AN INVESTIGATION OF DAMASCENINE BIOSYNTHESIS

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SCOPE AND CONTENTS: Aspartic acid-4-C^{14} and succinic acid-1, 4-C^{14} were administered to Nigella damascena L. plants. Damascenine was isolated from the ripe seeds obtained from the tracer-fed plants, but was non-radioactive. On the assumption that damascenine synthesis was taking place at the time of feeding, aspartic and succinic acids do not serve as precursors of damascenine.

Several steps in a degradation sequence, designed to isolate individual carbon atoms of damascenine, have been successfully completed, and the alkaloid has been synthesized by an improved procedure.

ACKNOWLEDGMENTS

The author wishes to express her sincere appreciation to Professor I. D. Spenser for his patient direction and encouragement during the course of the investigation.

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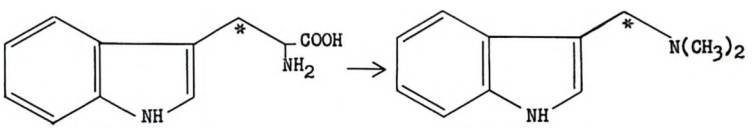
INTRODUCTION

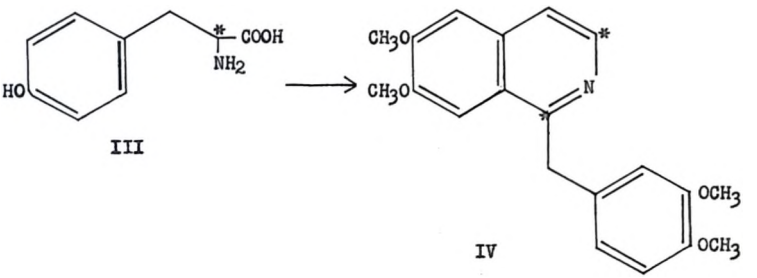
The view, first put forward by Robinson (1917), that in growing plants alkaloids are derived by metabolic processes from amino acids has been substantiated in the last decade by experiments with predicted precursors labelled with carbon, hydrogen, or nitrogen. The labelled compounds are administered to plants and, after a suitable length of time, the alkaloids are isolated and subsequently degraded to find the site of isotope incorporation.

Thus, when barley was germinated in the presence of tryptophan-β-C^{14} I, the gramine II which was isolated was found to be active in the position shown (Bowden & Marion, 1951 a, b; Leete & Marion, 1953).



I II

Tyrosine-2-C^{14} III was incorporated into the alkaloid papaverine IV (Battersby & Harper, 1959). Experiments with the Nicotiana alkaloids showed that ornithine-2-C^{14} V is a precursor of the pyrrolidine ring of nicotine VI (Leete, 1955, 1956; Leete & Siegfried, 1959; Lamberts,



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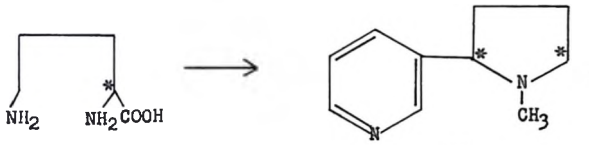

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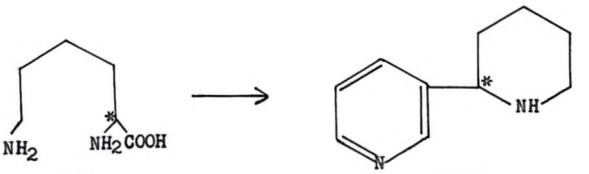


VII VIII

Dewey & Byerrum, 1959; Dewey, Byerrum & Ball, 1955) while lysine-2-C^{14} VII was found to be a precursor of the piperidine ring of anabasine VIII (Leete, 1956). These are but a few of the many experiments which show that amino acids serve as direct precursors of alkaloids, as predicted

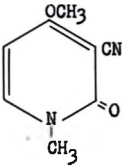
by the classical hypothesis of Robinson (1917).

It has been found, however, that some alkaloids are not derived from the predicted amino acid precursor. One such group of alkaloids is that which contains a substituted or modified pyridine ring.

LYSINE AS A POSSIBLE PRECURSOR OF THE PYRIDINE RING

Robinson (1955) and Leete (1956) proposed schemes for the formation of nicotine VI according to which the pyridine ring was derived from the amino acid lysine VII. It had been shown in earlier work that ornithine V was a precursor of the pyrrolidine ring of nicotine (Leete, 1955; Dewey et al. 1955) and thus lysine appeared to be a possible precursor of the pyridine ring. The lysine hypothesis was subjected to experimental test and it was shown that this pathway does not operate in higher plants. When lysine-ϵ-N^{15} was fed to N. tabacum, nicotine with little activity was obtained. This activity was located not in the pyridine but in the pyrrolidine ring (Bothner-By, Dawson & Christman, 1956). Similarly, lysine-2-C^{14} was not incorporated into anabasine VIII (Aronoff, 1956), although it is now known that lysine is a precursor of the piperidine ring of anabasine. Lysine-2-C^{14} was reported by Tamir and Ginsberg (1959) to be specifically incorporated into the α-pyridone ring of ricinine IX, but reinvestigation by Juby and Marion (1961) showed that the ricinine had little activity. The percentage incorporation of lysine into ricinine was of the same order of magnitude in both experiments,

but the latter researchers showed that the activity was randomly distributed and that incorporation was much lower than that obtained from several other precursors. The evidence would suggest that lysine, as such, is not incorporated into the pyridine ring.



IX

TRYPTOPHAN AS A POSSIBLE PRECURSOR OF THE PYRIDINE RING AND OF HYDROXYANTHRANILIC ACID

It has been conclusively demonstrated that in mammals and in Neurospora, tryptophan is converted to nicotinic acid by a metabolic pathway which includes 3-hydroxyanthranilic acid as one of the intermediates (Fig.1) (Dalgleish, 1955). Since many of the biochemical processes which occur in plants are similar to, or identical with, those occurring in mammals or Neurospora, tryptophan would also be predicted to be the source of nicotinic acid and of other pyridine derivatives in plants.

To test whether the tryptophan-nicotinic acid pathway is operative in plants, radioactive tryptophan and 3-hydroxyanthranilic acid were fed, the pyridine ring compounds isolated, and their activity tested. It was shown in every case that this pathway does not operate in higher plants. Waller and Henderson (1961 a) have shown that

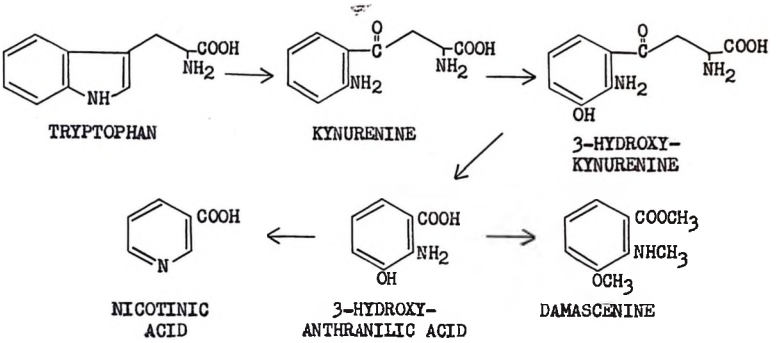
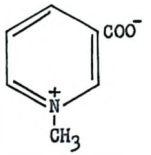
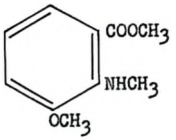


Figure 1

tryptophan-7a-C^{14} was not incorporated into ricinine IX and Leete (1957) proved that when DL-tryptophan-7a-C^{14} was fed to N. tabacum plants, the isolated nicotine VI was completely inactive. Bowden (1953) also showed that DL-tryptophan-β-C^{14} gave inactive nicotine. These results indicate that in N. tabacum and Ricinus communis L, the pathway tryptophan $\longrightarrow$ nicotinic acid is not available and the question arises whether this pathway exists in any higher plants. When labelled 3-hydroxyanthranilic acid was administered to soybean leaves, the isolated trigonelline X was inactive (Aronoff, 1956) and when tryptophan-3-C^{14} was fed to intact peas the trigonelline isolated here also showed no activity (Leete, Marion & Spenser, 1955). These experiments indicate that neither tryptophan nor 3-hydroxyanthranilic acid serve as precursors of trigonelline. Grimshaw and Marion (1958) tested whether a plant which could not utilize tryptophan in its synthesis of the pyridine ring might convert anthranilic acid into the pyridine ring since initial oxidation of the tryptophan would be by-passed. They tested this possibility with



X



XI

anthranilic acid-1-C^{14} but no activity was found in the isolated nicotine. Hence, anthranilic acid was also discarded as a precursor of the pyridine ring.

The alkaloid damascenine XI is of interest due to its structural relationship to 3-hydroxyanthranilic acid, of which it is a logical metabolite (Fig.1). Both tryptophan-3-C^{14} (Leete et al, 1955) and anthranilic acid-^{14}COOH (Vishen, Mothes, Engelbrecht & Schröter, 1960) failed to serve as precursors of damascenine in Nigella damascena L. Thus, Barger's (1938) prediction that this alkaloid arises from tryptophan was not substantiated.

It has thus been proven that in plants, nicotinic acid is not a product of tryptophan metabolism. In certain species of micro-organisms similar observations have been made. Neither kynurenine nor 3-hydroxyanthranilic acid, intermediates in the formation of nicotinic acid from tryptophan in mammals and Neurospora (Fig.1), could replace nicotinic acid as a growth factor for some nicotinic acid-requiring species of bacteria (Volcani & Snell, 1948). In other bacteria, it was shown that when tryptophan was used as the sole carbon source, the enzyme needed for oxidation of 3-hydroxyanthranilic acid was lacking

and apparently here nicotinic acid cannot be formed from tryptophan (Stanier & Tsuchida, 1949). Other experiments have confirmed the fact that certain bacteria cannot utilize tryptophan or any of the intermediates on the "tryptophan pathway" in the production of the pyridine ring (Yanofsky, 1954). When tryptophan-C^{14} was supplied to Escherichia coli and Bacillus subtilis, the nicotinic acid formed by these species was not radioactive (Yanofsky, 1954). Thus, similar to the case in plants, tryptophan does not serve as precursor of the pyridine nucleus in certain species of bacteria.

NICOTINIC ACID AS THE PRECURSOR OF NICOTINE AND RICININE

There can be little doubt that nicotinic acid is directly incorporated into the pyridine nucleus of both nicotine and ricinine. When nicotinic acid-2-H^3 was administered to tobacco plants, over 98% of the label was recovered in position 2 of the pyridine ring of nicotine (Dawson, Christman, D'Adamo, Solt & Wolf, 1960). Similarly the labels from nicotinic acid-4-^3H and nicotinic acid-5-^2H were quantitatively recovered in the corresponding positions of nicotine. When nicotinic acid-6-^3H was fed, loss of tritium took place, and it is therefore likely that in the course of its conversion to nicotine, nicotinic acid undergoes an oxidation-reduction reaction at carbon 6 (Dawson et al, 1960). When nicotinic acid labelled in the carboxyl group was administered to castor plants, all of the activity of the isolated ricinine was found in the cyano group (Leete & Leitz, 1957) and when carboxyl labelled nicotinic acid was fed to tobacco plants,

inactive nicotine was isolated (Dawson, Christman & Anderson, 1953). Activity from nicotinic acid universally labelled in the pyridine ring was incorporated into the pyridine nucleus of ricinine (Waller & Henderson, 1961 a), while N^{15} from nicotinamide labelled in the amide group was incorporated into the cyano group of ricinine (Waller & Henderson, 1961 a). All the available evidence thus supports the view that the pyridine nucleus of ricinine and of nicotine is derived specifically from nicotinic acid. It follows that an investigation of the origin of ricinine, nicotine, and presumably other pyridine alkaloids will throw light on the biosynthesis of nicotinic acid, and conversely, that findings regarding the origin of nicotinic acid are relevant to the problem of ricinine and nicotine biosynthesis.

