Diurnal Fluctuations of Cocaine and Potential Precursors in Leaves of *Erythroxylum coca*

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In memory of Prof. Dr. Edward Leete, Chemist, Natural Products Laboratory, Department of Chemistry, University of Minnesota, Minneapolis, Minnesota, U.S.A.

Diurnal Fluctuation, *Erythroxylum coca*, Cocaine, Arginine, Phenylalanine

Cocaine is abundant in leaves of *Erythroxylum coca* var. *coca* Lam. Consequently, cocaine and two of its early biosynthetic precursors were monitored for 24 h to determine whether they fluctuated diurnally. *E. coca* was grown under controlled environmental conditions in a growth chamber in soil at pH 3.5. After 17.5 months of growth, leaves not less than 35 days old were harvested every 2 and 4 h for 24 h for arginine, phenylalanine and cocaine content, respectively. The content of cocaine was determined by GC/MS and amino acid content by HPLC. Diurnal fluctuation of cocaine occurred during the 24 h cycle. Cocaine was highest in leaves of *E. coca* at 8 and 16 h where its content was 7.67 and 9.45 $mg \cdot g^{-1}$ dry weight, respectively. Arginine and phenylalanine in leaves of *E. coca* also displayed diurnal rhythmic patterns of fluctuation. The content of arginine declined from hours 6 to 12 and increased to the highest content at 13 h (21.8 $mg \cdot g^{-1}$ dry weight). Overall, phenylalanine content was lower than arginine, but had two peak periods of accumulation during the 24 h cycle, occurring at 8 and 14 h. The content of phenylalanine in leaves of *E. coca* during the peak hours was 6.98 and 6.54 $mg \cdot g^{-1}$ dry weight, respectively.

Introduction

It has been shown that many alkaloids are metabolically active and display active states of fluctuation in terms of their concentration and rate of turnover in plants [1, 2]. Thus, the quinolizidine alkaloids are one of several alkaloids which have been observed to fluctuate diurnally [3]. Further, the quinolizidine alkaloids are reported to be formed in the chloroplast and transported through the phloem to leaves, pods and maturing fruits of *Lupinus polyphyllus* L. [4].

Putrescine, a precursor of the pyrrolidine moiety of cocaine (*Erythroxylum coca* var. *coca* Lam. Erythroxylaceae) is believed to be derived from ornithine and/or arginine and through a series of reactions is metabolized into the pyrrolidine rings of cuscohygrine and to cocaine [5, 6]. However, in *Datura stramonium* (Solanaceae) the alkaloid hyoscyamine was shown to be derived through arginine *via* agmatine rather than ornithine [7], and a balance has been detailed for both ornithine and arginine as precursors for hyoscyamine in *Datura* [8]. It is conjectured that the nitrogen derived compounds of *Erythroxylum* arise through a biosynthetic path similar to that demonstrated for *Datura* [9]. Finally, it is known that the benzoyl moiety of tropane and cocaine is derived from phenylalanine [10, 11].

It is not known whether cocaine and two of its precursor metabolites, arginine and phenylalanine, fluctuate diurnally. In this study, cocaine, arginine and phenylalanine were monitored for 24 h to determine possible fluctuations.

Materials and Methods

Ripened fruit of *Erythroxylum coca* var. *coca* Lam. (Erythroxylaceae) were obtained from the Yungas region of Bolivia, South America. The pericarps were removed as previously described [12], and the seed planted in 10 cm plastic pots containing Jiffy-Mix (Premier Brand Inc., New Rochelle, N.Y.). The plants were grown in a con-

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trolled environment chamber with 27/22 °C (± 0.5 °C) day:night temperatures, and a 14 h photoperiod supplied by General Electric F72T12/CW/VHO 160 W fluorescent lamps plus 60 W Extended Life tungsten incandescent lamps (General Electric Corp., U.S.A.). After four months of growth, the plant number were reduced to one plant per pot. At eight months, plants with an average height of 17.5 cm, were transplanted into 15 cm pots containing a silt loam soil with the pH adjusted to 3.5 with sulfuric acid and fortified with Osmocote 13-13-13 (N:P:K) slow release fertilizer (Sierra Chemical Co., Dallas, T.X.).

