Recent Developments in the Biosynthesis of the Tropane Alkaloids[1]

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Abstract

Recent work on the biosynthesis of the tropane moiety of cocaine, hyoscamine, scopolamine, and related alkaloids is reviewed. Revision of the generally accepted biosynthetic pathway to these alkaloids is now proposed in the light of new discoveries. New information on the biosynthesis of some of the acid moieties (benzoic, tiglic and tropic acid) of the tropane ester alkaloids is also discussed.

Key words

Biosynthesis, tropane alkaloids, cocaine, hyoscyamine, scopolamine, tropane ester alkaloids.

Introduction

There are currently about 10,000 alkaloids of known structure (1). Over the last 40 years much has been learned about the biosynthesis of this group of secondary natural products. They arise from a relatively small number of organic compounds which are found in almost all living systems (acetic acid, anthranilic acid, arginine, lysine, mevalonic acid, nicotinic acid, ornithine, phenylalanine, tryptophan, and tyrosine). Fig. 1 illustrates, in abbreviated form, the metabolic relationship of alkaloids to these precursors. Unlike some classes of natural products, such as the terpenes, alkaloids are formed by many diverse biosynthetic routes.

Once the biosynthetic route to a certain alkaloid has been elucidated it is tempting to generalize this result to include other alkaloids of the same class. Thus it is now considered that most indole alkaloids containing a monoterpene residue are modifications of strictosidine (3) derived from tryptamine (1) and secologanin (2), as illustrated in Fig. 2 (2, 3), even though experimental biosynthetic work has been carried out on only a small fraction of the known monoterpene indole alkaloids. However there can be variations in the way in which an alkaloid skeleton is assembled. This is true of the tropane alkaloids and the recent modifications to the generally accepted biosynthesis of this

ring system will be discussed in this article. Several significant discoveries have been made since our last review on this subject (4). Some of these advances have been recorded recently in accounts of the chemistry and biochemistry of the tropane alkaloids (5−7).

Structural Variations in the Tropane Alkaloids

By definition, the bicyclic heterocyclic compound 8-azabicyclo[3.2.1]octane (4) is present in all the tropane alkaloids. Tropane (5) is its 8-methyl derivative. The six-membered piperidine ring is usually depicted in the chair form (as in 6), however in some of its reactions it adopts the boat conformation (as in 7). Over 150 (7) alkaloids are known which contain the bicyclic ring system 4. Some of these alkaloids are depicted in Fig. 3. The absolute configuration of the more common alkaloids has been elucidated and the chirality, using the (R), (S) symbolism, is indicated on the structural formulas. The numbering of the tropane nucleus in hyoscyamine will be used for all the tropane alkaloids so that their biosynthetic relationship can be more easily understood. According to IUPAC rules, scopolamine should be numbered with the (R)-bridgehead carbon as C-1 (R having a higher priority than S in such a symmetrical molecule). The majority of the tropane alkaloids are esters of hydroxytropanes with a wide variety of carboxylic acids (7), some of which are unique to this class of alkaloid [e.g. (S)-tropic acid]. Others, such as cinnamic acid and benzoic acid, are more widespread in nature and are found in other types of natural products.

Biosynthesis of the 1-Methyl-Δ^1-pyrrolinium Salt

All the experimental evidence is consistent with the 1-methyl-Δ^1-pyrrolinium salt (30) being a precursor of the tropane nucleus. The various biosynthetic routes which have been established for its formation are summarized in Fig. 4. Initially it was thought that the double bond in the iminium salt could isomerize between its C-2 and C-5 positions, which would result in the scrambling of any isotopic label (e.g. ^{14}C) which might be present at either of these positions. However this was shown not to occur in either acidic or basic media (8). Deprotonation of **30** affords
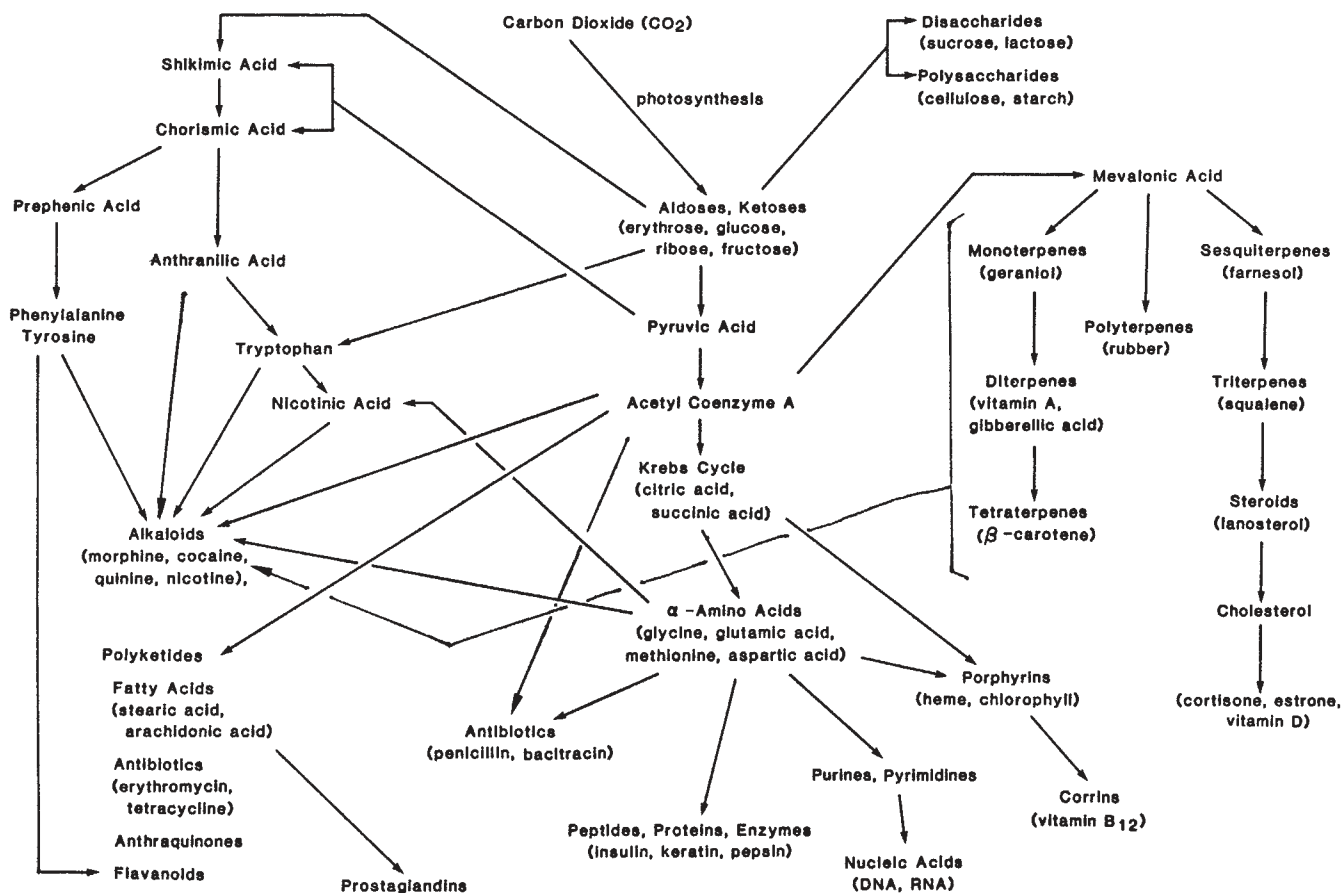
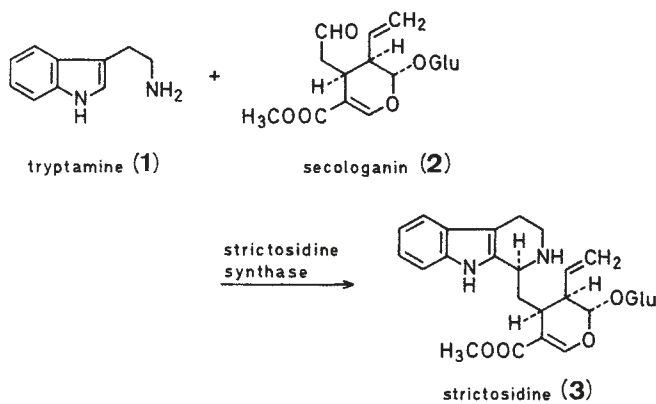**Fig. 1** Biosynthetic relationships of natural products.