BIOSYNTHESIS OF THE PYRIDINE RING FROM 2, 3, AND 4 CARBON UNITS

Attempts have been made to identify the precursors of nicotinic acid in microorganisms which do not synthesize nicotinic acid from tryptophan. Ortega and Brown (1960) presented evidence that in E. coli the precursors of nicotinic acid are a 4-carbon dicarboxylic acid and glycerol or one of its metabolites. They found that glycerol-1, 3-C^{14} and succinate-2, 3-C^{14} were effectively incorporated into nicotinic acid and that the synthesis of nicotinic acid by these organisms is aided if either a 3-carbon compound (glycerol, pyruvate) or an acid from the tricarboxylic acid cycle (Fig. 12) is present.

In other work with microorganisms, DL-aspartic acid-4-C^{14} was administered to Mycobacterium tuberculosis and active nicotinic acid was subsequently isolated (Mothes, Gross, Schutte & Mothes, 1961).

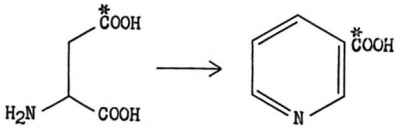


Figure 2

Decarboxylation of the nicotinic acid indicated that all of the radioactivity was located in the carboxyl group with no activity in the pyridine nucleus. The authors conclude that the carboxyl group of nicotinic acid arises from carbon-4 of aspartic acid (Fig. 2).

A large number of compounds have been tested as precursors of the pyridine ring of nicotine and ricinine, and therefore of nicotinic acid, in plants.

(1) Acetate as precursor

Leete (1958 a) administered acetate-2-C^{14} to N. tabacum plants and his work indicated that the acetate was incorporated into the isolated nicotine but that the activity was randomly distributed. Iljin (1960) also showed that acetate-C^{14} was incorporated into nicotine, but the location of activity in the nicotine molecule was not determined. Acetate-1-C^{14} and acetate-2-C^{14} were fed to tobacco plants by Griffith and Byerrum (1959 a, b) and by Griffith, Hellman and Byerrum (1960). Acetate-2-C^{14} was incorporated to a greater extent than acetate-1-C^{14}. Partial degradation indicated that when acetate -1-C^{14} was administered, only 4% of the total activity of the isolated

nicotine was located in the pyridine ring. When acetate-2-C^{14} served as precursor, approximately 40% of the nicotine activity was found in the pyridine ring, half of which (20%) was located at carbon 3.

Acetate-1-C^{14} and acetate-2-C^{14} were fed to Ricinus Communis L. by several groups of workers. Juby and Marion (1961) found greater incorporation of acetate-2-C^{14} than of acetate-1-C^{14} and when the latter compound was administered, 93% of the total activity of the isolated ricinine was found in the nitrile group and the remainder in the O-methyl and N-methyl groups. When acetate-2-C^{14} was fed, about 20% of the total ricinine activity was found in the nitrile group, about 1% in the O-methyl and N-methyl groups and approximately 80% in the ring carbons. Anwar, Griffith and Byerrum (1961) report that 90% of the activity from acetate-1-C^{14} was found in the nitrile group of ricinine while 25% was found in the nitrile after feeding acetate-2-C^{14}. Waller and Henderson (1961 b) found greater incorporation of acetate-2-C^{14} than acetate-1-C^{14} into ricinine.

The above results can be rationalized if it is assumed that acetate undergoes its normal metabolic fate, entering the tricarboxylic acid cycle to be converted to succinate, fumarate, or oxaloacetate, one of which then serves (with the loss of one carboxyl group) as the precursor of nicotinic acid.

Acetate-1-C^{14} would yield 1, 4-C^{14} succinate, fumarate, or oxaloacetate (Fig. 5), which on going through the next turn of the cycle with inactive acetate would lose all its radiocarbon as CO_2, or on recycling with a further molecule of acetate-1-C^{14} would be regenerated. α-Ketoglutarate-5-C^{14} would be formed as an intermediate

in this process (Fig. 3). Since this compound is convertible to

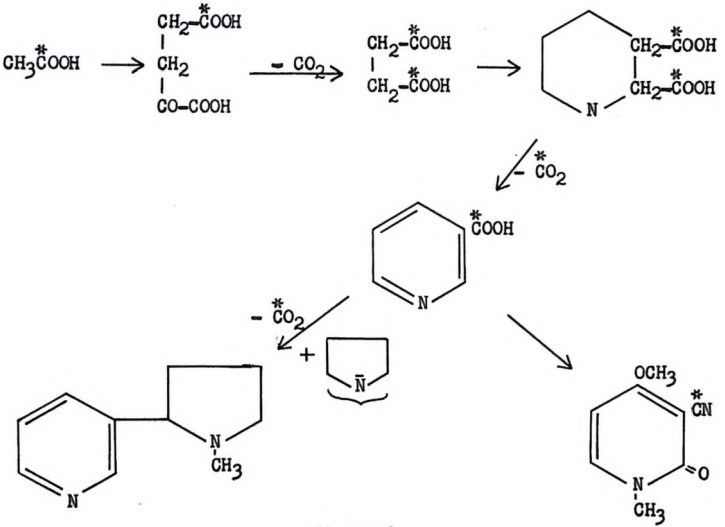


Figure 3

glutamate-5-C^{14} and ornithine-5-C^{14}, both of which can serve as precursors of the symmetrical intermediate which yields the pyrrolidine ring of nicotine (Leete, 1958 b; Leete & Siegfried, 1957; Lamberts & Byerrum, 1958; Lamberts et al, 1959), the major activity in nicotine on feeding acetate-1-C^{14} would be predicted to be at carbons 2' and 5', as was indeed observed. Since some C^{14} is detached as $C^{14}O_2$, methyl groups could be labelled, as was found in the case of ricinine (Table 1).

Acetate-2-C^{14} would yield 2,3-C^{14} labelled succinate, fumarate,

or oxaloacetate on its first turn through the cycle (Fig. 5). These on a further turn with inactive acetate would become equally labelled at positions 1, 2, 3, and 4 (Fig. 4). On further turns with inactive acetate, activity from positions 1 and 4 would be lost more quickly

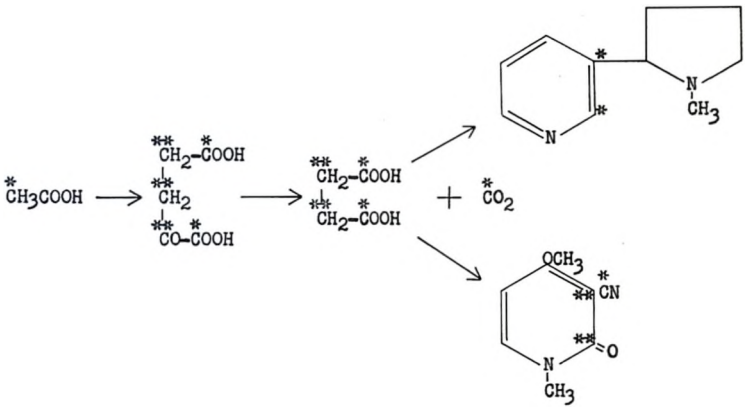
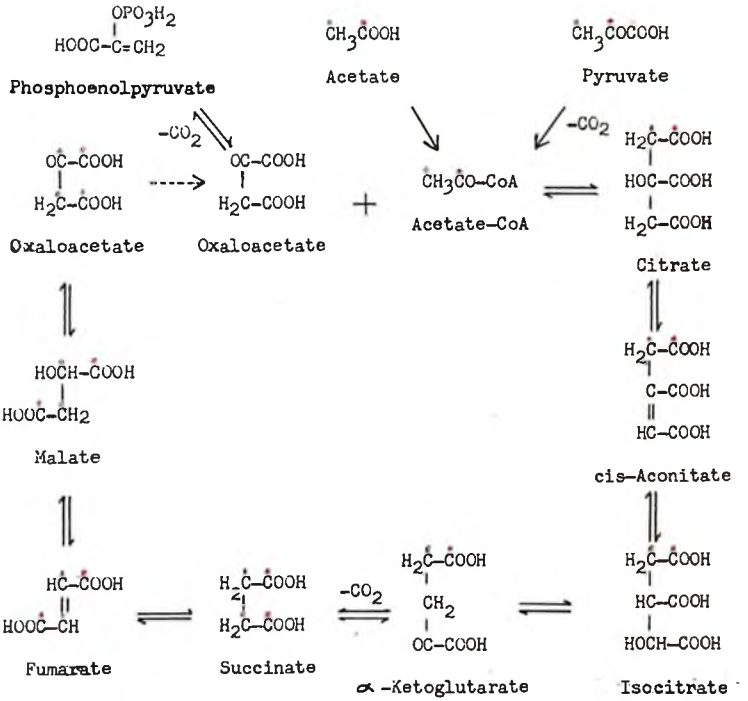


Figure 4

than from positions 2 and 3. On further turns with acetate-2-C^{14}, activity at positions 2 and 3 would increase relative to that at positions 1 and 4 (Fig. 4). Thus in nicotine, activity would be expected in the pyrolidine ring, less at $C_{2'}$ and $C_{5'}$ than at $C_{3'}$ and $C_{4'}$, and in the pyridine ring at C_2 and C_3. In ricinine some activity would be expected in the nitrile group, a little in the methyl groups, and the majority at C_2 and C_3. Partial degradation results are in agreement with this prediction.



Labelled Acetate and Pyruvate in the Tricarboxylic Acid Cycle

(dotted line indicates start of second cycle)

Figure 5

(2) Succinate as precursor

Juby and Marion (1961) fed succinate-2, 3-C^{14} to castor plants and isolated active ricinine with a distribution of activity identical to that obtained from acetate-2-C^{14}, in accord with the acetate-succinate hypothesis. Each succinate-2, 3-C^{14} molecule would result in succinate equally labelled on all four carbons after only one operation of the cycle (Fig. 12). These molecules would mix with 2, 3 labelled succinate, yielding succinate-1, 2, 3, 4-C^{14}, with activity predominating at 2 and 3. This type of randomization accounts for the activity found in the nitrile group which was lower than in the ring. The ring activity would be expected to be located at the 2 and 3 positions. Waller and Henderson (1961 b) found activity in the ring only on feeding succinate -2, 3-C^{14}.

Succinate-1, 4-C^{14} was found to be a less efficient precursor of ricinine than succinate-2, 3-C^{14} (Waller & Henderson, 1961 b). Approximately 25% of the activity of ricinine derived from succinate 1, 4-C^{14} was found in the nitrile and 75% in the ring. This result appears to be anomalous and cannot be reconciled with the acetate-1 -C^{14} results. A possible explanation may be that part of the succinate was converted to pyruvate and hence by reversal of glycolysis to glyceroaldehyde phosphate and glycerol.

(3) Aspartate as precursor

Byerrum and Griffith (Chem. & Eng. News, 1961) fed aspartic acid-3-C^{14} to tobacco plants and degradation of the active nicotine showed that only one half of the activity of the pyridine ring was

concentrated at carbon 3 (Fig. 6). Since this is the case, then either

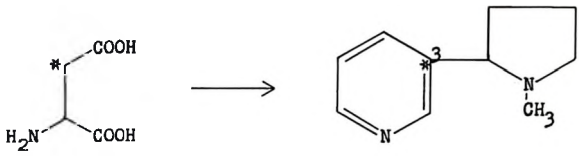


Figure 6

randomization of carbon from position 3 to other ring positions must have occurred or else the incorporation of aspartic acid into nicotine is slow relative to its conversion to the acids of the tricarboxylic acid cycle. In the former case, aspartic acid may be converted to some other molecule with randomization of carbon perhaps between C_2 and C_3. For example, aspartate may be converted to fumarate or succinate in the tricarboxylic acid cycle (Fig. 12), and randomization of carbon would occur, and one of these compounds would be an intermediate between aspartic acid and the pyridine ring. If, however, the conversion of aspartate to the acids of the cycle is much faster than its conversion to the pyridine ring, then aspartate-3-C^{14} would be quickly converted to the 2, 3 labelled compound and thus direct incorporation would still be possible.

