The Biosynthesis of Conium Alkaloids Using Carbon-14 Dioxide. The Kinetics of ^{14}C Incorporation into the Known Alkaloids and Some New Alkaloids*

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ABSTRACT: The kinetics of ^{14}C incorporation into the alkaloidal fraction of two varieties of *Conium maculatum* (10–12-weeks old) were followed for periods of 0–7 days after 1-hr photosynthesis in an atmosphere of $^{14}CO_2$. The specific activity time curves, their crossover points, and specific activity to pool ratios were consistent with the biosynthetic sequence: $CO_2 \rightarrow \rightarrow$ γ-coniceine → coniine → *N*-methylconiine. Minimum calculated rates of synthesis for γ-coniceine and coniine were about 4 mμmole/hr per g of fresh plant for Leetes' Minnesota variety and 35 mμmole/hr per g for our California variety. The oxygenated bases conhydrine and pseudoconhydrine were not synthesized at a detectable rate, though similar as yet unidentified compounds were. A rapidly labeled compound, accounting for 95% of the ^{14}C in the alkaloidal fraction and turning over completely in 1–2 days was detected in both varieties of *Conium* as well as in *Sedum sarmentosum* and *Punica granatum*, strongly suggesting it may be an intermediate prior to γ-coniceine. No trace was found of the reported precursors, cadaverine, or Δ^1-piperideine, or of valerolactam or 3-ketoindolizidine.

One of the structurally simplest groups of alkaloids known are the propylpiperidine bases γ-coniceine, coniine, *N*-methylconiine, conhydrine, and pseudoconhydrine (see Chart I) of the poisonous hemlock, *Conium maculatum L.* (family Umbelliferae). Similar compounds occur in unrelated plants such as *N*-methyldihydroisopelletierine in *Sedum sarmentosum* (family Crassulaceae), or isopelletierine and 2-(2-propenyl)-Δ^1-piperideine in *Punica granatum* (family Punicaceae).

The biosynthesis of *Conium* and other piperidine alkaloids has been covered in reviews by Gupta (1968) and Spenser (1968) while studies have been reported for *Sedum* (Gupta and Spenser, 1968) and for *Punica* (O'Donovan and Keogh, 1968; Liebisch *et al.*, 1968). Most investigations to date have dealt largely with trying to answer broad "biogenetic" questions (For example, can acetate or lysine, etc., serve as precursors of the piperidine ring and/or the propyl side chain?).

The importance of establishing the order in which individuals, in a family of structurally related compounds, are synthesized (*i.e.*, primacy) is seldom appreciated and is necessary to answer other than broad "biogenetic" questions and indeed to give significance to the latter. This is discussed with particular reference to the opium alkaloids by Stermitz and Rapoport (1961).

A primary role for γ-coniceine in the formation of the other *Conium* alkaloids seems clear from (a) studies on alkaloid variation with plant age (Cromwell, 1956) and within 1 day (Fairbairn and Suwal, 1961), (b) feeding results with specifically labeled γ-coniceine (Leete and Adityachaudhury, 1967), and (c) $^{14}CO_2$ kinetic studies by Dietrich and Martin (1968).

Using the $^{14}CO_2$ kinetic approach we set out to confirm our earlier results supporting the primacy of γ-coniceine, to extend them to the other known alkaloids in two varieties of *C. maculatum* and to search for possible early intermediates[1] formed prior to γ-coniceine. In addition, this approach is unique in providing information on *in vivo* rates of *de novo* alkaloid formation and turnover.

The power of the $^{14}CO_2$ kinetic approach applied to problems of alkaloid biosynthesis is amply illustrated in the opium series (Stermitz and Rapoport, 1961; Martin *et al.*, 1967; Blaschke *et al.*, 1967), in the nicotine series (Liebman *et al.*, 1967) even if controversial (Zielke *et al.*, 1968), and in the *Conium* series (Dietrich and Martin, 1968).

Methods

Alkaloid Standards and Reagents. Coniine was purchased from the Aldrich Chemical Co. *N-Methylconiine* was synthesized as follows. Coniine (2 mmoles), formic acid (10 mmoles), and formaldehyde (2.4 mmoles) (as formalin) were mixed in that order in an ice bath. Refluxing overnight on a steam bath, acidification with 1 N HCl, and evaporation *in vacuo* yielded a clear syrup which crystallized on cooling. Ethanol wash, followed by recrystallization from acetone, gave *N*-methylconiine hydrochloride in 70% yield: mp 187–189°, lit. mp 189–190° (Ahrens, 1902).

γ-Coniceine was synthesized from coniine *via* the *N*-

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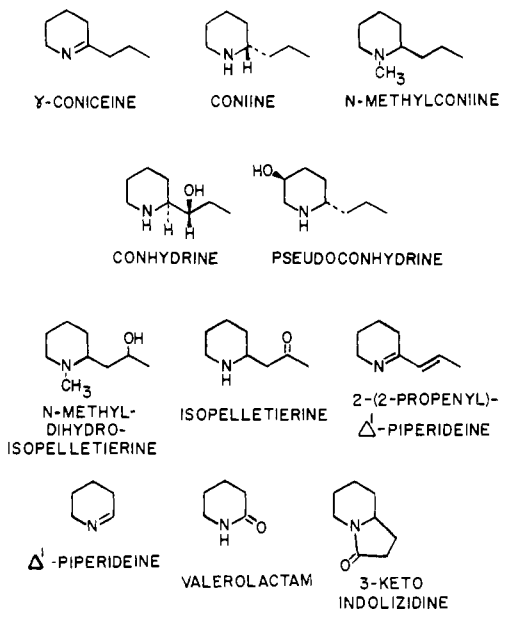
[1] The distinction proposed by Davis (1954) is made between the terms *precursor* as "any substance, whether endogenous or exogenous, that can be converted by an organism into some product" and intermediate as "a compound formed and converted by the organism into a product." This should be extended to provide that the latter is "formed and converted (turned over) at a sufficient rate to account for its proposed products."

