THE BIOSYNTHESIS OF THREE MACROCYCLIC PYRROLIZIDINE ALKALOIDS RETRORSINE, SENECIPHYLLINE AND SENECIONINE USING SENECIO VULGARIS

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SUMMARY

Senecio vulgaris, a plant containing 3 macrocyclic pyrrolizidine alkaloids (PAs) was grown under atmospheric CO_2, $^{14}CO_2$, and $^{13}CO_2$ conditions. This report describes the plant growth chamber, the growing conditions the plants were subjected to and the amounts of macrocyclic PAs isolated from the plants. In addition, a number of radiolabelled experiments are described including a single 25 mCi pulse $^{14}CO_2$ experiment, a dual $^{13,14}CO_2$ experiment and an 800 mCi $^{14}CO_2$ experiment. The ^{14}C labelled macrocyclic PAs isolated from these experiments (retrorsine, seneciphylline and senecionine) are presently being used in various metabolism studies.

Key Words: Pyrrolizidine Alkalolids, Senecio vulgaris, Retrorsine, Seneciphylline, Senecionine

INTRODUCTION

Plants containing pyrrolizidine alkaloids (PAs) are found worldwide and include such diverse botanical families as the Compositae, Leguminosae and Borginaceae (1). In the western United States plants which contain PAs such as Amsinckia intermedia (fiddleneck), Senecio vulgaris (common groundsel) and S. jacobaea (tansy ragwort) are rapidly spreading. These plant derived toxins have been shown to contaminate human and animal feed sources (2). This contamination may be highly significant, since PAs are responsible for numerous toxic syndromes (3,4) and are proven mutagens and carcinogens (5-10).

One of the greatest drawbacks in PA research has been the lack of ^{14}C radiolabelled compounds, especially macrocyclic PAs. Senecio vulgaris for example, contains at least 3 known toxic macrocyclic PAs, seneciphylline, senecionine and retrorsine (fig. 1), which may be rapidly isolated using high
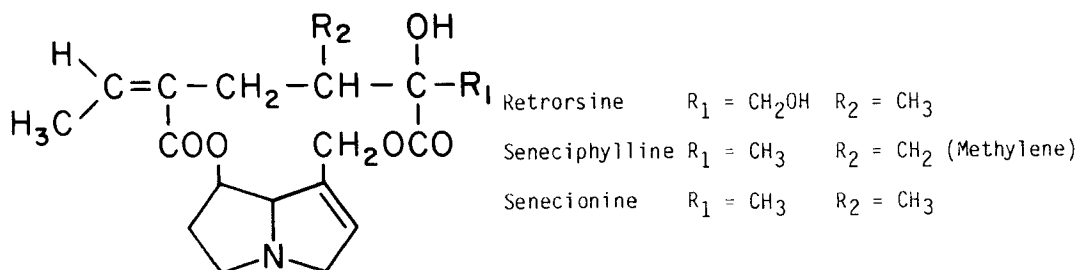

Retrorsine $R_1 = CH_2OH$ $R_2 = CH_3$

Seneciphylline $R_1 = CH_3$ $R_2 = CH_2$ (Methylene)

Senecionine $R_1 = CH_3$ $R_2 = CH_3$

Figure 1

performance liquid chromatography (11). None of these macrocyclic PAs have been previously ^{14}C-radiolabelled via chemical synthetic methods. Only recently have two macrocyclic PAs, dicrotaline and crobarbatine acetate, been successfully synthesized (12,13). Unfortunately, dicrotaline and crobarbatine acetate differ structualy from the PAs found in the western U.S. and may not exhibit the same toxic effects (1,2).

A number of laboratories have attempted to bypass the problem of obtaining radiolabelled macrocyclic PAs by ^{3}H labelling the C-9 position of either retronecine, retronecine derivatives or indicine N-oxide (14-16). The disadvantage of potential ^{3}H exchange(s) in future metabolism and toxicological studies was a strong advocate to proceed with ^{14}C labelling.