(4) Pyruvate as precursor

Griffith and Byerrum (1959 a) fed pyruvate-1-C^{14} and pyruvate -3-C^{14} to tobacco plants and active nicotine was isolated. Pyruvate -1-C^{14} was incorporated to a very small extent, while pyruvate-3-C^{14}

was incorporated to a much greater extent, its incorporation being even greater than that of acetate. Pyruvate-2-C^{14} was also reported by these same authors to undergo less dilution than pyruvate-1-C^{14}, but greater dilution than pyruvate-3-C^{14} on incorporation. For the most part, the results indicate that pyruvate was metabolized to acetate since, on the whole, pyruvate was incorporated to a smaller extent than acetate and the order of incorporation of the pyruvates would be consistent with their conversion to acetate and hence to the pyridine ring. However, the fact that pyruvate-3-C^{14} was efficiently incorporated and the small but significant incorporation of pyruvate -1-C^{14} indicates that not all pyruvate was converted to acetate before being utilized for nicotine synthesis. Another possibility is that pyruvate is converted to glycerol by a reversal of glycolysis. This could account for the incorporation of the 1-labelled compound into the pyridine ring of nicotine.

(5) Glycerol as precursor

Glycerol-1, 3-C^{14} was fed to tobacco plants and the isolated nicotine was found to be active (Griffith et al, 1960). Subsequent degradation showed that a significant quantity of activity (60%) was located in the pyridine ring. When acetate-2-C^{14} was administered, 40% of the radioactivity of the isolated nicotine had been found in the pyridine ring (section 1). If glycerol were converted to acetate before incorporation into the pyridine ring of nicotine, no more than 40% of the activity of nicotine should be found in the pyridine ring on feeding radioglycerol. Since 60% of the activity was found in the

pyridine ring after administering glycerol-1, 3-C^{14}, glycerol must be utilized for pyridine ring synthesis either entirely, or at least partially, by a pathway which excludes acetate. The authors suggest that glycerol is the source of carbons 4, 5, and 6 of the pyridine ring, but they drew no definite conclusions since these carbons were not individually isolated.

Juby and Marion (1961) fed glycerol-1-C^{14} and glycerol-2-C^{14} to castor plants and isolated active ricinine. The results indicated that glycerol-1-C^{14} was incorporated to the greater extent. Again it was suggested that glycerol may give rise to the 4, 5, and 6 carbons of the pyridine nucleus.

Waller and Henderson (1961 b) also showed that glycerol-1, 3-C^{14} was incorporated more efficiently into ricinine than glycerol -2-C^{14}. The results may be explained by the conversion of glycerol to acetate through glycolysis. Glycerol-1, 3-C^{14} and glycerol-2-C^{14} would give rise to acetate-2-C^{14} and acetate-1-C^{14} respectively.

In Chemistry and Engineering News (1961), Dawson and Christman consider glycerol as precursor of carbons 5 and 6 only of the pyridine ring of nicotine, but as yet these carbons have not been isolated by a stepwise degradation of the pyridine ring. Such a degradation scheme is necessary to resolve the conflict as to whether the 3 carbons of glycerol are incorporated as such, or whether glycerol is converted to a 2-carbon unit before its conversion to the pyridine ring.

(6) Glutamate as precursor

Glutamate-2-C^{14} was administered to castor plants and ricinine

isolated (Anwar et al, 1961). About 90% of the total ricinine activity was found in the nitrile group. If glutamate-2-C^{14} were involved in a transamination reaction, the resulting α-ketoglutarate-2-C^{14} would give rise to carboxyl labelled succinate (Fig. 12). Once again ricinine with a high proportion of activity in the nitrile would result.

(7) Propionate as precursor

Propionate-1-C^{14} and propionate-2-C^{14} were administered to tobacco plants by Griffith et al (1960). They reported greater incorporation with propionate-2-C^{14} than with the carboxyl labelled compound and when the nicotine isolated from plants fed propionate -2-C^{14} was degraded, the latter was shown to contribute significant quantities of activity to the pyridine ring. The results indicated that carbon 2 of the pyrrolidine ring contained 12% of the activity whereas carbons 3, 4, and 5 contained 42%. If one assumes a symmetrical intermediate then the activity of carbon 2 would equal that of carbon 5. This would be consistent with propionate being converted to acetate and then to glutamate on the way to the pyrrolidine ring. Approximately 38% of the total activity was found in the pyridine ring. In the case of propionate-2-C^{14}, as was the case with acetate -2-C^{14}, 50% of the activity in the pyridine ring was located at carbon 3, although the total incorporation of propionate was greater than that of acetate. The authors suggest that propionate donates its 2 and 3 carbons to the 3 and 2 positions respectively of the pyridine ring (Fig. 7). If the 2 and 3 carbons of propionate are incorporated into the pyridine ring, it is possible that the carboxyl carbon of propionate would become the carboxyl carbon of nicotinic acid. If this were so, no

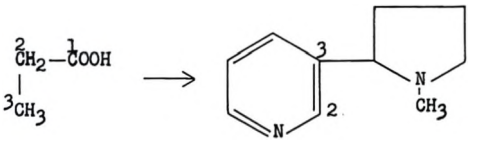


Figure 7

activity from propionate-1-C^{14} would be found in the pyridine ring. Griffith et al (1960) have shown this to be the case.

Anwar et al (1961) report that when both propionate-1-C^{14} and propionate-3-C^{14} were administered to castor plants, 90% of the total activity of the isolated ricinine was found, in each case, to be in the nitrile group. This would indicate that propionate is not a direct precursor of ricinine and the results could be explained by assuming β-oxidation of propionate to malonate with subsequent decarboxylation to acetate (Fig. 8).

$$\overset{3\ 2\ 1}{CH_3CH_2COOH} \longrightarrow \overset{3\ 2\ 1}{HOOCCH_2COOH} \longrightarrow \overset{2\ 1,3}{CH_3COOH}$$

Figure 8

Waller and Henderson (1961 b) reported that their results of the incorporation of propionate into ricinine (propionate-3-C^{14} > propionate -2-C^{14} > propionate-1-C^{14}) indicated that this compound may give rise

to succinic acid via a β-oxidative pathway (Fig. 9)(Giovanelli & Stumpf, 1957). Activity from propionate-1-C^{14} would not enter the tricarboxylic acid cycle (Fig. 9), propionate-2-C^{14} would give acetate-2-C^{14} and thus be incorporated to a greater extent than acetate-1-C^{14} which would be formed from propionate-3-C^{14}. Waller and Henderson (1961 b) have indicated in these experiments that the percentage incorporation of propionate into ricinine depended on the position of the propionate label with non-flowering plants, but with flowering plants the incorporation was identical for all three labelled propionates.

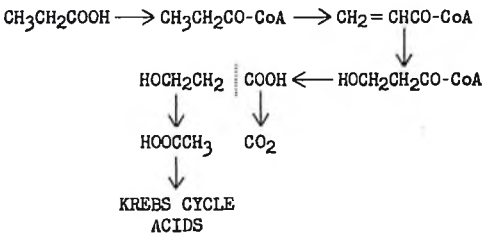


Figure 9

Thus, the above evidence would indicate that propionate, as such, is converted into the pyridine ring or that it is converted by way of succinate or a 4-carbon dicarboxylic acid of the tricarboxylic acid cycle. Propionate may be converted to succinate via methyl malonic acid (Flavin, Ortiz & Ochoa, 1955; Flavin & Ochoa, 1957). Propionate-1-C^{14} would give carboxyl labelled succinate while the 2 and 3 labelled propionate would give succinate labelled in the 2 and

3 positions. The results of Anwar et al (1961) indicate, however, that propionate is converted to acetate.

(8) β-Alanine as precursor

It was shown by Waller and Henderson (1961 b) that β-alanine -2-C^{14} was incorporated into ricinine to a larger extent than β-alanine-1-C^{14}. Griffith and Byerrum (Chem. & Eng. News, 1961) showed that approximately 50% of the activity from β-alanine goes into one carbon atom (C_3 of the pyridine ring) of nicotine. Dawson and Christman (Chem. & Eng. News, 1961) showed that another 25% of the activity was located at C_2 of the pyridine ring. Thus, from the reported results, β-alanine may be converted into propionate before being incorporated into the pyridine ring or it could be converted to malonate. If it were converted to malonate, then β-alanine-2 -C^{14} would give rise to malonic acid labelled in position 2 which in turn would give acetate-2-C^{14}. This is known to be incorporated to a greater extent than acetate-1-C^{14} which would be derived either from β-alanine-1-C^{14} or β-alanine-3-C^{14}. Dawson and Christman (Chem. & Eng. News, 1961) suggest that β-alanine is incorporated by way of citrate (section 9).

(9) Other compounds as precursors

Dawson and Christman (Chem. & Eng. News, 1961) consider citrate asprecursor of carbons 2, 3, and 4 of the pyridine ring (Fig. 10). The fact that $^{(1)}$carbons 2 and 3 of succinate, fumarate, and aspartate enter the pyridine ring much more readily than do the carboxyl carbons of these same compounds and that $^{(2)}$carbon 2 of acetate and $-CH_2-$ of

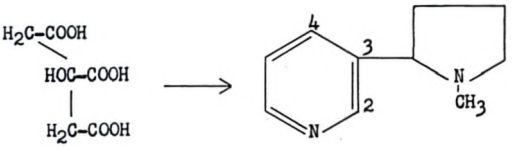


Figure 10

malonate enter the pyridine ring much more readily than their carboxyl carbons indicates that citric acid could well be a precursor of the pyridine ring.

Since, as mentioned under (8), 50% of the activity of β-alanine was found in carbon 3 of the pyridine ring and 25% was found in carbon 2, if the remainder of the activity were found at carbon 4, the citric acid hypothesis would be strengthened. During the one week incubation period inactive acetate might enter the tricarboxylic acid cycle and as a result, citric acid would become less heavily labelled at carbons 2 and 4 than at carbon 3. This would explain the percentage distribution of the carbon-14 in the pyridine ring. Further degradation work on the pyridine ring is necessary to settle the problem.

D-ribose-1-C^{14}, D-glucose-1-C^{14}, sodium formate-C^{14} and glycine-2-C^{14} have been fed to Ricinus communis L. (Waller & Henderson, 1961 a). The percentage incorporation into ricinine was of a low order of magnitude when these compounds were tested as precursors of the

pyridine ring.

The foregoing investigation of nicotine and ricinine biosynthesis has shown that the pyridine ring of these alkaloids is synthesized from simple metabolic intermediates rather than from the amino acid precursors, tryptophan and lysine.

The general observation that in all cases acetate-2-C^{14} is incorporated into the pyridine ring to a greater extent than acetate -1-C^{14} and that the efficiency of pyruvate incorporations is in the order pyruvate-3-C^{14} > pyruvate-2-C^{14} > pyruvate-1-C^{14} and, indeed, the finding that in all cases discussed, carboxyl labelled compounds were incorporated to a lesser extent than the 2 and 3 labelled compounds, can be explained by reference to the metabolic relation of the tested precursors to the tricarboxylic acid cycle (Fig. 12). As an illustration, Fig. 12 shows the path of succinate-2, 3-C^{14} and succinate-1,4-C^{14} in the first pass through the cycle.

It is difficult to draw definitive conclusions as to pathways in the formation of the pyridine ring since conflicting results are reported by different research groups. As yet the conflict has not been resolved as to whether the five carbons of the pyridine ring are [1]derived from the 3 carbons of glycerol and 2 of the carbons of a dicarboxylic acid of the tricarboxylic acid cycle or are [2]derived from 2 of the carbons of glycerol and the 3 carbons of citrate. Two groups (Griffith *et al*, 1960; Juby & Marion, 1961) favour glycerol as donating its three carbons, as such, with the two remaining carbons

of the ring arising from perhaps propinate or succinate while two other groups (Waller & Henderson, 1961 b; Dawson & Christman, Chem. & Eng. News 1961) favour glycerol as a source of two of the carbons of the pyridine ring. The last mentioned group favours citrate as a source of the remaining three carbons of the ring.

From a consideration of the 2, 3, and 4 carbon precursors, it would appear that the most consistent results for incorporation of precursors into the pyridine ring can be illustrated as shown in Fig. 11.