CHART I



chloro compound, by the procedure of Grundon and Reynolds (1964) with the following modifications. (a) Methylene chloride was used in place of ether for the chlorination step and subsequent extractions; (b) in the final step instead of removing the methylene chloride and distilling the γ-coniceine, dry HCl gas was passed in the solution until acid followed by evaporation *in vacuo* to give 85% yield of pure γ-coniceine HCl (hydroscopic). Picrolonate had mp 141°.

Conhydrine (racemic) was synthesized by catalytic hydrogenation of 2-pyridyl ethyl ketone (Galinovsky and Mulley, 1948) and recrystallized from ether to give 31% yield (mp 99°).

Pseudoconhydrine was a gift of Professor E. Leete. *Isotripiperideine* (a polymer of Δ^1-piperideine) was prepared according to Schöpf *et al.* (1952) from piperidine using *N*-chlorosuccinimide in place of *t*-butylhypochlorite as the chlorinating agent.

Valerolactam was synthesized from the oxime of cyclopentanone (Scott *et al.*, 1954).

3-Ketoindolizidine was prepared in 87% yield from β-(α-pyridyl)propionic acid (King *et al.*, 1951) by hydrogenation over PtO_2.

Cadaverine was purchased from Sigma Biochemicals. The purity of all compounds was checked in at least two thin-layer chromatography systems, electrophoresis, and on gas–liquid partition chromatography. Satisfactory infrared, nuclear magnetic resonance, and mass spectra were obtained for all compounds and will be published elsewhere. All solvents were reagent grade. Methylene chloride and ethanol were redistilled through a 70-cm column filled with glass helices.

Plant Growth. Seeds of *C. maculatum* were obtained from two sources; those from Berkeley, Calif., kindly supplied by Professor R. Ornduff, Botany Department, University of California, Berkeley, and those from Minnesota, kindly supplied by Professor E. Leete, Chemistry Department, University of Minnesota, Minneapolis, Minn. Since the designation of "variety" is of horticultural rather than taxonomic significance, plants from these seeds will be designated variety California and variety Minnesota respectively with no taxonomic implications. Voucher specimens of the variety California plants grown to flowering and fruiting are on file at the W. P. Fraser Herbarium (SASK.) at the University of Saskatchewan.[2]

All plants were grown from seeds in vermiculite in a greenhouse and were irrigated twice a week with Hoaglands nutrient solution. Plants between 2- and 3-months old were used for all photosynthesis studies.

Sedum sarmentosum was propagated from cuttings kindly supplied by Professor I. D. Spenser, Department of Chemistry, McMaster University, Hamilton, Ont. Biosynthesis experiments were carried out on plants 2–3 months after cutting and planting.

Punica granatum plants were grown to an age of 2–3 months from seeds of fresh pomegranate fruits obtained at a local market.

Biosynthesis Experiments Using $^{14}CO_2$. Clear polyethylene (0.001 in.) bags served as flexible, disposable photosynthesis chambers.[3] Rubber tubing for gas circulation was attached to opposite sides of the polyethylene bag by sandwiching the latter in between male and female polyethylene hose connectors ("Quick Disconnects" Bel-Art Products) then clearing the trapped plastic film from the central hole. $^{14}CO_2$ was generated by injecting 10% $HClO_4$ into a preweighed amount of $Ba^{14}CO_3$ (10.2 mCi/mmole). The latter was contained in a 5-ml tube with a top rubber septum and two side arms to connect with the rubber tubing from the chamber. A diaphragm circulating pump was inserted in between the plastic bag and the $^{14}CO_2$ generator. A fourway bypass valve was inserted in the hose system to permit diverting of the circulating atmosphere through a KOH trap at the end of the $^{14}CO_2$ exposure period and for removal of initial $^{12}CO_2$.

Plants were placed inside the bag, the top of the bag twisted and then sealed with tape. Any bag seams were sealed with tape as a precaution. The whole assembly was placed between two banks of six 30-W fluorescent lamps giving 1000-lx luminosity at the plant surface. The dilution of the $^{14}CO_2$ by the $^{12}CO_2$ initially present in the system was between 10 and 20% depending upon the bag size and amount of $Ba^{14}CO_3$ used. This dilution was avoided in latter experiments by pumping the initial atmosphere through the KOH trap prior to introduction of $^{14}CO_2$. The final concentration of CO_2 in the bag never exceeded 0.18%. After a period of exposure to $^{14}CO_2$, usually 1 hr, the bag was cut open and the plants were immediately extracted or restored to the normal atmosphere for various periods of time before extraction. Counting of an aliquot of the KOH trapping solution gave an estimate of the $^{14}CO_2$ assimilated.

Plant Feedings via Cut Petiole. The petiole of a plant was immersed in 0.25 ml of nutrient media containing the ^{14}C-

[2] Our appreciation to Professor G. Argus of this University and Professor L. Constance of the Botany Department, University of California, Berkeley, for identification of these specimens.