Fig. 2 The first step in the biosynthesis of the mono-terpene indole alkaloids.
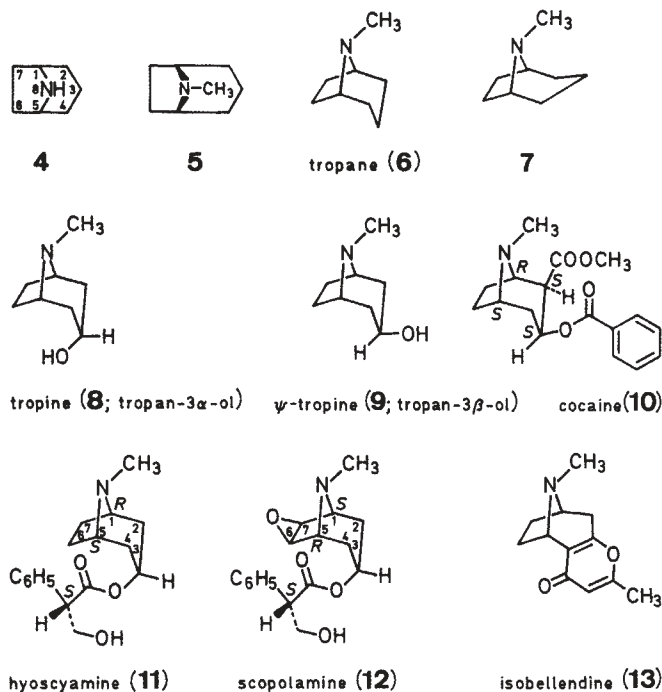
tryptamine (**1**)

secologanin (**2**)

strictosidine synthase

strictosidine (**3**)

4

5

tropane (**6**)

7

tropine (**8**; tropan-3α-ol)

ψ-tropine (**9**; tropan-3β-ol)

cocaine (**10**)

hyoscyamine (**11**)

scopolamine (**12**)

isobellendine (**13**)

Fig. 3 Structural features of some tropane alkaloids.
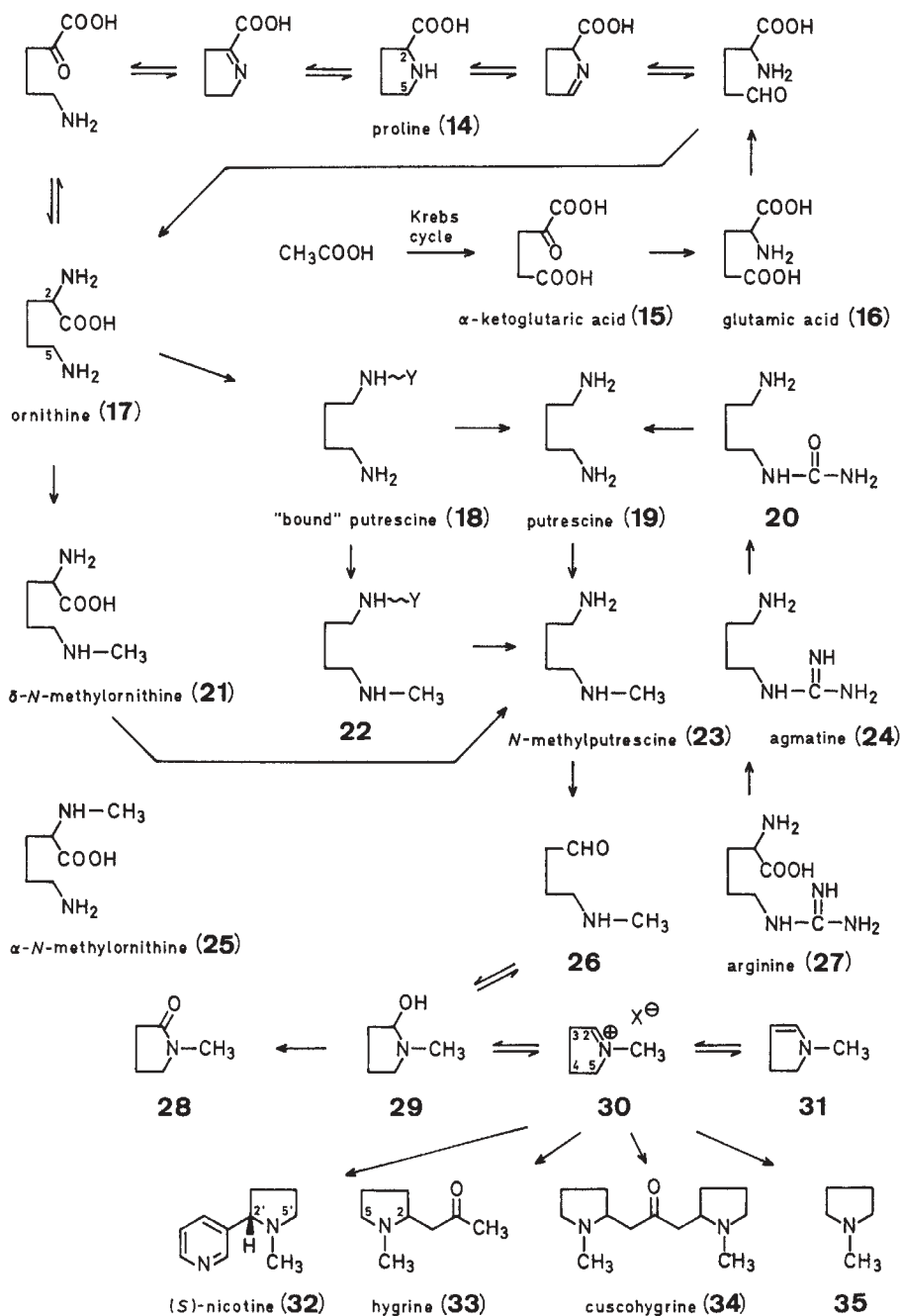
Fig. 4 Biosynthetic routes to the 1-methyl-Δ^1-pyrrolinium salt (**30**).

1-methyl-Δ^2-pyrroline (**31**) which is in equilibrium with **30**. The iminium salt **30** is also a precursor of the *N*-methylpyrrolidine rings of nicotine (**32**) (8, 9), hygrine (**33**) (10, 11), and cuscohygrine (**34**) (12) as well as hyoscyamine and other tropane alkaloids. The iminium salt is formed by the cyclization of 4-methylaminobutanal (**26**) via the carbinolamine, 2-hydroxy-1-methylpyrrolidine (**29**). 1-Methyl-2-pyrrolidinone (**28**) has been detected in *Atropa belladonna* (13, 14) and is plausibly formed by oxidation of the carbinolamine. 1-Methylpyrrolidine (**35**) which is a minor alkaloid of *Atropa belladonna* (15) and tobacco (16) is presumably formed by the reduction of the iminium salt **30**. Labeled 4-methylaminobutanal was detected in *Datura stramonium* plants which had been fed [2-^{14}C]ornithine (9).

4-Methylaminobutanal is formed by the oxidation of *N*-methylputrescine and the enzyme which catalyzes this oxidation has been isolated from *Nicotiana tabacum* (17–21), *Datura stramonium* (20), and *Hyoscyamus niger* (22). The enzyme, *N*-methylputrescine oxidase, isolated from tobacco roots, was not specific for *N*-methylputrescine. Other diamines such as putrescine and cadaverine (1,5-diaminopentane) were oxidized to 4-aminobutanal and 5-aminopentanal, respectively.

The stereochemistry of the oxidation of *N*-methylputrescine has been studied in *Nicotiana tabacum* (23) and it was established that it is the *pro-S* hydrogen which is lost from the carbon carrying the primary amino
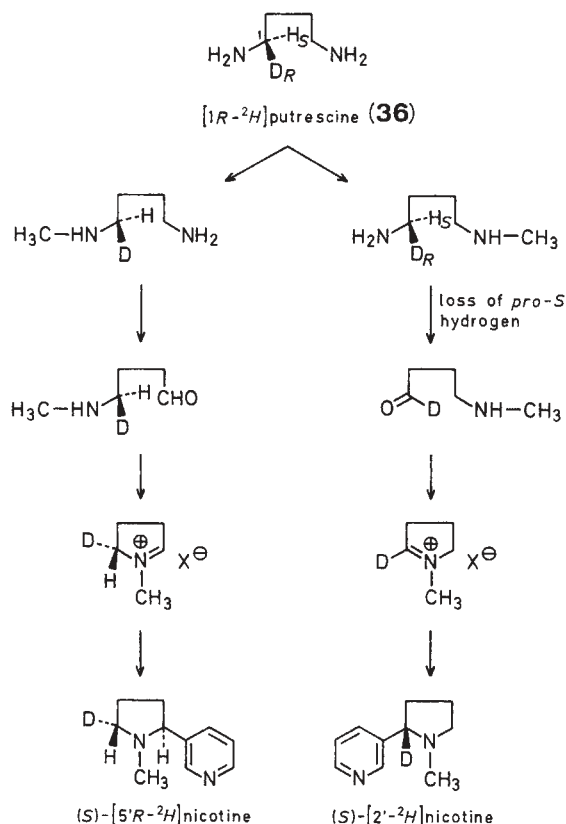
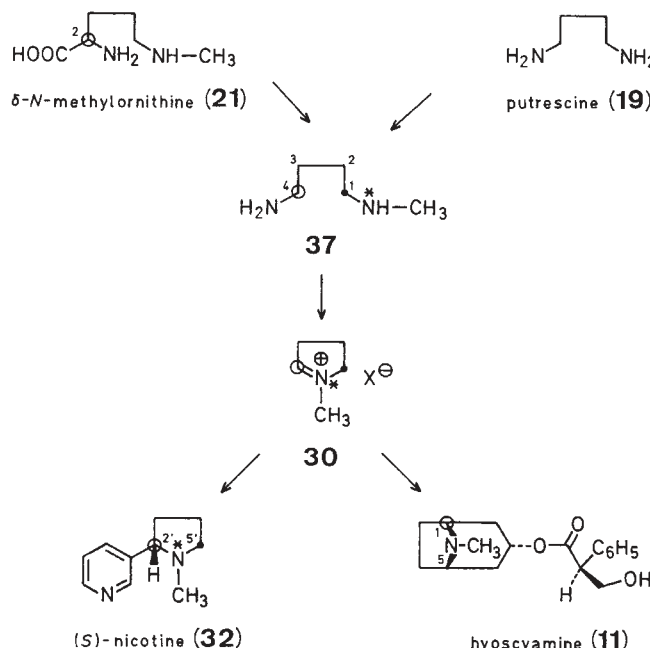**Fig. 5** Stereochemistry of the oxidation of *N*-methylputrescine.

