly labelled DL-[2-^{3}H,5-^{14}C]ornithine[16] yielded a sample of nicotine whose ^{3}H/^{14}C ratio was approximately half that of the labelled substrate. Thus, only one tritium per two ^{14}C atoms entered the product. The ^{2}H-n.m.r. experiment shows that the *pro*-R proton at C-1 of putrescine is retained and the *pro*-*S* proton is lost in the course of nicotine biosynthesis. Thus, when L-ornithine (**1**) is converted into putrescine (**2**) *en route* into the pyrrolidine ring of nicotine (**7**), the α-proton of L-ornithine gives rise to that proton at C-1 of putrescine which is lost, that is, the *pro*-*S* proton. It follows that the decarboxylation of ornithine to putrescine in intact *N. tabacum* takes place with retention of configuration. The decarboxylation of ornithine, catalysed by ornithine decarboxylase of bacterial origin, likewise takes place with retention of configuration.[10]

The stereochemistry of the final step in nicotine biosynthesis follows from the chirality of the product, (*S*)-nicotine (**7**). Attack by the nicotinic acid-derived fragment (**6**) on the *N*-methyl-1-pyrrolinium ion (**5**) occurs from the 1-*si*,2-*re* face of the latter.

The four stereochemical ambiguities of nicotine biosynthesis are thus resolved.

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