[3] The ability of these chambers to expand or contract with temperature changes eliminated leaking problems encountered with previous rigid lucite chambers. In addition to being disposable their size could be varied at will depending on the size of the available starting bags.

labeled compound. After 1 hr, 0.5 ml of water was added to the nearly empty container to "wash in" residual precursor.

Alkaloid Extraction. Whole fresh plants (*ca.* 20 g) were rapidly washed free of adhering vermiculite, blotted "dry," weighed, then extracted with 35 ml of 95% ethanol in a 50-ml Swedish oil extraction tube (Galve Rostfrei, Sweden) containing three 0.5-in. ball bearings and sealed with a Teflon plug. Vigorous shaking in a reciprocating floor shaker for 20 min reduced the plants to a fine suspension, suitable for pipetting aliquots for counting after solubilization, to give a reliable measure of the $^{14}CO_2$ assimilated (fixed). The suspensions were filtered, washed, and then extracted with a modification of Cromwell's (1956) procedure using methylene chloride instead of chloroform. Owing to the volatility of γ-coniceine and coniine, the final, sodium sulfate dried, extract was flushed with dry HCl gas prior to volume reduction *in vacuo* to 1.0–0.1 ml. The HCl salts were used directly for thin-layer chromatography and electrophoresis.

Since the HCl salt of pseudoconhydrine was insoluble in methylene chloride, resulting in losses of up to 90%, a portion of the final extract, prior to acidification, was reserved for thin-layer chromatography and gas–liquid partition chromatography. For gas–liquid partition chromatography of the remaining alkaloids, the HCl salts in the approximately desired volume of methylene chloride were flushed with dry ammonia gas, the final volume was adjusted with solvent, and any precipitated ammonium chloride was removed by centrifugation. The free bases thus formed were directly injected into the gas–liquid partition chromatography counting apparatus.

Chromatographic and Electrophoretic Systems. THIN-LAYER CHROMATOGRAPHY was carried out using three systems: system A: cellulose layers developed in *t*-pentyl alcohol–*t*-butyl alcohol–1 N HCl (9:3:2, v/v) (Cromwell, 1956); systems B and C: silica G layers developed in chloroform–methanol–concentrated ammonia (35:14:1, v/v) and ethyl acetate–methanol–ammonia (35:10:5, v/v), respectively (Sharma *et al.*, 1965). Alkaloids were detected using iodine vapor and/or Dragendorff's reagent (Waldi, 1964); 0.3% ninhydrin–1% acetic acid in butyl alcohol was used to detect secondary amines and cadaverine. γ-Coniceine was specifically detected when necessary using the alkaline nitroprusside reagent of Cromwell (1956). Preparative paper chromatography was carried out using Whatman No. 3MM paper developed (descending) in solvent system A (above). Bands were eluted with 0.01 N HCl.

ION-EXCHANGE CHROMATOGRAPHY of small radioactive samples were carried out on columns (0.4 × 5 cm) of either Dowex 50-X12 (H^+) or Dowex 1-X10 (formate).

THIN-LAYER ELECTROPHORESIS was carried out on cellulose layers in pyridine–acetic acid–water (5:1:100, v/v, pH 5.8) for 2 hr at 300–400 V.

GAS–LIQUID PARTITION CHROMATOGRAPHY–CONTINUOUS COUNTING of the alkaloid extracts using the gas–liquid partition chromatography–combustion–continuous-counting system of Martin (1968) permitted multiple, simultaneous mass, and radioactivity analysis of microgram amounts of all the piperidine alkaloids. The useful mass range was from 0.5 to 500 μg and activities greater than 150 dpm were detectable in any one peak.

Complete resolution of all the known *Conium* alkaloids was achieved on a $^3/_{16}$ in. i.d. × 4 ft glass column (5% Carbowax 20M, 4% KOH on Silanized Chromasorb W) (Smith and Radford, 1961). Conditioning was at 230° overnight (10 cc/min of He flow). Samples were injected with the inlet at 125°; He, 40 cc/min. Column was kept at 40° for the first 10 min then programmed at 5°/min to 210°. Compounds not eluted from this column were successfully eluted from one filled with 1% XE-60 on silanized Chromosorb W.

Counting Procedures. Macerated plant suspensions were counted either by first combustion to CO_2[4] or by solubilization with NCS solubilizer (Nuclear-Chicago Co.) then direct scintillation count (Hansen and Bush, 1967).

Results

Alkaloid Extraction. Within the 2% error for the gas–liquid partition chromatography area measurements, the recovery of the nonoxygenated bases, *via* the extraction scheme described, was quantitative and was >90% for the oxygenated bases.

Chromatographic and Electrophoretic Behavior of Alkaloids. These are tabulated in Table I, for both known and suspected compounds.

Comparison of the Alkaloid Content of C. maculatum var. California and var. Minnesota. In as much as these were the first alkaloid studies on var. California and var. Minnesota was used by Leete in his feeding experiments, a preliminary comparison was made of the alkaloid spectrum in fresh 2.5-month-old plants of both varieties using gas–liquid partition chromatography. The concentration of known alkaloids in var. California was about 400 μg/g (fresh weight) with γ-coniceine accounting for 75% with the remaining 25% about equally divided among the other four bases. Var. Minnesota, on the other hand, contained only about 30 μg/g with coniine accounting for somewhat less than half of this.[5] In addition both plants contained varying amounts of unidentified compounds eluted from the gas chromatograph.

