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BIOSYNTHESIS OF SYNEPHRINE IN CITRUS*

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Abstract—Synephrine is formed in citrus by a pathway involving tyramine and N-methyltyramine. Octopamine is probably not an important intermediate. ^{14}C-labeled tyramine was converted to N-methyltyramine, hordenine, octopamine, and synephrine in one of the citrus cultivars studied. Tyrosine was a less efficient precursor and phenylalanine, serine, ethanolamine, and epinephrine were ineffective.

INTRODUCTION

OCTOPAMINE and synephrine (Fig. 1), well-known sympathomimetic amines in animal metabolism, were only recently isolated from a plant source.[1,2] In citrus, in addition to

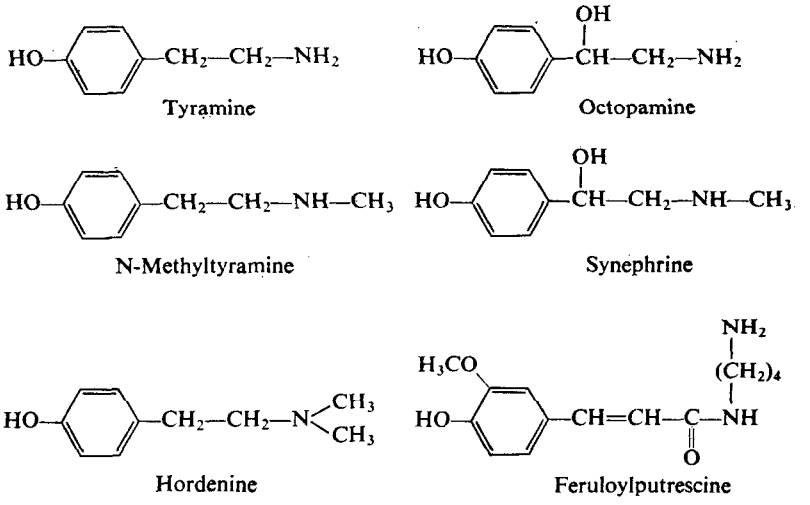

FIG. 1. PHENOLIC AMINES IN CITRUS.

octopamine and synephrine, we found tyramine, N-methyltyramine, hordenine, and feruloylputrescine (Fig. 1). The relative amounts of these compounds among citrus cultivars vary widely.[3] The principal intermediates in the conversion of tyrosine to synephrine in animals appear to be tyramine and octopamine, although alternate pathways have been proposed.[4]

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[1] I. STEWART, W. F. NEWHALL and G. J. EDWARDS, *J. Biol. Chem.* **239**, 930 (1964).
[2] I. STEWART and T. A. WHEATON, *Science* **145**, 60 (1964).
[3] T. A. WHEATON and I. STEWART, *Anal. Biochem.* **12**, 585 (1965).
[4] J. AXELROD, *Science* **140**, 499 (1963).

The major purpose of this study is to establish the sequence of steps involved in synephrine formation in plants. A minimum of three steps are involved in the conversion of an aromatic amino acid to synephrine: decarboxylation, N-methylation, and β-hydroxylation. Several biosynthetic sequences in plants involving one or more of these steps have been studied previously. In barley, hordenine is formed by the decarboxylation of tyrosine to tyramine followed by methylation of the latter compound to N-methyltyramine, hordenine, and candicine.[5] Similar steps are involved in the formation of hordenine and mescaline in peyote cactus.[6] Syntheses requiring β-hydroxylation have received less study in plants but are involved in the formation of compounds such as ephedrine in *Ephedra*,[7] *p*-hydroxymandelonitrile in *Sorghum*,[8] and norepinephrine in banana.[9] Our results indicate that one of the citrus cultivars studied can rapidly carry out the three steps required for synephrine synthesis, but that one or more steps may be absent in other cultivars.

