EXPERIMENTS ON THE BIOGENESIS OF THE PYRIDE RING IN HIGHER PLANTS

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That tryptophan may be a precursor of nicotine 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 N-methyl nicotinamide-7-C14.

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 nicotine acid accumulate as trigonelline (N-methyl nicotinic 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 [β-(α-α-nitrobenzyl)-pyridine] is synthesized solely by the roots of the tobacco plant, while its isomer, abanabase [β-(α-piperidyl)-pyridine] is synthesized both in roots and leaves. Independent studies (3, 9) have shown that the pyridoline 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 pipelic 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 abanabase biosynthesis. A similar situation exists with regard to hydroxylysine. Anaabase made by photosynthesis with C14O2 is uniformly labeled.

METHODS

Excised leaves of Nicotiana glauca were used for experiments with abanabase. 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 C14O2 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, lipidal pigments extracted with petroleum ether, the solution made alkaline (pH about 10 to 11) and the alkaloids extracted with diethyl ether. Anaabase was obtained chromatographically (20) (racemization occurs, as well as partial separation of the racemates) and purified by rechromatography. Part of the anaabase 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 CO2 and pyridine (3), the latter being caught in a solution of picric acid. The CO2 is about 20% contaminated with CO2 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 NH4OH 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 (Rf = 0.08) was almost to the edge, the trigonelline (Rf = 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 → sodium m-sulfo benzoic acid (16, 19) → m-hydroxybenzoic acid → m-methoxy benzoic acid (5) → 2-nitro-3-methoxybenzoic acid (15) → 2-amino-3-hydroxybenzoic acid. The final yield was 550 mg 3-hydroxyanthranilic-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 difficultly soluble in water. It oxidizes slowly in neutral solutions, and rapidly in alkali. Three different solutions were used to feed excised leaves: 1) One mg 3-HAA suspended in 5 ml H2O. To 0.5 ml were added one drop conc 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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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 green house. 2) A solution of 4 mg 3-HAA required about 3 ml of 0.1 M 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 anabasine is shown in Table I, assuming biogenic equivalence of the carbons in each of the rings. With lysine-2-C¹⁴ (obtained from Tracerlab, Boston Massachusetts) and an initial activity of the 80% ethanol extract of 2.1 x 10⁶ cpm, the radioactivity in anabasine, though positive, was so small as to be negligible.

Hydroxylysine-6-C¹⁴ (0.4 μc) resulted in an initially very low, but active anabasine 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 anabasine 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 nicotinic 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 hydroxyllysine, is not a precursor of the piperidyl ring. The small amounts of activity found in anabasine 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 anabasine is still unknown. The possibility of the transformation of γ-methylene glutamine (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 α-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 anabasine 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. Anabasine [β-(α-piperidyl)-pyridine] formed during photosynthesis in C¹⁴O₂ is uniformly labeled.
2. Glucose-U-C¹⁴ may be used as an ultimate precursor for anabasine.
3. Neither lysine-2-C¹⁴ or hydroxyllysine-6-C¹⁴ are definitive precursors for anabasine.
4. Radiotrigonelline (x-methylnicotinic acid) is not formed during photosynthesis of excised soy bean leaves in C¹⁴O₂.
5. 3-Hydroxyxanthranilic-7-C¹⁴ acid does not appear to be a precursor of trigonelline in the soybean leaves.

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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 Beavers (6) observed 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 respiratory 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...