Numerous investigators have attempted to determine via ^{14}C biosynthetic studies which compound(s) (ornithine, putrescine, arginine, proline, spermidine, spermine) are the specific precursors of the PA retronecine (17-25). These investigators have not had as their primary objective the

isolation of ^{14}C-radiolabelled macrocyclic PAs, exhibiting the specific activity and quantities required for use in metabolic and toxicological studies. Specific precursors such as ornithine or putrescine have the disadvantage of being expensive and limit the amount of ^{14}C PA that may be obtained. In addition, the vast majority of either ornithine or putrescine is incorporated within the necine base (20), which could potentially limit an investigator searching for an elusive metabolite. To obtain uniformly ^{14}C-radiolabelled PAs from S. vulgaris in the quantity and specific activity necessary for metabolism as well as toxicological studies, a series of CO_2 biosynthetic experiments using S. vulgaris were performed.
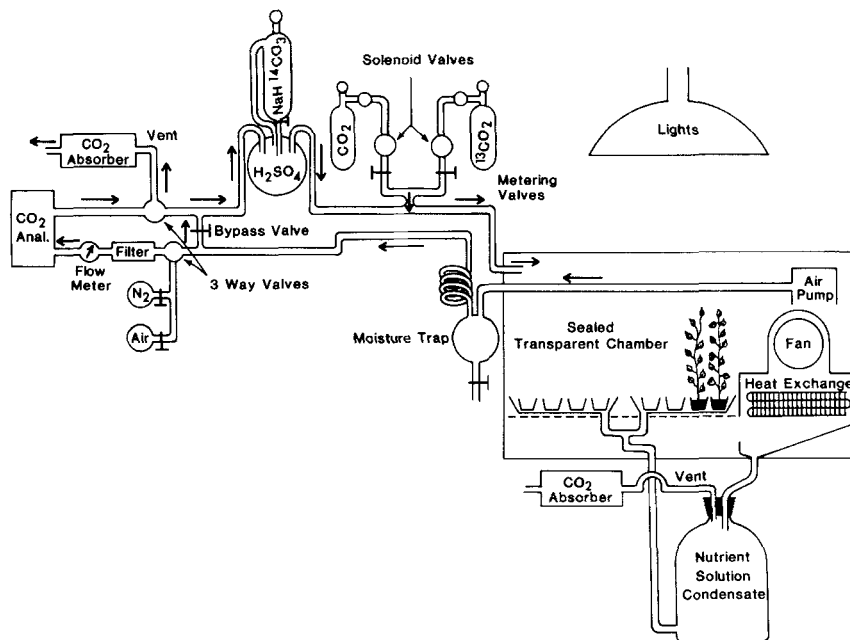

Figure 2

Illustration of plant growth chamber used to grow nonradiolabelled and radiolabelled S. vulgaris plants.

MATERIALS AND METHODS

Chamber (fig. 2):

A 124 cm x 71 cm x 59 cm chamber (511 liters) was constructed of 1/4 in plexiglas with a removable, gasketed lid held in place by ten draw catches. A 30 cm x 55 cm x 7.5 cm finned heat exchanger, made to order by Colmac Coil, Inc., Colville, WA. was mounted at one end with a centrifugal blower. Temperature control was achieved by pumping chilled water from a refrigerated water bath through the heat exchanger, while the blower circulated chamber air over the fins. The sensing bulbs of a "day" and a "night" thermostat were located in the air flow, and one or the other was selected by a 24-hour timer to control power to the pump, thus maintaining the chosen temperature. A heater was not needed for the temperature range of these experiments.

Illumination was accomplished with a single 1500-watt metal halide lamp, and four 100-watt incandescent lamps. Photoperiod was controlled with a 24-hour timer. A light diffuser of transluscent plexiglas (pyramid pattern) was hung 30 cm below the halide lamp.