Fig. 6 The mode of incorporation of *δ-N*-methylornithine into nicotine and hyoscyamine.

group, during its oxidation to the aldehyde. This result was deduced by using $[1R\text{-}^2H]$putrescine (**36**, Fig. 5), as a substrate. The location of deuterium in the ultimate nicotine was determined by ^{2}H-NMR spectroscopy. *N*-Methylputrescine has been established as a direct precursor of the iminium salt and the alkaloids derived from it (24–26). As expected, it is incorporated unsymmetrically into the ultimate alkaloids. For example, $[1\text{-}^{13}C, {}^{14}C, 1\text{-}^{15}N]$-*N*-methylputrescine (**37**) afforded nicotine which was labeled solely at its C-5′ position with ^{13}C and ^{14}C and with ^{15}N on its pyrrolidine nitrogen. This same precursor was incorporated unsymmetrically into the tropane moiety of scopolamine in *Datura innoxia* (24). However the method used to determine the location of the ^{13}C (NMR spectroscopy) did not establish whether the ^{13}C was at C-1 or C-5. Another established source of *N*-methylputrescine is *δ-N*-methylornithine (**21**). However the status of this methylated amino acid as an authentic natural product is in doubt. It was found that the administration of $[2\text{-}^{14}C, N\text{-methyl-}^{14}C]$-*δ-N*-methylornithine to *Datura stramonium* plants (by hydroponics) afforded radioactive hyoscyamine which was labeled at its C-1 and *N*-methyl positions (the ratio of activity at these two positions was the same as the ratio of the analogous positions in the *δ-N*-methylornithine (27).

However for several reasons, to be discussed, I now consider that this formation of *N*-methylputrescine by decarboxylation of *δ-N*-methylornithine is an aberrant biosynthetic reaction. It is proposed that a non-specific *α*-amino acid decarboxylase catalyzes this reaction. This is

definitely the case in *Nicotiana* species (28) since the nicotine derived from the $[2\text{-}^{14}C, N\text{-methyl-}^{14}C]$-*δ-N*-methylornithine was labeled only at its C-2′ and *N*-methyl positions, (Fig. 6). This result is inconsistent with feedings of unsymmetrically labeled ornithine to tobacco. In all cases the pyrrolidine ring of nicotine was labeled symmetrically (29). These results indicate that unsymmetrically labeled ornithine such as $[2\text{-}^{14}C]$ornithine is decarboxylated to putrescine prior to *N*-methylation. Attempts to show the presence of *δ-N*-methylornithine in *Datura* species have also been unsuccessful (30). It also could not be detected in labeled form when $[5\text{-}^{14}C]$ornithine was administered to a root culture of *Hyoscymus albus* (31).

However one experiment (32) is inconsistent with these observations. $[5\text{-}^{14}C]$- and $[5\text{-}^3H]$-ornithine were administered to *Atropa belladonna* plants for 2 days. The plants were then harvested with the addition of nonradioactive DL-*δ-N*-methylornithine. The recovered *δ-N*-methylornithine was labeled, its activity being equivalent to absolute incorporations of 0.37 % and 0.02 % for the ^{14}C and ^{3}H labeled *δ-N*-methylornithines, respectively. Oddly the hyoscyamine and scopolamine isolated from these *A. belladonna* plants, which were fed the ^{14}C- or ^{3}H-ornithines, were unlabeled. $[2\text{-}^{14}C, N\text{-methyl-}^{14}C]$-*α-N*-Methylornithine (**25**) did not serve as a precursor of hyoscyamine when fed to *Datura stramonium* plants (27). When this same compound was fed to tobacco plants (28), the resultant nicotine was labeled at C-2′ and C-5′ (equal labeling) and on its *N*-methyl group. This result was interpreted by proposing that

25 is demethylated to yield ornithine, which decarboxylates to yield putrescine. Methylation of putrescine from the C-1 pool then affords *N*-methylputrescine and then to nicotine via **30** as illustrated in Fig. 7. As expected from such a proposed metabolism, the ratio of ^{14}C activity on the *N*-methyl group of nicotine to the activity at C-2' + C-5' was different from that in the analogous positions of the administered α-*N*-methylornithine. The absolute incorporation of the α-*N*-methylornithine into nicotine was much lower than that of its δ-isomer.

The reason for first proposing the *N*-methylornithines as intermediates in the biosynthesis of the tropane alkaloids, was to rationalize the unsymmetrical incorporation of DL-[2-14C]ornithine into hyoscyamine (**11**), all the ^{14}C being at the C-1 bridgehead carbon. These experiments were carried out with intact *Datura stramonium* plants (33, 34). The administration of [2-14C]ornithine to a root culture of *Datura stramonium* also yielded hyoscyamine labeled solely at its C-1 position (35). We now suggest that the integrity of the C-2 and C-5 carbons of ornithine is maintained by the mechanism illustrated in Fig. 4. Ornithine is decarboxylated to afford a "bound-form" of putrescine (18). This bound form is then methylated to afford a bound form of *N*-methylputrescine (**22**). Pyridoxal phosphate is the coenzyme involved in the decarboxylation of α-amino acids and this could be an integral part of this bound form of putrescine as illustrated in Fig. 8. By this route, [2-14C]ornithine affords [4-14C]-1-methylamino-4-aminobutane. Other labeled compounds which yielded unsymmetrically labeled hyoscyamine in the indicated species were [5-14C]proline (*Datura metel* root culture) (36) and sodium [1-14C]acetate (*Datura stramonium* root culture) (37). The latter compound enters the Krebs cycle via acetyl coenzyme A ultimately affording [1,5-14C]-α-ketoglutaric acid. This then affords [1,5-14C]ornithine as illustrated in Fig. 4. The ultimate hyoscyamine is then labeled at C-5 with no activity at C-1. Radioactivity was also found in the three-carbon bridge but this was to be expected since these carbons are acetate derived.

It has recently been discovered that hyoscyamine formed in a root culture of *Hyoscyamus albus* from DL-[5-14C]ornithine was labeled equally at its C-1 and C-5 positions (30). It has also been shown that cocaine formed from [5-14C]ornithine in *Erythroxylum coca* was labeled equally at its C-1 and C-5 positions (38). *Duboisia* species are of considerable interest from a biosynthetic point of view since they contain both the tobacco alkaloids (nicotine and nornicotine) and the tropane alkaloids (hyoscyamine and scopolamine) (39, 40). Radioactive (*S*)-nicotine and scopolamine were isolated from a root culture of *Duboisia leichhardtii* which had been fed DL-[5-14C]ornithine. Degradations indicated that both alkaloids were labeled symmetrically in their pyrrolidine rings (41). This result is consistent with both these alkaloids arising from a common intermediate, the iminium salt **30**, via a symmetrical intermediate, namely putrescine. Another species in which ornithine is incorporated via a symmetrical intermediate is *Nicandra physaloides*. Hygrine (**33**) derived from [5-14C]ornithine was labeled equally at its C-2 and C-5 positions (11). Earlier work (10) in which the unsymmetrical incorporation of ornithine into hygrine was reported has been shown to be invalid (11). The cuscohygrine (**34**) isolated from *Ery-*

throxylum coca plants which were fed [5-14C]ornithine was also labeled symmetrically in its pyrrolidine rings. These results are illustrated in Fig. 9.

In summary, ornithine is incorporated into the pyrrolidine rings unsymmetrically in *Datura innoxia, Datura metel,* and *Datura stramonium.* In *Nicotiana, Erythroxylum coca, Duboisia leichhardtii, Hyoscyamus albus,* and *Nicandra physaloides,* a symmetrical intermediate is involved. A considerable amount of free putrescine was isolated from root cultures of *Hyoscymus albus,* along with the
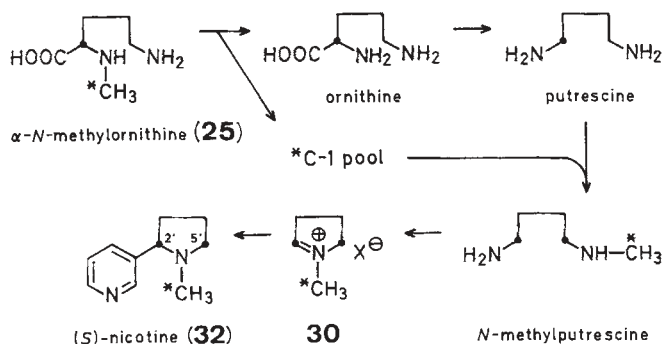

