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THE SEQUENTIAL APPEARANCE AND METABOLISM OF ALKALOIDS IN *HEIMIA SALICIFOLIA*

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Key Word Index—*Heimia salicifolia*; Lythraceae; sequential appearance of alkaloids; $^{14}CO_2$ feeding; catabolism; alkaloid interconversion; lyfoline; cryogenine; lythrine; sinicuichine; nesodine.

Abstract—The seeds of *Heimia salicifolia* do not contain alkaloids. Two unidentified alkaloids were detected in 1-week-old seedlings; these alkaloids were absent from older plant samples. Lyfoline, cryogenine, and lythrine were first detected in 2-week-old plants. Sinicuichine was first observed in 3-week-old plants and nesodine in 2-month-old plants. The maximum rates of synthesis for most of these alkaloids occurred in 1- to 2-month-old plants. Following administration of $^{14}CO_2$ to *H. salicifolia* plants, small quantities of alkaloids were purified to constant specific activity without alkaloid dilution; 95·6% of the administered $^{14}CO_2$ was assimilated and up to 0·16% of this activity was incorporated into known alkaloids. Sinicuichine and lyfoline were shown to undergo catabolism, while cryogenine was degraded very slowly, if at all. Evidence is presented for the conversion of lyfoline to lythrine.

INTRODUCTION

Several possible biogenetic schemes have been proposed for the formation of the alkaloids of *Heimia* and *Decodon* species (Lythraceae) [1]. Preliminary biosynthetic studies have shown that the aromatic groups of these alkaloids arise from phenylalanine [2–4] and that the quinolizidine moiety of the decodon alkaloids is partially derived from lysine [1]. However, the biosynthetic interrelationships between individual alkaloids have not been established. Work in other laboratories has shown that a study of the sequential formation of alkaloids can often suggest biosynthetic interrelationships which may not be obvious from a study of structures alone [5, 6]. Furthermore, kinetic studies of the incorporation of $^{14}CO_2$ into alkaloids have recently been used to help delineate biosynthetic pathways [7–14]. Leete [7] has stated that this is probably the ideal way to study alkaloid biosynthesis, since the normal physiology of the plant is maintained if the amount of ^{14}C fed is kept low enough to avoid radiation damage.

Two experiments were conducted in an attempt to determine the biosynthetic interrelationships among the alkaloids of *Heimia salicifolia* Link and Otto. In the first experiment, the sequential formation of alkaloids was investigated by examining plants at weekly intervals to the age of 4 weeks, and at monthly intervals to the age of 7 months. In the second experiment, *H. salicifolia* plants were exposed to $^{14}CO_2$ for 2 hr, at a time of maximum alkaloid synthesis and plant samples were collected after various periods of ^{14}C metabolism.

In both experiments, all plants were harvested at the same time of day (11 am) to avoid the effects of diurnal variation which have been observed in a number of other alkaloidal plants. After the alkaloids were extracted, they were separated by preparative TLC and quantitated by UV analysis.

RESULTS AND DISCUSSION

Sequential appearance of alkaloids

Of the eight known alkaloids only lyfoline (**1**), lythrine (**2**), cryogenine (**4**), nesodine (**7**), and sinicuichine (**8**) were observed during the present study. The occurrence of lythridine (**3**) in these young plants had been anticipated since it had

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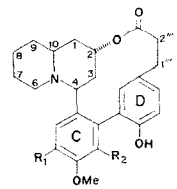
been detected by us in a preliminary investigation using 10-week-old plants. Its absence is however, consistent with an earlier finding that the presence of lythridine in *H. salicifolia* 'is dependent on climatic and/or other environmental conditions' [15]. Six new alkaloids were detected during this investigation.