A perforated steel mat with 1.25 cm x 1.25 cm openings was mounted ten cm from the chamber floor to support the plant trays while permitting free air circulation.

A plexiglas condensate trough was positioned under the heat exchanger and fitted with a drain tube which led to a 5 gallon polyethylene carboy of nutrient solution. The carboy had a tube connected to the bottom of each of the four plastic trays holding the potted plants, and a vent connected to a drying tube with ascarite carbon dioxide absorbant. During operation, the plants were watered as necessary with Hoagland's solution (26) by raising the carboy sufficiently to flood the trays, thus saturating the soil in the pots. Upon lowering the carboy, the excess solution drained from the trays to

the carboy. The water that transpired and evaporated from the pots condensed on the cool heat exchanger, dripped into the condensate trough, and returned to the carboy. The carboy was protected from light to minimize algae growth. Any dissolved carbon dioxide was thus retained in the system.

A aquarium air pump was mounted within the chamber and connected to the carbon dioxide analyzer and control system via bulkhead fittings through the chamber wall. The air flow first passed through a glass water trap followed by a heated 6 ft copper coil to raise the temperature of the air above the dew point. It then progressed through a particle filter, a flow meter, the sample tube of a Horiba model PRI-2000 infrared gas analyzer, a $^{14}CO_2$ generator and returned to the chamber. In addition, there were two three-way valves for the entrance and exit of calibration gases, a bypass for shunting flow in excess of 1 liter per min around the infrared analyzer, and a side channel for admission of CO_2 and $^{13}CO_2$ from the compressed gas cylinders.

The $^{14}CO_2$ Generator:

The $^{14}CO_2$ generator consisted of a one liter round bottom 3-neck pyrex flask equipped with an addition funnel, air inlet, air outlet and a Teflon coated stirbar. The flask was mounted inside a plastic spillage container over a magnetic stir plate.

Summary of the CO_2 System:

This system fulfills 3 functions as it analyzes the overall carbon dioxide concentration of the air in the chamber; controls the overall carbon dioxide concentration by the addition of CO_2, $^{13}CO_2$ or a mixture of both and provides for the addition of $^{14}CO_2$. The operation of each function is as follows:

CO_2 Analysis:

The infrared analyzer was calibrated by operating the two three-way valves to connect the entrance port to the nitrogen and air calibration tanks and the exit port to the atmospheric vent (an ascarite column was connected to the vent to absorb any released $^{14}CO_2$ or $^{13}CO_2$). The flow of nitrogen was set at 1 liter per min and the analyzer was adjusted to give a zero reading.

Approximately 330 ppm of CO_2 in compressed air was similarly admitted and used to set the analyzer's span at a known, but arbitrary percentage of full scale. The analyzer is fitted with a linearizer, and therefore this two-point calibration is sufficient to determine the CO_2 concentration of the air from the chamber.

CO_2 Control:

The electrical output signal of the analyzer is connected to a Linear Instruments Model 555 chart recorder and a Calex voltsensor. The voltsensor contains an electronic comparator which compares the analyzer's signal to a potentiometer-selected reference voltage, and pulls in relay contacts if the CO_2 reading is too low. These contacts in turn switch power to two solenoid valves admitting CO_2 and $^{13}CO_2$ from compressed gas cylinders. The flow rate of each is proportional to the setting of the cylinders' pressure regulators and the associated Nupro needle valves. Reproducible ratios between the additions of the two isotopes are thus possible.

CO_2 Regulation and Generation of $^{14}CO_2$:

The CO_2 set point was adjusted to maintain total CO_2 concentration in the air between 350 and 550 ppm during the day. The timer was set to turn off the CO_2 before the lights went off, allowing the CO_2 concentration to drop to about 100 ppm in anticipation of respiratory CO_2 buildup during the night. As the plants approached maturity, night time concentration would reach levels in excess of 1000 ppm and would require from 45-90 min after the onset of the photoperiod, before CO_2 addition would be triggered at the lower set point.