Biosynthesis Experiments with Conium var. California. The results of 2-, 4-, and 6-hr exposures to $^{14}CO_2$ with no return to normal atmosphere have been published (Dietrich and Martin, 1968). Further similar experiments carried out for 0.25, 0.5, and 1 hr showed γ-coniceine as the only radioactive alkaloid by gas–liquid partition chromatography. Another compound referred to as "D" did appear much more active than γ-coniceine (thin-layer chromatography system A). Since the total ^{14}C assimilated leveled off at *ca.* 60–70% after 1–2 hr and to avoid leaving the plants in an atmosphere of abnormally low CO_2 concentration, all subsequent exposures were for 1 hr followed by a return to normal air.

BIOSYNTHESIS I (0–35 HR). Seventy plants (45-g total fresh weight) were exposed to 8.55 mCi of $^{14}CO_2$ for 1 hr. Groups of three to five plants were killed immediately (0 hr) and at intervals up to 35 hr after. The total $^{14}CO_2$ assimilated in 1 hr was 75% or 4.69×10^8 dpm/g of which 1.66×10^8 dpm/g remained by 35 hr. The gas–liquid partition chromatography counting results for the alkaloids are tabulated in Table IIA together with the calculated specific activities. Note particu-

[4] Kindly carried out by Mr. J. Dyck of the Prairie Regional Laboratory. Results using the two methods agreed within ±5% so the solubilization procedure was used routinely.

[5] Our *Conium maculatum* var. Calif., with γ-coniceine as the major alkaloid in the vegetative stage is apparently similar to the var. "Chelsea" used by Professor Fairbairn (Leete and Adityachaudhury, 1967).

TABLE I: Chromatographic and Electrophoretic Behavior of Known, Unknown, and Suspected Alkaloids of *C. maculatum*.

Compound	R_F on Thin-Layer Chromatography System			Electrophoretic Mobility toward the Cathode at pH 5.8 (cm)	R_t on Gas–Liquid Partition Chromatography[a] (min)
	A	B	C		
γ-Coniceine	0.33	0.75	0.94	2.5	4.0
Coniine	0.65	0.32	0.75	2.1	2.0
N-Methylconiine	0.58	0.55	0.18	2.1	1.6
α-Conhydrine	0.46	0.15	0.54	2.1	12.2
Pseudoconhydrine	0.67	0.36	0.64	2.1	14.5
Compound D	0.89	0.60	0.74	0.0–0.3[c]	(13)[b]
Hydrolyzed D	0.06	0.03		−1.4	
Valerolactam	0.91	0.32	0.61	0.0–0.3	27.5
Isotripiperideine	0.85	0.65	0.89		30.0
3-Ketoindolizidine	0.98	0.80	0.74	0.0–0.3	

[a] Column 5% Carbowax, 4% KOH on Chromasorb W. [b] Not eluted from Carbowax column. Retention time is for a 1% XE-60 column started at 60°. [c] Mobility unchanged at pH 2 acetic–formic acid buffer or at pH 8.5 0.1 M $(NH_4)_2CO_3$.

larly the low recoveries of radioactivity from the gas–liquid partition chromatography in the early time samples. Autoradiograms of thin-layer chromatography in system A and B for all time samples are shown in Figure 1. Note particularly the heavily labeled, early appearing compound called "D", and the increase in ^{14}C in γ-coniceine in latter time samples. As a check on the homogeneity of the γ-coniceine area, this was eluted from a preparative thin-layer chromatography run, reduced with sodium borohydride (pH 8.5) in methanol followed by thin-layer chromatography to confirm complete reduction to coniine as the sole product. In addition, the eluted material on gas–liquid partition chromatography gave single, coincident mass and radioactivity peaks identical with γ-coniceine and containing >95% of the original radioactivity.

BIOSYNTHESIS II (0–7 DAYS). Since in the previous experiment the specific activity of *N*-methylconiine was still increasing at 35 hr and conhydrine and pseudoconhydrine were not yet labeled, a longer experiment was carried out to obtain a more complete kinetic picture and in an attempt to obtain labeled oxygenated bases. The results of a 1-week-long experiment in which a similar group of plants (99 g fresh weight) were exposed to 2.52 mCi of $^{14}CO_2$ are tabulated in Table IIB and the time course of the alkaloid specific activities is shown in Figure 2. The total ^{14}C assimilated in 1 hr was 72% or 4.05×10^7 dpm/g of which 1.70×10^7 dpm/g remained by 7 days. Again, no radioactivity could be detected by gas–liquid partition chromatography in pseudoconhydrine and only a small amount was detected in conhydrine on thin-layer chromatography by long-term autoradiography (4 weeks).

Biosynthesis Experiments with Conium var. Minnesota. BIOSYNTHESIS III (0–48 HR). To verify the observations made on var. California including the occurrence and kinetic behavior of compound D, 50 plants (50-g total fresh weight) of var. Minnesota were exposed for 1 hr to 5.70 mCi of $^{14}CO_2$ with samples taken from 0 to 48 hr. The total ^{14}C assimilated in 1 hr was 50% or 1.72×10^8 dpm/g of which 0.59×10^8 dpm/g remained by 48 hr. The gas–liquid partition chromatog-

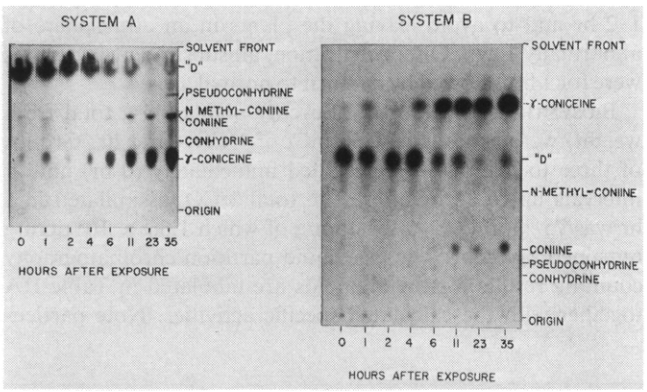


FIGURE 1: Distribution, after thin-layer chromatography, of ^{14}C among the alkaloids of *C. maculatum*, var. California, 0–35 hr after exposure to $^{14}CO_2$ (biosynthesis I). Approximately equal amounts of total radioactivity from each time sample were spotted. Autoradiogram: 1-week exposure.