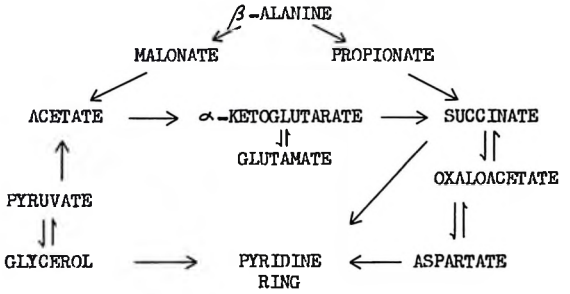


Figure 11

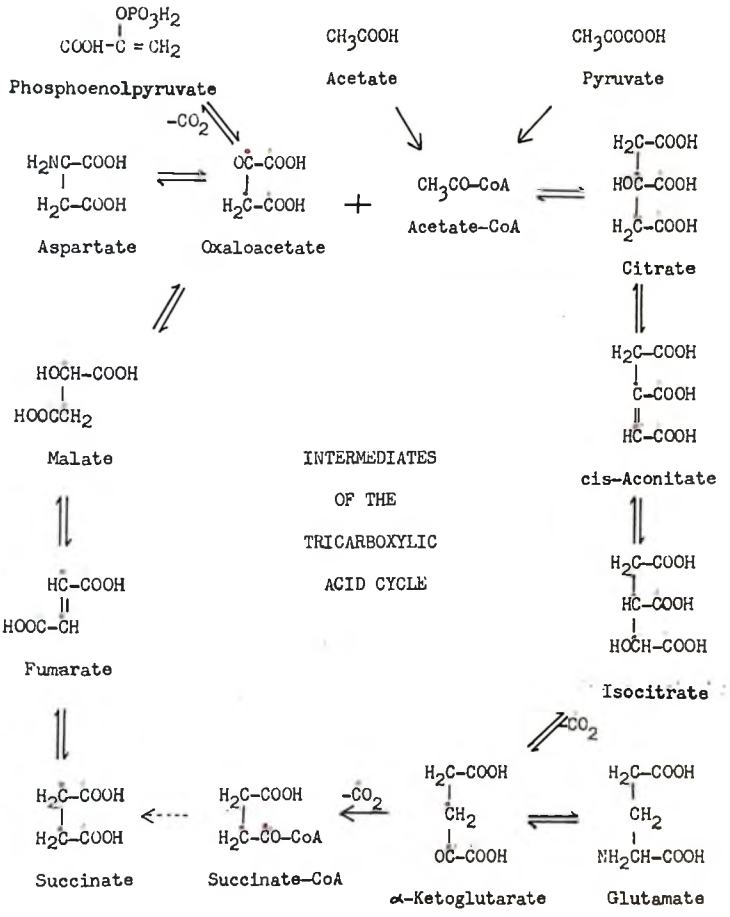
A summary of the important precursors and distribution of activity in the isolated nicotine and ricinine is given in Table 1.

The alkaloid damascenine XI is a substituted anthranilic acid derivative, containing a phenyl and not a pyridine nucleus. It would nevertheless be expected to be metabolically related to the pyridine

compounds. It has been shown earlier that in mammals and certain microorganisms, 3-hydroxyanthranilic acid is a metabolic intermediate in the biosynthesis of nicotinic acid from tryptophan (Fig. 1). Damascenine is of interest due to its structural relationship to 3-hydroxyanthranilic acid from which it is presumably derived.

Although it has been shown that 3-hydroxyanthranilic acid is neither incorporated into the pyridine ring of trigonelline (Aronoff, 1956) nor into the pyridine nucleus of nicotinamide in maize (Henderson et al, 1959) and that the pyridine ring does not therefore arise from 3-hydroxyanthranilic acid in plants, the possibility does exist that nicotinic acid and 3-hydroxyanthranilic acid are biochemically related and arise from a common precursor. If this were the case, then compounds which act as precursors of the pyridine ring might also act as precursors of 3-hydroxyanthranilic acid.

Tracer work has now established that the pyridine ring in plants is built up from 2, 3, and 4-carbon compounds. If 3-hydroxy-anthranilic acid and therefore damascenine were related to nicotinic acid by way of a common precursor, the same small fragments should be biochemically incorporated into damascenine. A convenient starting point for an investigation of damascenine biosynthesis, therefore, is the feeding of known precursors of the pyridine ring in plants to Nigella damascena L.



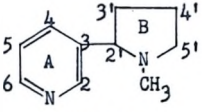
Succinate-1,4-C^{14} and Succinate-2,3-C^{14} in the Tricarboxylic Acid Cycle (dotted line indicates start of second cycle)

Figure 12

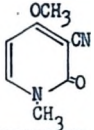
COMPOUND FED	DILUTION[1] OF RADIOACTIVITY IN NICOTINE	(Ref.)	DILUTION OF RADIOACTIVITY IN RICININE	(Ref.)
$CH_3\overset{*}{C}OOH$	950	(a)	8,000	(c)
Acetate-1-C^{14}	946	(h)	5,620	(d)
$\overset{*}{C}H_3COOH$	324	(a)	6,880	(c)
Acetate-2-C^{14}	436	(h)	3,810	(d)
$\overset{*}{C}H_2COOH$			11,600	(c)
$\overset{*}{C}H_2COOH$	——		7,030	(d)
Succinate-2,3-C^{14}				

[1] Dilution = $\frac{\text{specific activity of precursor}}{\text{specific activity of product}}$

TABLE 1



NICOTINE (Ref.)



RICININE (Ref.)

$C_{2'}$ = 47%

$C_{3'+4'+5'}$ = 40%

Ring A = 4% (b)

CN = 93% (c)
= 90% (f)

$N-CH_3$ and $O-CH_3$ = 6% (c)

$C_{2'}$ = 8%

$C_{3'+4'+5'}$ = 40%

Ring A = 40% (C_3 = 20%) (b)

CN = 21% (c)
= 25% (f)

$N-CH_3$ and $O-CH_3$ = 1% (c)

Ring = 80% (c)

—

CN = 20% (c)

$N-CH_3$ and $O-CH_3$ = 1% (c)

Ring = 80% (c)
= 100% (d)

$CH_2{}^{*}COOH$ $CH_2{}^{*}COOH$ Succinate-1,4-C^{14}	----		32,550	(d)	----	CN = 25% Ring = 75%	(d)
$^{*}CH_2COOH$ $NH_2CHCOOH$ Aspartate-3-C^{14}	----		----		C_3 = 50% Of Activity Of Ring A (g)	----	
$CH_3CO{}^{*}COOH$ Pyruvate-1-C^{14}	8,370	(a)	----		Distribution Of Activity Not Reported	----	
$^{*}CH_3COCOOH$ Pyruvate-3-C^{14}	243	(a)	----		"	----	
$CH_3{}^{*}COCOOH$ Pyruvate-2-C^{14}	3,370	(a)	----		"	----	

TABLE 1

(continued)

CH_2^*OH | CHOH | CH_2^*OH Glycerol-1,3-C^{14}	138	(h)	6,630 61,300	(c) (c)	$C_{2'}$ = 6% $C_{3'+4'+5'}$=27%($C_{5'}$=6%(?)) Ring A = 56% (C_{4+5+6}=56%(?))(h)		N-$CH_3$and O-CH_3 =35% CN =10% Ring = 45% (c)
CH_2OH | $CHOH^*$ | CH_2OH Glycerol-2-C^{14}	——		8,640 123,000		——		CN = 20% N-$CH_3$and O-CH_3 =35% Ring =55% (c)
CH_2COOH | CH_2 | $NH_2^*CHCOOH$ Glutamate-2-C^{14}	——		115,000	(e)	——		CN =90% (e) =90% (f)
$CH_3CH_2^*COOH$ Propionate-1-C^{14}	9,238	(h)	78,400	(d)	Ring A=0%	(h)	CN=90% (f)

TABLE 1

(continued)

$CH_3\overset{*}{C}H_2COOH$ Propionate-2-C^{14}	203 (h)	4,535	(d)	$C_{2'}$=12% $C_{3'+4'+5'}$=42%($C_{5'}$=12%(?)) Ring A=38%(C_3=19%) (h)	CN=25%	(f)
$\overset{*}{C}H_3CH_2COOH$ Propionate-3-C^{14}	——	3,085	(d)	——	CN=90%	(f)
$H_2NCH_2CH_2\overset{*}{C}OOH$ β-Alanine-1-C^{14}	——	4,020	(d)	——	Distribution Of Activity Not Reported	
$H_2NCH_2\overset{*}{C}H_2COOH$ β-Alanine-2-C^{14}	——	4,810	(d)	C_3=50% Of Activity Of Ring A C_2=25% Of Activity Of Ring A C_4=25% Of Activity Of Ring A(?)	"	(g)

(a) Griffith & Byerrum, 1959a
(b) Griffith & Byerrum, 1959b
(c) Juby & Marion, 1961
(d) Waller & Henderson,1961b
(e) Waller & Henderson,1961a
(f) Anwar et al, 1961
(g) Chem. and Eng. News, 1961
(h) Griffith et al, 1960

TABLE 1

(continued)

AN INVESTIGATION OF DAMASCENINE BIOSYNTHESIS

CHOICE OF PRECURSORS

The present investigation was undertaken to test the hypothesis that an anabolic relationship exists between damascenine and pyridine derivatives occurring in higher plants. Damascenine biosynthesis from known precursors of the pyridine ring was therefore investigated.

Succinic acid and aspartic acid were selected for investigation. Both of these 4-carbon dicarboxylic acids are known as efficient precursors of the pyridine ring in plants.

Succinic acid is an intermediate in the tricarboxylic acid cycle (Fig. 12) and aspartic acid is closely related to oxaloacetic acid, another link of the cycle. From Fig. 12 it can be seen that after going through one complete turn of the cycle, each succinic acid-1, 4-C^{14} molecule would have lost all of its original activity. It would be expected, therefore, that the specific activity of succinic acid would decrease rapidly, but that no metabolic randomization of activity would take place. The fact that activity from succinate-1, 4-C^{14} was incorporated into ricinine (Waller & Henderson, 1961 b) appeared to indicate that the relative rates of incorporation and decarboxylation favoured synthesis and would allow isolation of active damascenine, if succinate did indeed serve as a precursor of the alkaloid.

Since direct incorporation would not be accompanied by randomization, activity from succinic acid-1, 4-C^{14}, if incorporated into damascenine, would be expected to be distributed equally between the carboxyl group and one ring carbon as shown in Fig. 13.

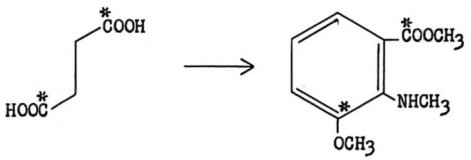


Figure 13

On decarboxylation of damascenic acid, obtained from damascenine, half of the radioactivity would be recovered as CO_2 while the other half could be found in the residual N-methyl-o-anisidine in a position fairly accessable to degradation.

If aspartic acid-4-C^{14} were incorporated directly into damascenine, decarboxylation of damascenic acid would yield carbon dioxide containing all the activity, and inactive N-methyl-o-anisidine (Fig. 14). Since aspartic acid is converted by metabolic transamination to oxaloacetic acid (Fig. 12), an intermediate of the tricarboxylic cycle, from which it can be regenerated, a dilution of activity would be expected. Each molecule of aspartic acid-4-C^{14} would lose all its activity as CO_2 within the first turn of the tricarboxylic acid cycle. However

de novo synthesis of oxaloacetic acid from phosphoenolpyruvate and this labelled CO_2 would yield oxaloacetate-4-C^{14} and thus aspartate -4-C^{14} and randomization of activity would therefore not be expected.

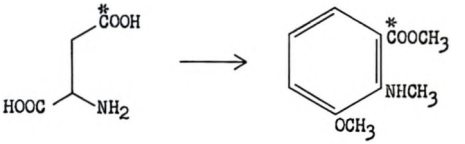


Figure 14

Succinic acid-1, 4-C^{14} and aspartic acid-4-C^{14} were selected as potential precursors of damascenine.

CHOICE OF FEEDING TIME

The precursors were fed at the time when the green seeds had just started to form, i.e., just after the flowering stage. It has been reported that this is the stage at which the intact plants begin to biosynthesize the alkaloid rapidly (Vishen *et al*, 1960). Since damascenine is found in the ripe seeds, and little, if any, is present in the young green seeds, the ripening period appeared to be the appropriate time for feeding the radioactive compounds. This was the stage chosen by Vishen *et al* (1960) for feeding anthranilic acid -^{14}COOH and by Leete *et al* (1955) for feeding tryptophan-3-C^{14} to *N. damascena* plants.

