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Metabolic Relations between Methylxanthines and Methyluric Acids in *Coffea* L.[1]

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ABSTRACT

Metabolism of purine alkaloids in the leaves of *Coffea dewevrei* De Wild et Durand var *excelsa* Chev, *Coffea liberica* Bull ex Hiern and *Coffea abeokutae* Cramer was studied by analyzing leaf discs collected during vegetative development and by feeding the following radioactive tracers: [^{14}C]theobromine, [^{14}C]caffeine, and [^{14}C]theacrine (1,3,7,9-tetramethyluric acid). Their principal metabolites were quantitatively and qualitatively determined. All three species convert the precursors to the same radioactive products, and proceed through the same four maturity stages characterized by the alkaloid accumulation pattern and by a particular transformation potency: (stage 1) young plant accumulating caffeine, transforms theobromine to caffeine; (stage 2) caffeine is gradually replaced by theacrine, theobromine and caffeine are converted to theacrine; (stage 3) theacrine disappears whereas liberine (*O*(2), 1,9-trimethyluric acid) accumulates, theacrine is metabolized to liberine; (stage 4) branched-out plant containing liberine but no theacrine, caffeine is converted rapidly to liberine via theacrine. Methylliberine (*O*(2),1,7,9-tetramethyluric acid), presumably the direct precursor of liberine, is occasionally found in low concentrations at stage 3 and 4.

The collective term '*liberio-excelsoid*' introduced by geneticists for the numerous races or species of *Pachycoffea* is in accordance with the phytochemical equality found in this work.

Scattered throughout the plant kingdom there are at least 30 genera and 90 species producing purine alkaloids, esteemed by many people for their stimulating effect. Primarily, these are the methylxanthines caffeine (Fig. 1, I) and theobromine (II) which are stored up to a few per cent of the plant dry weight. Less important, as regards concentration in such plants, is theophylline (III) which in tea leaves (*Camellia sinensis* L. O. Kuntze) can occur at up to 0.001%. The list of purine alkaloids has recently been extended by the discovery of three methylated uric acids in the genus *Coffea* (12, 13). As regards the 1,3,7,9-tetramethyluric acid (IV), one should properly use the expression 'rediscovery,' because Johnson (7) in 1937 reported its presence in tea. His observation resulted from a unique situation—he collected this compound in crystals from "residues which came from several million pounds of tea"—, which may explain the little attention paid in the literature to this pioneering study. Biological effects of this uric acid derivative have been reviewed recently (9). The rediscovery of the 1,3,7,9-tetramethyluric acid (named theacrine, due to its occurrence in the top tea leaves) was favored by the fact that it is the main purine alkaloid (1) at a certain growth stage of the tall, West-African coffee species belonging to the subsection *Pachycoffea* (3). Besides theacrine, these plants form two methoxiuric acids, named liberine (V; *O*(2),1,9-trimethyluric acid) and methylliberine (VI;*O*(2),1,7,9-tetramethyluric acid) due to their discovery in *Coffea liberica* Bull ex Hiern (12, 13).

Although the *Pachycoffea* are of minor economic importance, they deserve the plant physiologist's attention for their ability to shift during vegetative development from a methylxanthine (caffeine) to a methyluric acid state and for the remarkable feature of resuming caffeine biosynthesis in the endosperm during seed formation. The present study was undertaken to examine qualitatively, and to some extent quantitatively, the metabolic transformations that occur in this systematic group during vegetative development.

MATERIALS AND METHODS

Plant Material and Cultivation Conditions. Plants of the following designation and origin were used: *Coffea dewevrei* De Wild et Durand var *excelsa* Chev from the Botanical Garden, Bogor, Indonesia; *Coffea liberica* Bull ex Hiern and *Coffea abeokutae* Cramer (*Gros Indénié* Portères) from the IFCC Experimental Station in Divo, Ivory Coast. They were cultivated in a greenhouse at an average temperature of 22 to 25°C and RH of 50 to 70%. To observe the alkaloid pattern over a period of time, plants were kept in a controlled-environment chamber (1000 E/OJU-PR, Weiss, Giessen, F.R.G.) under the following conditions: a 12-h light period; PAR on the top of the plants of 500 to 550 μE m^{-2} s^{-1} (Philips fluorescent lamps TLMF 115 33RS); day/night temperatures of 29 ± 0.5/22 ± 0.5°C and day/night vapor deficits of 6.0/1.5 mg l^{-1}.