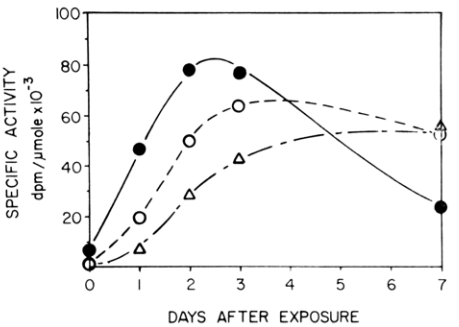


FIGURE 2: Specific activity–time course of the alkaloids of *C. maculatum*, var. California 0–7 days after exposure to $^{14}CO_2$ for 1 hr. Date obtained, biosynthesis II, Table IIB (●) γ-coniceine, (○) coniine, and (△) *N*-methylconiine.

TABLE II: Gas–Liquid Partition Chromatographic–Continuous-Counting Results and Specific Activities for the Alkaloids of *C. maculatum*, var. California and var. Minnesota, at Increasing Periods after Exposure to $^{14}CO_2$ for 1 hr: (A) Biosynthesis I, (B) Biosynthesis II, and (C) Biosynthesis III.

		Concentrations of Alkaloids (μmole/g of plant)[a]				Specific Activities (dpm/μmole × 10^{-3})				
	Time after Exposure	γ-Coniceine	Coniine	*N*-Methyl-coniine	Conhydrines[b]	γ-Coniceine	Coniine	*N*-Methyl-coniine	Conhydrines	% Recov of Radioactivity[e]
A, Var. California	0 hr	2.42	0.11	0.10	0.1	48.6	<6[c]	<6.5[c]	<6[c]	5
	1 hr	1.46	0.06	0.06	0.06	20.1	<6	<6.5	<6	9
	2 hr	2.90	0.06	0.16	0.11	26.4	<6	<6.5	<6	7
	4 hr	3.64	0.05	0.07	0.06	195	6.4	<6.5	<6	7.5
	6 hr	2.54	0.04	0.07	0.07	199	31.9	<6.5	<6	78
	11 hr[d]	0.8	0.16	0.21	0.04	291	221	7.1	<6	85
	23 hr[d]	1.21	0.10	0.02	0.08	468	408	26.3	<6	70
	35 hr	1.43	0.07	0.02	0.05	440	454	403.6	<6	95
B, Var. California	0 days	1.64	0.04	0.02	0.02	6.3	5.0	<5.0	<6	4
	1 day	2.55	0.03	0.05	0.04	46.4	19.4	6.4	<6	95
	2 days	2.32	0.04	0.11	0.01	77.9	48.9	27.0	<6	100
	3 days	2.14	0.08	0.10	0.03	75.0	62.9	40.2	<6	100
	7 days	1.78	0.19	0.15	0.02	22.6	50.9	55.3	<6	95
C, Var. Minnesota	0 hr	0.16	0.44	0.01	0.01	79.5	<1.0	<1.0	<6	10
	1 hr	0.07	0.12	<0.01	0.02	116	2.5	2.0	<6	10
	4 hr	0.13	0.44	0.01	0.02	489	19.6	2.0	<6	11
	9 hr	0.05	0.24	0.05	0.01	790	45.6	2.0	<6	67
	24 hr	0.13	0.47	<0.01	0.01	685	98.2	2.0	<6	75
	30 hr	0.03	0.08	<0.01	0.01	685	146	2.0	<6	
	48 hr	0.02	0.22	0.09	0.01	685	145	2.0	<6	70

[a] Fresh weight. [b] Values for both conhydrine and pseudoconhydrine were nearly the same. [c] Figures represent minimum radioactivity detectable for any one peak (150 dpm) divided by measured mass. [d] Samples taken during dark period. [e] From gas–liquid partition chromatography.

TABLE III: Per Cent of Total Fixed ^{14}C in Alkaloids of *Conium*, *Sedum*, and *Punica*.