Alkaloid	Stereochemistry at ring juncture	1‴ – 2‴	R_1	R_2
(1) Lyfoline	*trans*	—CH=CH—	—OH	—H
(2) Lythrine	*trans*	—CH=CH—	—OMe	—H
(3) Lythridine	*trans*	—CHOH—CH_2—	—OMe	—H
(4) Cryogenine	*cis*	—CH=CH—	—OMe	—H
(5) Heimidine	*cis*	—CHOH—CH_2—	—OMe	—H
(6) Dehydrodecodine	*trans*	—CH=CH—	—H	—OH
(7) Nesodine	*trans*	—CH=CH—	—H	—OMe
(8) Sinicuichine	*cis*	—CH=CH—	—H	—OMe

Seeds were found to be devoid of alkaloids. Known alkaloids could not be detected in 1-week-old seedlings, but two polar, Dragendorff-positive compounds were detected, each of which gave a fuchsia color with diazotized *p*-nitroaniline [16] (phenol reagent). Although the quantities of these two alkaloids permitted only chromatographic studies, one of them gave the same color with the phenol reagent and had the same chromatographic mobility as both the synthetic axial and equitorial isomers of (4′-hydroxy-5′-methoxy)-4-phenylquinolizidin-2-ol [17]. Both of these alkaloids were absent from plants older than two weeks.

Lyfoline (**1**), lythrine (**2**), and cryogenine (**4**) were first isolated and determined quantitatively in 2-week-old plants. Sinicuichine (**8**) was first observed in 3-week-old plants, and nesodine (**7**) in 2-month-old plants. This would tend to rule out bioconversion of nesodine (**7**) or sinicuichine (**8**) to the other three alkaloids. It is possible that lyfoline (**1**), lythrine (**2**), or cryogenine (**4**) are converted to nesodine (**7**) or sinicuichine (**8**). However, this seems unlikely since such conversions would require rupturing the biphenyl bond, rotating ring *C*, and reforming the biphenyl bond. It seems more likely that there is a branch point in the biosynthetic pathway, possibly at the point of phenyl-coupling. Thus, the two series of alkaloids, one substituted at R_1, the other substituted at R_2, could arise from a common uncoupled precursor. The results indicate that alkaloids with R_1 substitution are formed earlier than those with substitution at R_2.

Although this sequential study did not provide conclusive evidence of alkaloid interconversions, changes in alkaloid concentrations during the course of this investigation did provide information about the rates of alkaloid biosynthesis (Table 1). All of the alkaloids except sinicuichine are synthesized at their maximum rates in 1- to 2-month-old plants. The alkaloid concentrations fluctuate significantly in the 4- to 7-month time interval. However with the exception of sinicuichine, the ratios of each individual alkaloid concentration to the total alkaloid concentration remain reasonably constant during this interval (Table 2). This indicates that, with the exception of sinicuichine, the rate of degradation of any alkaloid is virtually identical to its rate of synthesis, and suggests that the observed alkaloid concentration changes are caused by fluctuations in nonalkaloidal constituents.

The concentration changes with plant age (Table 1) clearly show that the maximum rates of

Table 1. Alkaloid changes in *H. salicifolia*

Age	Alkaloid concentration (mg/g plant dry wt)				
	Lyfoline	Cryogenine	Lythrine	Sinicuichine	Nesodine
1 Week	—	—	—	—	—
2 Weeks	0·27	0·85	0·31	—	—
3 Weeks	0·84	1·40	0·15	0·36	—
1 Month	1·42	1·93	0·25	0·80	—
2 Months	6·70	8·67	1·73	1·11	0·03
3 Months	6·98	8·55	1·22	0·98	0·05
4 Months	6·16	8·55	0·72	2·17	0·02
5 Months	7·39	10·60	1·45	1·13	0·03
6 Months	4·93	6·21	1·12	0·24	0·03
7 Months	6·55	8·63	0·66	1·50	0·09

Table 2. Relative alkaloid concentration changes in *H. salicifolia*

Age	Weight of individual alkaloid/weight of total alkaloid				
	Lyfoline	Cryogenine	Lythrine	Sinicuichine	Nesodine (× 10)
1 Week	—	—	—	—	—
2 Weeks	0·19	0·60	0·22	—	—
3 Weeks	0·31	0·51	0·05	0·13	—
1 Month	0·32	0·44	0·06	0·18	—
2 Months	0·37	0·48	0·09	0·06	0·01
3 Months	0·39	0·48	0·07	0·06	0·03
4 Months	0·35	0·49	0·04	0·12	0·01
5 Months	0·36	0·51	0·07	0·06	0·01
6 Months	0·39	0·50	0·09	0·02	0·03
7 Months	0·38	0·50	0·04	0·09	0·05

alkaloid synthesis occur in plants much younger than those usually used in precursor feeding experiments [2, 4].