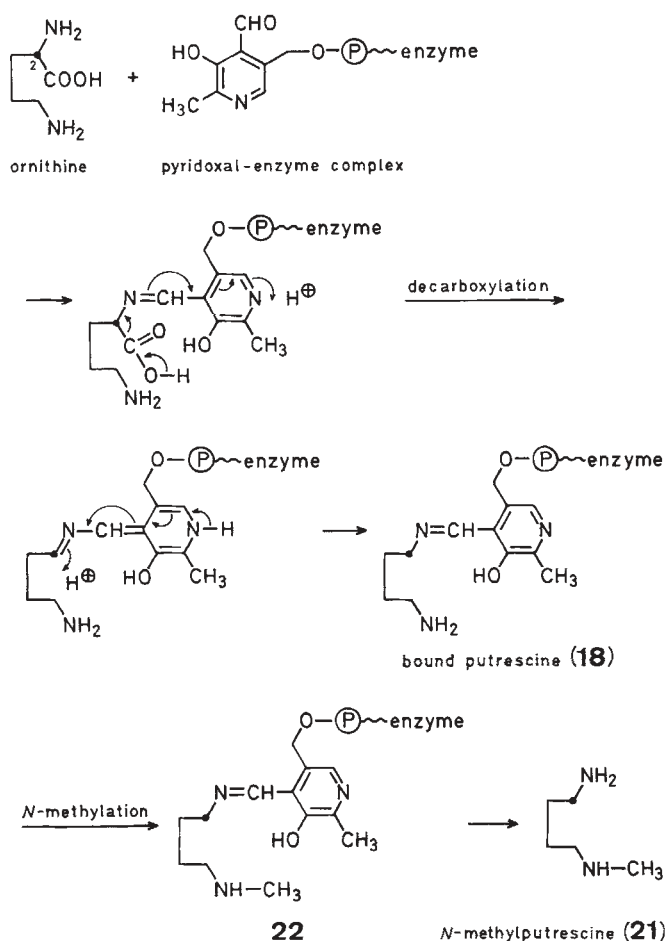**Fig. 7** The mode of incorporation of α-*N*-methylornithine into nicotine.
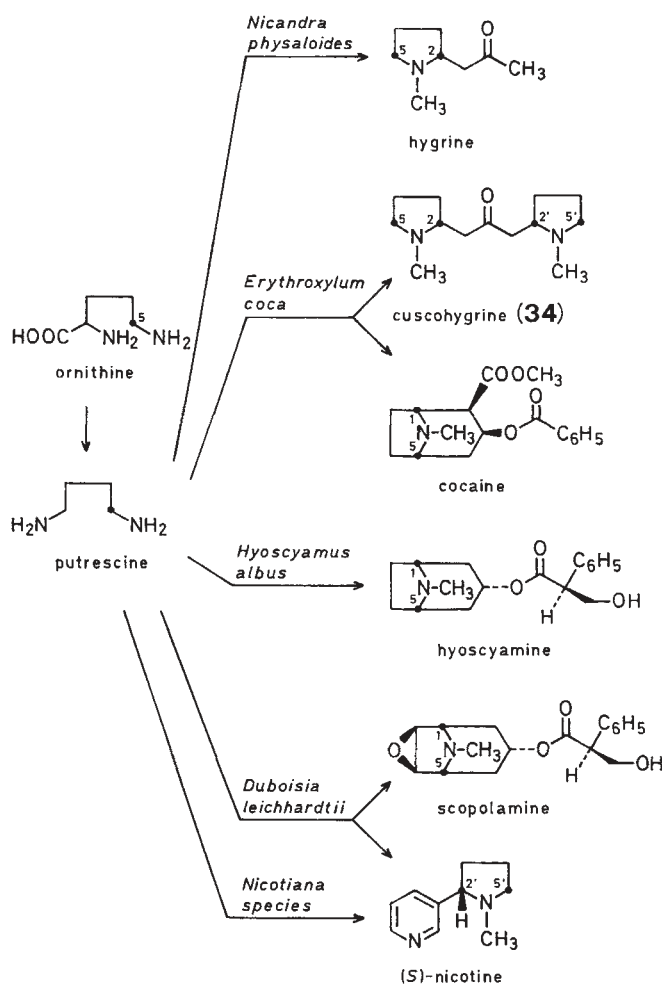

Fig. 8 Hypothetical nature of the bound putrescine.

Fig. 9   Incorporation of [5-^{14}C]ornithine via a symmetrical intermediate.
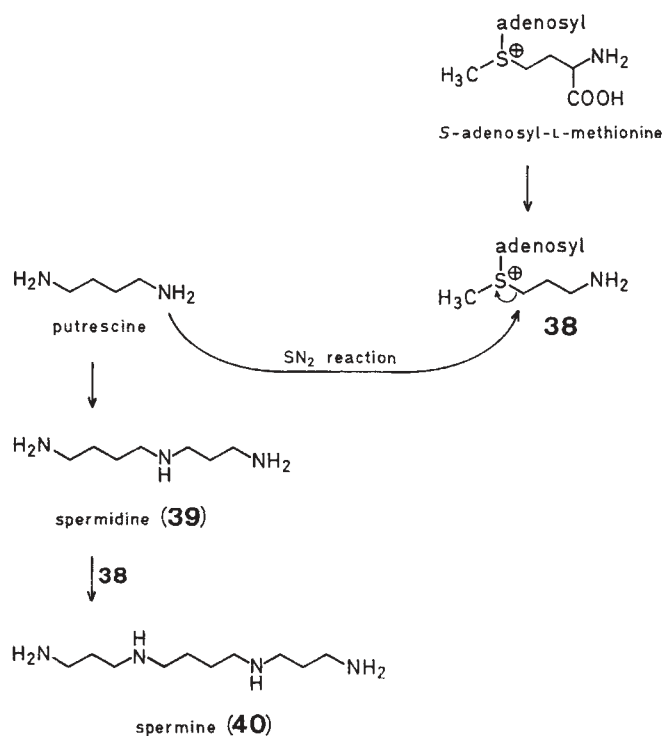
Fig. 10   Biosynthesis of spermidine and spermine.

polyamines, spermidine (**39**) and spermine (**40**), which are formed from putrescine and decarboxylated S-adenosyl-L-methionine as illustrated in Fig. 10 (42). Even though all the evidence precludes free putrescine as an intermediate between ornithine and the tropane alkaloids in *Datura* species, it has been found that the administration of [1,4-^{14}C]putrescine to *Datura* plants afforded labeled tropane alkaloids (43−45). It is proposed that its incorporation is another example of an aberrant biosynthesis, entering the normal biosynthesis via the bound form of putrescine (**18**, Fig. 3). In the species in which free putrescine is an intermediate between ornithine and the iminium salt **30**, the methylation to *N*-methylputrescine involves reaction with S-adenosyl-L-methionine. The enzyme which catalyzes this reaction is putrescine *N*-methyl transferase. It has been isolated from *Nicotiana* species (18, 46, 47), *Datura stramonium* (46), and *Hyoscyamus albus* (31). It is paradoxical that this enzyme should be found in *Datura stramonium*, when all the current evidence is against the intermediacy of free putrescine in this species. It may be that the main function of this enzyme in *Datura* is to catalyze the methylation of the bound form of putrescine.

      The formation of putrescine by the decarboxylation of ornithine has been widely investigated and

the enzyme which catalyzes this reaction is ornithine decarboxylase (E.C. 4.1.1.17). This enzyme had been isolated from tobacco (18, 48, 49), *Hyoscyamus albus* (31), and several other unrelated plant species (50, 51). Arginine (**27**) can also serve as a precursor of putrescine. Arginine decarboxylase (E.C. 4.1.1.19) catalyzes the formation of agmatine (**24**) which is converted to putrescine via carbamoylputrescine (**20**). The activity of the arginine decarboxylase in a root culture of *Hyoscyamus albus* was twice that of ornithine decarboxylase (31). DL-[2,3-^{3}H]Arginine when fed to this root culture also resulted in labeling of putrescine, *N*-methylputrescine, and hyoscyamine (31). α-Difluoromethylornithine (**41**, Fig. 11), is a suicide inhibitor of ornithine decarboxylase (51). It was thus found that this compound reduced significantly the incorporation of label from [5-^{14}C]ornithine into putrescine, *N*-methylputrescine, and hyoscyamine in *Hyoscyamus albus* roots (31). This compound also inhibited the formation of nicotine in a *Nicotiana* callus tissue (52). However the inhibiton of nicotine formation was greater with α-difluoromethylarginine (**42**). This compound is a selective inhibitor of arginine decarboxylase, and this result suggests that the major pathway to putrescine, at least in this tobacco callus tissue, is from arginine rather than ornithine. Although it seems like a circuitous route it is possible that putrescine is

formed from ornithine by the sequence: ornithine → δ-carbamoylornithine → arginine → agmatine → carbamoylputrescine → putrescine. This route apparently operates in a root culture of *Senecio vulgare* (53). This species contains senecionine *N*-oxide, a pyrrolizidine alkaloid which is derived form two molecules of putrescine. It was found that α-difluoromethylarginine completely inhibited the incorporation of radioactivity from [^{14}C]arginine and [^{14}C]ornithine into this alkaloid and the polyamines, spermidine and spermine which are also found in this root culture. On the other hand α-difluoromethylornithine, the inhibitor of ornithine decarboxylase, had no effect on the incorporation of [^{14}C]arginine or [^{14}C]ornithine into these alkaloids.
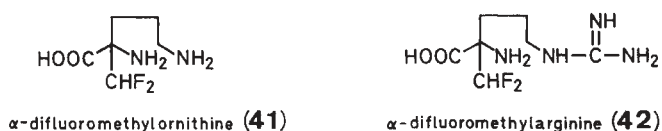
α-difluoromethylornithine (**41**)

α-difluoromethylarginine (**42**)

Fig. 11 Inhibitors of ornithine and arginine decarboxylase.

Incorporation of the 1-Methyl-Δ^1-pyrrolinium Salt into the Tropane Alkaloids

It has been shown that acetic acid is incorporated into carbons 2, 3, and 4 of the tropine moiety of hyoscyamine (54, 55) and these results were interpreted as illustrated in Fig. 12. Two molecules of acetyl coenzyme A (derived from acetic acid) condense to yield acetoacetyl coenzyme A (**43**). This β-keto thioester then condenses at its C-2 position with the 1-methyl-Δ^1-pyrrolinium salt **30** to yield the intermediate **46**. Hydrolysis of this ester then affords hygrine-1'-carboxylic acid (**47**). Expected facile decarboxylation of this β-keto acid then yields hygrine (**50**). Attempts to demonstrate an enzymatic condensation between the iminium salt **30** and acetoacetic acid or its esters, using a cell-free extract of *Duboisia leichhardtii*, were unsuccessful (56). Hygrine synthesis did indeed occur, however its formation was probably non-enzymatic, since it also occurs with denatured enzyme. A non-enzymatic reaction was also presumably involved when a *Nicotiana tabacum* root culture was incubated with acetoacetic acid or acteone-1,3-dicarboxylic acid. These compounds apparently reacted with the endogenous 1-methyl-Δ^1-pyrrolinium salt present in the tobacco roots (56). Hygrine is not an alkaloid normally found in tobacco roots. The biomimetic synthesis of hygrine from the iminium salt **30** and acetonedicarboxylic acid or ethyl acetoacetate has been studied extensively (8, 57–59). Reaction of ethyl [2-^{13}C]acetoacetate (**44**, R = C$_2$H$_5$) with the iminium salt **30** in aqueous ethanol yielded hygrine in which all the excess ^{13}C was located at the C-1' position of the side chain (60). Feeding experiments carried out in *Nicandra physaloides* were in agreement with this biomimetic result (61). A chemical degradation of the radioactive hygrine, obtained after feeding sodium [4-^{14}C]acetoacetate (**44**, R = Na) to this species, indicated that it had all its activity at C-2' or C-3' (presumably the latter position). This result should be interpreted with caution, since the labeled hygrine could have arisen by a non-enzymatic reaction occurring in the plant.