Excess 18N H_2SO_4 was placed in the generator flask. A Kontes addition funnel (#K 63620, Kontes Glass, Vineland, NJ.) was modified by removing the screw thread drop control mechanisms. The upper glass thread was replaced by a 24/40 joint and stopper. The inner glass valve seat was removed and replaced by a Pyrex No. 2 stopcock with Teflon plug and in tandem below it a Teflon plug stopcock equipped with needle valve control (AB Glass Inc. Vineland, NJ.) The high specific activity $NaH^{14}CO_3$ solution was transferred from the ampules in which they were received into the addition funnel with the aid of sufficient 0.1N NaOH, to dilute it to a convenient concentration. The drop rate was set to deliver $^{14}CO_2$ at a constant rate during the hours when CO_2 and $^{13}CO_2$ were being added. The $^{14}CO_2$ was turned on and off manually at the beginning and end of the period.

Lighting

Lighting was adjusted to be fairly uniform over the area occupied by the plants, and provided an intensity of 500-700 μEinsteins/m^2/sec at pot height and 600-800 μEinstein/m^2/sec at 23 cm above pot height. The day length was set at 12 hr light and 12 hr darkness. The temperature inside the chamber was set for 26°C day and 15°C night.

Plant Nutrient Solution:

In these experiments, 800 ml Hoagland's solution (10X) was diluted to 16 liters with distilled water to provide 1/2 X Hoagland solution. The plants required watering approximately once a week during their early growth stages and as often as every 48 hr when fully grown. Small amounts of the 10X solution were added to the carboy as deemed necessary according to the appearance of the plants.

Radioactive and Stable Isotope Sources.

The $NaH^{14}CO_3$ used in the dual label (250 mCi) and 25 mCi pulse experiments was purchased from Amersham Corp, Arlington Heights, IL. The $NaH^{14}CO_3$ used in the 800 mCi experiment was obtained from Rose Chem, Los Angeles, CA. Carbon-^{13}C dioxide was purchased from Prochem, Summit, NJ.

Radioactive Safety Precautions:

Large polyethylene bags were placed in the hood to receive solid waste. Prior to and during the harvest of the plants, radioactive safety precautions in compliance with NIOSH regulations and the University of California Environmental Health and Safety Committee were performed.

The soil in the pots was somewhat damp to prevent any dust on the day of harvest. The chamber was opened to air out one hour prior to harvesting the plants. The trays containing the potted plants were quickly disconnected from the drains and transferred to the spillage trays in the hood. A quick survey with a geiger counter indicated that both the plants and the soil were intensely radioactive. The pots were placed one by one into a bag, the pot knocked away from the root ball and the plants quickly separated from the vermiculite and sand. No attempt was made to save the fine roots which were discarded along with the pots and soil.

The plants, after being quickly cut with scissors into pieces about 1" long into 4 liter beakers, were covered with methanol and minced with a homogenizer. At this stage the beakers were set aside, the area decontaminated and the bags of radioactive waste sealed and disposed. After 24 hours the methanol was decanted through a paper filter with the aid of a siphon into 5 liter round bottom flasks and fresh methanol added to the plant residue. This process was repeated each day for a total of 4 methanol changes. The last methanol extract plus the plant residue was transferred to a soxhlet apparatus containing a cellulose thimble. The beakers and filter were rinsed with enough fresh methanol to fill the soxhlet. The plant material was extracted another 48 hours on the soxhlet, the methanol extract siphoned into a single neck 5 liter round bottom flask and solvent removed under reduced pressure.

The concentrated extract was worked up in the standard manner and purified by HPLC (27).