RESULTS AND DISCUSSION

The phenolic amine content of citrus cultivars varies widely. "Cleopatra" mandarin, for example, contains tyramine, N-methyltyramine, hordenine, octopamine, and synephrine. Grapefruit and some other cultivars, however, do not have detectable levels of any of these compounds. For this study, "Duncan" grapefruit, "Avon" and "Meyer" lemons, and "Cleopatra" mandarin were selected. The phenolic amines are absent in "Duncan" and "Avon"; "Meyer" has moderate levels of tyramine, octopamine, and synephrine; and "Cleopatra" accumulates large quantities of synephrine (Table 1).

TABLE 1. PHENOLIC AMINES IN LEAVES OF CITRUS CULTIVARS

Plant*	Tyramine	N-methyl-tyramine	Hordenine	Octopamine	Synephrine
			mg/kg fresh weight		
"Meyer" lemon	4	—	—	31	50
"Cleopatra" mandarin	28	31	Trace	12	2215

* "Duncan" grapefruit and "Avon" lemon contain no detectable amounts of any amine.

The administration of labeled compounds to woody plants is difficult. Methods attempted with citrus included floating stem segments or leaf discs, petiole feeding of excised leaves, feeding through the primary root of seedlings, wick feeding of intact seedlings, and introduction of compounds into detached fruit. The use of stem segments, leaf discs, and fruit was unsatisfactory. Excised leaves were found to take up material rapidly through the petiole, and this method was used in several experiments. Other satisfactory methods included supplying the labeled compounds to the roots of intact seedlings and absorption of compounds into the stem by a wick feeding method.

During these preliminary studies, a number of feeding experiments were carried out using nonlabeled phenolic amines. In one experiment, the wick feeding method was used to introduce either water, tyramine, N-methyltyramine, or synephrine into seedlings of each of the cultivars studied. Approximately 10 mg of the test compound was introduced during a

[5] E. LEETE and L. MARION, *Can. J. Chem.* **31**, 126 (1952).
[6] J. L. MCLAUGHLIN and A. G. PAUL, *Lloydia* **30**, 91 (1967).
[7] I. IMASEKI, S. SHIBATA and M. YAMAZAKI, *Chem. & Ind.* 1088 (1958).
[8] J. E. GANDER, *Fed. Proc.* **18**, 232 (1959).
[9] W. J. SMITH and N. KIRSHNER, *J. Biol. Chem.* **235**, 3589 (1960).

48 hr period. The seedlings were observed and leaves analyzed for phenolic amine content during the following 4 months. No morphological changes or deleterious effects were observed. Although the large amounts of test compounds introduced must have resulted in nonphysiological levels in the seedlings, conversion patterns to other phenolic amines were apparent and were substantiated by the subsequent work using labeled precursors.

A number of labeled compounds were evaluated as potential precursors of synephrine in excised leaves of "Cleopatra" mandarin (Table 2). Tyramine was the most effective precursor

TABLE 2. LABELED COMPOUNDS AS PRECURSORS OF PHENOLIC AMINES IN "CLEOPATRA" MANDARIN EXCISED LEAVES

Compound administered†	Specific activity mc/mM	% Incorporation*				
		Tyramine	N-methyl-tyramine	Hordenine	Octopamine	Synephrine
Tyramine-α-^{14}C	4·71	36·16	39·7	0·03	0·6	4·4
Tyrosine-β-^{14}C	6·85	2·4	1·2	—	—	—
Methionine-$^{14}CH_3$	14·5	—	0·4	—	—	—

* Based on radioactivity in 80% ethanol fraction. (DPM in the phenolic amine ÷ total DPM in 80% ethanol fraction) × 100. The leaves were allowed to metabolize in continuous light for 48 hr from time of administering the tracer.

† Phenylalanine-α-^{14}C, Serine-3-^{14}C and Epinephrine-β-^{14}C were not incorporated into any of the amines examined.

of synephrine and other phenolic amines. Tyrosine was much less effective. Although N-methyltyramine was the only labeled phenolic amine detected after feeding methionine-$^{14}CH_3$, the methyl group of synephrine probably would become labeled with a longer incubation period.

