EXPERIMENTS ON THE BIOGENESIS OF THE PYRIDINE RING IN HIGHER PLANTS.1,2

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That tryptophan may be a precursor of nicotinic acid in rats and Neurospora was shown in the former by feeding experiments with nicotine-deficient animals (13) and in the latter by studies with mutants requiring 3-hydroxyanthranilic acid (1), a metabolite known to arise from tryptophan. The mammalian experiments have been substantiated with isotopic experiments (8, 7) where tryptophan-3-C14 and 3-hydroxyanthranilic-7-C14 acid have resulted in the excretion of \( \text{N-methylpicolinamide-7-C}'4 \). Equivalent experiments with higher plants do not provide similar results. For example, in excised peas, where a steady state of nicotinic acid exists, so that precursors of nicotinic acid accumulate as trigonelline (\( \text{N-methylnicotinic acid} \)), no accumulation of the latter occurred when the plants were fed ornithine, tryptophan, citrulline or pyridoxine (22). More directly, the trigonelline from garden peas fed tryptophan-3-C14 was inactive (11). We have also synthesized 3-hydroxyanthranilic acid-7-C14 and fed them to excised soybean leaves. The lack of conversion of this compound to trigonelline is reported here.

Grafting experiments by Dawson (2) have shown that nicotine \( \left[ \beta-(\alpha-\text{N-methylpyrrolidyl})-\text{pyridine} \right] \) is synthesized solely by the roots of the tobacco plant, while its isomer, abasabine \( \left[ \beta-(\alpha-\text{piperidyl})-\text{pyridine} \right] \) is synthesized both in roots and leaves. Independent studies (3, 9) have shown that the pyrrolidine ring of nicotine may be synthesized from ornithine via a symmetrical precursor (e.g., putrescine or pyrrolidine), which, however, contributes no activity to the pyridine portion of the ring. The conversion of lysine to pypecolic acid occurs in both plants and animals (6, 12, 18). However lysine is not converted to the pyridine portion of nicotine (10) nor, as will be shown below, is it utilized for anabasine biogenesis. A similar situation exists with regard to hydroxyllysine. Anabasine made by photosynthesis with \( \text{C}^{14} \text{O}_2 \) is uniformly labeled.

METHODS

Excised leaves of Nicotiana glauca were used for experiments with abasabine. Petioles of the leaves were placed in small test tubes containing the solution being assimilated. An average leaf of this plant transpires approximately 2 ml/hr. Since this requires five to six replenishments of the available volume of solution in the tube, essentially quantitative uptake is assured. In \( \text{C}^{14} \text{O}_2 \) assimilation, the leaves were permitted to photosynthesize until \( > 95 \% \) of the atmospheric radioactivity was utilized (about 3 hrs). Following both types of feeding the excised leaves were then permitted to sit in the laboratory in tap water for 48 hours, following which they were extracted with 80% aqueous ethanol, lipoidal pigments extracted with petroleum ether, the solution made alkaline (pH about 10 to 11) and the alkaloids extracted with diethyl ether. Abasabine was obtained chromatographically (20) (racemization occurs, as well as partial separation of the racemates) and purified by rechromatography. Part of the abasabine was characterized by conversion to its dipicrate, the remainder oxidized to nicotinic acid with permanganate (17). The nicotinic acid was purified by sublimation and a portion decarboxylated (450 °C) to \( \text{CO}_2 \) and pyridine (3), the latter being caught in a solution of picric acid. The \( \text{CO}_2 \) is about 20% contaminated with \( \text{CO}_2 \) from the partial degradation of pyridine.

Hawkeye soybeans, grown in a greenhouse under conditions of vegetative photoperiod were found to be a satisfactory source of trigonelline. The concentrated 80% aqueous ethanol leaf extract was ion-exchanged on IR-120. The 2 N \( \text{NH}_4\text{OH} \) eluate, combined with a subsequent water wash, was discarded; trigonelline was then removed with 5% diethylamine in water and run descendingly in Munier's (14) solvent, until the nicotinic acid (\( R_F = 0.08 \)) was almost to the edge, the trigonelline (\( R_F = 0.02 \)) then being about one-fourth of the way down. The trigonelline area (made visible with Dragendorff's reagent) was eluted with hot water, followed by ethanol, and repeated with hot water. The entire extract was banded on a strip for paper electrophoresis, along with trigonelline and 3-hydroxyanthranilic acid reference spots. When run at pH 8.6 (0.1 M citrate buffer) and 800 v (about 0.5 ma), the trigonelline moves cathodically about 1.5 cm, while the 3-hydroxyanthranilic acid moves anodically about 5.0 cm.