^{14}C-*Carbon dioxide feedings*

Since the sequential appearance study showed that most of the alkaloids were being synthesized at a nearly maximum rate in 6-week-old plants, a preliminary feeding experiment was conducted to develop experimental techniques for purification of the alkaloids to constant specific activity. In this feeding of 6-week-old plants, 90·8% of the administered $^{14}CO_2$ was assimilated during the 2-hr feeding period. The incorporation of $^{14}CO_2$ into known alkaloids was 0·002% for a plant sample harvested immediately after feeding, and 0·026% for a sample harvested after an additional 48 hr of ^{14}C metabolism. In another feeding of 6-week-old plants, 95·6% of the administered $^{14}CO_2$ was assimilated. One plant sample was harvested immediately, the other plants were allowed to metabolize ^{14}C for additional periods of three, six, nine, or fourteen days. The small weight of plant material extracted precluded purification of the alkaloids by crystallization; furthermore, in several cases a lack of pure reference alkaloids did not permit carrier dilution. Purification was achieved by a com-

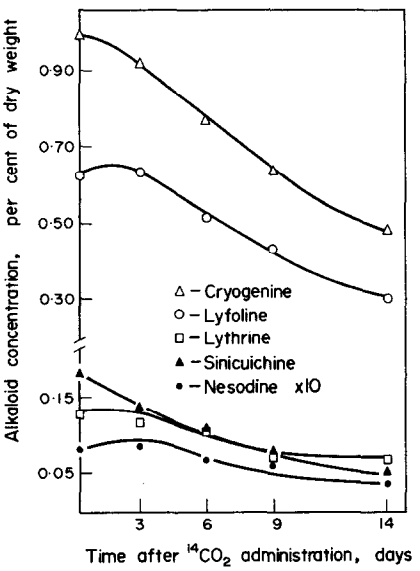


Fig. 1. Alkaloid concentration in *H. salicifolia* after exposure to $^{14}CO_2$.

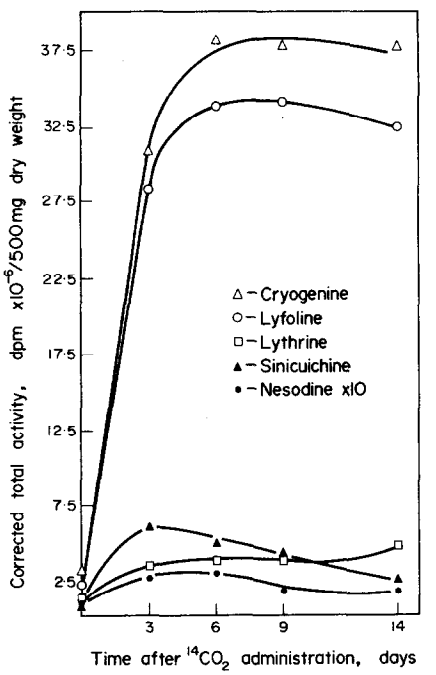


Fig. 2. Corrected total activities of alkaloids from *H. salicifolia* after exposure to $^{14}CO_2$.

bination of preparative high-voltage electrophoresis and repeated TLC. The following % total incorporations of assimilated $^{14}CO_2$ into known alkaloids were obtained: time 'zero', 0·016; 3 days, 0·155; 6 days, 0·154; 9 days, 0·123; and 14 days, 0·088.

