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BIOSYNTHESIS OF PIPERIDINE ALKALOIDS*—I.

EVIDENCE FOR USE OF THE DIAMINOPIMELIC ACID PATHWAY FOR LYSINE SYNTHESIS IN *NICOTIANA GLAUCA*

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Abstract—Acetate-2-^{14}C, alanine-2-^{14}C, succinate-2-^{14}C, aspartate-4-^{14}C or lysine-2-^{14}C was supplied to growing *Nicotiana glauca* L. plants and anabasine [2-(3′-pyridyl)-piperidine] was isolated from the plants after 7 days. The labelled alkaloid was degraded to isolate as $BaCO_3$ carbon 2 and a mixture of carbons 3, 4, 5, and 6 of the piperidine ring. Isotope distribution in the isolated radioactive anabasine was compatible with the formation of lysine (and subsequently anabasine) via 2,6-diaminopimelic acid as an intermediate rather than 2-aminoadipic acid.

INTRODUCTION

A METABOLIC precursor-product relationship between lysine and the piperidine moiety of anabasine has been assumed on the basis of the following evidence. Growing *Nicotiana glauca* incorporates lysine into the piperidine ring asymmetrically, i.e. lysine-2-^{14}C affords anabasine labelled almost exclusively in the 2 position of the piperidine ring.[1] Cadaverine-1,5-^{14}C yields anabasine with half of its ^{14}C at C-2 of the piperidine ring. The remainder of the ^{14}C is also in the piperidine ring, presumably at C-6.[2] Further, of the pair lysine-α-^{15}N and lysine-ϵ-^{15}N only the ϵ-labelled compound results in ^{15}N in the anabasine piperidine ring when fed to intact plants.[3] It appears that carbons 2, 3, 4, 5, and 6 and the ϵ-nitrogen of lysine form the correspondingly numbered carbons and the nitrogen of the piperidine ring of anabasine (Fig. 1.) The present study was undertaken to provide information about the biochemical transformation of smaller metabolites to lysine and ultimately to anabasine in higher plants. Acetate-2-^{14}C, alanine-2-^{14}C, aspartate-4-^{14}C, succinate-2-^{14}C and lysine-2-^{14}C were supplied to whole *N. glauca* plants and anabasine was isolated after 7 days in each case. The alkaloid was chemically degraded to isolated and assay for ^{14}C in carbon 2 of the piperidine ring and carbons 3, 4, 5, and 6 of the piperidine ring. Labelling patterns were consistent with the hypothesis that lysine furnishes the carbon skeleton of anabasine and is synthesized by *N. glauca* by decarboxylation of 2,6-diaminopimelic acid (DAP) rather than through 2-aminoadipic acid (AA).

RESULTS AND DISCUSSION

The incorporation and distribution of radioactivity in anabasine isolated 7 days after feeding specifically labelled metabolites to *N. glauca* plants are shown in Table 1. Succinate-2-^{14}C and alanine-2-^{14}C are efficient precursors of the alkaloid. Lysine-2-^{14}C, the most efficient precursor, enters anabasine readily and labels the alkaloid in position 2 of the piperidine ring, as has been shown by Leete.[1]

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[1] E. LEETE, *J. Am. Chem. Soc.* **78**, 3520 (1956).
[2] E. LEETE, *J. Am. Chem. Soc.* **80**, 4393 (1958).
[3] E. LEETE, *J. Am. Chem. Soc.* **86**, 3907 (1964).