The biosynthetic route to cocaine was initially thought to involve the intermediate **46** in which C-1 of the acetoacetate ultimately becomes C-9 of cocaine. Feeding experiments carried out in *Erythroxylum coca* with sodium [1-^{14}C]acetate were consistent with this hypothesis (62). The bulk of the ^{14}C was at C-3 (48%) and C-9 (38%) with smaller amounts (4%) at C-1 and C-5. The incorporation into the bridgehead carbons being in agreement with the formation of [1,5-^{14}C]ornithine via Krebs cycle intermediates.

Labeled hygrine served as a precursor of the tropine moiety of hyoscyamine and other tropane alkaloids (10, 36, 63–65). In *Datura innoxia* the (2R)-hygrine (**50**, depicted in Fig. 12) was a more efficient precursor of hyoscyamine (by a factor of 5) than its (2S)-isomer (63). In this species the (2R)-hygrine was also a better precursor of other tropane alkaloids (scopolamine and 3α,6β-ditigloyloxytropane) than the (2S)-isomer. In other species (*Atropa belladonna, Hyoscyamus*, and *Physalis alkekengi*) both isomers of hygrine were equally efficient precursors of the tropane skeleton. As pointed out before (4) hygrine is readily racemized in alkaline solution. The previously discussed observations on the unsymmetrical incorporation of ornithine into hyoscyamine in *Datura* species requires that the (2R)-isomer of hygrine is the precursor of the tropane nucleus. Thus the sequence from [2-^{14}C]ornithine is → [4-^{14}C]-*N*-methylputrescine → [1-^{14}C]-4-methylaminobutanal → [2-^{14}C]-1-methyl-Δ^1-pyrrolinium salt → (2R)-[2-^{14}C]hygrine → (1R)-[2-^{14}C]tropine, as depicted in Fig. 12.

The formation of the tropane nucleus from hygrine is considered to proceed via the 5-acetonyl-1-methyl-Δ^1-pyrrolinium salt (**49**). The final cyclization is a Mannich reaction affording tropinone (**52**). The iminium salt **49** has not yet been isolated from plant sources. However circumstantial evidence which favors its intermediacy was the isolation of two alkaloids from *Datura innoxia*, which are apparently diastereoisomers which were assigned the structure **54**, 4-(1-methyl-1-pyrrolidinyl)hygrine (66). A plausible biosynthesis of this alkaloid is illustrated in Fig. 13. Loss of a proton from the iminium salt **49** yields **53**. Reaction with a molecule of **30** yields **55** which is then reduced to afford structure **54**.

We (67) have achieved a biomimetic synthesis of tropinone by the oxidation of hygrine with mercuric acetate in boiling 2% acetic acid. Another compound produced in this reaction is 2,1'-dehydrohygrine (**58**). The mechanism is considered to proceed via the quaternary ammonium ion **56** which eliminates a proton yielding **49** or **57**. These compounds then yield tropinone and the new dehydrohygrine as illustrated in Fig. 14.

The biosynthetic route to 2-methoxycarbonyl-3-tropinone (**51**), the established precursor of cocaine (68), was formerly considered to proceed by a similar sequence of reactions. Oxidation of **46** yields the iminium salt **45** which cyclizes to the tropinone derivative **48**. Conversion of the thioester to the methyl ester affords 2-methoxycarbonyl-3-tropinone. However when [2-^{14}C]-1-methyl-Δ^1-pyrrolinium chloride or [1-^{13}C, ^{14}C, ^{15}N]-4-methylaminobutanal diethyl acetal [this acetal is considered to undergo hydrolysis to 4-methylaminobutanal in the
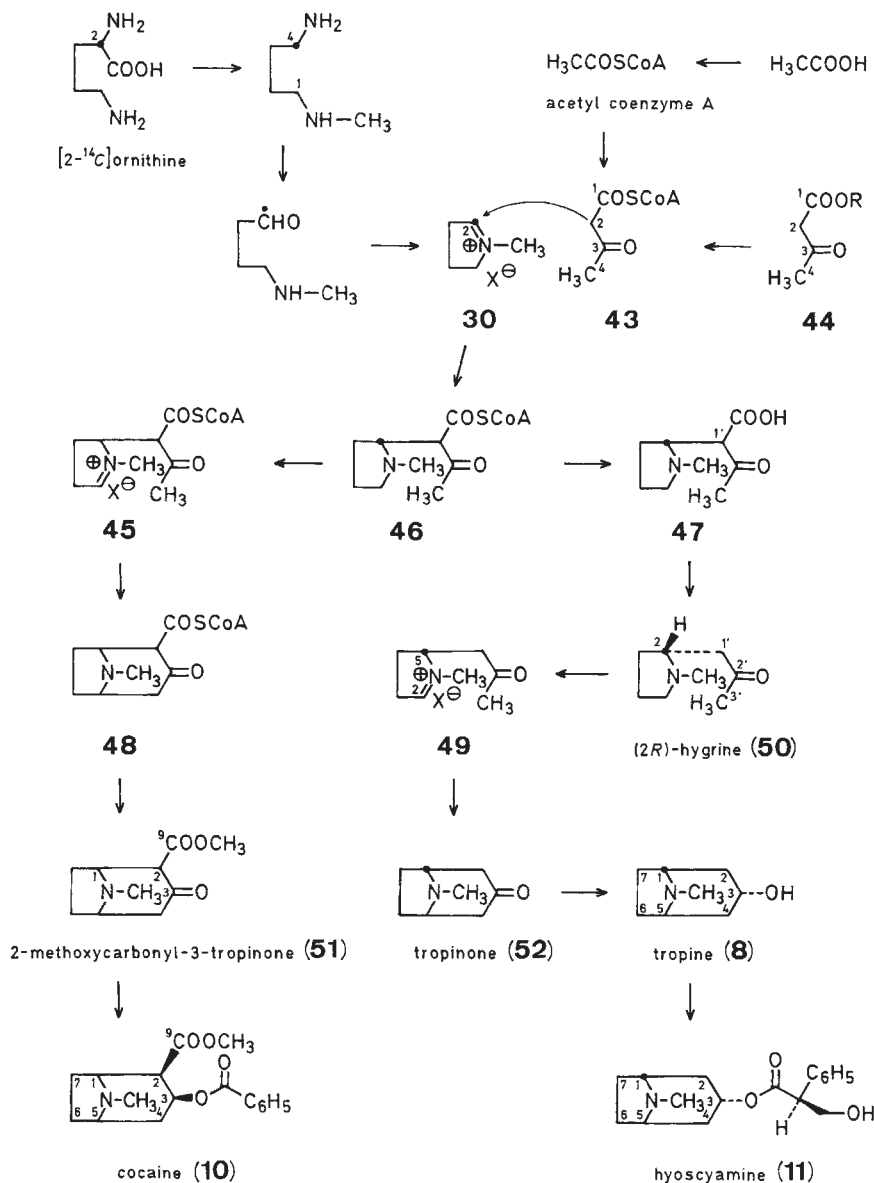
Fig. 12 Old hypothesis for the biosynthesis of cocaine and hyoscyamine.
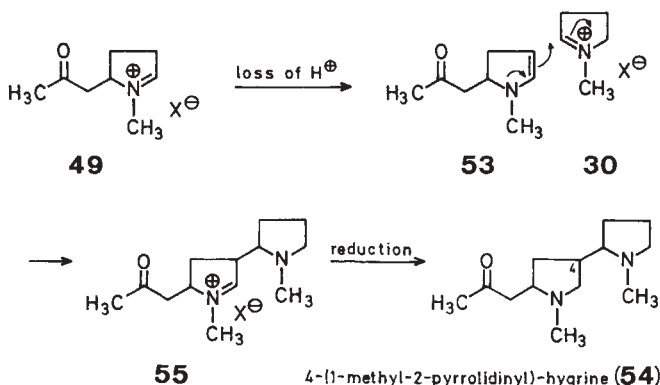

Fig. 13 Hypothetical biosynthesis of 4-(1-methyl-2-pyrrolidinyl)hygrine.