The changes in alkaloid concentrations in the second experiment with 6-week-old plants are shown in Fig. 1. The observed decrease in the total alkaloid concentration in the plant was unexpected, since it had increased during the same time interval in the previous sequential study. Moreover, this decrease was the same (within experimental error, 50 ± 4%) for lyfoline, cryogenine, lythrine, and nesodine; the decrease in sinicuichine was 73%. If the alkaloid concentration decreases were caused by net alkaloid degradation, then the ratio of degradation to synthesis was nearly identical for all alkaloids except sinicuichine. A more plausible explanation is that the nonalkaloidal plant constituents increased more rapidly than the alkaloids, causing a decrease in alkaloid concentrations. For this reason, the total activities of all alkaloids were corrected to account for the alkaloid decrease. Sinicuichine was assumed to undergo a net degradation of 23% (73–50%). These corrected total activities are shown in Fig. 2.

Figure 2 shows that the rate of synthesis of the alkaloids is in the order of cryogenine > lyfoline > sinicuichine > lythrine > nesodine, and that the rates for lyfoline and cryogenine are very similar. The study of sequential alkaloid formation had shown that lythrine was synthesized more rapidly than sinicuichine. The reason for this discrepancy is not known. However, the concentration of sinicuichine appears to be more variable than those of the other alkaloids.

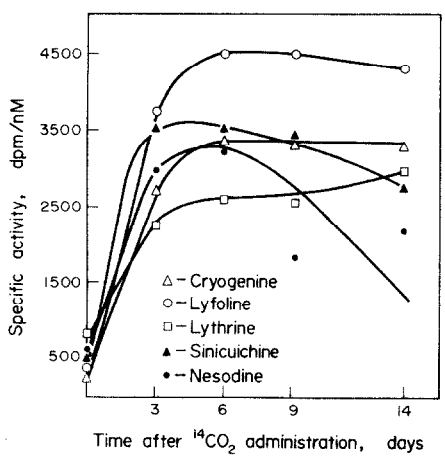


Fig. 3. Specific activities of alkaloids from *H. salicifolia* after exposure to $^{14}CO_2$.

The corrected total activity data also show that sinicuichine and lyfoline underwent net degradation, while cryogenine was degraded very slowly, if at all. No conclusions can be drawn about lythrine or nesodine degradation, since the total activity of lythrine was still increasing at the end of the experimental sequence, and since nesodine is probably still radiochemically impure (Experimental).

Alkaloid interconversions

Based on theoretical considerations, several requirements have been established in order to demonstrate precursor relationships in a $^{14}CO_2$ feeding experiment. If A is a precursor of B, and if there are no stable intermediates between them, then the specific activity of A is greater than that of B before B reaches its maximum specific activity; when B has reached its maximum specific acitivity, the specific activities of A and B are equal. Finally, after B has reached its maximum specific activity, the specific activity of B is greater than that of A [18]. In addition, in a short-term feeding experiment the specific activity ratio of A–B should exceed the inverse mole ratios of the compounds in the plant, this difference becoming greater with shorter exposure time [8]. If these conditions are not met, then A cannot be a precursor of B.

The specific activity changes for the alkaloids are shown in Fig. 3. These data suggest that sinicuichine is a precursor of cryogenine; however, the specific activity ratio does not exceed the inverse molar ratio. Cryogenine can also be isolated from the plant in relatively large quantities before sinicuichine can be detected.*

The only interconversions which meet both of the prerequisite specific activity relationships are the conversion of lyfoline or cryogenine to lythrine, if the specific activities at time "zero" are not considered. The conversion of lyfoline to lythrine would only require the methylation of a phenolic group (R_1) while the transformation of cryogenine to lythrine would require a *cis–trans* isomerization

* The maximum amount of sinicuichine which could escape detection was less than 1 μg.