In another experiment, labeled tyramine, tyrosine, phenylalanine, serine, epinephrine, and ethanolamine were supplied to grapefruit, "Avon", "Meyer", and "Cleopatra" seedlings by root feeding. Tyramine was the most effective precursor in all cultivars; tyrosine was slowly converted to tyramine and N-methyltyramine; and the other tested compounds were ineffective. The synephrine formed from tyramine-1-^{14}C by "Cleopatra" seedlings was isolated, diluted with cold- (−)synephrine, and recrystallized to constant specific activity. This diluted synephrine was degraded by alkaline fusion giving methylamine and *p*-hydroxybenzoic acid as the principal products. Synephrine was also oxidized with periodate yielding the α-carbon as formaldehyde which was trapped as the formaldemethone. The label in synephrine was found to be in the α-carbon as would be expected using tyramine-α-^{14}C as the precursor (Fig. 2).

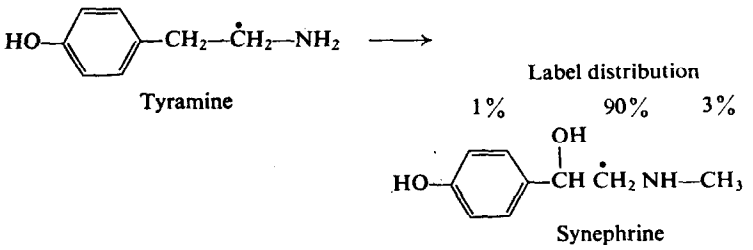

FIG. 2. DISTRIBUTION OF LABEL IN SYNEPHRINE FORMED FROM TYRAMINE-1-^{14}C.

Cultivars differed in their ability to metabolize labeled tyramine (Table 3). Neither grapefruit nor "Avon" lemon normally contain detectable levels of the phenolic amines. Tyramine was slowly converted to other phenolic amines in grapefruit, but in "Avon" lemon

TABLE 3. TYRAMINE-1-^{14}C METABOLISM IN EXCISED LEAVES OF FOUR CITRUS CULTIVARS

Plant	Distribution of label (%)*				
	Tyramine	N-methyl-tyramine	Hordenine	Octopamine	Synephrine
"Duncan" grapefruit	96·8	1·0	—	2·2	—
"Avon" lemon	36·8	61·3	—	1·9	—
"Meyer" lemon	94·8	0·7	—	4·5	—
"Cleopatra" mandarin	35·9	40·3	0·1	1·0	22·7

* Based on radioactivity in phenolic amines. (DPM in each phenolic amine ÷ total DPM in all phenolic amines) × 100. The leaves were allowed to metabolize in continuous light for 48 hr from time of administering the tracer.

was rapidly converted to N-methyltyramine. "Meyer" lemon leaves accumulated radioactive octopamine more rapidly than N-methyltyramine. The appearance of radioactive octopamine and absence of labeled synephrine in these three cultivars suggest a slow conversion of tyramine to octopamine. In "Cleopatra" mandarin leaves, tyramine was very rapidly converted to N-methyltyramine and synephrine with little label appearing in octopamine. Except for "Avon" lemon, the labeling patterns generally reflect the accumulation of phenolic amines in untreated plants. The rapid conversion of tyramine to N-methyltyramine in "Avon" lemon indicates the presence of an active tyramine methylpherase in these leaves, although N-methyltyramine is not normally accumulated.

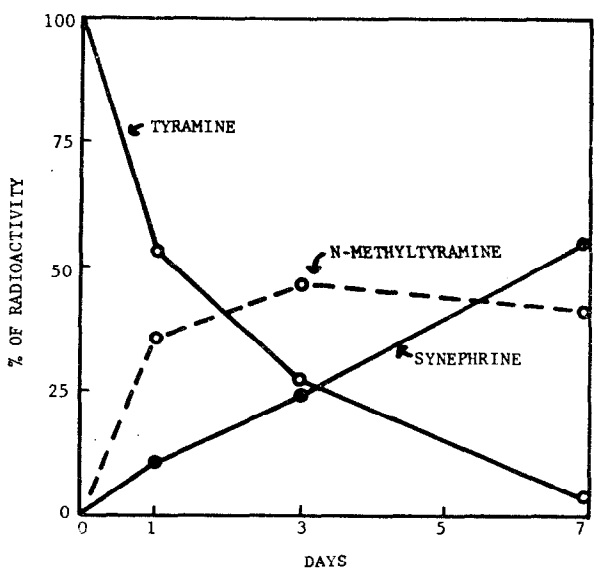

FIG. 3. DISTRIBUTION OF RADIOACTIVITY AFTER FEEDING TYRAMINE-1-^{14}C TO SEEDLINGS OF "CLEOPATRA" MANDARIN.

