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ALKALOID CATABOLISM AND MOBILIZATION IN *CATHARANTHUS ROSEUS*

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Key Word Index—*Catharanthus roseus*; Apocynaceae; biosynthesis; catabolism; alkaloids; vindoline; catharanthine.

Abstract—The catabolic turnover of vindoline and catharanthine, indole-dihydroindole alkaloids produced by *Catharanthus roseus*, occurs much more rapidly in apical cuttings than in intact plants. The implication of the results is that such alkaloids may participate actively in the plant's biochemical processes under certain conditions, in contradistinction to the general belief that plant alkaloids are metabolically insignificant.

INTRODUCTION

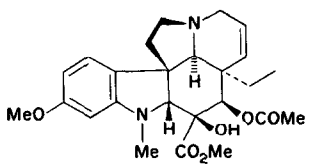
Robinson [1] has recently reviewed current knowledge about the metabolism and function of alkaloids in plants wherein he emphasizes that the available data, although generally circumstantial, are in accord with an active metabolic role for alkaloids. For example, a marked diurnal change in the alkaloid content of *Papaver somniferum* has been observed [2]; the half-life of the alkaloid morphine has been reported to be 2 [3] to 7.5 hr [2]. It also has been reported that radioactive carbon from nicotine finds its way into protein and many other metabolites in tobacco plants [cf. 1]. In regard to this, Leete [4] does not agree with certain of Robinson's conclusions, for he has observed that 60% of radioactively labeled nicotine is recovered either unchanged, or as closely related alkaloids after 3 days metabolism in *Nicotiana glauca* [5].

A hypothesis has been put forth by Miller *et al.* [3] that the opium alkaloids of *Papaver somniferum* serve as sources of C_1 units *in vivo*, since in the biosynthetic sequence thebaine → codeine → morphine → *N*-demethylmorphine there are successive losses of methyl groups. Waller and co-workers [6] also have shown that a methylation-demethylation sequence occurs for the plant alkaloid, ricinine, as a function of leaf age. Overall, there is much circumstantial evidence supporting both rapid and slow turnover of alkaloidal pools in plants.

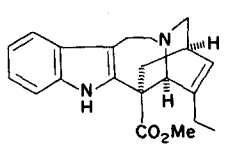
We became intrigued with the problem of alkaloid turnover quite accidentally. During preliminary studies designed to assess the utility of highly enriched $^{13}CO_2$ as a biosynthetic probe of secondary plant metabolites (Hutchinson *et al.* submitted for publication), we examined the time variation of the percentage specific incorporation of $^{14}CO_2$ into alkaloids in several different plants, one of which was *Catharanthus roseus* G. Don (Apocynaceae). This plant produces about 60 alkaloids biosynthetically derived from tryptophan and mevalonic acid [7] of which vindoline (**1**) and catharanthine (**2**) are the major ones. As far as it is known, **1** and **2** are terminal alkaloids in the plant, except for their possible dimerization to form vincaleukoblastine [8]. Since studies of their biosynthesis frequently have been carried out using apical cuttings or intact plants, we looked at both systems to see which would yield the higher specific incorporation of carbon for the $^{13}CO_2$ work. A dramatic difference between the two systems in the turnover rates of **1** and **2** was observed, which suggests that **1** and **2** can be utilized actively in the plant's biochemical processes under certain conditions.

RESULTS

We chose to follow the passage of ^{14}C radioactivity through the pools of **1** and **2** in the apical cuttings of *C. roseus* from zero to three days after exposure to $^{14}CO_2$, because it was known from biosynthetic studies that the maximal total incorporation of radioactivity from various specifically labeled organic compounds usually was obtained over a three day total metabolism period [9]. On the other hand, Scott *et al.* [10] had reported that in intact *C. roseus* seedlings total incorporation of radioactivity from labeled tryptophan into **1** and **2** was still increasing rapidly six days after an initial 10-hour pulse feeding period. Thus, we decided to follow



(**1**)



(**2**)

the feeding experiments with intact plants over several weeks.