acid conditions (pH 4.6) prevailing in the leaves of the *coca* plant] was fed to *Erythroxylum coca*, the resultant cocaine was labeled with ^{13}C or ^{14}C at its C-5 position, not the C-1 position as would be required by the hypothesis illustrated in Fig. 12 (69). This surprising result was established by ^{13}C-NMR spectroscopy and confirmed by a chemical degradation. If acetoacetate was involved in the biosynthesis of cocaine this result would mean that a reaction had to take place between the iminium salt **30** and C-4 of the acetoacetate. This reaction has no chemical or biochemical precedence. We were thus led to propose a new biosynthetic scheme for cocaine which is illustrated in Fig. 15. It is suggested that the iminium salt **30** reacts with one molecule of acetyl coenzyme A (possibly activated by carboxylation to malonyl coenzyme A as in fatty acid biosynthesis) to yield the coenzyme A thioester of 1-methylpyrrolidine-2-acetic acid (**59**). Reaction with a second molecule of acetyl coenzyme A then affords the β-keto thioester **60**. Oxidation then produces the iminium salt **62** which by a Mannich reaction
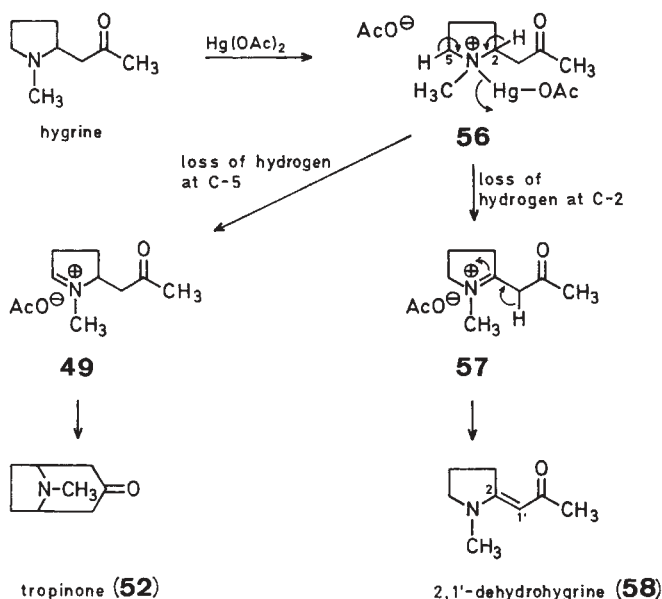
Fig. 14 Biomimetic synthesis of tropinone from hygrine.

yields the tropinone derivative **48**. Ester interchange produces 2-methoxycarbonyl-3-tropinone (**51**) which is reduced to methyl ecgonine (**63**) and thence to cocaine by benzoylation. One piece of evidence in favor of this new biosynthetic scheme was the isolation of radioactive 1-methylpyrrolidine-2-acetic acid (**61**) from the *Erythroxylum coca* plants which had been fed [2-^{14}C]-1-methyl-Δ^1-pyrrolinium chloride. This 1-methylpyrrolidine-2-acetic acid had all its ^{14}C located at the C-2 position (70) consistent with this scheme. The methyl ester of the (2*S*)-isomer of **61** has been isolated from the plant *Solanum sturtianium* (71) providing circumstantial evidence in favor of the intermediacy of **61** or its thioester in the biosynthesis of cocaine.

It remains an open question as to whether hygrine is formed by a similar biosynthetic route in species such as *Datura, Hyoscyamus,* or *Nicandra*. Mechanistically it would be quite reasonable for intermediate **60** to undergo

hydrolysis to the corresponding β-keto acid which on decarboxylation would yield hygrine.

No biosynthetic work has been carried out on alkaloids such as isobellendine (**13**). Speculations have been made on the biosynthesis of these alkaloids (7). Based on our new knowledge of the biosynthesis of cocaine, two alternate biosynthetic routes, illustrated schematically in Fig. 16, are plausible. Route A is analogous to the old biosynthetic scheme for hygrine. In route B, the iminium salt **30** serves as a "starter unit" for a polyketide involving four acetate units. I intuitively favor the latter route.

Reduction of Tropinone and Related Compounds

Tropinone (**52**) is a key intermediate in the biosynthesis of many of the more complex tropane alkaloids. It has been found in *Nicandra physaloides* (72), *Atropa belladonna* (13), *Cyphomatra betacea* (73), and *Crossostylis biflora* (74). Reduction of tropinone affords tropine (**8**) or ψ-tropine (**9**, Fig. 3). The major tropane alkaloid hyoscyamine, is an ester of tropine with (*S*)-tropic acid. However many tropane alkaloids are esters of ψ-tropine and its derivatives. A recent investigation of the alkaloids of *Datura innoxia* (66) revealed the presence of both tropine and ψ-tropine along with various esters of both isomers. It was proposed (65) that perhaps tropine could be formed from hygrine without involving tropinone as an intermediate, as illustrated in Fig. 17. In this scheme hygrine is reduced to hygroline (**64**) followed by a rather implausible cyclization affording tropine (**8**). This hypothesis was tested by feeding [2'-^{14}C, 2'-^3H]hygroline (a mixture of four stereoisomers) to *Datura innoxia* plants (75). The resultant alkaloids (hyoscyamine, scopolamine, and 3α,6β-ditigloyltropan-7β-ol) were labeled with ^{14}C but only retained about 10 % of the ^3H. The location of the residual tritium was not determined, but I suggest that it is at C-3 of the tropine moiety of these alkaloids. These results indicate that hygroline is not a direct precursor of tropine. Its incorporation probably proceeds by oxidation to hygrine with NAD^+ or $NADP^+$. These coenzymes will then become labeled with tritium at their C-4 positions. The [2-^{14}C]hygrine is then incorporated into
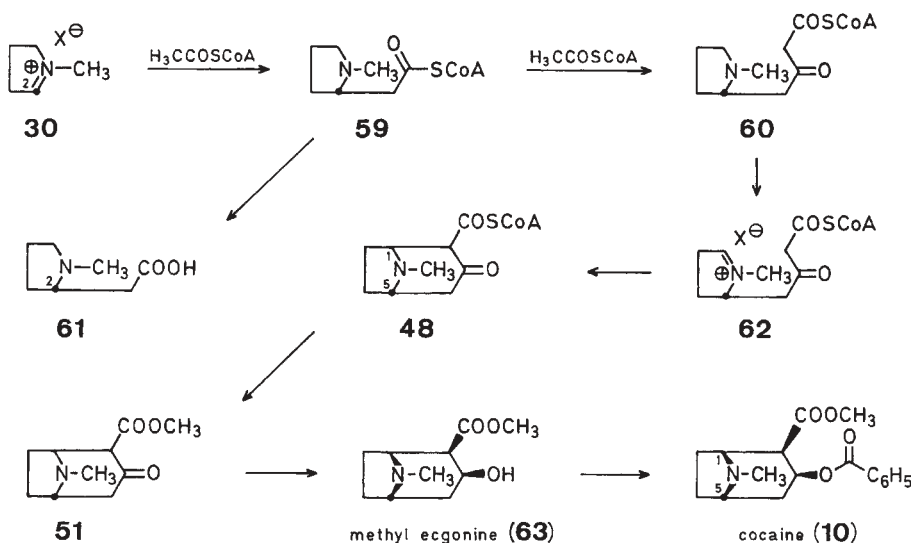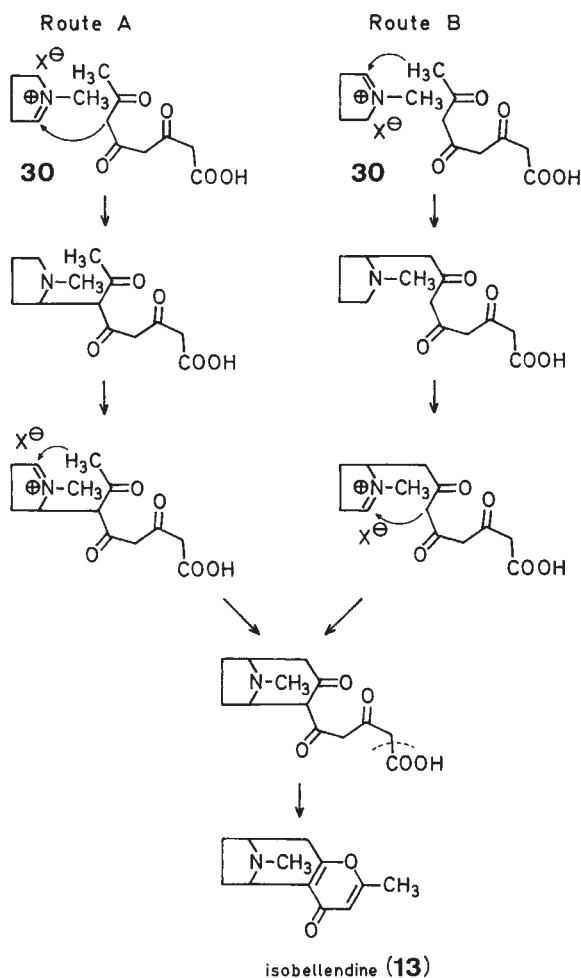

Fig. 15 New hypothesis for the biosynthesis of cocaine.

Fig. 16 Hypothetical biosynthetic routes to isobellendine.

The administration of [*N-methyl*-^{14}C]tropinone to the roots of intact *Datura innoxia* plants growing in hydroponics, afforded labeled hyoscyamine and scopolamine, specifically labeled at their *N*-methyl groups (76). The reduction of tropinone has also been studied in cell-free systems (76–78). The results so far obtained are contradictory. This could be due to different sources of the tropinone-reductase. Also none of the studies have progressed to the stage where a pure enzyme can be claimed. A cell-free extract obtained from a root culture of *Datura stramonium* was dependent on NADPH as a coenzyme and the sole product obtained was tropine (78). These same authors were unable to detect this enzyme activity in a root culture of *Atropa belladonna*. Yamada and coworkers (77) obtained a cell-free system from a root culture of *Hyoscyymus niger* which in the presence of NADPH catalyzed the reduction of tropinone to ψ-tropine. In later work (79) Yamada reported that their system did produce some tropine (10–30%) but the major product was ψ-tropine. Both these research groups monitored their crude enzymes by measuring the change in UV adsorption when NADPH is oxidized to its pyridinium form (NADP$^+$). We have obtained a cell-free system from the roots of intact *Datura innoxia* plants which were grown either in hydroponics or soil in a greenhouse (76). With NADPH as a coenzyme and [*N-methyl*-^{14}C]tropinone as a substrate we also found that ψ-tropine was the main product, with a smaller amount of tropine. When NADH was used as a coenzyme the production of tropine was favored over ψ-tropine. This interesting result suggests that probably two different enzymes are involved in the reduction of tropinone, one catalyzing the formation of tropine and the other ψ-tropine.