RESULTS AND DISCUSSION

Non Radioactive Plant Experiment - Optimization of Conditions

To establish optimum growing conditions and PA yield prior to $^{14}CO_2$ radiolabelling studies, $\underline{S}$. $\underline{vulgaris}$ was grown using the plant growth chamber. Studies by A. Kery which had examined the PA content of $\underline{S}$. $\underline{vulgaris}$ through its various growth stages were carefully consulted (28), although no prior studies regarding the growing of $\underline{S}$. $\underline{vulgaris}$ in a sealed plant growth chamber were found.

$\underline{Senecio}$ $\underline{vulgaris}$ seeds were collected from plants native to the Davis area and planted in peat pots. Approximately 14 days later when the seedlings had reached the 4 leaf state (1.5 cm high), they were transplanted, 4 seedlings per 3" plastic pot to a mixture of 50% vericulite-50% sand. The plants were grown in the greenhouse an additional 8 days.

TABLE 1

Growth Trial of Senecio vulgaris

Harvest Number	Days in Chamber	Number of Pots Per Harvest	Plant wet wt in grams per 12 pots			Crude PAs in mg per 12 pots			PA yield as % of wet weight	
			Tops	roots	total	tops	roots	total	tops	roots
1	12	10	65.9	35.3	101.2	18.1	7.3	25.4	.028	0.21
2	18	12	92.3	45.6	137.9	30.6	-	-	.033	-
3	21	12	112.1	48.4	160.5	30.7	11.2	41.9	.027	.023
4	26	12	129.6	57.3	186.9	47.5	11.1	58.6	.037	.019
5	30	12	166.3	56.2	222.5	50.7	13.7	64.4	.030	.024
6	34	12	190.7	55.5	246.2	66.2	10.1	76.3	.035	.018
7	39	10	153.0	35.8	188.8	80.9	12.7	93.6	.053	.036

Top = leaves and stems

- = PA concentration not determined

Eighty pots (320 plants), 22 days old, each approximately 2.5 cm high and possessing several leaves, were transferred to the plant growth chamber. The plants were grown in the chamber according to the techniques previously described. The chamber was opened at selected intervals and pots removed from random positions in the chamber (Table 1). The PA contents were determined at 7 separate time points with the final harvest using plants 61 days old (39 + 22).

$^{13}C, ^{14}C$ Senecio vulgaris Experiment

To determine if the carbons of the macrocyclic PAs of S. vulgaris would be uniformly radiolabelled in $^{14}CO_2$ experiments, a dual labelled $^{13}CO_2$ (2.94 mol) and $^{14}CO_2$ (250 mCi) experiment was performed.

Senecio vulgaris plants were started from seed, the plants transferred to the chamber and grown as previously described. After three days in the chamber, the $^{14}CO_2$ was added from days 3 through 21 in fairly even increments. The plants were allowed to metabolize any remaining $^{14}CO_2$ for 3 days prior to harvesting them. The $^{13}CO_2$ was started simultaneously with the $^{14}CO_2$ and delivered over the same period of time as the $^{14}CO_2$, but in proportion to the CO_2 used by the plants.

Following the standard workup, 223 mg crude alkaloids were isolated. In addition, 100 mg of non-radiolabelled mixed PAs were added to the hexane and ethyl ether residues to yield a radiolabelled PA of lower specific activity (^{13}C and ^{14}C). An aliquot of the crude PAs yielded by HPLC analysis senecionine 26.4%, seneciphylline, 22.3%, and retrorsine, 9.8% (27). The specific activities were senecionine 0.52 µCi/ mol, seneciphylline 0.40 µCi/ mol, retrorsine 0.34 µCi/ mol. Radiolabelled yield based on 174 µCi total in the 3 purified alkaloids was 0.07%. Uniform incorportion of ^{13}C based on ^{13}C-NMR varied slightly for each carbon atom in the molecule with ^{13}C enrichment ranging from 5.25 to 6.5% (29-31).

Pulsed $^{14}CO_2$ Experiment:

To determine the optimum conditions to produce the highest specific activity and yield of the PAs, a single 25 mCi pulse of $^{14}CO_2$ was provided to S. vulgaris plants.