Plant	hr	Total ^{14}C Fixed[a] (dpm/g per mCi $\times 10^{-7}$)	% of Fixed ^{14}C in Alkaloids and Compd D	% of Alkaloids ^{14}C in Compd D
Var. California	0	5.5	0.23	95
Var. California	4	4.6	0.68	75
Var. Califoria	23	3.2	0.64	20
Var. Minnesota	0	2.3	0.24	90
Var. Minnesota	4	2.1	0.1	90
Var. Minnesota	24	1.6	0.20	25
Sedum	0	1.3	0.76	90
Sedum	3	1.3	0.17	40
Sedum	0[b]	0.8	1.53	>95
Sedum	24[b]	0.8	6.4	<1
Punica	0	3.5	0.01	50
Punica	17	4.6	0.1	<5

[a] Total disintegrations per minute fixed (or remaining after removal from $^{14}CO_2$) and expressed in disintegrations per minute per gram of fresh weight per millicurie fed (to correct for different amounts of $^{14}CO_2$ fed). [b] Initial exposure to $^{14}CO_2$ was for only 15 min.

raphy results are tabulated in Table IIC; note the low ^{14}C recovery in early time samples. The distribution of ^{14}C among the various alkaloids resolved by thin-layer chromatography systems A and B revealed by autoradiography were essentially the same as in var. California. Compound "D" showed identical behavior with that from var. California in all chromatographic systems and in its kinetics of labeling. Again, conhydrine and pseudoconhydrine were without detectable labeling. The gas–liquid partition chromatography results, however, revealed the appearance of significant activity in two compounds with retention times near conhydrine and pseudoconhydrine but definitely not either of the latter as checked by cochromatography with the authentic compounds.

Biosynthesis Experiments with Sedum and Punica. Since the kinetic behavior of compound D in both *Conium* varieties suggested the possibility of its being an intermediate in the biosynthesis of other propylpiperidine alkaloids, *Sedum sarmentosum* and *Punica granatum* were examined for its presence and behavior.

BIOSYNTHESIS IV: *Sedum*. Two separate experiments were carried out, each using about 90 g of plants exposed to about 2 mCi of $^{14}CO_2$. The first group was exposed for 1 hr to $^{14}CO_2$ with samples taken at 0 and 3 hr. The second was given only a 15-min exposure with samples taken immediately and 24 hr after. A comparison of the total ^{14}C assimilated, of the per cent of this in the alkaloids, and of the per cent of [^{14}C]-alkaloid in D is tabulated in Table III along with representative results from both *Conium* varieties and *Punica*.

Analysis of the alkaloids by all the chromatographic systems and electrophoresis showed the presence of a substance identical in all respects with compound D from *Conium*, being the first major radioactive compound to appear, again with no detectable mass. After 3 hr more than half the [^{14}C]alkaloid was in the major known bases such as *N*-methyldihydroisopelletierine and by 24 hr >90% was in the latter compound(s).

BIOSYNTHESIS V: POMEGRANATE. A similar experiment on *Punica* with samples taken at 0 and 17 hr showed comparable $^{14}CO_2$ assimilation to *Conium*, but with only about $^1/_{20}$ the amount of ^{14}C incorporated into the alkaloids in the zero-time sample. Insufficient activity was available for a gas–liquid partition chromatography count. On electrophoresis and thin-layer chromatography in systems A and B half of the alkaloid ^{14}C, in the zero-time sample, was in a spot identical with D from *Conium* while at 17 hr none remained in "D" but was in two distinctly different compounds, possibly pelletierine and pseudopelletierine.

PRELIMINARY STUDIES ON COMPOUND D. Preparative paper chromatography of pooled 0- and 2-hr samples of [^{14}C]-alkaloids from *Conium* var. California gave a single highly radioactive compound in all the chromatography systems described and on electrophoresis. See Table I for R_F values, etc. Only by autoradiography could "D" normally be detected, since no iodine or Dragendorff's reaction could be observed, although in case of heavier sample loads, ultraviolet absorption could be associated with the ^{14}C "D" area.

ACID HYDROLYSIS OF D. About 200,000 dpm of D hydrolyzed (sealed tube) in 1 N HCl at 100° for 1 hr resulted in its almost complete conversion with <10% loss of ^{14}C, to a polar, anionic compound (by thin-layer chromatography and electrophoresis) which was no longer extractible into methylene chloride at pH 11. A control kept at room temperature showed no change.

Ion-Exchange Behavior of D and Its Hydrolysis Product. Both were examined by passing about 50,000 dpm of each through separate columns of Dowex 1-X10 ($HCOO^-$) and Dowex 50-X12 (H^+). Neither were retained at all on Dowex 50 whereas hydrolyzed D was completely retained by Dowex 1. A control of γ-coniceine was completely retained by Dowex 50. This behavior was the basis for a subsequent large-scale purification of D.

Recoveries of any retained compounds were quantitative when columns were eluted with 5 N NH_4OH for Dowex 50 or 2 N HCl for Dowex 1.

Alkaline hydrolysis with 0.8 N NaOH under the same conditions as above gave very similar results.

Feeding of Compound D and Acetate to Intact Plants. Two petiole feeding experiments were carried out for 8 hr on two individual 3-month-old plants; 1.25×10^6 dpm of compound D was fed to one and 12.5×10^6 dpm (0.09 mg) of [1-^{14}C]-acetate to the other. Slightly more than 30% of the acetate was "fixed" in the total plant emulsion, the remaining 70% presumably having been partly respired as CO_2. On reduction in volume (*in vacuo*) of the acidic aqueous methanol extract after filtering only 4–5% remained. This loss could be due to evaporation of [^{14}C]acetate "in the plant" but not yet incorporated or incorporated into insoluble products. Only 640 dpm was found in the alkaloids which is 0.13% of the ^{14}C fixed. No ^{14}C was detectable in compound D. With compound D, 0.4% was incorporated with 2000 dpm (*ca.* 0.2%) being in γ-coniceine and 2000 dpm in compound D.