Extraction Techniques. Leaf discs and whole leaves were dried to constant weight at 80°C. The finely ground material was extracted for 20 min with boiling 0.0125 N H_2SO_4 (10 ml/100 mg). As a consequence of methodical progress during this work, two different kinds of extract purification were used:

(a) '*Magnesium Oxide Method.*' The hot extract was mixed with heavy MgO (1.1 g/10 ml), cooled, and filtered through glass filter G4. The filtrate was extracted three times with 4 ml $CHCl_3$. The solvent was evaporated under low pressure.

(b) '*Siliceous Earth Method.*' After cooling, the extract was applied to a column, packed with siliceous earth (Extrelut; Merck, Darmstadt, F.R.G.: 0.7 g/ml extract) which after 10 min was eluted with a 4-fold volume of $CHCl_3$. The eluate was brought to dryness.

Chromatography. *TLC.* The residue was dissolved in 1 ml chilled $CHCl_3$ and an aliquot (10–250 μl) spotted onto the silica gel layer (Aluminum foil, Kieselgel 60F 254; Merck). Separation of all compounds was achieved by two consecutive chromatographic runs. After development in $CHCl_3$:CH_3OH (95:5, v/v), the upper part (above R_F 0.25) was rechromatographed in ethyl acetate:H_2O:HCOOH (60:35:5, v/v/v, upper phase). The R_F val-

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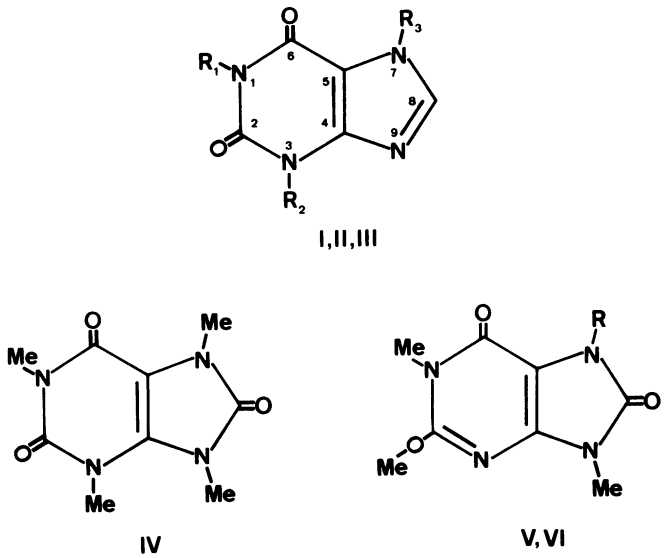

FIG. 1. I, Caffeine, $R_1 = R_2 = R_3 = Me$; II, theobromine, $R_1 = H$, $R_2 = R_3 = Me$; III, theophylline, $R_1 = R_2 = Me$, $R_3 = H$; IV, theacrine (1,3,7,9-tetramethyluric acid); V, liberine (O(2),1,9-trimethyluric acid), R = H; VI, methylliberine (O(2),1,7,9-tetramethyluric acid), R = Me.

ues were 0.15, 0.24, 0.53, 0.58, and 0.72 for theobromine, liberine, theacrine, caffeine, and methylliberine, respectively. For quantification, the corresponding spots, localized under UV light, were cut out and the amount of substance eluted (with CH_3OH) was determined spectrophotometrically. To ensure proper identification, authentic samples were co-chromatographed and UV spectra of the eluates (CH_3OH) were recorded regularly. For spectral data, see Wanner *et al.* (13).