Seedlings from S. vulgaris were raised as previously described. On day 0, the plants were pulsed with 25 mCi $^{14}CO_2$ over a half-hour period. The chamber was briefly opened for partial harvests and ten pots (40 plants) removed from random locations in the chamber at each interval (Fig. 3). If the plants required watering on the day of harvest, the watering was performed immediately after resealing the chamber.
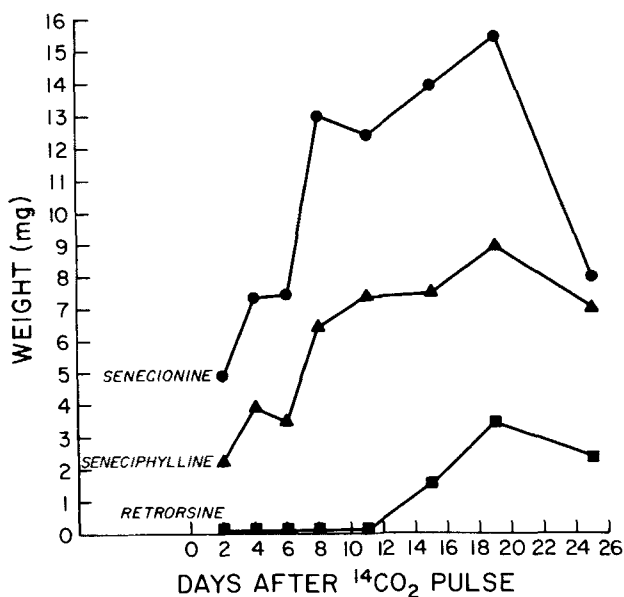
Figure 3

Total pyrrolizidine alkaloids (retrorsine, seneciphylline and senecionine) isolated at 8 different intervals following a single 25 mCi pulse of $^{14}CO_2$. The maximum pyrrolizidine alkaloid concentration occurred 19 days following the $^{14}CO_2$ pulse.

The dried crude PAs from each harvest were isolated as previously described, dissolved in distilled methanol, and weighed. Two ml aliquots were weighed, evaporated to dryness and reweighed. Aliquots were subjected to analytical HPLC measurements of the PAs made at 218 nm UV and refractive index (27). The concentrations of the PAs (senecionine, seneciphylline and retrorsine) were calculated using PA standards. The maximum yield of purified PAs by weight occurred 48 days following planting, or 19 days from the $^{14}CO_2$ pulse (fig. 3). Aliquots from the original solutions and from the HPLC fractions were counted for ^{14}C activity to obtain the radiolabelled yields and the specific activities.

From Figs. 4,5 the maximum radiolabelled yield of the purified PAs appeared to occur 8 days after the $^{14}CO_2$ pulse. However, the maximum radioactivity may be as late as day 15 if the total radioactivity of the crude extract, the three PAs plus the non-macrocyclic PA peak (which may contain valuable radioactive compounds such as retronecine and the necic acids) are included.
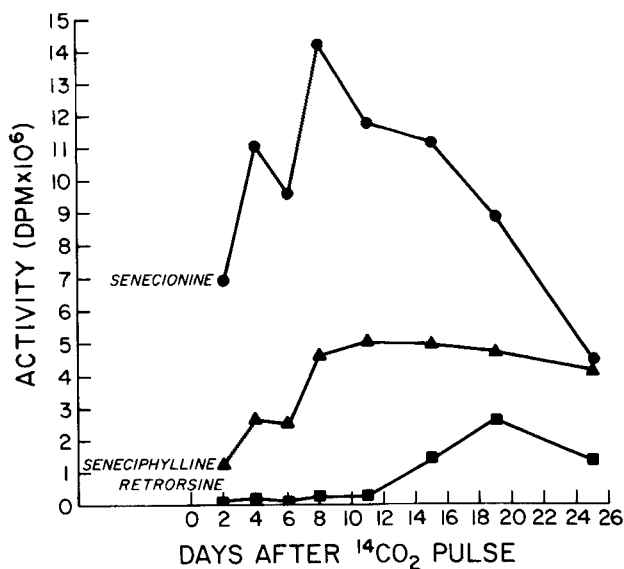