An increase, then decrease in the total radioactivity present in a metabolite's pool with time as a pulse of label passes through the pool, is usually acceptable evidence for turnover of a metabolite. As shown by Fig. 1a, the total dpm of ^{14}C (from $^{14}CO_2$) isolable in **1** increased at about the same rate for both cuttings and intact plants independent of the $^{14}CO_2$ exposure method (pulse or pseudosteadystate). In marked contrast, the total ^{14}C radioactivity isolable in **1** then declined much more rapidly in the apical cuttings than in the intact plants, again independent of the $^{14}CO_2$ exposure method. This relationship was also true for **2** (Fig. 1b), where it appears there may have been a cyclic fluctuation in the passage of ^{14}C radioactivity through the pool of **2** in the apical cuttings. This cyclic fluctuation did not appear to be occurring in the intact plants, although the sampling frequency for **2** was too low to permit a valid assessment, nor did it appear to be occurring with **1**, but again with similar data inadequacy. The comparative accuracy of data based on the total dpm's of ^{14}C isolable in **1** and **2** suffers from the lack of quantitative isolation of both alkaloids; however, the excellent isolation reproducibility is justification for the comparative precision of the data shown in figures 1a and 1b. The results based on total dpm's of ^{14}C also could be expressed as the percentage decrease from a maximal value in the total radioactivity of **1** and **2**. The lack of more sampling points does not allow a secure determination of this maximum, so we do not feel it sensible to consider the results in such a way. On the other hand, it is useful to consider the variation in the percentage total incorporation of $^{14}CO_2$ into **1** and **2** with time (Table 1), since this gives a measure of the amount of the CO_2 fed to the plants that was used for alkaloid synthesis over the metabolic period. We show such data only for one set of apical cuttings vs intact plants experiments, although comparable results were obtained for the other set.

The variation of the specific radioactivity of **1** (Fig. 1c) and **2** (Fig. 1d) with time in both apical cuttings and intact plants mirrored the changes observed for the total radioactivity. Although a metabolite's specific radioactivity fluctuation as a function of time is not a direct measure of its turnover rate in a pulse feeding experiment, it is obvious that the specific radioactivity of **1** and **2** changes much more rapidly in the apical cuttings than in the intact plants, independent of the $^{14}CO_2$ feeding method. Since the latter data do not con-