HPLC. The residue from method b was dissolved in water (10 ml/100 mg plant material) and samples (10–100 μl) were chromatographed with 25% CH_3OH (v/v) on a data processing HPLC-system (Waters Associates) equipped with a 4- × 300-mm column (10 μm, μBondapack RP-18; Waters) and with a UV detector monitoring the eluent at 271 nm. The k′ values were 1.4, 2.7, 5.0, 6.3, 13.2, and 16.0 for theobromine, theacrine, caffeine, liberine, methylliberine, and phenacetin, respectively. Phenacetin was added to the crude H_2SO_4 extract (1 mg/100 mg dry plant material) and used as an internal standard (recovery, 79 ± 4%).

Tracer Syntheses and Feeding Technique. Caffeine (6) and theobromine (5) were synthesized by methylation with dimethyl sulfate from [2-^{14}C]xanthine (CEA, Paris, France; 1.88 gigaBq mmol^{-1}), theacrine and methylliberine (4) from [2-^{14}C]uric acid (RCA, Amersham, England; 1.88 gigaBq mmol^{-1}). The tracers were fed by vacuum infiltration. Infiltrated leaf discs were transferred into Petri dishes (5–10/dish) with the cover and lid lined with wet filter paper and exposed to light (Philips fluorescent lamps TL 40 W/33, PAR of 70 μE m^{-2} s^{-1}). At intervals, plant material was collected and processed as described above. Radioactivity distribution on the layer was monitored by radiochromatogram scanning (Berthold, Karlsruhe, F.R.G.). After elution of the corresponding zones with CH_3OH, radioactivity (Bq) was determined by liquid scintillation counting.

RESULTS AND DISCUSSION

Purine Alkaloid Accumulation Pattern during Plant Development. The *pachycoffea* is a group of races or of closely related species within the genus *Coffea*—a typical member is *C. liberica* Bull ex Hiern—which all hybridize frequently and transmute from one to another (3). Therefore these plant 'species,' sharing a great number of characteristics but exhibiting a continuous phenotypic variation, were recently collectively termed '*liberio-excelsoids*' (2).

In an earlier investigation in which a large number of morphologically heterogeneous specimens of *C. liberica* was screened for purine alkaloids, we noticed a mutual, qualitative alkaloid pattern in the leaves that changes with plant development (1). In an alternative approach, we monitored individual plants for their alkaloid pattern over a period of several weeks, analyzing small discs, taken at 1-week intervals from the emerging leaves. An example is presented in Figure 2 (*C. liberica*, extraction method b) which demonstrates clearly a shift from theacrine to liberine in the upper part of the plant during the time of observation. Similar studies were performed on other *liberio-excelsoids* with the following designations: *C. aruwimiensis* De Wild, *C. abeokutae* Cramer, and *C. dewevrei* De Wild et Durand var *excelsa* Chev. Based on all these studies, the vegetative development of the *liberio-excelsoids* is characterized qualitatively by means of purine alkaloids as follows: the seedlings up to the stage of two leaf pairs contain mainly caffeine (stage 1). In the succeeding leaves, caffeine is gradually replaced by theacrine (stage 2). Later in development, theacrine disappears whereas liberine is accumulated (stage 3). This 'final transition' is usually completed when the plant branches out and results in a liberine-containing coffee tree (stage 4). During this latter process, methylliberine is occasionally detectable in very low concentrations (less than 0.1% of plant dry weight). It should be mentioned that alkaloid accumulation is restricted to the newly formed plant parts (Fig. 2) and that quantitative differences exist between individuals or between species.

These experiments with plants kept under controlled environmental conditions confirm that internal plant factors, such as the age of the individual leaf, its position on the tree, and the age of the plant itself, rather than external factors, control the enzymic system of alkaloid metabolism in this taxonomic group.

Tracer Experiments. Tracer studies were performed to examine whether the observed change in alkaloid content from methylxanthines to methyluric acid and the shifting within the different uric acid derivatives (as described in the previous experiments) are the result of corresponding metabolic conversions. We noticed that the radioactive tracer had to be applied to plants exhibiting only a very small endogenous pool of the relevant substance. Otherwise, no conversion could have been detected because of an extremely large decrease in the specific radioactivity. This effect was amplified by the small turnover typical for secondary metabolism. Under these conditions, the conversion of caffeine to theacrine in theacrine-containing plants as well as that of theacrine to liberine in liberine-containing plants is demonstrated.