Figure 4

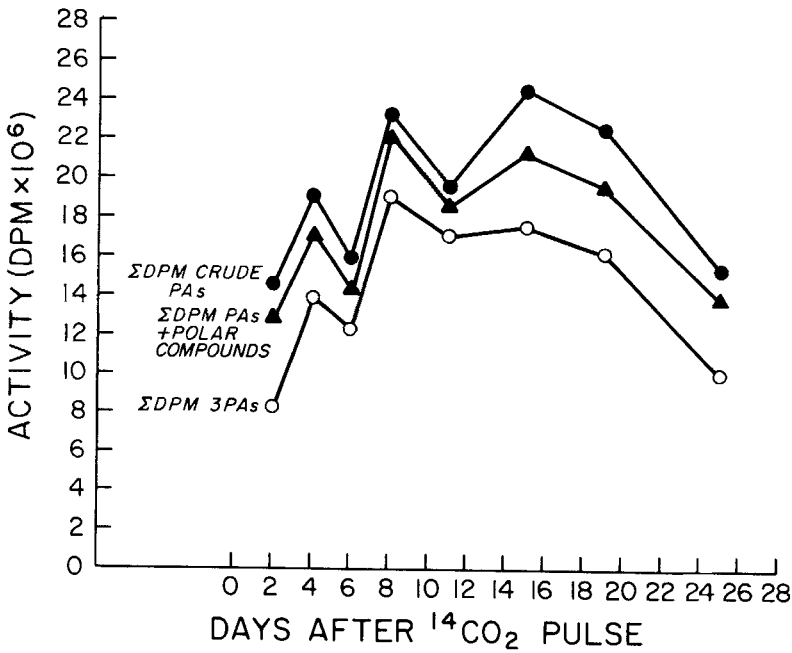The total radiolabelled activity of each of the 3 PAs following a single 25 mCi $^{14}CO_2$ pulse.

Figure 5

The total radiolabelled activity ΣDPM crude pyrrolizidine alkaloids,
  ΣDPM pyrrolizidine alkaloids plus polar compounds and the ΣDPM of
the 3 pyrrolizidine alkaloids (retrorsine, seneciphylline and senecionine),
following a single 25 mCi $^{14}CO_2$ pulse.

The maximum specific activity occurred between days 3 and 4 following the
$^{14}CO_2$ pulse (Fig. 6). The plants in late maturity had ceased biosynthesizing
PAs approximately day 19 (day 48 after planting) as verified by the constant
specific activity and the drop in PA yield by weight between day 19 and day
25. When the curve for the DPM/μg for the three alkaloids is further
analyzed, it is evident that the ^{14}C is incorporated into the plant tissues
very rapidly and that the anabolism of the alkaloid is also rapid (T 1/2 < 1
day). However, the decay of the specific activity is slower with an apparent
half-life of two weeks and an apparent turnover time of 3 weeks. The slope of
the decay probably indicates not so much a slow catabolism of the alkaloids,
as a continued availability of ^{14}C from its numerous precursors, some of which