Discussion

Biosynthesis of Nonoxygenated Alkaloids. A comparison of the specific activities of γ-coniceine, of coniine, and of *N*-methylconiine from var. California (Table II, biosynthesis I) shows γ-coniceine is the first to appear radioactive, followed by coniine, then *N*-methylconiine. Since γ-coniceine is present in the highest concentration of the three, no corrections are necessary for pool dilution, whereas they are for coniine and its *N*-methyl derivative (Rapoport *et al.*, 1960). Only by 11 hr (Table II) was activity measurable in *N*-methylconiine at which time the coniine:*N*-methylconiine specific activity ratio was 31:1; by 23 hr it was 15:1 with the pool ratios being 1:1.3 and 5:1, respectively. By 35 hr the reverse trend had set in. The above data confirm our earlier findings (Dietrich and Martin, 1968) and the condition is met that specific activity ratios exceed the inverse molar ratios, this difference becoming greater at earlier times (Zilversmit *et al.*, 1943) (Martin *et al.*, 1967) which is consistent with the biosynthetic sequence: γ-coniceine → coniine → *N*-methylconiine.[6]

A 1-week experiment (biosynthesis II) showed the specific activity curve for γ-coniceine crosses over almost at the maximum of the coniine curve (Figure 2), which is consistent with there being no (stable) intermediate between the two (Zilversmit *et al.*, 1943). Likewise the coniine curve appears to intersect the maximum of that of *N*-methylconiine.[7]

In the case of var. Minnesota in which coniine was the major alkaloid during the vegetative stage, γ-coniceine was again the first known base to become labeled (biosynthesis III, Table IIC). In this case however pool dilutions have to be taken into consideration. At 1 hr the γ-coniceine:coniine:*N*-methylconiine specific activity ratios were in round numbers 58:1:<1 and at 4 hr 245:9:<1. The corresponding pool ratios were 50:100:1 and 12:38:1, respectively. Even though no ^{14}C was detectable in *N*-methylconiine the same conclusions may be drawn as for the variety California.

Rates of Synthesis of γ-Coniceine and Coniine. Table II shows that after 1-hr exposure to $^{14}CO_2$ (specific activity 10 μCi/μmole, diluted 20% to 8 μCi/μmole), γ-coniceine has a specific activity of 0.02 μCi/μmole which is $^1/_{3200}$ of the calculated maximum specific activity (64 μCi/μmole) in this compound if all eight carbons had the same specific activity as the fed $^{14}CO_2$ and if no dilution other than the initial 20% dilution of the label in the chamber had occurred. This value corresponds to a total of 0.05 μCi of carbon-14 incorporated into γ-coniceine (2.4 μmoles) per gram of fresh plant in 1 hr, or 0.8 mμmole of γ-coniceine of maximum specific activity, synthesized per hr per g of plant. These figures represent the *minimum* amount of γ-coniceine synthesized by the plants during the 1-hr exposure to $^{14}CO_2$ under the conditions of biosynthesis expt I.

Kinetic data for var. Minnesota (calculated on the same basis as for var. California) indicate a minimum rate of synthesis of 0.1 mμmole/hr per g of plant for γ-coniceine.

A calculation of the rate of synthesis of coniine, based on the observed specific activity of γ-coniceine rather than of $^{14}CO_2$ gives a more reliable figure, since there appear to be no intermediate pools, in which label dilution would occur between these two compounds. For var. California, using the specific activity of γ-coniceine at 6 hr and that of coniine at 11 hr, the average rate for this 5-hr period was 35 mμmoles/hr per g. During the 4–9-hr interval for var. Minnesota an average rate of about 4.3 mμmoles/hr per g is obtained. Since γ-coniceine is synthesized prior to coniine, its rate of synthesis in var. California is at least 35 mμmoles/hr per g or 35 times greater than that calculated on the basis of $^{14}CO_2$ specific activity. Likewise the var. Minnesota rate would be at least 4.3 mμmoles/hr per g or 30 times greater than the calculation based on the fed $^{14}CO_2$. These represent *minimum* rate values in which no account has been taken of possible "bound" pools (*cf.* next section).

Although no experiments were carried out for longer than 7 days to determine if *N*-methylconiine was turning over or merely accumulating as an "end product," calculated rates for *N*-methylconiine gave values of from 1 mμmole/hr per g (biosynthesis I, 11–23 hr) to 5 mμmole/hr per g (biosynthesis II, 1–2 days) which means between only 1 and 4 days would be required to synthesize the "extractable" pool, which may be converted into other compounds or terminate as a "bound" form.

On the basis of these rates the pool of γ-coniceine in var. California would take about 4 days to synthesize *de novo* and since the pool size does not increase over the 7-day period, its turnover[8] must be at about this rate. This is consistent with the observation that the γ-coniceine specific activity has decreased to 20% of its 2.5-day maximum by 7 days. This would not be consistent with appreciable conversion of coniine by the reverse reaction and should be contrasted to the observations of Fairbairn and Ali (1968b) that ^{14}C from coniine was incorporated into γ-coniceine[9] and that little decrease in the specific activity of fed [γ-^{14}C]coniceine occurred between 1 and 19 days in umbels of fruits.

For var. Minnesota, calculation of the γ-coniceine pool turnover time gives a value of 1 day (24 hr). The lack of any appreciable drop in γ-coniceine specific activity by 48 hr would indicate a reserve ^{14}C pool of some prior intermediate turning over slower than in var. California.