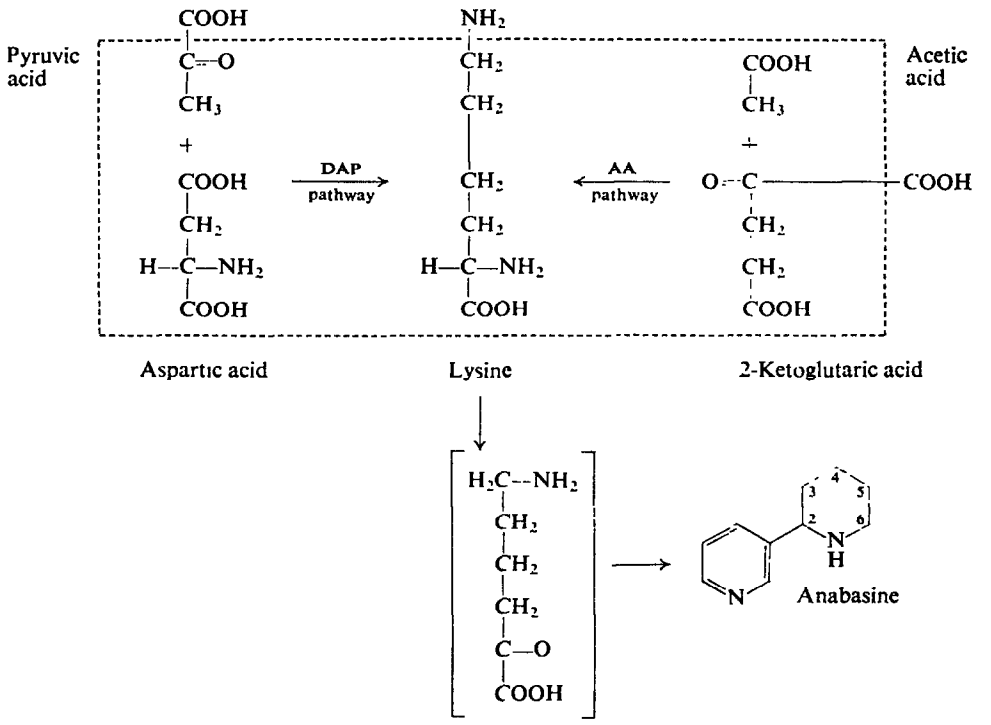

FIG. 1. BIOSYNTHETIC RELATIONSHIPS AMONG PRIMARY METABOLITES, LYSINE, AND ANABASINE.

Lysine is synthesized in plants by two distinct pathways.[4] The first involves decarboxylation of DAP, which is formed by condensation of pyruvate with aspartate (or a derivative) and has been shown to function in bacteria, green algae, and higher plants. In the second, the key intermediate is AA, whose carbon chain is formed from 2-ketoglutarate and acetate in a series of reactions homologous to those of the tricarboxylic acid cycle. AA may be further transformed to lysine through an ϵ-N-glutaryl derivative (saccharopine). This pathway has been found to operate in certain fungi (notably *Neurospora*) and euglenids.

Anabasine, the major alkaloid of *N. glauca*, appears to have its piperidine ring synthesized rather directly from lysine. Since lysine-ϵ-^{15}N supplies isotope to the piperidine ring of anabasine and lysine-α-^{15}N does not, compounds related to 6-amino-2-ketocaproic acid must be intermediate in the transformation.[3] Although cadaverine is an efficient precursor of the piperidine ring of anabasine it cannot be an intermediate in the biosynthetic pathway as the free diamine since this would necessitate equal labelling of the 2 and 6 positions of the ring when lysine-2-^{14}C is fed to *N. glauca*.[2] Further experiments are needed to delineate the biochemical steps from lysine to anabasine. A brief illustration of the biosynthetic routes to lysine and anabasine is shown in Fig. 1.

Aspartate-4-^{14}C

Aspartate-4-^{14}C will lose its ^{14}C before entering anabasine through the AA pathway, since its conversion to fumarate (with equilibration of label between the 1 and 4 positions) or

[4] For a review of lysine biosynthesis see A. MEISTER, *Biochemistry of the Amino Acids*, p. 928. Academic Press, New York (1965).

oxalacetate will result in loss of label either in the tricarboxylic acid cycle or in the conversion of 2-ketoglutarate to lysine. Acetate produced from aspartate-4-^{14}C would be unlabelled. Thus if the plant used the AA pathway there should be no radioactivity in the piperidine ring of anabasine when aspartate-4-^{14}C is fed except that amount which enters through photosynthetic fixation of $^{14}CO_2$ derived from aspartate oxidation. ^{14}C does appear in the piperidine ring, however, and amounts to about half of the total ^{14}C in the anabasine molecule (Table 1). Utilization of the DAP pathway, on the other hand, would result in anabasine labelled predominantly in position 4 of the piperidine ring. Again, aspartate-4-^{14}C would lose its label if it goes through the tricarboxylic acid cycle or is converted to pyruvate. Degradation of the anabasine-^{14}C shows that only 7 per cent of the ^{14}C of the piperidine ring residues in the 2 position. The remainder could reside in any or all the remaining positions.