Feeding of [^{14}C]Theobromine. ^{14}C-Labeled theobromine was fed to *C. liberica* of different maturity stages, with the assumption that theobromine is the direct precursor of caffeine as in *C. arabica* (10). Within 7 h, the tracer was metabolized exclusively to caffeine by caffeine-containing leaves, or to caffeine and theacrine by theacrine-containing leaves (extraction method b). Plants of the third developmental stage however did not transform the applied [^{14}C]theobromine to liberine as expected, even after 72 h (*C. abeokutae*, extraction method a). Radioactive metabolites were found in the aqueous phase after extraction with $CHCl_3$. We assume that at this maturity stage the plant has lost the ability to methylate theobromine to caffeine and further to oxidize it to methyluric acid.

Feeding of [^{14}C]Caffeine. The conversion of caffeine to theacrine, indicated by the previous results, was repeatedly verified. For example, when labeled caffeine was fed to the youngest leaves, which contain mainly theacrine (*C. abeokutae*, extraction method b), the radioactivity in theacrine increases at the same ratio as it decreases in caffeine within the first 8 h after infiltration

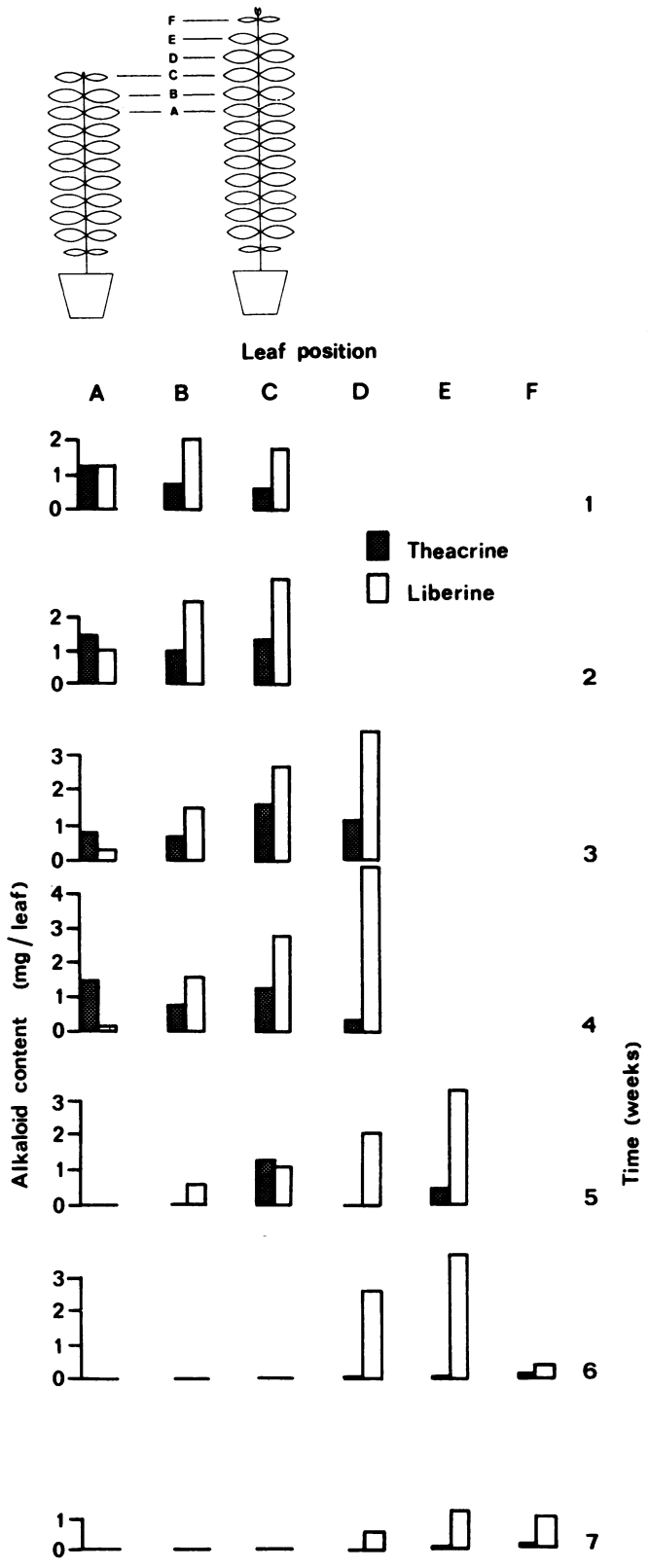