MODE OF ADMINISTRATION

The radioactive precursors were fed to *Nigella* plants by a

Figure 15

"wick" method (Fig. 15)(Comar, 1955). The active solution was placed in a small vial anchored in the soil near the plant. An ordinary sewing needle attached to a double strand of heavy thread was passed through the stem, the needle removed, and the four thread ends immersed in the solution as illustrated in Fig. 15. The solution moves into the plant by capillary action.

ISOLATION OF ALKALOID

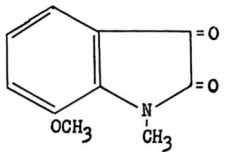
Damascenine was isolated from ripe seeds of Nigella plants. Reported work indicated that the alkaloid is contained mainly in the ripe seeds (Tyler, 1955; Vishen et al, 1960) and this was confirmed in the present investigation by extracting various parts of Nigella and taking ultraviolet absorption spectra of the extracts. Damascenine absorption was found only in the extract from the ripe seeds.

Ether was found to be the most efficient solvent for extracting the alkaloid (Leete et al, 1955). Chloroform (containing a few drops of ammonia) and petroleum ether (Ewins, 1912) extracted damascenine, contaminated by substances which absorbed in the ultraviolet region of the spectra.

LABORATORY SYNTHESIS OF DAMASCENINE

Damascenine was synthesized using a modification of the method of Kaufmann and Rothlin (1916), who prepared damascenine by oxidation of 8-methoxyquinoline methosulfate, followed by hydrolysis and esterification of N-formyldamascenic acid so obtained. Improved yields of N-formyldamascenic acid were obtained by oxidation of 8-methoxyquinoline methochloride, which was obtained from the corresponding methiodide. The oxidation product was not contaminated with

the isatin derivative XII as found by Kaufmann and Rothlin.



XII

Since oxidation of the quinoline derivative to N-formyldamascenic acid was known to give poor yields, an oxidation in two stages was attempted. 8-Methoxyquinoline methochloride was oxidised with potassium ferricyanide to give 1, 2-dihydro-1-methyl-8-methoxy-2-oxoquinoline. This oxidation was adapted, on a much smaller scale, from the procedure of Perkin and Robinson (1913) for the oxidation of 1-methylquinoline to 1, 2-dihydro-1-methyl-2-oxoquinoline. The reaction sequence is shown in Fig. 16. The carbostyril derivative

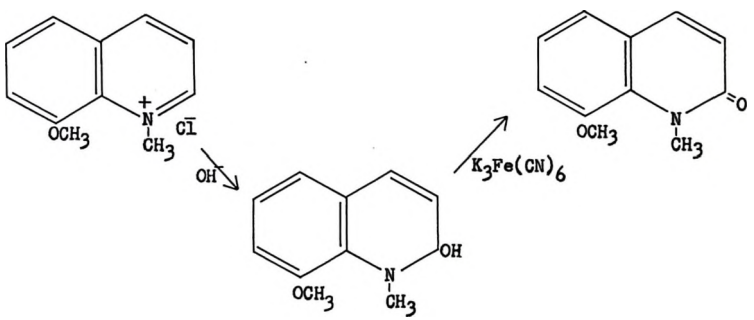


Figure 16

proved stable to oxidation even under vigorous conditions.

The single step oxidation which was finally adopted was a modification of the method of Kaufmann and Rothlin (1916). Permanganate oxidation of 8-methoxyquinoline methochloride was carried out very slowly and gave an increased yield of N-formyldamascenic acid.

Ultraviolet absorption spectra in both acid and base indicated that damascenic acid is not formed as readily from N-formyldamascenic acid as was reported by Kaufmann and Rothlin; it was prepared from the N-formyl derivative by alkaline hydrolysis. The alkaloid itself, damascenine, was prepared from N-formyldamascenic acid according to Kaufmann and Rothlin (1916). The reaction sequence is given in Fig. 17.

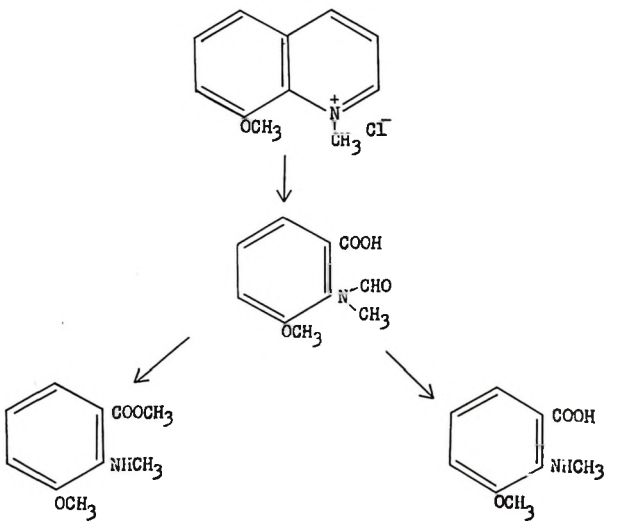
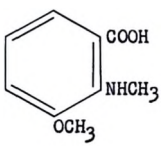


Figure 17

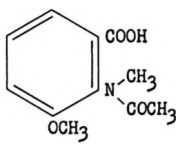
SYSTEMATIC DEGRADATION OF DAMASCENINE

The isolation of an active product from a metabolising system to which a tracer has been administered, is not in itself proof of a direct precursor-product relationship. Definite conclusions can only be drawn when it is shown that the precursor has been incorporated into the product without scrambling. For this purpose, the product must be systematically degraded to locate the positions of activity. Thus, a stepwise degradation of damascenine was devised and carried through a number of steps.

Damascenine was hydrolyzed with base to the corresponding acid, damascenic acid XIII, and the N-acetyl derivative XIV of the acid was prepared. This was a high melting compound, which could be prepared



XIII

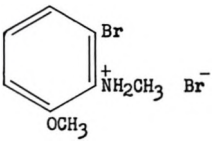


XIV

on a very small scale, and would be a useful derivative for radioactive assay.

An attempt to decarboxylate damascenic acid by heating the sodium salt of the acid *in vacuo* failed to produce any N-methyl-o-anisidine and although an attempt to decarboxylate the acid in glycerol was

moderately successful, a control experiment indicated that part of the liberated carbon dioxide arose by decomposition of the glycerol. Bromodecarboxylation proved successful. The liberated carbon dioxide was collected as barium carbonate while the residue, 2-bromo-6-methoxy -N-methylaniline was isolated as the hydrobromide XV and characterized as the picrate. Thus, since succinic acid-1, 4-C^{14} was expected to give

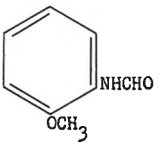


XV

damascenine labelled equally in the carboxyl group and in the ring, and aspartic acid-4-C^{14} to give damascenine labelled in the carboxyl group only, isolation of carbon dioxide and of 2-bromo-6-methoxy-N-methylaniline for radioactive assay would constitute a complete analysis for radioactivity in aspartate-derived damascenine, but only a partial analysis for the succinate-derived alkaloid.

To locate the expected activity in the benzene nucleus from the succinate experiment, further degradation was necessary. Decarboxylation of damascenic acid gave N-methyl-o-anisidine. To prepare a sub-

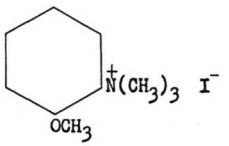
stantial amount of this compound for use in degradation experiments, it was synthesized from o-anisidine. o-Anisidine was converted to the N-formyl derivative XVI which showed the expected ultraviolet absorption, unaffected by pH. N-Formyl-o-anisidine was reduced with lithium aluminum hydride to N-methyl-o-anisidine which was character-



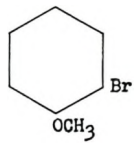
XVI

ized as the hydrobromide and as the picrate.

Hydrogenation of N-methyl-o-anisidine in glacial acetic acid gave 2-methoxy-N-methylcyclohexylamine which was suitable for entry into the ring. Exhaustive methylation and elimination of the quaternary nitrogen gave a methoxycyclohexene. To obtain larger amounts of the quaternary compound XVII, it was prepared from 1-bromo-2-methoxycyclohexane XVIII, which on treatment with trimethylamine gave a white oily quaternary bromide, whose infrared spectrum bore a close resemblance to that of the quaternary iodide prepared above. A Hofmann elimination

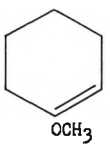


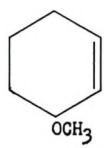
XVII



XVIII

of either the quaternary bromide or the quaternary iodide gave an unsaturated product, which could be methoxy-1-cyclohexene or methoxy -2-cyclohexene XIX or a mixture of the two isomers. Knowledge of the





XIX

absolute identity of the unsaturated product is not necessary for further degradative steps. An outline of the degradation procedure is given in Fig. 18.

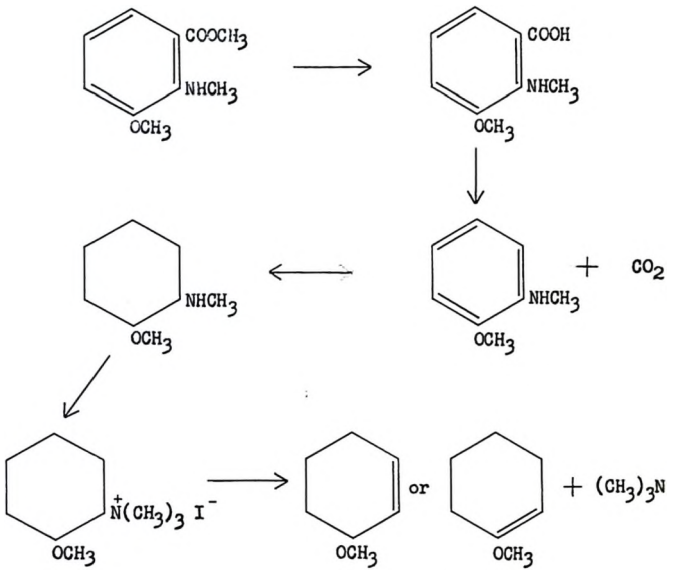


Figure 18

The actual experimental work of the degradation procedure has been carried no further. However, a possible outline for the carbon by carbon degradation of the methoxycyclohexene(s) has been formulated. The scheme in Fig. 19a outlines the sequence of reactions. This sequence (Fig. 19a) allows the unequivocal isolation of carbons 3 and 6, and the separation of carbons 1+5 from carbons 2+4. Degradation of N-methyl-2-methoxycyclohexylamine according to the outline in Fig. 19b would permit the isolation of carbons 2+3, 4+1, and 5+6.

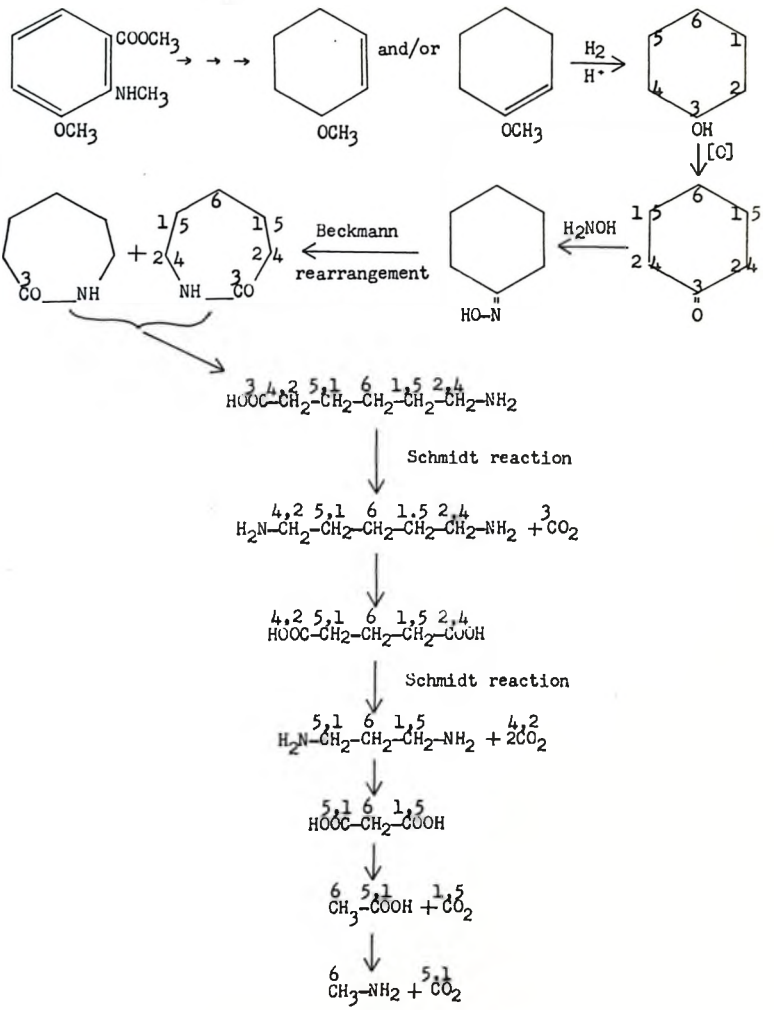

COOCH3
NHCH3
OCH3
and/or
OCH3
OCH3
H2
H+
OH
[C]
O
H2NOH
HO-N
Beckmann
rearrangement
CO NH
NH CO
HOOC-CH2-CH2-CH2-CH2-CH2-NH2
Schmidt reaction
H2N-CH2-CH2-CH2-CH2-CH2-NH2 + CO2
HOOC-CH2-CH2-CH2-COOH
Schmidt reaction
H2N-CH2-CH2-CH2-NH2 + 2CO2
HOOC-CH2-COOH
CH3-COOH + CO2
CH3-NH2 + CO2