Table 3. UV and chromatographic data of heimia alkaloids

Alkaloid	λ_{max}	Color with *p*-nitroaniline spray	R_f*	Average TLC recovery
Dehydrodecodine	287	Fuchsia	0·08	†
Lyfoline	282	Gray	0·15	98·7 ± 1·9%
Heimidine	292	Orange	0·21	†
Lythridine	292·5	Orange	0·28	†
Cryogenine	285	Purple	0·54	98·3 ± 1·7%
Lythrine	285	Gray–lavender	0·68	97·0 ± 2·7%
Sinicuichine	285	Purple	0·78	97·4 ± 2·8%
Nesodine	281	Gray–lavender	0·86	96·4 ± 3·0%

* Al_2O_3–GF_{254}; NH_3-saturated C_6H_6–MeOH (19:0·6).
† Since these alkaloids were absent from all plant samples used in this study, they were not included in the TLC recovery study.

of the quinolizidine ring junction. Although the latter conversion can not yet be ruled out, neither a *cis–trans* nor *trans–cis* isomerization has yet been reported *in vitro* for the biphenylquinolizidine alkaloids. The data do not prove that lythrine originates from either lyfoline or cryogenine; however, the specific activity of lythrine is still increasing after the specific activities of the other alkaloids have either reached a maximum or decreased. This suggests that lythrine is derived from a highly labeled precursor which is relatively stable metabolically. Both lyfoline and cryogenine meet this requirement. However, the specific activity of lythrine is larger than the specific activity of either lyfoline or cryogenine at time "zero".

Data obtained from $^{14}CO_2$ feedings of other alkaloid-containing plants strongly suggest the formation of a rapidly saturated C_1 pool from $^{14}CO_2$ [8, 13, 19], i.e. the specific activity of a methyl carbon atom is greater than that of the average carbon atom after a short period of $^{14}CO_2$ metabolism. The relatively high proportion of activity in methyl groups tended to decrease rapidly as other parts of the molecule became labeled. If a high proportion of the activity of the heimia alkaloids also occurs in methyl groups after a short period of $^{14}CO_2$ metabolism, then any alkaloid with more methyl groups than lyfoline might also be expected to have a larger specific activity at time "zero". Lythrine, cryogenine, nesodine, and sinicuichine each contain one more methyl group than lyfoline, and each of them except cryogenine does have a larger initial specific activity than lyfoline (Fig. 3). The slightly lower specific activity of cryogenine compared to that of lyfoline could indicate that cryogenine has a smaller pool of an immediate precursor. It therefore appears that methyl group carbons of heimia alkaloids are also labeled to a greater extent than other carbon atoms at time "zero". Since the time "zero" specific activity of lythrine can thus be expected to be larger than that of lyfoline, the data are now fully consistent with the methylation of lyfoline to form lythrine. Although the conversion of cryogenine to lythrine requires only an isomerization, the threefold higher specific activity of lythrine as compared to that of cryogenine at time "zero" appears to rule out the possibility of this transformation. These same conclusions are also supported by the data obtained from the preliminary $^{14}CO_2$ feeding experiment.

An attempt to feed G-^{14}C-cryogenine, isolated during this investigation to intact plants showed that the hydroponically administered cryogenine was extensively degraded in the plant (*ca* 98%). Another feeding technique will therefore have to be developed before the conversion of lyfoline to lythrine can be conclusively established.

Since the methylation of lyfoline to form lythrine is the only interconversion which is strongly supported by these preliminary data, it appears that there may be one or more branch points in the biosynthetic pathway of the alkaloids. However, additional work will be needed to solve this problem.

EXPERIMENTAL

General methods. Heimia alkaloids were shown to produce somewhat distinctive colors with diazotized *p*-nitroaniline (phenol reagent) which aided in their identification. Sensitivity of the alkaloids to this reagent was found to be 100× greater than to Dragendorff's reagent. The colors produced with the phenol reagent, the R_f values in the primary TLC system, and the average recovery of alkaloids from five duplicate TLC plates are given in Table 3. The overall recovery of lyfoline after a second TLC purification was 96·7 ± 1·7%.

Reagent grade MeOH was purified by adding Norit-A (500 mg/l.) and boiling for 10 min. The hot suspension was then filtered through Whatman No. 50 filter paper. The TLC adsorbents were purified by boiling a 50% w/v slurry of adsorbent in purified MeOH for 5 min, filtering the slurry and washing the adsorbent 3× with portions of fresh solvent. This procedure was repeated 3×; the air-dried adsorbent was further dried in an oven at 100° for 3 hr. Whatman 3 MM chromatography paper for electrophoresis was washed successively with redistilled C_6H_6, 0·1 N aq HCl, dis H_2O, and purified MeOH by allowing the solvent to flow downward over the paper in a chromatography jar.