After 17.5 months of growth, new leaves were tagged and dated as they appeared on the main stem and branches. Labeling of new leaves continued for four months, and leaf harvesting began six weeks after the last leaves were tagged. This was to ensure that no leaves harvested were less than 35 days old and fully expanded.

Before harvesting, two groups of 12 plants were arranged to provide three replicates for each sampling time. Leaves from the first group of plants were harvested for cocaine content, while leaves harvested from the second group of plants were used to determine arginine and phenylalanine content. Four labeled leaves were harvested from each plant of the row at its designated hour of harvest. Leaves for cocaine analyses were harvested every 4 h and for the amino acid analyses every 2 h for 24 h. The leaf harvest for amino acids and cocaine commenced at 8 h and ended the following day at 8 h.

Harvested leaves were chilled on ice and immediately lyophilized or stored at −20 °C. The lyophilized leaves were placed in 7.6 cm × 15.2 cm cotton mesh bags and stored over silica gel at −20 °C until analyzed for cocaine or amino acid content.

Alkaloid extraction and analysis

Analyses of *E. coca* leaves for cocaine content were performed by GC/MS. A modification of the methods of Turner *et al.* [13] and Johnson and Elsohly [14] were used to extract and determine the cocaine content in leaves of *E. coca*. Lyophilized samples of 1.2 g (± 20 mg d. wt.) of leaves were combined with 70 ml of EtOH and refluxed for 15 min at 80 °C, and then acid/base partitioned. The residue containing the basic extract was dissolved in 5 ml of EtOH containing 2 μg of d_3-cocaine as an internal standard (20 μl of methanolic solution containing 100 μg ml^{-1}). GC/MS analyses was performed using 1 μl injections of sample extracts.

Amino acid extraction and identification

Amino acids were extracted from lyophilized leaves of *E. coca* by methods of Johnson *et al.* [15]. On column derivatization with orthophthalaldehyde and mercaptopropionic acid (OPA/MPA) was used to derivatize the primary amino acids, and 9-fluorenylmethylchloroformate (FMOC) to derivatize secondary amino acids. This was accomplished with the use of Hewlett-Packard's (H-P) 1090 M Liquid Chromatography Analyzer and ChemStation equipped with an H-P 1046 A Fluorescence detector and AminoQuant Program (Hewlett-Packard, Palo Alto, C.A.). The column used was a H-P Hypersil ODS (5 μm) 200 × 4.6 mm (i.d.) maintained at 35 °C: The following conditions were used: Program: Gradient; Eluents: Solvent A: NaOAc 30 mM, pH 7.2 + 0.5% THF; Solvent B: NaOAc 100 mM, pH 7.2 + 80% ACn; Elution profile: 0 min, 100% A; 0−8 min, 70% A; 9−13 min, 50% A; 14−17.5 min, 100% B; 18−19 min, 100% A. Flow rate 0.450 ml/min (0−14 min), 0.800 to 1.2 ml/min (15−17.5 min) and 0.450 ml/min (18−19 min). Fluorescence detection: E_{x230}, E_{m455} (primary amines) E_{x266}, E_{m305} (secondary amines). The injection volume was 1 μl and the total run time was 19 min.

Results and Discussion

In a previous study *Erythroxylum coca* var. *coca* Lam. was grown in soil where the soil pH ranged from 3.0 to 7.0 [16]. The results showed that optimum growth and alkaloid content occurred in leaves of plants grown in soil with pH's between 3.5 and 4.7. In this investigation, *E. coca* was grown in soil with a pH 3.5 under controlled environmental conditions to minimize stress associated with plants grown under greenhouse conditions [17]. Thus, observation of patterns of diurnal fluctuation of cocaine, arginine and phenylalanine were attributed to their activity during the diurnal cycle rather than a stress response. To ensure that

leaves were fully expanded and within the time period in which they are harvested for cocaine, leaves less than 35 days old were not used.