TABLE 1. INCORPORATION AND DISTRIBUTION OF RADIOACTIVITY IN ANABASINE ISOLATED 7 DAYS AFTER FEEDING ^{14}C LABELLED METABOLITES TO *N. glauca* PLANTS

Compound fed	% Incorporation*	Specific activity (cpm/mmole × 10^{-4})		
		Anabasine	Carbons 3, 4, 5 and 6	Carbon 2
Acetate-2-^{14}C	0·062	8·05	3·24	0·875
Aspartate-4-^{14}C	0·078	4·61	2·44	0·187
Alanine-2-^{14}C	0·61	31·7	12·4	7·15
Succinate-2-^{14}C	1·69	50·8	17·4	5·80
Lysine-2-^{14}C	4·18	25·2	0·723	23·4

*$\frac{\text{total cpm in anabasine}}{\text{total cpm fed}} \times 100$.

Acetate-2-^{14}C and Succinate-2-^{14}C

Formation of anabasine from acetate-2-^{14}C using the AA pathway would introduce ^{14}C into the 2 and 5 positions of the piperidine ring since 2-ketoglutarate would become labelled at C-4 during operation of the tricarboxylic acid cycle. A second and subsequent turns around the tricarboxylic acid cycle would label positions 3 and 4 of the piperidine ring. Succinate-2-^{14}C, since it is a symmetrical molecule, would label positions 3 and 4 in the piperidine ring if the AA pathway operates, and this labelling would not change if the succinate was further oxidized through the tricarboxylic acid cycle before entering 2-ketoglutarate and anabasine. It would be expected, therefore, that acetate-2-^{14}C would furnish ^{14}C to the 2 position of the piperidine ring of anabasine whereas succinate-2-^{14}C would not. However, Table 1 shows that succinate-2-^{14}C supplies the 2 position of anabasine with 25 per cent of the total ^{14}C in the piperidine ring and that acetate-2-^{14}C supplies it with 21 per cent of the total piperidine ring label.

In the case of the DAP scheme, acetate-2-^{14}C and succinate-2-^{14}C would both necessarily produce oxalacetate-2,3-^{14}C and pyruvate-2,3-^{14}C (by oxalacetate decarboxylation) when metabolized in the tricarboxylic acid cycle. Anabasine formed from these precursors should be labelled in positions 2, 3, 5 and 6 and the labelling pattern should be similar when ^{14}C is supplied by acetate-2-^{14}C or succinate-2-^{14}C. As noted above, the two precursors supply nearly the same percentage of the total radioactivity to the 2 position of the piperidine ring.

The difference in absolute percent incorporation of isotope from acetate-2-^{14}C and succinate-2-^{14}C into anabasine probably reflects differences in the pool sizes and turnover rates of the two metabolites.

It has been suggested that acetate-2-^{14}C might enter anabasine through the AA pathway since about one-fifth of the ^{14}C of the piperidine ring resides in the 2 position after metabolism by *N. glauca*. It was assumed that the isotope would become equally distributed among the ring carbons through randomization in the tricarboxylic acid cycle[5]. In light of the present evidence it seems more probable that the DAP pathway is operative.

Alanine-2-^{14}C

Alanine-2-^{14}C may enter into the biosynthesis of anabasine by the DAP pathway by three routes, the first through pyruvate-2-^{14}C, the second through aspartate-2-^{14}C via carboxylation of pyruvate to malate or oxalacetate, and the third by decarboxylation of pyruvate to acetate-1-^{14}C and then through the tricarboxylic acid cycle to oxalacetate. A combination of these processes results in anabasine labelled in positions 2, 4, and 6 of the piperidine ring.

Alanine-2-^{14}C may enter the AA scheme by conversion to acetate-1-^{14}C as above or to 2-ketoglutarate-3,5-^{14}C by carboxylation of pyruvate to malate or oxalacetate and tricarboxylic acid cycle intermediates and subsequently be metabolized to anabasine labelled in positions 3 and 6. Further metabolism of 2-ketoglutarate through the tricarboxylic acid cycle would result in ^{14}C entering C-4 of the piperidine ring, but in any case carbon 2 would not become labelled. However, alanine-2-^{14}C furnishes 2 of the piperidine ring of anabasine with 37 per cent of the ^{14}C of the ring and thus it appears that the DAP pathway of lysine synthesis is followed by *N. glauca*.