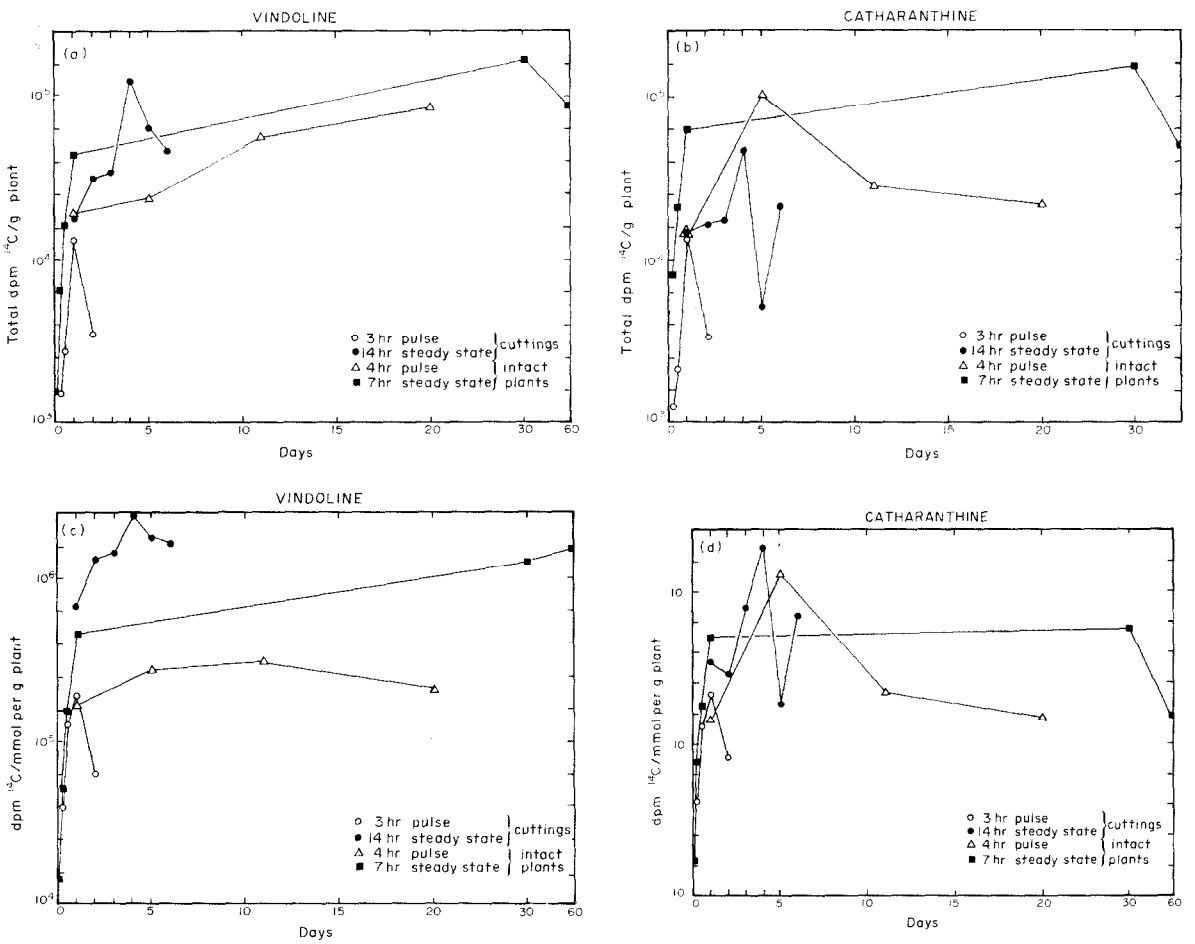


Fig. 1. Change in the specific and total ^{14}C radioactivity isolable in vindoline (**1**) and catharanthine (**2**) as a function of the metabolism period. Following single pulse or pseudosteady state exposure to $^{14}CO_2$ (dpm/mmol: ○, 3.0×10^{10}; ●, 2.5×10^{9}; △, 3.0×10^{10}; ■, 1.4×10^{10}) the plants were allowed to continue metabolism in the normal atmosphere.

Table 1. Variation in the percentage total incorporation of radioactivity* from $^{14}CO_2$ into vindoline (**1**) and catharanthine (**2**)

	Metabolism period (days)	Total incorporation* 1	2
apical cuttings	0.25†	0.0013	0.0008
(3 hr pulse	0.5	0.0031	0.0024
feeding)	1	0.0095	0.0084
	2	0.004	0.003
(7 hr steady	0.12	0.004	0.007
state feeding)	0.25	0.010	0.010
	0.30	0.018	0.029
	1	0.035	0.043
	30	0.20	0.17
	60	0.13	0.09

* Total dpm of ^{14}C in **1** or **2** divided by the total dpm of $^{14}CO_2$ taken up by the plant material times 100. † For periods longer than the $^{14}CO_2$ feeding period, the plants were grown in $^{12}CO_2$ in a growth chamber.

tain the relative inaccuracy of that shown in Figs 1a and 1b (from losses of **1** and **2** due to the isolation procedures), it is reassuring to see the same trend in these results as in the former.

Although we did not sample frequently enough in the intact plant experiments to permit an accurate determination of the curve maxima in figures 1a–1d, this does not invalidate the fact that there is a significant difference between the results for the apical cuttings and for intact plants.

DISCUSSION

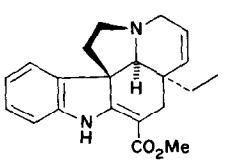
Since it seems to be well established that plant alkaloids undergo metabolism, perhaps as a function of the plants' ontogeny [1], it is not surprising that we observed evidence for active turnover of **1** and **2** in *C. roseus*. These alkaloids appear to be turned over considerably less rapidly than has been reported for the alkaloids in the opium poppy [2,3], since the fluctuation in their total radioactivity did not occur in small hourly intervals, but as in the case of the apical cuttings, occurred over at least a 48 hour period.