$Ba^{14}CO_3$ (25·32 μCi/mg and 255·10 μCi/mg) was obtained from New England Nuclear Corp., and ^{14}C-toluene standard ($8·29 \times 10^5$ dpm) was purchased from Amersham/Searle Corp.

Growth and harvesting of H. salicifolia. For the sequential study, seeds obtained from plants grown in the greenhouse were germinated in sterile Petri dishes containing filter paper and sterile tap H_2O. The dishes were wrapped in aluminum foil and placed in a controlled environment chamber (Sherer, model CEL 37-14, 12-hr day temp 21°, and night temp 16°). The water was decanted and replaced with sterile tap H_2O every 2 days. After germination occurred (4 days), the aluminum foil was removed and the seedlings were exposed to light (photoperiod, 12 hr, 15500 lx). Microbial growth was not visible at any time. After 1 month, plantlets were transferred to soil and placed in a greenhouse (day temp 21°, and night temp 16°; natural illumination).

After harvesting, all plants were immediately dried at 40° for 8 hr. Because of the small size of plants harvested before 3 months of age, the entire plant was used for extraction. After the plants were 3-months-old, only the above ground portions were used.

For the $^{14}CO_2$ feedings, seeds obtained from plants grown in the greenhouse were germinated in soil and grown in a greenhouse (day temp 21°, and night temp 16°; natural illumination).

Alkaloid extraction. Dried plant material (*ca* 500 mg dry wt per sample) was ground with sand, and 1% w/v HCl (100 ml) was added. After heating to 100°, the mixture was blended at high speed in a Waring blender for 2 min. The mixture was again heated to 100° and filtered through Whatman No. 1 filter paper. The marc was washed three times with hot 1% HCl (20 ml portions). The combined acid extracts were cooled to room temp, brought to pH 8·5 with $NaHCO_3$ and extracted repeatedly with $CHCl_3$–MeOH (3:1) (50 ml portions) until the organic phase no longer gave a positive Dragendorff or phenol test. The combined organic phases were evaporated to dryness *in vacuo* at 30°, the residue was dissolved in 1 ml of MeOH, and EtOAc was added (50 ml). This soln was repeatedly extracted with acetate buffer (pH 5·0, 25 ml portions) until the organic phase gave a negative alkaloid test. The pH of the combined aq phases was adjusted to 8·5 with $NaHCO_3$ and this soln was again repeatedly extracted with $CHCl_3$–MeOH (3:1) as before. The combined organic phases were concentrated to dryness *in vacuo* at 30° and the resulting residue was dissolved in MeOH and divided into two parts from which the alkaloids were separated and quantitated separately.

Alkaloid separation. The alkaloids were chromatographed (TLC) on Al_2O_3–GF_{254} using NH_3-sat C_6H_6–MeOH (19:0·6). The zones were visualized by quenching of UV fluorescence, removed with a micro-type vacuum zone collector and eluted with MeOH by shaking the mixture on a rotary shaker for 2 hr. The mixture was filtered through Whatman No. 50 filter paper, and the Al_2O_3 was washed 3× with MeOH. The lyfoline fraction was rechromatographed (SiO_2–GF_{254}, NH_3-sat C_6H_6–MeOH 9:2), visualized, removed from the plate, and eluted as before.

Alkaloid quantitation. The UV absorbance of each appropriately diluted eluate was determined at the λ_{max} (Table 3) of the alkaloid contained therein. The absorbances were corrected by subtracting the absorbance of a similarly diluted extract of TLC adsorbent obtained from the same plate on which the alkaloids were separated. The concn of each alkaloid was determined by comparison with a standard curve.