Figure 19a

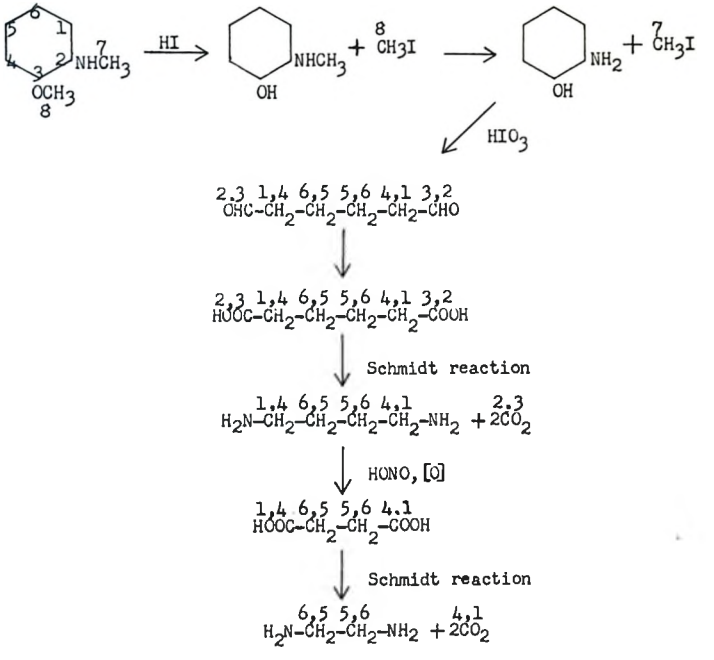

HI
NHCH3
OH
8
CH3I
NH2
7
CH3I
HIO3
2,3 1,4 6,5 5,6 4,1 3,2
OHC-CH2-CH2-CH2-CH2-CHO
2,3 1,4 6,5 5,6 4,1 3,2
HOOC-CH2-CH2-CH2-CH2-COOH
Schmidt reaction
1,4 6,5 5,6 4,1
H2N-CH2-CH2-CH2-CH2-NH2
2,3
+2CO2
HONO, [O]
1,4 6,5 5,6 4,1
HOOC-CH2-CH2-COOH
Schmidt reaction
6,5 5,6
H2N-CH2-CH2-NH2
4,1
+2CO2

Figure 19b

Since in a degradation of labelled damascenine, the activity of carbon atom 3 and of carbon atom 6, obtained by the cyclohexanone sequence, are known, that of carbons 2 and 5 can be calculated from the data of the aminocyclohexanol reaction sequence. Knowing these, the activity of carbons 1 and 4 can now be obtained from sequence 19 a. In this way, the activity of each of the carbons of damascenine can be deduced in an experiment using carbon-14 tracers.

RESULTS OF FEEDING EXPERIMENTS AND CONCLUSIONS

Ripe seeds, obtained from Nigella plants, which had been grown in contact with succinate-1, 4-C^{14} and aspartate-4-C^{14} were extracted, and damascenine purified as described earlier. The damascenine concentration of the extract was determined by ultraviolet spectrometry and it was found that approximately 4% of the dry seed weight in each case was damascenine. The original extracts were radioactive showing the following specific activities (counts/min/mg): 47 for extract from seeds of 1st harvest of plants fed succinic acid-1, 4-C^{14}, 134 for extract from 2nd harvest of seeds fed succinic acid-1, 4-C^{14}, 189 and 120 for extracts of 1st and 2nd harvests of seeds from aspartic acid-4-C^{14}-fed plants.

A sample of each of the four extracts was chromatographed and the damascenine spots obtained were eluted with ethanol, plated, and counted. In each case damascenine was totally inactive. All activity appeared to be located in a nonbasic oil but time did not permit investigation of this material.

It has been reported that damascenine is synthesized in the

ripening seeds of Nigella (Vishen et al, 1960). Labelled succinic and aspartic acids were administered during this period of development. It would therefore appear that neither of these compounds can serve as a precursor of damascenine, since they were available at the correct time and were not incorporated.

The possibility that the alkaloid is synthesized in other parts of the plant and then transported to the ripening seeds is remote. If this were the case however, then actual synthesis would not take place at a time when the seeds are ripening and a more opportune time would have to be found for feeding the expected precursors. This could be done by keeping the intact plant in an atmosphere of radioactive carbon dioxide, isolating the alkaloid, and determining its activity. Vishen et al (1960), have subjected both the leaves and green seeds to radioactive carbon dioxide, but not the intact plant.

Although in the present investigation inactive damascenine was isolated from the ripe seeds, a highly radioactive oily, ether soluble, material of unknown constitution was obtained from the extract, showing that other biosynthetic processes were taking place during the chosen period.

Taken at face value, the results indicate that known precursors of the pyridine ring are not incorporated into damascenine and the question of its origin is still unanswered. It is conceivable that damascenine may be built up from acetate units, which may be incorporated into the alkaloid by a route that differs from the route of

acetate incorporation into the pyridine ring. This possibility should be tested by feeding acetate-1-C^{14} and acetate-2-C^{14} to N. damascena.

Since all experiments with this plant have so far proved negative, a thorough study of the timing of damascenine synthesis would appear to be required before incorporation of further possible precursors is investigated.

EXPERIMENTAL

SYNTHESIS OF DAMASCENINE

(1) Preparation of 8-methoxyquinoline methiodide

8-Hydroxyquinoline (25 g) was dissolved in methanol (300 ml) which contained potassium hydroxide (11.7 g). Methyl iodide (40 ml) was added and the mixture was heated under reflux for 12 hours. Initially, the mixture turned very dark but during the reflux period the solution became lighter and at the end of 12 hours it was a very pale orange-yellow. The solution was concentrated *in vacuo* to about 150 ml, and cooled overnight, yielding the product as a yellow solid. A second crop of crystals was obtained by further concentration of the mother liquor. The product was twice recrystallized from ethyl alcohol yielding yellow crystals of 8-methoxyquinoline methiodide (39 g, 75%), melting at 147-151° (Claus & Dewitz, 1890).

(2) Preparation of 8-methoxyquinoline methochloride from the methiodide

The methiodide (5 g) was dissolved in distilled water (100 ml) and an excess of freshly precipitated silver chloride (4 g) was added. The mixture was shaken on the mechanical shaker for about 1 hour. The yellow precipitate of silver iodide was filtered off, more freshly precipitated silver chloride was added, and shaking continued overnight. The mixture was then filtered and the solution concentrated *in vacuo* until approximately 1 ml of liquid remained. On standing

crystals separated which were filtered off and the filtrate concentrated to dryness. The solids were digested with ethanol and an insoluble white inorganic salt was filtered off. The ethanolic solutions containing the product were combined and concentrated. Since the methochloride did not crystallize from this solution a little ether was added and the solution cooled to 5° when crystals of the product separated. These were recrystallized by dissolving in ethanol and adding ether until a cloudiness appeared. On cooling, 8-methoxyquinoline methochloride separated as a deep yellow solid (2.9 g, 83%), melting at 102-104° (lit. 100° (Bedell & Fischer, 1881)).

The product was also purified on a cation exchange column (Dowex 1-X8). The salt was applied in aqueous solution, the column washed with water and the product eluted with 5N HCl. The movement of the product down the column was followed with an ultraviolet lamp. The yellow solution was evaporated to dryness and the product re-crystallized as above.

(3) Oxidation of 8-methoxyquinoline methochloride (cf. Kaufmann & Rothlin (1916))

(a) Oxidation of 8-methoxyquinoline methochloride to 1, 2 -dihydro-1-methyl-8-methoxy-2-oxoquinoline

8-Methoxyquinoline methochloride (1 g) and potassium ferricyanide (6 g) were dissolved in water (100 ml). Ether was added, and a solution of potassium hydroxide (10%) was added in small portions, with shaking after each addition until the mixture was strongly alkaline. The mixture was shaken for a further 10 minutes, when the ether layer was separated, dried over anhydrous K_2CO_3 and evaporated to dryness, yielding the product as a yellow crystalline solid which

was insoluble in water but soluble in ethanol. A small amount of the product was recrystallized from 30% ethanol but was still pale yellow. The product was distilled (1.5×10^{-3} mm, 80°) and a white crystalline sample of 1, 2-dihydro-1-methyl-8-methoxy-2-oxoquinoline (71 mg, 79%) was obtained melting at 74-75° (lit. 70° (Fischer & Chur, 1916)).

Ultraviolet absorption (λ_{max}, mμ)

in 0.1N HCl: 337, 288, 260, 238

in 0.1N NaOH: 337, 288, 261, 237.

(b) Attempted oxidation of 1, 2-dihydro-1-methyl-8-methoxy-2-oxoquinoline

(i) in acetone at room temperature

1, 2-Dihydro-1-methyl-8-methoxy-2-oxoquinoline (500 mg) was dissolved in acetone (20 ml) and a small amount of powdered potassium permanganate was added. At 0° (ice-salt bath) the purple color of the permanganate did not disappear indicating that oxidation was not taking place at this temperature. At room temperature, the permanganate color disappeared and more potassium permanganate was added in small portions to the stirred solution. After permanganate (1 g) had been added (2 days), the manganese dioxide was filtered off and washed with small portions of acetone. The acetone solution was evaporated. The residue was unchanged starting material.

(ii) in boiling acetone

A solution of the carbostyril (160 mg) and potassium permanganate (275 mg) in acetone was heated under reflux at 60°. After the purple permanganate color had disappeared, the manganese dioxide was removed by filtration and the acetone solution evaporated to dryness, when

starting material was recovered as a white crystalline solid.

(iii) in water

The carbostyril (700 mg) was dissolved in a small amount of acetone and the solution diluted with distilled water. The pH was adjusted to pH 10 with 5% NaOH. A solution of potassium permanganate (1.25 g) was added slowly at room temperature to the stirred mixture. The permanganate color disappeared very quickly and the entire permanganate solution was added in 3 hours.

Manganese dioxide was removed by filtration, the acetone evaporated, and the residual aqueous alkaline solution extracted with ether. The ether extract was evaporated leaving a white crystalline residue of starting material, melting at 65°. The aqueous solution was acidified and extracted with ether. The extract was dried (Na_2SO_4) and evaporated to dryness, giving a small amount of residue which was also unchanged starting material.

In each case identity of the product with unchanged starting material was demonstrated. The products were nitrogenous (sodium fusion), nonbasic and nonacidic (ether extraction from acid and alkaline solution), and stable to acid hydrolysis and high vacuum distillation (2×10^{-3} mm, 85°). The ultraviolet absorption spectra of the purified materials were identical with those of 8-methoxy-1-methyl-2-quinalone.