The differences observed between the results of the two apical cuttings feeding experiments regarding the total and/or specific radioactivity of **1** and/or **2** are as would be expected when it is realized that our pseudosteady state feeding method was really only a pulse exposure to $^{14}CO_2$ of 5–7 times longer duration than the single 3-hr pulse feeding. Thus, since a larger quantity of ^{14}C entered the precursor pools of **1** and **2** in the former case, it should take longer to pass through the pools of **1** and **2**, and these pools should acquire a greater total and specific radioactivity than in the latter case. Regardless, the total radioactivity of **1** and **2** dropped at about the same rate in either case, supporting an equally rapid turnover of the two alkaloids.

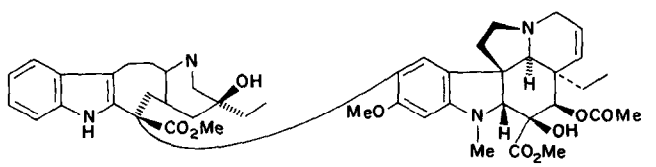
We do not attach much significance to the apparent cyclic variation in the specific and total radioactivity of **2**, which seems to rise and fall about every 48 hours. Since we did not follow the changes for **2** seen in the two experiments over several weeks, the observed, rather unusual cycling may be artifactual. However, such rapid daily fluctuations in the alkaloid levels of several plants have been reported by Fairbairn and co-workers [2,11,12]; thus, it is possible that our data are real.

Before discussing our results in terms of the catabolism of **1** and **2**, we must question if the observed turnover of **1** and **2** is catabolism in the sense of metabolic breakdown, or anabolism to other alkaloids that are known constituents of *C. roseus*. If we accept the position that alkaloid biosynthesis is not reversible in *C. roseus*, which seems sensible, although there is some evidence [13] that **2** can be transformed back to tabersonine (**3**), thence to **1** in *C. roseus* [10,13], the alkaloids into which **1** and **2** might be transformed are the dimeric indole-dihydroindole alkaloids represented by vincaleukoblastine (**4**). However, **4** appears to be formed very slowly in *C. roseus*, based on how rapidly precursors are incorporated into it [8,14], its pool size is about 10^{-2} × that of **1** or **2**, and it is questionable that **2** is even a precursor of **4**. We do not feel, then, that utilization in the formation of **4** or other minor dimeric alkaloids could account for the observed rapid turnover of **1** and **2** in the apical cuttings. Among the known minor alkaloids of *C. roseus*, there do not appear to be any that should be considered to be derived from **1** or **2** via biosynthetic, i.e. energy requiring, processes. It is possible that the observed turnover of **1** and **2** is due to neither catabolism or anabolism, but to their transformation into "storage forms" such as glucosides, which would not have been extracted by our isolation procedures. Such "bound forms" of alkaloids have been found in hemlock [15] and opium poppies [16], although it was not determined if these were a result of alkaloid catabolism. In the sense that **1** and **2** may have been turned over by oxidative, i.e. energy yielding, processes to other alkaloidal substances known or unknown, this would fit into the hypothesis we propose below.

The similarity in the biosynthetic rate, but the difference in the turnover rate of **1** and **2** between apical cuttings and intact plants of identical age and occurring under (hopefully) identical environmental conditions is remarkable to us. We propose that the stress imposed on the plants by apical scission caused many biochemical and physiological changes in response, whose purpose was to repair the damage done to the plants, e.g. to begin root regeneration, which appear in about 20 days for *C. roseus*. Since new tissue synthesis would require energy as well as carbon and nitrogen, it is reasonable to speculate that the alkaloids (as well as other plant



(3)



(4)

metabolites) could be oxidatively catabolized, thus providing reducing equivalents for general anabolism in the plant. This would necessitate the presence of, or genetic capability for, enzymes capable of catabolizing **1** and **2** to substances more oxidized, perhaps eventually leading to re-entry of fragments of **1** and **2** into the plant's primary metabolic pathways. Certainly in the case of nicotine in tobacco plants, such extensive breakdown and re-entry into primary plant metabolites has been observed [17–20]. Mothes, *et al.* [21] have reported that in *C. roseus*, the seeds do not contain alkaloids; these appear rapidly on germination, then almost *disappear* from the plant between about the fourth and eighth week of growth only to subsequently reappear in older plants. Such observations seem to indicate a dynamic role for the alkaloids of *C. roseus*, which nicely corroborate our present findings. We believe that while such alkaloid mobilization could occur, this does not necessarily imply a role for these alkaloids in ontogeny. We still have to show that **1** and **2** undergo rapid oxidative transformation as a result of apical plant scission, and that fragments of them re-enter the plant's primary metabolic pathways. This possibility should be investigated, since similar results of future research could lead to a clearer understanding of the biochemical relationship between primary and secondary plant metabolism.