may have slow turnovers. In any case, the true catabolism was substantially

slower than the true anabolism as shown by the almost linear increase in the

weight of the isolated PAs from day 2 until day 19 during the active growth of
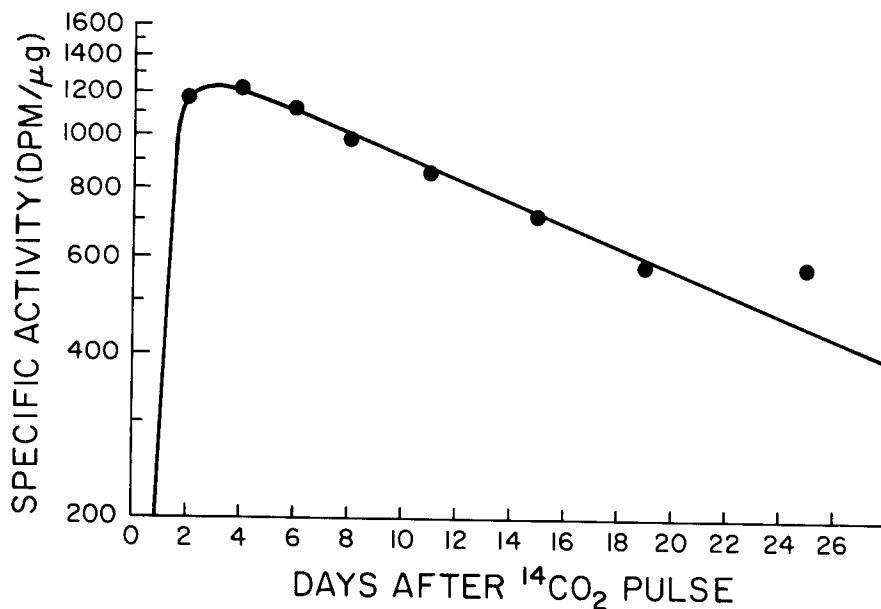
the plants.

Figure 6

The specific activity of the total pyrrolizidine alkaloids on a
semilog plot.

800 mCi Experiment-[14]CO_2:

The 800 mCi experiment was planned following the results of the 25 mCi

pulsed experiment. The seedlings were grown and transplanted as previously

described. The plants were grown under atmospheric CO_2 concentrations in the

chamber for 12 days to insure the viability of the plants and to check all

equipment. The harvest was timed so that the plants would reach maturity, but

not be in a declining growth phase. The first 500 mCi of $^{14}CO_2$ was administered as 50 mCi followed by 75 mCi on alternate days for 8 days. The remaining 300 mCi was provided at a rate of 75 mCi per day for 4 days. The 12 days (inclusive) of $^{14}CO_2$ addition was expected to coincide with active plant growth, quasilinear increase in total PA content and to span somewhat less than one specific activity decay half-life. The plants were harvested five days following the last $^{14}CO_2$ addition, to allow the residual radioactivity in the chamber to subside and to coincide as nearly as possible with the expected time of maximum radiolabelled yield of the PAs.

The results of the 800 mCi experiment are shown in Table II. The yield of purified PAs by weight was rather disappointing, since it was only about one fourth of what we were led to expect by the preliminary experiments. We do not know whether the plants did not produce the PAs in the quantity we expected, or whether a substantial amount was lost during workup.

Analytical HPLC of the senecionine on a Waters C-18 μBondapak column run isocratically with 50% methanol, 50% 0.01 M phosphate buffer (pH 6.43) gave a single peak with the 218 nm UV detector and a radioactive peak beneath the UV peak amounting to 97% of the total radioactivity. A minor radioactive peak corresponding to 1.2% of the total had a retention time corresponding to seneciphylline. Radiopurity checks using different solvent systems and HPLC columns exhibited a radiopurity of 97%, confirming the above result (27).

The goal of obtaining pure PAs of high specific activity in quantities sufficient for numerous metabolic experiments in vitro and in vivo was accomplished. This has allowed the identification of a new PA metabolite, 19-hydroxysenecionine isolated from in vitro mouse hepatic microsomes (32). The use of ^{14}C-radiolabelled PAs should aid a great deal in the isolation of numerous PA metabolites which remain to be identified.

TABLE II

Yield from the 800 mCi $^{14}CO_2$ Experiment

Pyrrolizidine Alkaloids	Total Yield mg	Specific Activity mCi/mmol
Retrorsine	5.5	3.43
Seneciphylline	12.8	2.49
Senecionine	30.3	3.42
Totals	48.6	

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