Diurnal fluctuation of cocaine, arginine and phenylalanine was observed in leaves of *E. coca* during the diurnal cycle (Fig. 1). Cocaine showed periods of fluctuation which were highest at 8 and 16 h where its content was 7.67 and 9.45 $mg \cdot g^{-1}$ dry weight, respectively (Fig. 1). Arginine a precursor of putrescine [7, 5] displayed diurnal and a rhythmic pattern of fluctuation during the 24 h period (Fig. 1). Declines in arginine occurred between 6 and 12 h where the content in leaves was lowest at 8 and 12 h (9.3 and 5.2 $mg \cdot g^{-1}$ dry weight, respectively; Fig. 1). Peak activity for arginine occurred at 6, 14 and 20 h where the content was 17.9, 21.8, 20.1 $mg \cdot g^{-1}$ dry weight, respectively (Fig. 1). Phenylalanine, the confirmed moiety of the tropane alkaloid [10, 11] through tropic acid [8], showed two peak periods of fluctuation during the diurnal cycle. The peak periods occurred at 8 and 14 h where the content of phenylalanine in leaves of *E. coca* was 6.98 and 6.54 $mg \cdot g^{-1}$ dry weight, respectively (Fig. 1).

Leete [6] suggested that L-ornithine was the precursor of putrescine (in leaves of *E. coca*) because the label for L-ornithine (^{14}C) when administered to leaves of *E. coca* was present in putrescine. Leete (personal communication) posited that arginine might be the precursor of putrescine and that the label (^{14}C) observed in putrescine after feeding labeled L-ornithine, may have been a bound form of putrescine. Leete [5] suggested that the pathway to δ-N-methylputrescine may by some circuitous route be formed from L-ornithine by the following sequence: Ornithine → δ-carbamoylornithine → arginine → agmatine → carbamoylputrescine → putrescine.

In conclusion, this study showed that cocaine, arginine and phenylalanine fluctuated diurnally and that the decline in arginine and phenylalanine was followed by an increase in cocaine.

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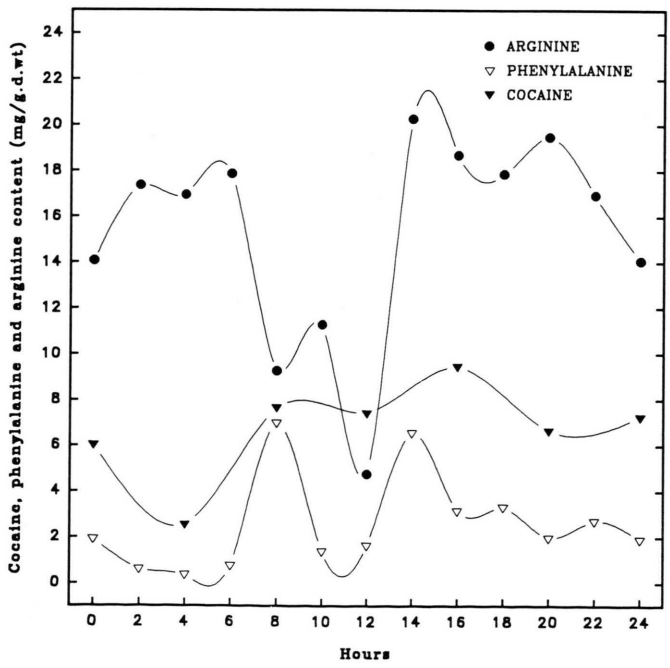

Fig. 1. Diurnal fluctuation and content of cocaine, phenylalanine and arginine in leaves of *E. coca*. The photoperiod commenced at 7 h and ended at 21 h. Each data point represents the mean average of three replicated samples ± *s.e.*

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