EXPERIMENTAL

Plants. Actively flowering *C. roseus* plants, *ca* 3 months old, were used from greenhouse grown stock. Apical cuttings were taken from intact plants and used immediately.

Pulse feeding of $^{14}CO_2$. A plastic bag growth and feeding chamber was constructed from a simple wooden framework surrounded tightly by a plastic bag of 3 mils thickness. The open end of the bag served as an access to the chamber and could be sealed air tight. A diaphram pump (600 ml/min capacity) was used to circulate air through Tygon tubing connected to the chamber at opposite ends by quick-fit connectors. The chamber was placed inside a growth chamber (Biotronette Mark III, Lab-Line, Chicago IL) equipped with Westinghouse Agro-Lite F40 lamps, which provided 300–400 μeinsteins M^{-2} sec^{-1} of photosynthetically active radiation; as measured by a Lambda Instruments Corporation LI-170 quantum photometer. The air inside the chamber, circulated with the aid of battery operated fans, either traveled directly back to the pump, or indirectly through a $Ba(OH)_2$ trap, which was used to selectively remove CO_2 from the air. The internal temperature varied between 21° and 26° at a saturating relative humidity. In a typical experiment, cut shoots of plants in Hoagland's medium [22] or intact plants potted in soil were placed inside the chamber. A sample of [^{14}C]-$BaCO_3$ was weighed into a small beaker and was placed inside the chamber near one wall with a septum fitted into the bag's wall by means of a quick-fit connector. The chamber was sealed and the circulating pump was started. Conc H_2SO_4 was slowly injected through the septum onto the [^{14}C]-$BaCO_3$ sample. The immediate evolution of $^{14}CO_2$ commenced the pulse feeding. After 3–4 hr, whence the CO_2 fixed by the plants was not replenished, the circulating air inside the system was diverted into the $Ba(OH)_2$ trap to remove any residual $^{14}CO_2$ before the chamber was opened. After the chamber was opened, the plants were harvested and extracted immediately or allowed to metabolize for various lengths of time in the Biotronette growth chamber: photoperiod 14 hr; day temp. 27°; night temp 18° at a relative humidity of 40–50%. Recovered [^{14}C]-$BaCO_3$, resulting from trapped residual $^{14}CO_2$ was washed with H_2O, MeOH and Et_2O then dried *in vacuo*. A suitably diluted aliquot was counted as a suspension in 4% Cab-O-Sil in a toluene + 0.5% w/v PPO scintillation solution on a Packard 3375 liquid scintillation counter. The difference between the total amount of ^{14}C radioactivity injected as $BaCO_3$ and the amount recovered as $BaCO_3$ was taken as the amount of $^{14}CO_2$ fed to the plants.