The synthesis of 3-hydroxybenzoic-7-C14 acid was accomplished by known procedures: benzoic acid \( \rightarrow \) sodium m-sulfobenzoic acid (16, 19) \( \rightarrow \) m-hydroxybenzoic acid \( \rightarrow \) m-methoxybenzoic acid (5) \( \rightarrow \) 2-nitro-3-methoxybenzoic acid (15) \( \rightarrow \) 2-amino-3-hydroxybenzoic acid. The final yield was 550 mg 3-hydroxyanthranilic acid-7-C14 acid from an initial 5 gm benzoic-7-C14 containing 1 mc C14. The last step is primarily responsible for the poor yield.

The 3-hydroxyanthranilic acid (3-HAA) is difficulty soluble in water. It oxidizes slowly in neutral solutions, and rapidly in alkalii. Three different solutions were used to feed excised leaves: 1) One mg 3-HAA suspended in 5 ml H2O. To 0.5 ml water were added one drop cone NH4OH and several drops ethanol. The solution was concentrated in vacuo to 0.14 ml, of which 0.13 was fed to the excised leaf. A

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2 The author gratefully acknowledges the partial support of this program by the National Science Foundation.
similar experiment with 4 mg of 3-HAA in 0.5 ml H₂O plus 2 drops NH₄OH resulted in a brownish solution which was still fluorescent in UV. The absorption of this solution by four leaves, resulted in no obvious ill effects to the leaves after 3.5 days in tap water in the greenhouse. 2) A solution of 4 mg 3-HAA required about 3 ml of 0.1 M pH 5.0 phosphate buffer. Uptake by the leaf was not too satisfactory. 3) Similarly, a solution of 4 mg of 3-HAA required about 3 ml of 0.1 M citrate buffer, pH 4.35. Although there was no browning, the large amount of citrate appeared somewhat deleterious to the plant, since proper turgor was never reestablished despite occasional petiole cutting to unplug xylem vessels.

Results and Discussion

The uniformity of distribution of radioactivity in abasine is shown in Table I, assuming biogenetic equivalence of the carbons in each of the rings. With lysine-2-C¹⁴ (obtained from Tracerlab, Boston Massachusetts) and an initial activity in the 80% ethanol extract of 2.1 x 10⁶ cpm, the radioactivity in abasine, though positive, was so small as to be neglected.

Hydroxylasine-6-C¹⁴ (0.4 μc) resulted in an initially very low, but active abasine dipicrate which successive recrystallizations showed to be active primarily as a result of contamination: initial activity, 2.16 cpm/mg; 1st recrystallization, 0.84 cpm/mg; 2nd recrystallization 0.28 cpm/mg. On the other hand, glucose-U-C¹⁴ (prepared photosynthetically), resulted in respective activities of 7.59, 6.71, and 6.44 cpm/mg, in a total yield of about 0.5%, based upon the activity of the 80% ethanol extract. The degradation of the abasine from glucose-U-C¹⁴ is in progress.

Radiotrigonelline could not be found either from C¹⁴O₂ photosynthesis or from administration of 3-HAA-7-C¹⁴.

The experiments with 3-HAA are not entirely satisfactory. Certainly a specific activity greater than 0.2 μc/mg would be desirable—although considering the uniqueness of the reaction, this should be ample. More perturbing is the question of oxidation of the compound to an unusable derivative. This can be answered only by stating that radioactive material, chromatographically identical with 3-HAA, could still be extracted from leaves fed 3-HAA within less than 24 hours after feeding. The possibility exists that the carboxyl group (and consequently the radioactivity) of 3-HAA is lost by exchange, or some other process, during transformation of 3-HAA to nicotine acid. However, in the light of the parallel mammalian experiments, this possibility must be rather remote. The experiments with C¹⁴O₂ are quite straightforward. In soybeans the ultimate precursors of trigonelline must be formed in the roots—unless excision alters the metabolism that the leaf is incapable of its biosynthesis. Direct experiments with tryptophan-3-C¹⁴ are in progress.