The labeling pattern obtained with "Cleopatra" suggested that, in contrast to animal systems, octopamine might not be an important intermediate in the synthesis of synephrine. To study this, several further experiments were carried out. In one experiment, "Cleopatra" seedlings were fed labeled tyramine through the roots. The distribution of activity among the phenolic amines 1, 3, and 7 days after feeding is shown in Fig. 3. Tyramine was rapidly converted to N-methyltyramine and more slowly to synephrine. A similar labeling pattern was observed after feeding tyramine to excised leaves of "Cleopatra" mandarin and sampling at intervals up to 48 hr.

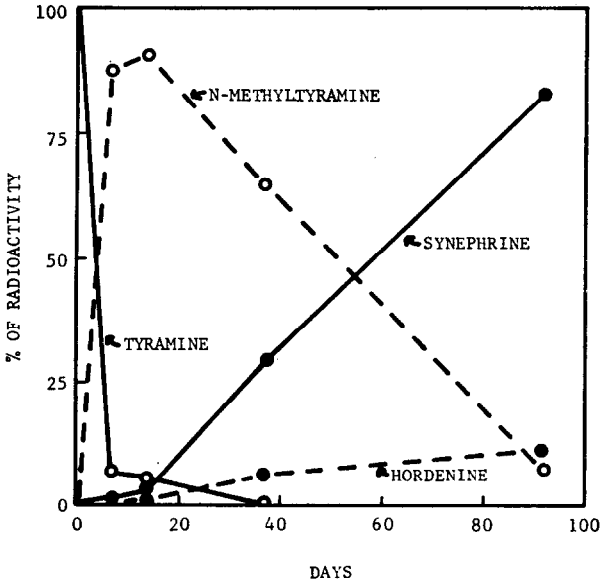

FIG. 4. DISTRIBUTION OF RADIOACTIVITY AMONG PHENOLIC AMINES DURING 3 MONTHS AFTER FEEDING TYRAMINE-1-^{14}C TO A "CLEOPATRA" MANDARIN SEEDLING.

The transfer of label with time among phenolic amines was even more apparent in a preliminary experiment with a "Cleopatra" seedling supplied with labeled tyramine by wick feeding. This seedling was maintained under growing conditions for 94 days and leaves sampled at intervals (Fig. 4). The amount of label in tyramine fell rapidly to a low level. Activity in N-methyltyramine increased rapidly and subsequently decreased as the activity in synephrine and hordenine increased. Radioactive octopamine was not detected in this experiment.

The results from these experiments indicate that the pathway in "Cleopatra" mandarin is probably tyramine→N-methyltyramine→synephrine (Fig. 5) rather than the tyramine→ octopamine→synephrine pathway believed to predominate in animals. This difference could result from differences in substrate specificities of the N-methylase found in animals and the tyramine methylpherase found in plants[10] or in the substrate specificity of the β-hydroxylase found in citrus. Although tyramine is better than N-methyltyramine as a substrate for dopamine β-hydroxylase in animals, it appears on the basis of labeling experiments that in citrus the most important substrate for the β-hydroxylation step is N-methyltyramine.

[10] J. W. DALY, in *Phenolic Compounds and Metabolic Regulation* (edited by B. J. FINKLE and V. C. RUNECKLES), p. 48, Appleton-Century-Crofts, New York (1967).