(c) Direct oxidation of 8-methoxyquinoline methochloride to N-formyldamascenic acid (cf. Kaufmann & Rothlin, 1916)

A solution of 8-methoxyquinoline methochloride (2 g) in water (125 ml) was kept at 0° in an ice-salt bath. The solution was contin-

uously stirred by a mechanical stirrer. A 4% solution of potassium permanganate (5 g in 125 ml) was added very slowly over a period of 8 days. Manganese dioxide was separated by centrifugation and the clear solution then filtered and concentrated under vacuum to approximately 15 ml. The solution was then acidified by addition of at least 1 ml of concentrated HCl. A white precipitate formed gradually and precipitation appeared to be complete after 5 minutes. Filtration gave N-formyldamascenic acid (1.0 g, 54%) melting at 194-195° (lit. 194-195° (Kaufmann & Rothlin, 1916)). Ultraviolet absorption (λ_{max}, mμ (log ϵ)):

in 0.1N HCl: 298, (3.53)

in 0.1N NaOH: 280, (3.36).

(4) Hydrolysis and esterification of N-formyldamascenic acid to damascenine

N-Formyldamascenic acid (75 mg) was dissolved in methanolic HCl (20 ml, 5%) and heated under reflux for 20 hours. The mixture was evaporated to dryness, the residue was dissolved in cold 1N NaOH (10 ml) and the ester (damascenine) was immediately extracted into ether. The ethereal solution was dried (Na_2SO_4), the ether was removed under vacuum, and the oily residue distilled (2×10^{-3} mm, 110°). Chromatographically pure damascenine (60 mg, 82%) was obtained as an oil, which on cooling solidified to a solid, melting at 23-24°. (lit. 24-25° (Kaufmann & Rothlin, 1916)). Ultraviolet absorption (λ_{max}, mμ (log ϵ)):

in 0.1N HCl: 298, (3.50)

in 0.1N NaOH: 336.

(5) Hydrolysis of N-formyldamascenic acid to damascenic acid

N-Formyldamascenic acid (600 mg) was hydrolyzed by heating under reflux for 3 hours with 20% NaOH (25 ml). The pH was adjusted to pH 4.7 (the isoelectric point as found by titrating a solution of damascenic acid at pH 7 with 0.1N HCl using an automatic titrator) and the solution extracted with ether, yielding a strongly fluorescent ether extract. The ether extract was dried (Na_2SO_4) and evaporated to dryness, yielding a yellow solid (300 mg, 52%) whose ultraviolet absorption spectrum corresponded to that of damascenic acid.

Ultraviolet absorption (λ_{max}, $m\mu$ (log ϵ)):

in 0.1N HCl: 298

in 0.1N NaOH: 310, (3.35).

High vacuum distillation (1×10^{-3} mm, 100-110°) yielded damascenic acid (250 mg, 44%) melting at 137-138° (lit. 78°, anhyd. 140-141°, (Pommerehne, 1900)) with pK values 7.3 and 2.55.

When larger quantities of the N-formyldamascenic acid were used, the final ether extraction was carried out on a liquid-liquid extractor. Extraction was continued until no more fluorescent material entered the ether layer.

EXTRACTION OF DAMASCENINE FROM SEEDS OF NIGELLA DAMASCENA L.

A number of methods for the extraction of the alkaloid from the seeds were tried. In the successful method, an ultraviolet absorption curve of the extract indicated that it contained substantially pure damascenine, uncontaminated by other materials absorbing in the ultra-

violet region of the spectrum.

The seeds (100 mg) were finely ground and extracted with ether (200 ml) for three days on a Soxhlet extractor. The ether solution was then evaporated to dryness on a rotary evaporator and the residue was dissolved in 1N HCl (10 ml) and then extracted with ether to remove acidic substances. The aqueous layer was made alkaline with 15M ammonia (1 ml) and the alkaloid was extracted into ether. The fluorescent ether solution was dried for several hours with Na_2SO_4 and the ether removed *in vacuo*. The residue was dissolved in 95% ethanol and diluted to 10 ml in a volumetric flask and the concentration of damascenine determined from the intensity of the ultraviolet absorption band at 298 mμ in 0.1N HCl. The alcohol was removed and the alkaloid was purified by distillation (2×10^{-3} mm, 110°). The product was pure and suitable for degradation, after dilution with synthetic damascenine.

Other attempted extraction procedures

The seeds were ground with 95% ethanol and the extractdiluted to a known volume in a volumetric flask. Other solvents which were tried were chloroform containing a little ammonia, and petroleum ether (Ewins, 1912). Even though the extracts were fluorescent under an ultraviolet lamp (Keller, 1908) showing the presence of damascenine, the ultraviolet absorption curves of the solutions did not give a definite damascenine peak at 298 mμ and there was some absorption due to other plant materials.

Location of damascenine in the plant

In order to confirm the results of other workers that damascenine

was found mainly in the ripe seeds (Tyler, 1955; Vishen et al, 1960), ultraviolet absorption curves of extracts of various parts of Nigella were taken. The seed pods, stalks, green seeds, ripe seeds, and cotyledons were ground and extracted with ethanol. Each alcoholic solution was diluted to 50 ml in a volumetric flask. Only the extract from the ripe seeds showed damascenine absorption, confirming that damascenine is found in the ripe seeds, and that little, if any, is present in other parts of the plant.

DEGRADATION OF DAMASCENINE

(1) Hydrolysis of damascenine to damascenic acid

Damascenine (40 mg) was hydrolyzed with 5% NaOH (10 ml) by heating under reflux for 10 hours. The pH of the solution was adjusted to 4.8 with HCl and the solution was extracted with ether. The ether extract was dried overnight with Na_2SO_4 and evaporated to dryness. The product, damascenic acid, (30 mg, 79%) was identified by means of its ultraviolet absorption and by ascending paper chromatography on Whatman No. 1 paper, buffered at pH 8 with phosphate buffer, using methanol as the developing solvent. The following R_f values were obtained: damascenine, 0.88; damascenic acid, 0.67. Butanol saturated with water and 50% aqueous methanol gave poorer separation of damascenine and damascenic acid.

(2) N-Acetyldamascenic acid

This was prepared by a procedure similar to that of Keller (1904). An alcoholic solution of damascenic acid (10 mg in 1 ml) was taken to dryness in vacuo. Acetic anhydride (5 ml) was added to the residue and the mixture heated under reflux for 1.5 hours. The solution was

concentrated *in vacuo* yielding a dark oily residue. This oily product was dissolved in hot water (3 ml), the solution was transferred to another small round-bottom flask to separate the supernatant from the insoluble residue, and again taken to dryness *in vacuo* (water pump), yielding an impure crystalline product. This was dissolved in a minimum amount of hot water (2 ml), the solution transferred to a 10 ml beaker and concentrated to a few drops on a hot plate. On cooling and scratching white crystals of N-acetyldamascenic acid (7 mg, 61%) appeared. When recrystallized from a small volume of water, the compound melted at 201-203° (lit. 203-204° (Keller, 1904)).

(3) Bromodecarboxylation of damascenic acid (cf. Keller, 1904)

(a) trapping of carbon dioxide

An alcoholic solution of damascenic acid (20 mg in 2 ml) was placed in a small (50 ml) three-necked flask. The centre neck of the flask was connected to two small gas traps in series containing saturated barium hydroxide. The purpose of the first trap was to absorb the carbon dioxide given off from the sample; the purpose of the second was to absorb any carbon dioxide that might enter from the air. A small dropping funnel containing 2 ml of a 2% ($^{v}/_{v}$) bromine in ethanol solution was fitted into the flask. A stream of nitrogen was passed by means of a capillary tube into the damascenic acid solution. The bromine solution was then added, drop by drop, until the bromine color no longer disappeared. The reaction was carried out at room temperature. Nitrogen was allowed to pass through the solution for 1 hour after the last addition of bromine. The liberated carbon dioxide was swept into the first trap where it was converted to

barium carbonate (17.5 mg, 81%) which was quickly filtered, washed with distilled water, dried, and weighed.

(b) Isolation of 2-bromo-6-methoxy-N-methylaniline hydrobromide

The solution remaining in the three-necked flask after removal of carbon dioxide was transferred to a 30 ml beaker and the ethanol removed. When the solution was concentrated to a drop or two, scratching produced a brown solid. This was separated from the remaining mother liquors, dried, and purified by washing with ether. A brown impurity entered the ether layer leaving a colorless sample of the bromo-hydrobromide (16 mg, 50%) melting at 202-203° after several ether washings (lit. 206-208° (Keller, 1904)).

(4) Picrate of 2-bromo-6-methoxy-N-methylaniline

The bromo-hydrobromide (10 mg) was dissolved in a minimum amount of distilled water and a saturated aqueous picric acid solution (0.5 ml) was added. The mixture became cloudy and a precipitate formed after a few seconds, yielding the picrate (11 mg, 73%) melting at 145° after recrystallization from water. (Found: C, 37.77; H, 3.04. $C_8H_{10}BrNO,C_6H_3N_3O_7$ requires C, 37.77; H, 2.94%).

(5) Attempted decarboxylation of damascenic acid

(a) Damascenic acid (10 mg) was dissolved in 0.1N NaOH (1 ml). The mixture was taken to dryness and high vacuum distillation (3×10^{-3} mm) of the residue attempted. Below 175° no distilate was obtained. At 300° (Wood's metal bath) a small amount of brown material distilled whose ultraviolet absorption spectra in 0.1N acid and in 0.1N base corresponded to those of an authentic sample of the expected

product, N-methyl-o-anisidine. Ultraviolet absorption spectra (λ_{max}, mμ(logϵ)) in ethanol: in 0.1N HCl: 270, (3.41).

(b) Damascenic acid (25 mg) was added to glycerol (10 ml) in a small two-necked flask. One neck of the flask was attached to a line consisting of two gas traps in series containing saturated barium hydroxide. Purified nitrogen was passed through the other neck by a capillary tube and bubbled gently through the mixture. The mixture was heated to 250° (Wood's metal bath) and decarboxylation took place. Barium carbonate corresponding to 75% of the theoretical yield was obtained. By a control experiment it was established, however, that part of the liberated carbon dioxide arose by decomposition of the glycerol itself.

To obtain N-methyl-o-anisidine two methods were tried.

(i) The glycerol solution was extracted with ether. Fluorescence in the ether layer indicated that some damascenic acid which had not undergone decarboxylation was extracted in this way. Since in an experiment with radioactive damascenine, complete separation of damascenic acid and N-methyl-o-anisidine would have been imperative, this method was unsatisfactory.

(ii) The cool glycerol solution was diluted with an equal volume of water and this solution was extracted several times with a total volume of about 50 ml of benzene. The benzene layer was in turn extracted with water to remove any glycerol which may have been extracted into the benzene. The benzene layer was concentrated to 1 ml, 3 drops of HBr (48%) were added, and the benzene layer was completely

evaporated on a water bath. The residue, containing N-methyl-o-anisidine hydrobromide now became very dark and viscous. It was cooled and on addition of acetone (2 ml), a small amount of product was obtained. Ultraviolet absorption spectra of the aqueous glycerol solution, the final product, and the acetone solution from which this product had been obtained showed (a) that N-methyl-o-anisidine had been quantitatively extracted into benzene, whereas undecarboxylated damascenic acid remained in the glycerol solution and (b) that most of the N-methyl-o-anisidine did not precipitate as the hydrobromide, since the acetone solution showed stronger absorption due to the product than was found in the solution of the hydrobromide derivative.

To obtain adequate amounts of N-methyl-o-anisidine for further degradation, the compound was synthesized and experiments were carried out with synthetic material.

(6) Synthesis and degradation of N-methyl-o-anisidine

(a) Preparation of N-formyl-o-anisidine

o-Anisidine (o-methoxyaniline) (2 g) was added to formic acid (15 ml) and the mixture was heated under reflux for 5 hours. Ultraviolet absorption spectra in 0.1N acid and 0.1N base indicated that the starting material was no longer present and acylation had taken place. Ultraviolet absorption (λ max, mμ) for starting material, o-anisidine:

in 0.1N HCl: 278, 270

in 0.1N NaOH: 284,235

ultraviolet absorption (λ max, mμ) after refluxing:

in 0.1N HCl: 282, 244

in 0.1N NaOH: 282, 244.

The solution was neutralized with 1N NaOH, extracted with benzene, evaporated to dryness, and the product recrystallized from water using decolorizing charcoal. After two recrystallizations, white needles of N-formyl-o-anisidine were obtained (2.1 g, 85%) which melted at 83-83.5° (lit. 83.5° (Diepolder, 1899)). Much larger amounts of starting material gave equally high yields of product.