In intact plants or root cultures the reduction of tropinone is not apparently reversible. [*N-methyl*-^{14}C, 3β-^{3}H]tropine was fed to *Datura meteloides* for seven days. The tropine was incorporated into hyoscyamine, scopolamine, 3α,6β-ditigloyltropan-7β-ol, and meteloidine with no loss of tritium. Also tropine reisolated at the end of the experiment had retained all its ^{3}H, even though its specific activity was reduced ten times (80). When [*N-methyl*-^{14}C]tropine and [*N-methyl*-^{14}C]-ψ-tropine were fed independently to a root culture of *Hyoscyamus niger*, only the former labeled compound yielded radioactive hyoscyamine and scopolamine (81). This result indicates that no oxidation of ψ-tropine to tropinone occurred in the course of the feeding experiment.

tropinone as previously described. Reduction with the tritium labeled pyridine nucleotides will then introduce some ^{3}H at C-3 of tropine. Another proposal that esters of hygroline such as its tigloyl ester (**65**) are involved in the biosynthesis of alkaloids, such as meteloidine (**66**), was also shown to be invalid (65). The results indicated that the ester **65** underwent hydrolysis in *Datura meteloides* prior to incorporation of its two components into meteloidine.
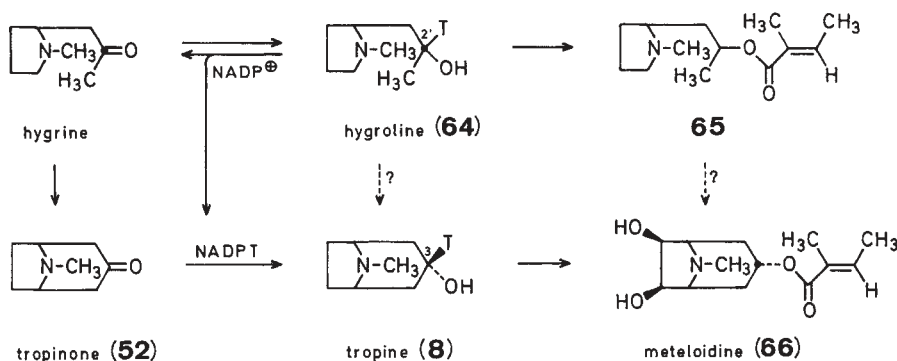

Fig. 17 Proposed biosynthesis of tropine via hygroline.

The penultimate step in the biosynthesis of cocaine is the reduction of 2-methoxycarbonyl-3-tropinone (**51**) to methyl ecgonine in which the hydroxyl group at C-3 is β as in ψ-tropine. Preliminary studies (82) using leaf disks of *Erythroxylum coca* and [9-^{14}C]-2-methoxycarbonyl-3-tropinone as a substrate have demonstrated the accumulation of labeled methyl ecgonine. The greatest amount of this compound (relative to cocaine) was obtained in a prolonged incubation, when presumably the source of the benzoyl moiety (probably benzoyl coenzyme A) was depleted. An alternate biosynthetic route to cocaine is illustrated in Fig. 18. It was proposed that the enol of 2-methoxycarbonyl-3-tropinone (**67**) was benzoylated to yield 2,3-dehydro-cocaine (**68**). Cocaine would then result from *cis*-addition of two hydrogens to **68**. This hypothesis was shown to be invalid by feeding (*RS*)-3-[4'-^{3}*H*]benzoyloxy-2-[*carbonyl*-^{13}*C*, ^{14}*C*]methoxycarbonyl-2-tropene (**68**) to *Erythroxylum coca*. The resultant cocaine was labeled with both ^{14}C and ^{3}H, but their ratio was significantly different from the ^{3}H/^{14}C in the administered compound, and varied with the duration of the feeding experiment (3 and 15 days). The results were interpreted by postulating hydrolysis of the dehydro-cocaine to 2-methoxycarbonyl-3-tropinone and benzoic acid (or its coenzyme A thioester) followed by utilization of these pieces as illustrated in Fig. 18.

Origin of the Acid Moieties of the Tropane Ester Alkaloids

Some of the acids which are present in the esters of the hydroxytropanes are very common in nature (formic, acetic, propanoic, and butanoic acid) and their biosynthesis requires no comment. Table 1 lists the acids found in tropane alkaloids whose biosynthesis has been studied, usually by feeding potential precursors, isotopically labeled, to intact plants. Very little work, if any, has been carried out on the origin of these acids in cell-free systems.

The administration of [4'-^{3}*H*]phenylalanine to *Erythroxylum coca* plants afforded labeled cocaine in which essentially all the tritium was located on the *para* position of its benzoyl moiety (62). This result is in agreement with earlier work in which it was shown that [3-^{14}*C*]phenylalanine yielded cocaine with all of its activity located on the benzoyl moiety (89). The biosynthetic route from L-phenylalanine (**39**) to benzoyl coenzyme A, the presumed immediate precursor of the benzoyl moiety of

Table 1 Origin of the acid moieties of some tropane alkaloids whose biosynthesis has been studied.

Acid	Alkaloid in which the acid is present[a]	Precursor	Ref.
Benzoic acid	Cocaine 3α-Benzoyloxytropane 3β-Benzoyloxytropane (tropacocaine) 3α-Benzoyloxytropan-6β,7β-diol	Phenylalanine	62, 89
m-Hydroxy-benzoic acid	Cochlearine	Phenylalanine *m*-Hydroxy-phenylalanine Shikimic acid	83 83 83
Phenyllactic acid	Littorine	Phenylalanine	84
Tiglic acid	Meteloidine 3α,6β-Ditigloyloxytropan-7β-ol	Isoleucine (*S*)-2-Methyl-butanoic acid	4, 85 4
(*S*)-Tropic acid	Hyoscyamine	Phenylalanine	4, 86–88
	Scopolamine Norhyoscyamine Norscopolamine 6β-Hydroxyhyoscyamine	Phenyllactic acid Phenylpyruvic acid	4 4

[a] This list is not inclusive, only representative examples are recorded.

cocaine, is illustrated in Fig. 19. The enzyme phenylalanine ammonia lyase (PAL) catalyzes the elimination of ammonia from phenylalanine to yield *trans*-cinnamic acid (**70**). Hydration of the cinnamic acid affords 3-hydroxy-3-phenylpropanoic acid (**73**) which is then oxidized to **72** (benzoylacetic acid in which the carboxyl group is esterified with coenzyme A to inhibit decarboxylation). Benzoyl coenzyme A (**74**) then arises by cleavage of this β-keto thioester. This sequence is analogous to the reactions involved in the β-oxidation of fatty acids. We found that the *N*-acetylcysteamine thioester of benzoic acid (**75**) was an excellent precursor (10 % specific incorporation) of the benzoyl moiety of cocaine when fed to intact *Erythroxylum coca* plants (90). Thus the incorporation of **75**, labeled with ^{13}C at the benzoyl carbonyl, into cocaine was easily established by ^{13}C-NMR spectroscopy of the enriched alkaloid. The cinnamoyl-cocaines (**71, 76**) are minor alkaloids of *coca*. It is proposed that *trans*-cinnamoylcocaine (**71**) is the initially formed alkaloid which on photolysis (by sunlight) is partially converted to *cis*-cinnamoylcocaine (**76**). This reaction is probably not controlled by an enzmye since we have obtained essentially the same mixture of *cis*- and *trans*-isomers
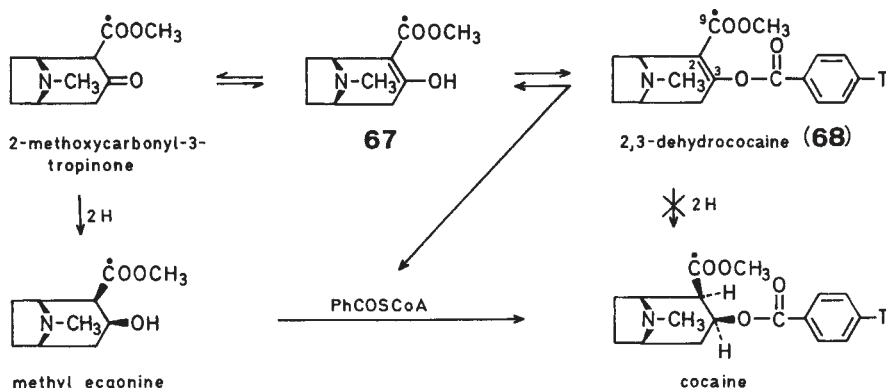

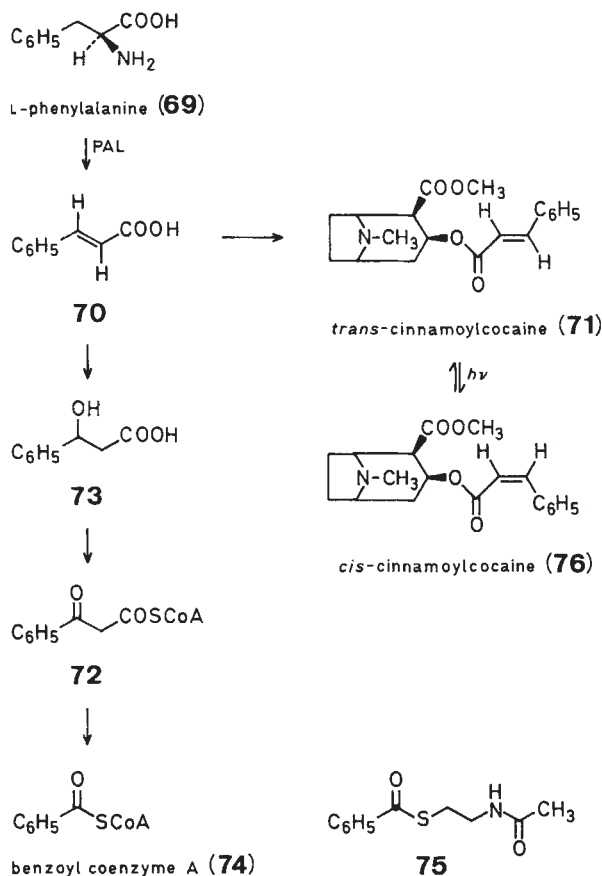**Fig. 18** Failure of 2,3-dehydrococaine to serve as a direct precursor of cocaine.