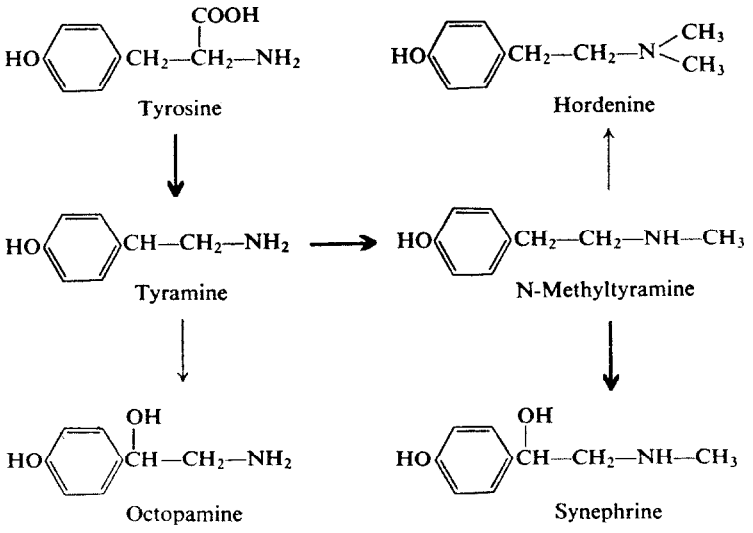

FIG. 5. MAJOR PATHWAYS OF PHENOLIC AMINE SYNTHESIS IN CITRUS.

Since a significant portion of the total soluble nitrogen in some citrus species is found in synephrine, the role of synephrine in nitrogen metabolism was evaluated. Alkaloids and other aromatic nitrogen compounds in plants have frequently been considered as end products of metabolism, and attempts to demonstrate any role of these compounds in metabolism or physiology have generally failed.[11] However, the presence of substantial quantities of octopamine and synephrine in actively metabolizing leaves and fruit of citrus and recent reports of the rapid turnover of other alkaloids[12] suggested that such nitrogen compounds may not always be just end products. An attempt to demonstrate turnover of synephrine by feeding labeled synephrine to excised leaves failed, however. No detectable activity was found in any compound except synephrine after 48 hr. In addition, the results from the long-term feeding experiment (Fig. 4) indicate a gradual increase in activity in synephrine over a 3-month period. That experiment indicated a rapid turnover of tyramine and a moderate turnover rate for N-methyltyramine with the resultant accumulation of synephrine and hordenine.

MATERIALS AND METHODS

Plant Materials

Seedlings of "Duncan" grapefruit (*Citrus paradisi* Macf.), "Meyer" and "Avon" lemon (*C. limon* Burm.), and "Cleopatra" mandarin (*C. reshni* Hort. ex Tan.) were propagated in a growth chamber with a 12-hr day, 30°-day, and 25°-night temperatures.

Administration of Labeled Compounds

For experiments using excised leaves, seedlings were cut and immediately recut underwater. A young fully-expanded leaf was cut underwater and immediately transferred to a 0·4 ml conical plastic centrifuge tube containing about 0·1 ml of an aqueous solution of the

[11] W. O. JAMES, in *The Alkaloids* (edited by R. H. F. MANSKE and H. L. HOLMES), Vol. 1, Academic Press, New York (1950).
[12] J. W. FAIRBAIRN and G. WASSEL, *Phytochem.* 3, 253 (1964).

radioactive compound to be fed. When approximately 0·05 μc had been absorbed (estimated by monitoring the leaf with a thin-window Geiger tube until 1,000 cpm was reached), the leaf was removed, the petiole rinsed twice in water, and the leaf placed in the light in a small beaker with sufficient water to cover the petiole. The feeding time required varied from 15 sec to a few min. Three replications were used, and results presented are averages of these.

For the root feeding experiments, three seedlings of each cultivar were removed from the soil, and the roots washed. The roots were then placed in a small plastic bag containing 10 ml of nutrient solution and the radioactive test compound. After this was taken up, additional nutrient solution was supplied as required. The seedlings were maintained in a laboratory hood on a 12-hr day.