Considering the ease of conversion of lysine to piperolic acid, it is surprising that the former, or hydroxylasine, is not a precursor of the piperidyl ring. The small amounts of activity found in abasine when these substrates are fed may be real; however, they are obviously not direct since the yield is far below that obtained by photosynthesis or with the feeding of glucose-U-C¹⁴. Consequently the origin of both the piperidyl as well as the pyridyl ring of abasine is still unknown. The possibility of the transformation of γ-methylene glutamic acid (which is known to occur in higher plants [21, 4]) to the pyridyl ring is being considered. This is, in a sense, the reversal of the known alkaline degradation of nicotinic acid to 2-hydroxymethyl glutaric acid, which, on vacuum distillation, is converted to the corresponding methylene.

R. F. Dawson, et al (Jour. Amer. Chem. Soc. 78: 2645-2646. 1956) have shown that nicotinic acid, with tritium substituted in the ring, is incorporated without loss of specific activity into tobacco root nicotine. It is suggested that nicotinic acid may also serve as the precursor of the pyridine ring of abasine and other pyridine-containing compounds. While this experiment does not resolve the problem of the mechanisms of the formation of the pyridine ring per se, it suggests that when the origin of nicotinic acid in higher plants is known, we will simultaneously have delineated the biogenesis of many naturally-occurring pyridines.

Summary

1. Abasine [β-(α-piperidyl)-pyridine] formed during photosynthesis in C¹⁴O₂ is uniformly labeled.
2. Glucose-U-C¹⁴ may be used as an ultimate precursor for abasine.
3. Neither lysine-2-C¹⁴ or hydroxylasine-6-C¹⁴ are definitive precursors for abasine.
4. Radiotrigonelline (N-methyl-L-nicotinic acid) is not formed during photosynthesis of excised soybean leaves in C¹⁴O₂.
5. 3-Hydroxyanthranilic acid is not formed as a precursor of trigonelline in the soybean leaves.

The writer wishes to acknowledge the skillful assistance of Messrs. D. Maier, J. Wickiff, and J. Hanson in various portions of this study.

Table I

<table>
<thead>
<tr>
<th>Substance</th>
<th>Specific Activity CPM/MGc*</th>
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<tbody>
<tr>
<td>Anabasine (picroate)</td>
<td>99.2</td>
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<tr>
<td>Nicotinic acid</td>
<td>100.0</td>
</tr>
<tr>
<td>Pyridine (picroate)</td>
<td>97.5</td>
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* CPM/mgC = counts per minute per mg carbon of the pertinent portion of the molecule.
Standard error < 3%.

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LITERATURE CITED


THE INFLUENCE OF SALTS ON THE ACTIVITY OF PARTICULATE CYTOCHROME OXIDASE FROM ROOTS OF HIGHER PLANTS1 2

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Recent investigations by Webster (21) have shown that cytochrome oxidase is widely distributed in higher plant species and it has been suggested (7) that this enzyme may play a major role in the terminal transfer of electrons in plant respiration. Lundegårdh (13) reported that the absorption spectrum of bundles of roots from wheat and other cereals revealed the existence of a complete cytochrome oxidase which was similar to that observed in animal tissues and many microorganisms. He calculated that this system was responsible for 50 to 75% of the total aerobic respiration. Fritz and Bevers (6) observed

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that extracts from etiolated pea, wheat and barley seedlings contained cytochrome oxidase at all stages of development. When the amount of enzyme in the tissues was evaluated in terms of activity at an infinite concentration of cytochrome c, it was concluded that shoots or roots of peas, and shoots of either barley or wheat contained sufficient amount of the enzyme to account adequately for the respiration of these tissues. The roots of barley and wheat also contained the enzyme but the amounts extracted were not capable of mediating all the respiratory oxygen absorption. The results with wheat were in good agreement with those reported by Lundegårdh. It would appear that the importance of cytochrome oxidase in many higher plant species may approach that of this enzyme in the respiration of animals and aerobic microorganisms.

Several investigators have observed that salts...