FIG. 2. Changes over a 7-week period in alkaloid content of the youngest leaves of *C. liberica*, kept under controlled environmental conditions. One leaf disc (1.0 cm diameter) per leaf was cut out every week and analyzed (extraction method b). Alkaloid concentration of the whole leaf was calculated by extrapolation to the actual leaf area. Plant illustration: Habitus at the beginning (left) and at the end, after 6 weeks (right), of the experiment.

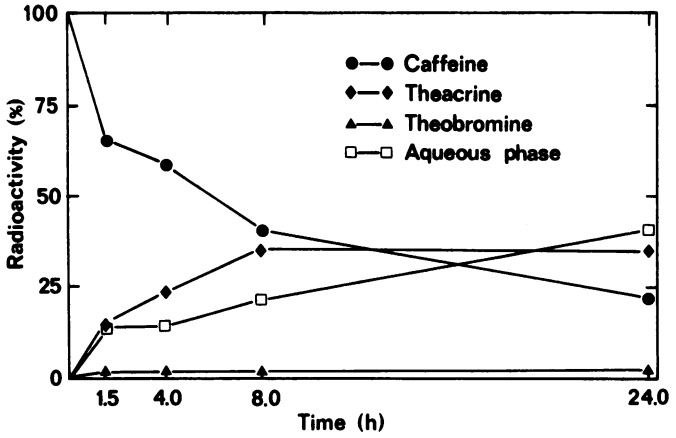

FIG. 3. Kinetics of [^{14}C]caffeine metabolism in the youngest leaf pair of *C. abeokutae* (stage 2). For each time of analysis recovery of radioactivity was set at 100%. Alkaloid concentration in the youngest leaves (extraction method a) was: theacrine, 0.91%; liberine, 0.18%.

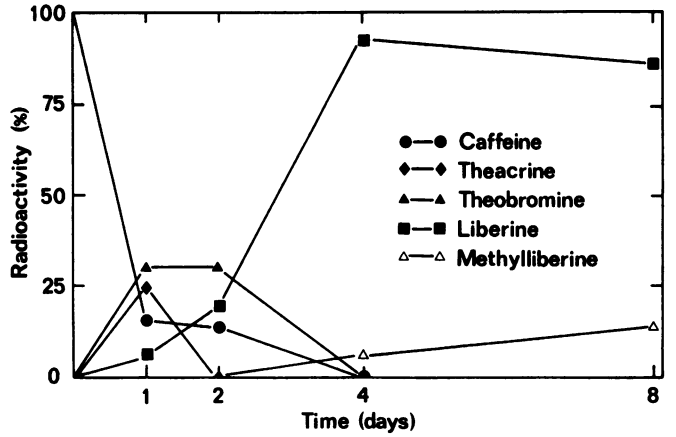

FIG. 4. Kinetics of [^{14}C]caffeine metabolism in the two youngest leaf pairs of the uppermost branch in *C. dewevrei* (stage 4). For each time of analysis radioactivity on the chromatogram was set at 100%. Alkaloid concentration in the youngest leaves (extraction method a) was: liberine, 0.16%; methylliberine, 0.01%.

Table I. *Distribution of Radioactivity after Infiltration of [^{14}C] Theacrine to Leaf Discs Cut Out from the Youngest Leaf Pair of C. liberica (Stage 3)*

For each time of analysis, recovery of radioactivity was set at 100%. Alkaloid concentration in the youngest leaves (extraction method b) was liberine 1.0%.