The wick feeding method[13] employed a short length of nonmercerized cotton thread on a needle forced through the stem of a growing seedling 2 in. above the ground. The ends of the thread were placed in a 0·4 ml conical plastic centrifuge tube containing the radioactive solution. After the solution was absorbed, it was washed in by adding water to the centrifuge tube. The seedling was maintained on a 12-hr day in a laboratory hood. Leaves were removed at various time intervals for determination of the distribution of radioactivity in the phenolic amines.

Analytical Methods

Leaves were cut into small pieces, homogenized in hot 80% ethanol, filtered, and the filtrate reduced to dryness. The residue was dissolved in 2·0 ml of a solution containing 3% sulfosalicylic acid and 10% sucrose. Aliquots of this solution were analyzed for radioactivity in a liquid scintillation counter.

The separation of phenolic amines for determination of incorporation of radioactivity was accomplished by an ion-exchange chromatographic system.[3] This system uses a strong cation exchanger in the ammonium form with an NH_4OH concentration gradient for the elution of the phenolic amines. The quantities of the individual phenolic amines present were determined by recording the u.v. absorption of the effluent at 238 nm with a flow cell in a spectrophotometer.

Along with the spectrophotometric determination of each compound as it was eluted from the column, radioactivity was determined with a flow cell in a liquid scintillation counter. A digital record of the counts accumulated in 1-min intervals was obtained throughout the chromatogram. The same channel of the liquid scintillation counter was also monitored with a rate meter and recorder so that corresponding u.v. absorbing and radioactive peaks could be readily observed. After calibration of the system with a standard tyramine-^{14}C solution, the dpm for each compound in the feeding experiments was calculated by accumulating the counts under each peak of a chromatogram. In most experiments, the distribution of radioactivity among the phenolic amines was expressed as a per cent of the total activity in all phenolic amines. Comparisons of specific activities were not very meaningful due to the great variation of the pool sizes of the individual phenolic amines, particularly in "Cleopatra" mandarin.

Degradation of Synephrine

For the alkaline fusion, the diluted radioactive synephrine was mixed with an excess of dry KOH in a tube and heated to 250° in N_2. The CH_3NH_2 was collected by sweeping with N_2

[13] C. L. COMAR, *Radioisotopes in Biology and Agriculture*, p. 151, McGraw-Hill, New York (1955).

into aqueous 1 M HCl. The solution was then evaporated to dryness and the CH_3NH_2 HCl counted in a dioxane scintillation solution. Less than 3 per cent of the activity was recovered in this fraction.

The *p*-hydroxybenzoate in the alkaline residue from the fusion was recovered by dissolving the residue in water, acidifying with H_2SO_4 ,and extracting the *p*-hydroxybenzoic acid into ether. The ether extract was reduced to dryness and the *p*-hydroxybenzoic acid vacuum sublimed. Paper chromatography of this sublimate in isopropanol:ammonia:water (80:10:10) indicated several phenolic compounds were present. The *p*-hydroxybenzoic acid was separated from these other components by preparative chromatography in the above system and counted. Less than 1 per cent of the label was recovered in this fraction. The methylamine and *p*-hydroxybenzoate accounted for all carbon of synephrine except the α-carbon. The low level of radioactivity found in these two degradation products indicated that little randomization of label occurred in the formation of synephrine from tyramine-1-^{14}C.

Proof of label location was obtained by recovering the α-carbon as formaldemethone following $NaIO_4$ oxidation of synephrine.[14] Undiluted radioactive synephrine was placed in 3 ml of 0·1 M phosphate buffer, pH 6·2, and 1 ml of 0·75 M $NaIO_4$ was added. After 5 min, 1·2 ml of 0·3 M HCHO was added, the pH adjusted to the methyl red end point with 10% HOAc and 40 ml of 0·4% 5,5-dimethyl-1,3-cyclohexanedione added. The formaldemethone was collected and recrystallized from alcohol plus water to constant specific activity. Over 90 per cent of the activity was found in this fraction.

[14] E. Y. LEVIN, B. LEVENBERG and S. KAUFMAN, *J. Biol. Chem.* **235**, 2080 (1960).