CONCLUSIONS

This evidence supports the observations by Vogel[6] that higher plants utilize aspartate and alanine for the synthesis of lysine. He has shown also that cell-free extracts of maize seedlings and *Agave toumeyana* leaf tissue contain a meso-DAP-decarboxylase.[7] Finlayson and McConnell[8] have demonstrated the formation of lysine-1-^{14}C from DAP-1,7-^{14}C in wheat plants. In addition, it has been observed that AA-6-^{14}C does not label lysine when administered to wheat, whereas lysine-U-^{14}C produces labelled AA, pipecolic acid and glutamic acid which are presumed to be catabolic products of lysine.[9]

The present experimental results can be consistently interpreted in terms of the operation of the DAP pathway for lysine synthesis in *N. glauca*. Further, they provide additional evidence for the postulate that lysine affords the carbon skeleton of the piperidine ring of anabasine in this plant.

EXPERIMENTAL

N. glauca seeds[10] were germinated under artificial light in sand and seedlings were transplanted into sand-filled clay pots when they reached a height of 2–4 cm. Plants were fed weekly with an inorganic nutrient solution[11] and watered daily. When the plants reached a

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[6] H. J. VOGEL, *Proc. Natl Acad. Sci.* **45**, 1717 (1959).
[7] Y. SHIMURA and H. J. VOGEL, *Federation Proc.* **20**, 10 (1961).
[8] A. J. FINLAYSON and W. B. MCCONNELL, *Biochem. Biophys. Acta* **45**, 622 (1960).
[9] S. N. NIGAM and W. B. MCCONNELL, *Can. J. Biochem. Physiol.* **41**, 1367 (1963).
[10] Obtained as a gift from Dr. T. C. TSO.
[11] S. A. BROWN and R. U. BYERRUM, *J. Am. Chem. Soc.* **74**, 1523 (1952).

height of about 60 cm (3–4 months), they were fed ^{14}C-labelled metabolites by the wick method.[5] The stem was pierced horizontally through the center with a needle threaded with a short piece of cotton string. Both ends of the string were placed in a tube containing 1 ml of solution containing the labelled metabolite. Each plant was supplied with 10 μc of ^{14}C. When the solution had been taken up by the plant, 0·5 ml of distilled water was added to the tube and allowed to be absorbed by the plant. This was followed by another washing with 0·5 ml of water. The feeding process was complete in 3–4 hr. Plants were harvested 7 days after administration of the isotopic compounds. Five plants were normally used, yielding 1·3 kg of plant material (fresh weight) in a typical experiment.

Isolation of Anabasine

The isolation procedure for anabasine was a modification of the procedure used by Dawson *et al.*[12] Minced plant material was mixed with 1 per cent by weight of trichloroacetic acid and extracted twice with hot water. The combined aqueous filtrates were reduced to 50 ml and extracted with ether. The ether layer was discarded. The aqueous residue was made basic with 10 M NaOH and extracted continuously for 72 hr with ether. The extract was placed on a rotary evaporator and ether was removed under reduced pressure. The residue containing anabasine was dissolved in chloroform and dried over sodium sulfate. The chloroform was removed by evaporating and the crude anabasine was distilled *in vacuo* (135°, 0·5 mm). Anabasine was isolated as the diperchlorate.[1] After recrystallization the anabasine diperchlorate yield was 1·028 g, m.p. 154–156°. Absence of other *Nicotiana* alkaloids was ascertained using paper and gas chromatographic methods.

Oxidation of Anabasine to Nicotinic Acid

Anabasine was oxidized with $KMnO_4$ to yield nicotinic acid and CO_2.[13] In a typical experiment 363 mg anabasine diperchlorate was dissolved in 50 ml water and neutralized with 0·1 M NaOH. Potassium permanganate (972 mg) was added over a 2 hr period. After oxidation was complete, the reaction mixture was acidified and CO_2 collected as $BaCO_3$. Nicotinic acid was isolated as the copper salt. After decomposition of the salt with H_2S, nicotinic acid was purified by sublimation and recrystallization from ethanol. The yield of nicotinic acid was 54 mg, m.p. 234–236°.

Decarboxylation of Nicotinic Acid

Nicotinic acid was decarboxylated by pyrolysis of its calcium salt.[13] Carbon dioxide from the carboxyl carbon was collected as $BaCO_3$.

Procedure for ^{14}C Assays

All samples were assayed for ^{14}C in a proportional flow counter and corrected for instrument efficiency and self-absorption. All samples were counted as $BaCO_3$.

Radiochemicals were purchased from Volk Radiochemical Company, Skokie, Illinois.

Acknowledgements—The authors gratefully acknowledge the excellent technical assistance of Miss Judith Zwemer and Mr. Jerry Stankorb.

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