Fig. 19 Biosynthesis of benzoyl coenzyme A from phenylalanine.

Fig. 20 Stereochemistry of the formation of tiglic acid from isoleucine.
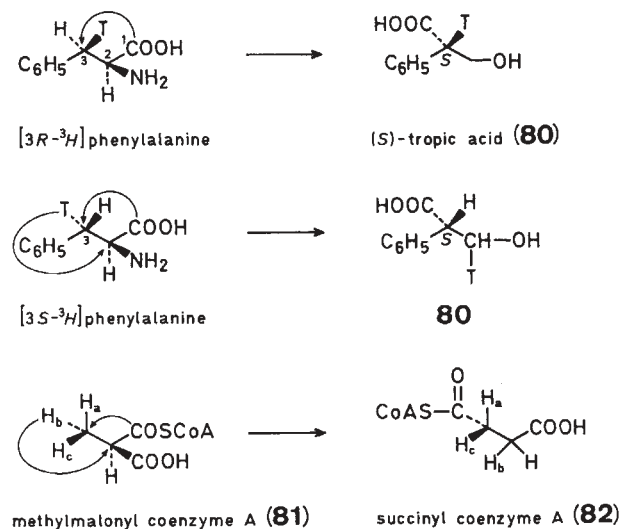
Fig. 21 Stereochemistry of the formation of tropic acid.
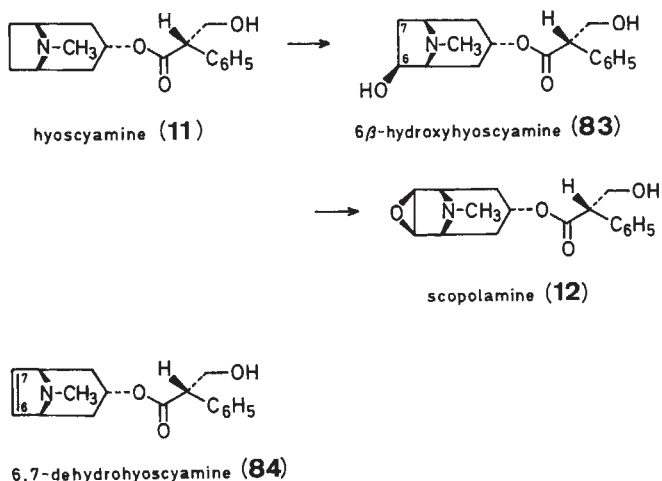
Fig. 22 Biosynthesis of scopolamine.

(30 : 70) as that found in the plant, by exposure of an ethanol solution of *trans*-cinnamoylcocaine to sunlight at 20 – 25 °C (91).

    Isoleucine is a well established precursor of tiglic acid (**79**) (4). By carrying out feeding experiments with isoleucine, stereospecifically labeled with tritium, it was established (85) that the dehydrogenation of the intermediate (*S*)-2-methylbutanoic acid (**78**) is *anti*-periplanar (i.e. *trans*). This result is illustrated in Fig. 20 in which the precursor was (2*RS*,3*S*,4*S*)-[2-^{14}C,4-^{3}H]isoleucine (**77**). The tiglic acid obtained by hydrolysis of the 3α,6β-ditigloyloxytropane isolated from *Datura innoxia* plants retained all its tritium relative to ^{14}C. A complementary experiment was carried out with (3*S*,4*R*)-[2-^{14}C,4-^{3}H]isoleucine. The resultant meteloidine (**66**, Fig. 17) isolated from *Datura meteloides* had lost essentially all of its tritium relative to ^{14}C.

    Since our previous review (4) on the origin of (*S*)-tropic acid (**80**), it has been discovered that the carbonyl group of phenylalanine undergoes the 1,2-migration from C-2 to C-3 with retention of configuration (88). At the same time the displaced hydrogen at C-3 moves to C-2 and ultimately becomes part of the hydroxymethyl group of tropic acid (**87**). These results were obtained by the use of stereospecifically labeled phenylalanines as illustrated in Fig. 21. Independently, Haslam and coworkers (86) fed
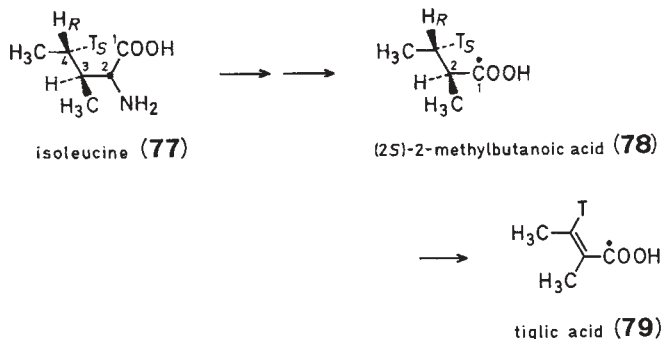
(2*S*,3*R*)- and (2*S*,3*S*)-[2-^{14}*C*,3-^{3}*H*]phenylalanine to *Datura stramonium* plants. They considered that their results were consistent with a stereospecific loss of the *pro-R* hydrogen at C-3 of phenylalanine, the carboxyl group migrating with inversion of configuration at this position. This conclusion is the direct opposite of the one illustrated in Fig. 21. However, the authors apparently failed to realize that tritium located at the chiral center of tropic acid is very labile. The mixture of alkaloids was hydrolyzed by refluxing with aqueous 10% sodium hydroxide for 30 min, conditions which would cause racemization of tropic acid. It was reported that this tropic acid derived from (2*S*,3*R*)-[2-^{14}*C*,3-^{3}*H*]phenylalanine (88% enantiomereic excess at C-3) had lost 88−93% of its tritium relative to carbon-14. The tropic acid derived from (2*S*,3*S*)-[2-^{14}*C*,3-^{3}*H*]phenylalanine (83% enantiomeric excess at C-3) lost 30−35% of the tritium. These experimental results are thus entirely consistent with ours, the retention of tritium in the tropic acid derived from the [3*S*-^{3}*H*]phenylalanine being due to its migration to the hydroxymethyl group, and not because it remained at the chiral center of tropic acid. These concurrent 1,2-migrations in opposite directions are analogous to the biochemical conversion of methylmalonyl coenzyme A (**81**) to succinyl coenzyme A (**82**) also illustrated in Fig. 21. In this conversion the thioester group moves with retention of configuration and is *intramolecular*. The migration of the hydrogen is *intermolecular* (92) in this transformation. The migration of the carboxyl group in the formation of tropic acid is *intramolecular* (93), but it is not known whether the migration of hydrogen from C-3 to C-2 is *inter-* or *intramolecular*. The stereochemistry of the migrated hydrogen in the *pro*-chiral methylene of the hydroxymethyl group is also unknown. The actual mechanism of the migration of the carboxyl group remains a mystery. It was shown that 3-hydroxy-3-phenylpropanoic acid (**73**, Fig. 19) is not a precursor of tropic acid (**94**). The microbial transformation of **81** to **82** is catalyzed by coenzyme B$_{12}$. However the presence of this coenzyme in higher plants is quite tenuous. There is one isolated report that it is found in comfrey (*Symphytum officinale*) (95).

Conversion of Hyoscyamine to Scopolamine

Recent results of Yamada and his coworkers (5) are now consistent with the biosynthetic scheme illustrated in Fig. 22. An enzyme preparation has been obtained from cultured roots of *Hyoscyamus niger* which catalyzes the formation of 6β-hydroxyhyoscyamine (**83**) from hyoscyamine (**11**) (96, 97). This hydroxylation occurs with retention of configuration and requires 2-ketoglutaric acid and oxygen as cofactors. The next step is not a dehydration to 6,7-dehydrohyoscyamine (**84**) as was previously considered (98). Thus ^{18}O was retained in the scopolamine isolated from a shoot culture of *Duboisia myoporoides* which had been fed [6-*hydroxy*-^{18}*O*]-6β-hydroxyhyoscyamine (99). Scopolamine (**12**) is thus formed by a direct attack of the 6β-hydroxyl group at C-7, displacing the 7β-hydrogen (100). This enzyme, 6β-hydroxyhyoscyamine epioxidase, also requires 2-ketoglutaric acid and oxygen as cofactors. Iron (FeSO$_4$) and ascorbic acid also promoted the activities of these two enzymes.

Conclusions

This review has been mainly concerned with the biochemical reactions which are involved in the biosynthesis of the tropane and related alkaloids. As with most alkaloids our knowledge of the enzymology of their production is very limited, although some progress in this area has been made in the last ten years. Considerable work has been published on the production of the tropane alkaloids in tissue and cell cultures derived from various parts of the intact plants. This work has not been included in this review. So far, the production of the economically valuable alkaloids, hyoscyamine and scopolamine, by the use of tissue culture methods has not been commercially successful. A recent development which has great potential for biosynthetic studies and for the production of medicinal agents is the use of "hairy root" cultures. These cultures are formed by infection of dicotyledonous plants with the bacterium *Agrobacter rhizogenes*. Roots form at the site of inoculation, and arise from cells which have been genetically transformed by the acquisition of DNA from a plasmid in the infecting organism. These "hairy roots" can be excised from the parent plant and cultured on a large scale in fermentors. Such cultures have been obtained from *Datura stramonium* (101) and *Duboisia myoporoides* (102). The production of alkaloids in these cultures was comparable to the amounts found in the intact plants.

Acknowledgements

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