	Radioactivity after Infiltration			
	1 hr	5.5 h	24 h	72 h
		%		
Aqueous phase	30	46	48	71
Theacrine	36	21	25	13
Liberine	0	7	9	16
8-Methoxicaffeine	34	26	18	0

(Fig. 3). This demonstrates a metabolic conversion of caffeine to theacrine. During the next 16 h, radioactivity increases in the aqueous phase, decreases in caffeine, and stays at a constant level in theacrine. This would seem to be due to an alternative catabolism of caffeine and/or to a catabolism of theacrine. During the experiment, theobromine represents about 2% of the applied radioactivity.

In plants of the fourth developmental stage, *i.e.* when the

young leaves mainly contain liberine, the synthesis of liberine, methylliberine, and occasionally of theobromine is observed. Distribution of radioactivity after feeding [^{14}C]caffeine to young leaves of a *C. dewevrei* plant indicates that theacrine is readily transformed to liberine, whereas theobromine is likely to be synthesized by demethylation of caffeine (Fig. 4). The results do not conclusively determine the metabolic position of methylliberine. It is remarkable that 1 and 2 d after the application of the tracer, we noticed on the chromatogram at least two unidentified radioactively labeled spots (R_F value, 0.09 and 0.62), which yielded 25% (day 1) and 33% (day 2) of the radioactivity isolated at this time. At each sampling time, during the experiment, 10% of the applied radioactivity was found in the aqueous phase (extraction method a).

Feeding of [^{14}C]Theacrine. To confirm the transformation of theacrine to liberine, assumed by [^{14}C]caffeine metabolism, plants of maturity stage one and three were fed with [^{14}C] theacrine. In young, caffeine-containing plants (*C. liberica*, stage 1) a substance represents the main metabolite of [^{14}C]theacrine, chromatographically identical with *O*(8),1,3,7-tetramethyluric acid (8-methoxicaffeine) (extraction method a). As this substance has never been isolated from a coffee leaf and as its appearance is not correlated to any developmental stage, an unspecific transmethylation is considered. Other labeled metabolites could be observed in traces, but obviously appropriate enzymes have not yet been synthesized or are deactivated. Liberine-containing plants (*C. liberica*, stage 3) transformed [^{14}C]theacrine to liberine, but even 72 h after its application not more than 20% of the [^{14}C]theacrine was transformed in [^{14}C]liberine (Table I, extraction method b), while the aqueous phase contained up to 71% of the applied tracer activity. Several experiments yielded similar results, so that we presume theacrine as a precursor of liberine, whereas the metabolic position of methylliberine is not yet confirmed. Methylliberine never represents more than 14% of the applied radioactivity after feeding [^{14}C]caffeine and never more than 3% after feeding [^{14}C]theacrine, which might be due to the very small pool of methylliberine as well as to a high metabolic turnover. We consider methylliberine as the direct precursor of liberine.

Results of these studies refer to the close relationship within and between the two groups of purine alkaloids, as there is not only a shifting in accumulation products from caffeine to theacrine and further to liberine and methylliberine, but also a shifting in metabolic turnover from one substance to the other.

Studies on caffeine catabolism of *C. arabica* by Kalberer (8) showed allantoin and allantoic acid as two of the principal catabolites, both known as accumulation products in the genus *Acer* (11). Species examined in this work are likely to follow this general degradation pathway of purines, leading finally to ammonia which can be re-used for amino acid synthesis. Accumulation products with comparatively high water solubility such as methylxanthines, methyluric acids, allantoin, and allantoic acid may therefore act as reserve stocks of nitrogen allowing a rapid access in case of high nitrogen demand. In experiments with *Coffea*, *Theobroma*, *Cola*, and *Camellia*, Weevers (14) found a decrease of xanthines, while the protein level increased and vice versa. From these results, he considered methylxanthines as an economical form of storing nitrogen. Further studies, concentrating on nitrogen metabolism within this group of plant products, must still be carried out to clarify this physiological aspect.

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