The chromatographic recovery of each alkaloid was determined by applying a soln containing an aliquot of a standard soln of each alkaloid to 5 chromatograms and by obtaining an alkaloid eluate as described. The corrected absorbance of each eluate was obtained and this was compared with the absorbance of an aliquot of unchromatographed standard soln.

$^{14}CO_2$ feedings. $^{14}CO_2$ was administered to plants in an 8·81 l. desiccator to which tygon tubing had been attached for gas circulation. $^{14}CO_2$ was generated by adding 3·75 N H_2SO_4 dropwise from a burette to $Ba^{14}CO_3$ (preliminary exp, 225·7 mg, 25·32 μCi/mg, CO_2 concn, 0·32%; second expt, 78·5 mg, 255·10 μCi/mg, CO_2 concn, 0·11%) contained in a 25 ml tube; this tube was located inside a 250 ml vacuum flask. (CO_2 concn in the atm is about 0·03%.) A diaphragm pump (flow rate 1·26 l./min) was inserted between the desiccator and the $^{14}CO_2$ generator. Two bypass valves were inserted in the hose system to permit the circulating atmosphere to be diverted through a NaOH (2·5 N) trap for removal of the initial $^{12}CO_2$ and the $^{14}CO_2$ remaining at the end of the exposure period. The entire feeding apparatus was placed inside the controlled environment chamber (26° ± 1°, 15000 lx). The r.h. in the feeding apparatus was about 78%.

Six-week-old plants were placed inside the desiccator and the lid was sealed with calking. After trapping initial $^{12}CO_2$ for 20 min, the $^{14}CO_2$ was generated and circulated through the system for 2 hr. At the end of this time, the remaining $^{14}CO_2$ was trapped for 40 min. Plants were either harvested at 11 am, immediately placed in a drying oven at 40° and dried for 8 hr, or returned to the greenhouse for additional ^{14}C metabolism.

Radioactive alkaloid purification. Each labeled alkaloid was further purified by preparative high voltage (Camag HVE system) electrophoresis (3000–3350 V, 95 mA) on Whatman 3 MM chromatography paper. Pyridine–acetic acid buffer (pH 4·5) was used as the electrolyte and the voltage was applied for 45 to 60 min. Alkaloid zones were visualized by spraying a 3·5 cm wide strip from one long edge of the pherogram with diazotized *p*-nitroaniline [16]. The corresponding alkaloid zone was cut from the paper and the alkaloids were eluted by allowing 50 ml purified MeOH to flow downward over this strip. An aliquot of the MeOH eluate was used for UV quantitation.

The following additional preparative TLC systems were used to purify each alkaloid to constant sp act:

SiO_2–GF_{254}: $CHCl_3$–MeOH, 9:1 (lyfoline and lythrine); EtOAc–EtOH, 9:1 (lyfoline); and NH_3-satd C_6H_6–MeOH, 9:2 (lyfoline and lythrine).

Al_2O_3–GF_{254}: NH_3-satd $CHCl_3$ (cryogenine and sinicuichine); and NH_3-satd C_6H_6–MeOH, 19:0·6 (cryogenine, lythrine, and sinicuichine).

Since the small quantities of nesodine isolated from the plant did not permit electrophoretic purification, it was purified by two-dimensional TLC on SiO_2 using $CHCl_3$–MeOH (9:1) followed by NH_3-satd C_6H_6–MeOH (9:2). However, the radiochemical purity of nesodine at this point was questionable.

Radioactivity measurements. An aliquot (*ca* 2,000 cpm) of a MeOH soln of each alkaloid was dissolved in modified Bray's soln* [20] and counted 4× to a s.d. of ± 1% or less in a Packard Tri-Carb Liquid Scintillation Spectrometer. An aliquot of

* Bray's soln was modified by substituting dimethyl POPOP for POPOP.

each chromatographic or electrophoretic blank was also counted to determine background. The counting efficiency of each sample was determined by spiking with ^{14}C-toluene standard.

Assimilation of $^{14}CO_2$ by plants was determined by counting an aliquot of the NaOH trapping soln in modified Bray's soln containing Cab-O-Sil [21]. Each counting sample was prepared in duplicate.

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