Pseudosteady-state feeding of $^{14}CO_2$. Our technique is a modification of that of Rapoport and coworkers [23]. Seven 4 mil thick plastic bag feeding and growth chambers, each one 18.5 × 35.5 × 45.7 cm, 30.0 l, were connected in parallel. With the system closed air tight, air was circulated by the diaphram pump past an inlet where radioactive CO_2 could be introduced into the system. The air then was pumped into all chambers, where it diffused throughout the chambers, and was pumped out at the opposite ends of the chambers. The air then flowed through an ionization chamber (250 ml) electronically connected to a vibrating reed electrometer (Applied Physics Model 31) so that the total radioactivity in the system could be monitored continuously. In a feeding experiment, approximately an equal number of plants were placed inside each chamber accompanied by [^{14}C] $BaCO_3$ placed into a small beaker. The chambers were sealed air tight, the diaphram pump was started and conc H_2SO_4 quickly was injected into each beaker of [^{14}C]-$BaCO_3$ in each chamber. The amount of $^{14}CO_2$ thus generated in each chamber, brought the level of total CO_2 to *ca* 0.05%. The ^{14}C radioactivity level was monitored immediately by the vibrating reed electrometer before the Biotronette's growth lights were switched on. A plateau level in the specific radioactivity was reached within minutes. As soon as the growth lights were turned on, a rapid drop in the total radioactivity was noted as photosynthesis commenced, which then was maintained close to the originally recorded level by careful addition of $^{14}CO_2$ of the same specific activity as initially present to the system from a gas cylinder. Monitoring the total radioactivity in the air allowed an indirect measurement of the concentration of CO_2, since the specific radioactivity was kept constant. Plants were removed at various time intervals after feeding commenced, by clamping the entrance and exit tygon tubing to the individual chamber at two places on each side, and cutting the tubing between the clamps. The chamber then was connected to a pump, and the air inside the chamber was rapidly drawn through a $Ba(OH)_2$ trap. The plants were removed from the bag, and their tops frozen in liquid N_2. The last four operations were done as rapidly as possible. Once the $^{14}CO_2$ feeding had been terminated the remaining plants were permitted to continue their metabolism in a normal atmosphere using the Biotronette growth chamber.

Alkaloid isolation. The frozen plant tops, or cuttings, were ground with methanol in a Sorvall Omni-mixer, and the marc was extracted repeatedly with hot methanol until blanched. The combined methanol extracts then were evaporated to dryness *in vacuo* at 40°. The residue was partitioned between equal vols of 1N HCl and EtOAc. The two layers were separated, and the organic phase was extracted with 1N HCl (2 vol.). The combined acidic aqueous fractions were made basic (pH 8) with $NaHCO_3$ and extracted with EtOAc (3 vol.). The organic phase then was dried, filtered, and evaporated to dryness *in vacuo* to give the total alkaloid extract fraction. Catharanthine (**2**) was isolated from the total alkaloid fraction by preparative TLC on Si gel in Et_2O–MeOH (4:1). The UV absorbing band corresponding to authentic **2** (R_f 0.8) was extracted with EtOAc–MeOH (9 : 1) followed by removal of the solvent *in vacuo*. The crude **2** isolated was replated, and the plates were developed in EtOAc, **2** was isolated as before, then characterized and quantitated by UV spectrophotometric analysis (E_{224} 34,000 and E_{283} 8,800 in EtOH). Formation of the hydrochloride of **2** by titration in MeOH with a 0.1N anhyd HCl–MeOH solution to pH 3.0 and crystallization from MeOH–Et_2O yielded crystals: mp 185–188° dec. Vindoline (**1**) was purified from the total alkaloid extract by preparative TLC on Si gel as for **2**. The UV absorbing band corresponding to authentic **1** (R_f 0.6) was extracted with EtOAc–MeOH (9:1) followed by removal of the solvent *in vacuo*. **1** was replated and the plates were developed in EtOAc. The

UV band (R_f 0.5) was eluted as before to yield **1**, which was characterized and quantitated by UV spectrophotometric analysis (E_{211} 33,000 and E_{251} 7100) in ethanol. Formation of the hydrochloride of **1** by bubbling dry HCl gas into a solution of **1** in Et_2O and recrystallization of the solid from MeOH–Et_2O yielded crystals: mp 168–170° dec. In both cases, the hydrochlorides of **1** and **2** were recrystallized to constant specific ^{14}C radioactivity (±3%) for three consecutive samples. Their specific radioactivity was measured by liquid scintillation counting in dioxane + 10% w/v naphthalene and 0.5% w/v PPO. Reproducibility and quantitization of the isolation methods for **1** and **2** were checked by the addition of a known quantity of [G-^{14}C] – **1** and – **2** to non-radioactive fresh plant tops and their reisolation according to the aforementioned scheme. Thereby, 77% of **1** and 65% of **2** could be recovered as TLC pure material with a reproducibility of ±2% (three repetitions).

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