(b) Reduction of N-formyl-o-anisidine to N-methyl-o-anisidine

Lithium aluminum hydride (4 g) was suspended in anhydrous ether (150 ml). The activity was measured by taking 0.5 ml of the solution and, by the use of an inverted graduated cylinder, measuring the amount of water displaced by the hydrogen (in this particular experiment, 0.5 ml of lithium aluminum hydride displaced 70 ml water).

An ether solution of lithium aluminum hydride (20 ml) was placed in a three-necked flask of 500 ml capacity fitted with a condenser. N-Formyl-o-anisidine (1 g) in anhydrous ether was added slowly from a dropping funnel. After complete addition, the mixture was heated under reflux for 2 hours.

The lithium aluminum hydride was destroyed by pouring the entire mixture slowly into 0.05N HCl (1 liter). The acidic solution was extracted several times with ether. The extracts were dried (Na_2SO_4) and concentrated, yielding a small amount of starting material. The acidic solution was warmed to remove any dissolved ether and was made basic with 2N NaOH. At pH 9-10, a gelatinous

white precipitate ($Al(OH)_3$ or LiOH) appeared which was removed by filtration. The basic solution was extracted with ether, the extracts dried (Na_2SO_4), and evaporated to dryness. A brown oil was obtained which crystallized on cooling, giving white needles of N-methyl-o-anisidine (1.2 g, 65%) melting at 32-33° (lit. 33-33.5 (Diepolder, 1899)). When larger amounts of the N-formyl compound were reduced similar yields were obtained, but the compound was added to the lithium aluminum hydride suspension from the thimble of a Soxhlet extractor, in order to avoid using a large volume of ether.

To prepare N-methyl-o-anisidine hydrobromide, the base (20 mg) was dissolved in benzene (0.5 ml) and 48% HBr (3 ml) was added. Two distinct layers resulted. The acid layer turned very dark. The benzene layer was completely removed by evaporation on a steam bath. The remaining dark acidic solution was cooled, when it became viscous, and white crystals were obtained upon scratching the sides of the beaker. Recrystallization from acetone yielded colorless crystals (17.5 mg, 52%) of the hydrobromide, melting at 118°.

To prepare N-methyl-o-anisidine picrate, the base (20 mg) was dissolved in ether (1 ml) and to this was added a saturated solution of picric acid in ether (0.5 ml). Immediately a precipitate formed, which was filtered and dried. The picrate (45 mg, 87%) melted at 140-141° (lit. 139° (Wedekind, 1906)).

(c) Hydrogenation of N-methyl-o-anisidine to N-methyl-2-methoxy-cyclohexylamine

Equal weights of N-methyl-o-anisidine and platinum dioxide catalyst (91.2 mg) were suspended in glacial HAc (5 ml) and diluted to

60 ml. After hydrogenating at 40 p.s.i.g. for 20 hours, a small sample was removed and an ultraviolet spectrum taken in 0.1N HCl. The sample did not absorb, indicating that the starting material had been hydrogenated. The catalyst was filtered off and the solution was evaporated to dryness. A viscous oily liquid was obtained, which was dissolved in ether, washed with NaOH (1N) and evaporated to dryness to give the product as an oil which was further purified by distillation (2×10^{-3} mm, 40-50°) *in vacuo* using a receiver cooled in dry ice-acetone. N-Methyl-2-methoxycyclohexylamine was collected as a colorless oil.

Hydrogenation with platinum oxide in methanol yielded unchanged starting material.

Attempts were made to prepare a solid derivative of N-methyl -2-methoxycyclohexylamine. The sulfate and perchlorate were oils, and although the hydrochloride did form as a solid melting at 100-102°, it was extremely hygroscopic. The picrate could not be obtained from aqueous or dilute ethanolic solution, but was prepared in the following manner: a few drops of N-methyl-2-methoxycyclohexylamine were dissolved in a minimum amount of ethanol and to this was added a saturated solution of picric acid in ethanol (0.5 ml). The ethanol was evaporated to two or three drops and ether (1 ml) was added. A very fine, bright yellow, oily precipitate settled out, which softened at 150°, turned black about 250° and decomposed completely at 330-335°. The product was not analyzed.

(d) *Exhaustive methylation of N-methyl-2-methoxycyclohexylamine*

N-Methyl-2-methoxycyclohexylamine (200 mg) was dissolved in

excess benzene (20 ml) and methyl iodide (2.5 ml) and 10% NaOH (5 ml) were added. This mixture was shaken for 24 hours on a mechanical shaker. Distilled water (10 ml) was then added and the benzene and basic layers were separated. To the aqueous solution was added 20% NaOH (5 ml) and the solution was heated under reflux for 4 hours. No basic vapours were liberated during this treatment.

The benzene layer, containing excess methyl iodide and any methoxycyclohexene which might have formed by elimination, was distilled using an efficient distilling head. The methyl iodide and benzene distilled off and a small amount of yellow solid residue remained which was not methoxycyclohexene since this compound is a liquid.

The aqueous layer was concentrated and an oil separated from the residual basic solution which, on cooling, solidified into a hard mass. The alkaline solution was decanted leaving N-trimethyl -2-methoxycyclohexylammonium iodide, melting at 215-218° (Found: C, 40.08; H, 7.41; I, 42.41; N, 4.68. $C_{10}H_{22}INO$ requires C, 40.14; H, 7.51; I, 42.21; N, 4.55%).

(e) Synthesis of N-trimethyl-2-methoxycyclohexylammonium bromide from cyclohexene

1-Bromo-2-methoxycyclohexane (cf. Winstein & Henderson, 1943)

N-Bromosuccinimide (47.2 g, 0.4 mole) was dissolved in cold anhydrous methanol (100 ml, 4 moles) and two drops of concentrated H_2SO_4 were added. When cyclohexene (25.3 ml, 0.4 moles) was added to the stirred solution a rather vigorous reaction ensued which was controlled by the use of an ice-salt bath for the first 15 minutes.

Stirring was continued 0.5 hours longer and the reaction mixture was left overnight. The solution was poured into aqueous sodium chloride (100 ml) and the organic layer was separated and washed with water. The aqueous layer was extracted with ether and this extract was partially dried over K_2CO_3. The ether was removed in vacuo. The residue was diluted with water and the organic layer was separated by extraction into ether. This ether solution was combined with the original organic layer and dried over K_2CO_3. The ether was removed under vacuum. A very pale yellow liquid remained which contained covalent bromine, did not decolorize bromine water and was the desired 1-bromo-2-methoxycyclohexane n_D^{25}, 1.4908 (lit. n_D^{25}, 1.4900; boiling point 75.0 -75.5° (10 mm) (Winstein & Henderson, 1943)).

N-Trimethyl-2-methoxycyclohexylammonium bromide

1-Bromo-2-methoxycyclohexane (1 ml) was dissolved in methanol and excess methanolic trimethylamine (30%) was added. The solution was kept in the incubator at 45° for 24 hours. The solvent was evaporated leaving a brown solid which was dissolved in water, the solution extracted with ether, and the aqueous layer filtered twice through decolorizing charcoal. The solution was evaporated to dryness and an oily white solid was obtained whose infrared absorption spectrum was very similar to that of the quaternary iodide obtained from N-methyl-o-anisidine (vide supra). Infrared absorption (Nujol) (cm^{-1}): quaternary bromide, 1562-1613 (shoulder), 1250, 1200, 1075 -1085 (shoulder), 983, 962, 943, 885, 847; quaternary iodide, 1560-1613 (shoulder), 1250, 1202, 1075-1087 (shoulder), 1055, 962, 945, 884, 850.

(f) Hofmann degradation of N-trimethyl-2-methoxycyclohexylammonium bromide and iodide

Sodium sulfate-dried tertiary butyl alcohol (15 ml) was placed in a two-necked flask fitted with a condenser holding a drying tube. Potassium metal was added until no more would dissolve. A drop of dry benzene was then added to ensure that the condenser would not be clogged by the tertiary butoxide. The quaternary bromide, dissolved in a small amount of tertiary butyl alcohol, was added and the mixture heated under reflux for 2 hours, when basic vapours were no longer given off. Some solid material, probably potassium bromide, settled out during the refluxing period. The solution was diluted with water and extracted with ether. The extract was dried, the ether evaporated, and a pale yellow oil was obtained which decolorized bromine water. The infrared absorption spectrum indicated that the product was contaminated with tertiary butyl alcohol since a strong -OH band was observed ($3420\ cm^{-1}$).

The same result was obtained when quaternary iodide was used in place of the quaternary bromide. In each case the product rapidly decolorized bromine water and was presumably either 1-methoxy-1-cyclohexene, or 1-methoxy-2-cyclohexene, or a mixture of the two, together with a small amount of tertiary butyl alcohol.

Attempted Hofmann elimination of the quaternary bromide or iodide using 20% alcoholic KOH proceeded slowly and gave a much poorer yield of product.

CULTIVATION OF NIGELLA DAMASCENA L. AND ADMINISTRATION OF RADIOACTIVE TRACER

Seeds of Nigella damascena L. were soaked in water overnight

and allowed to germinate in earth in the greenhouse. Approximately 50 seeds were planted in each pot but, after germination, the young shoots were thinned out so that about three or four plants were allowed to mature in each pot of soil. After sixteen to eighteen weeks the plants began to flower. At the end of the flowering stage, i.e. when the seed pods were just beginning to form, aspartic acid-4-C^{14} and succinic acid-1, 4-C^{14} were fed using a "wick" method. Doubled threads of No. 10 mercerized cotton were passed with a needle through the stem about two inches above soil level and then cut so that 3 inch lengths of four threads protruded on the same side of the stem (Fig. 15). The ends were placed in a small glass thimble (capacity 1.5 ml). The thimble was then filled with the radioactive solution which was supplied in two portions - one week apart for aspartic acid-4-C^{14} and five days apart for succinic acid-1, 4-C^{14}. When all of the solution had been absorbed by the plant (3 hours), the holder was filled with water several times to ensure that all of the activity had been absorbed by the plant. The plants were allowed to grow until the seeds had formed and ripened, at which time they were harvested.

The first batch of seeds from aspartic acid-fed plants was harvested 21 days after feeding. Growing was continued until unripened pods had matured, and a second drop of seeds was harvested 45 days after feeding. The seeds from the plants fed with succinic acid were also harvested in two groups; one, 10 days after feeding and the other, 34 days after feeding (total feeding times are calculated from the date of the last addition of precursor). Ten plants were fed with

DL-aspartic acid-4-C^{14} and each plant received 10 μc of the radioactive solution. The same number of plants were fed with succinic acid-1, 4-C^{14} and each received 10 μc of the tracer solution. At the conclusion of the experiment, the wicks themselves were extracted with ethanol and a known volume of the solution was counted to ascertain what portion, if any, of the activity had not entered the plant. Activity corresponding to 3% of the original aspartic acid solution had not been absorbed by the plant and only 0.5% of the original succinic acid solution had not been taken up. The data of the two feeding experiments are given in Table 2.

ISOLATION OF DAMASCENINE FROM THE SEEDS OBTAINED FROM TRACER-FED PLANTS

The 4 crops of seeds (see Table 2) were extracted separately by the procedure described under Section II, page 53. The extracts were chromatographed along with standard damascenine and fluorescent spots corresponding to the R_f of damascenine were obtained. These were eluted with ethanol, counted, and in all cases the damascenine was found to be inactive. Since the original extract was active, all of the activity must be in a nonbasic fraction.

The sample to be counted was placed on an aluminum planchette, 2 drops of 1% sucrose were added (2 drops of a 1% solution of collodion in dimethylformamide was used for the radioactive oily material), the solution covered with a circle of lens tissue, dried, and counted on a Beckman/Berkeley Electronic Counter (Model 7060) using a Nuclear Chicago low background automatic sample changer (Model C 115).

Compound Fed	Weight (mg)	Specific Activity counts/min/mM	Total Activity counts/min	Number of Plants Fed	Feeding Time (days) 1st harvest	2nd harvest
Aspartic acid-4-C^{14}	12.66	3.3×10^8	3.12×10^7	10	21 (3.2383 g)[1]	45 (1.1221 g)[1]
Succinic acid-1, 4-C^{14}	2.36	11.0×10^8	2.17×10^7	10	10 (1.9504 g)[1]	34 (1.1690 g)[1]

[1]Weight of seeds when the plants were harvested.

Table 2

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