Finally some interesting comparisons of rates of alkaloid formation in *Conium*, *Sedum*, and *Punica* can be made from Table III, keeping in mind that the major component of the early-time samples was compound D. *Punica* at this early vegetative stage shows a considerably lower rate of *de novo* synthesis and *Sedum* as good or in one case 10–15 times higher than either *Conium* variety. Also it is interesting that the ^{14}C in compound D does not follow the total $^{14}CO_2$ fixation pattern but rather the alkaloid incorporation; *e.g.*, with

[6] N demethylation rather than methylation appears to be the natural pathway in *Nicotiana glutinosa*, nicotine being demethylated to nornicotine (Alworth and Rapoport, 1965), thus there is no *a priori* basis for knowing which process to expect.

[7] No degradations were carried out on *N*-methylconiine to determine the *N*-methyl to ring ^{14}C ratio, however, this would not effect the above conclusion. The apparent high specific activity value for *N*-methylconiine after 7 days may be due to still increasing $^{14}CH_3$ specific activity, derived from an independent source.

[8] See Reiner (1953) for definitions of turnover, turnover rate, and turnover time.

[9] Since no data were given, the extent of the reverse conversion is not known. The local high concentration of coniine at the site of feeding could upset an equilibrium normally favoring the reduction of γ-coniceine. Also the ability to carry out the reverse oxidation reaction may be a function of age and/or site of feeding.

Punica, even though the total $^{14}CO_2$ fixation was as good as *Conium*, var. California, the ^{14}C incorporation into the alkaloids and compound D was low.

Variations in Alkaloid Concentrations and Their Turnover. The data presented in Table II, over the course of the individual biosynthesis experiments, show large, inconsistent variations in the concentrations of the individual alkaloids which may partly reflect both biological variability, in as much as different groups of plants (though in duplicate) were taken for each time sample and variations inherent in plant fresh weight determinations. In addition, consideration should be given to Fairbairn and Challen's (1959) observations on *ripening Conium fruits* of hour-to-hour and day-to-day alkaloid variations which Fairbairn and Ali (1968a,b) have attributed to the existence of bound and unbound forms of the alkaloids which are freely and rapidly interconverted. If γ-coniceine were synthesized *via* the "bound" pools then its specific activities would be expected to fluctuate accordingly. However, since the specific activities were independent of pool fluctuations, the free (extractable) pool of γ-coniceine most likely represents the newly (*de novo*) synthesized material and its removal to bound pools would not affect its specific activity. Similar considerations would apply to coniine and *N*-methylconiine.

With the sequence of interrelations clear, a comparison of the *total activity* ratios (since all three compounds were recovered to the same extent) is indicative of the relative turnover of these three compounds (Martin *et al.*, 1967). For var. California, the total activity ratios γ-coniceine:coniine:*N*-methylconiine were 1770:1:1 at 4 hr, 165:26:1 at 11 hr, and 80:40:1 at 35 hr. These ratios indicate at least that γ-coniceine is not turning over faster than coniine, nor the latter faster than its *N*-methyl derivative and that γ-coniceine is not being converted into other (similar?) compounds at any rate comparable with its conversion into coniine.

For var. Minnesota the same total ^{14}C ratios were 500:15:<1 at 1 hr, 2500:100:1 at 4 hr and 3800:2300:<1 after 24 hr, indicating γ-coniceine as having a turnover rate near to that of coniine and possibly limited by its rate of reduction to the latter. Likewise, the turnover rate of coniine would appear to be limited by its N methylation which was imperceptibly slow. That the latter conversion is too low to be detected would account for the reverse trend by 48 hr where the total ^{14}C in coniine is greater than in γ-coniceine.

These conclusions should be contrasted with the reticuline:thebaine ratios observed in poppies (Martin *et al.*, 1967) where reticuline, the more rapidly synthesized alkaloid, was found with lower *total* activity than thebaine, a fact accountable for by the conversion of reticuline into other related compounds *via* routes not involving thebaine as an intermediate.

The *total alkaloid concentration* and the relative amounts of the nonoxygenated bases in both varieties of *Condium* are deserving of comment since they are a reflection of both the rate of synthesis as well as turnover (Martin *et al.*, 1967). For example, the accumulation of large amounts of a compound in a plant may reflect a high rate of synthesis and slower turnover or a low rate of synthesis with less or no turnover. The large pool of γ-coniceine in var. California compared with var. Minnesota would appear to be due to a combination of a faster rate of synthesis (35 mμmole/hr per g) and a slower turnover (4 days) compared with the slower rate of synthesis (4.5 mμmole/hr per g) and a faster turnover (1 day) for var. Minnesota. In either case the methylation of coniine does not appear to be limiting since it did not occur at a measurable rate in var. Minnesota which had total activity and pool ratios consistent with a rapid conversion of γ-coniceine into coniine.

The Oxygenated Bases. During an 8-day petiole feeding experiment with 8-month-old *Conium*, var. Minnesota plants, Leete and Adityachaudhury (1967) obtained good, specific incorporation of ^{14}C from [γ-1-^{14}C]coniceine into (±)-pseudoconhydrine and (+)-coniine. All our attempts to find the oxygenated alkaloids, conhydrine and pseudoconhydrine, labeled were unsuccessful with var. California, even 1 week after exposure of the plants to $^{14}CO_2$ (biosynthesis II) or with var. Minnesota (biosynthesis III) after 48 hr. One must conclude then that the rate of synthesis of these alkaloids at this early vegetative stage was too slow to allow incorporation of detectable amounts of ^{14}C, or *de novo* synthesis did not occur at all, the amounts of these oxygenated alkaloids in the plants being a remnant of those present in the seeds.

Radioactive compounds which gas chromatographed with retention times very similar to those of the two known oxygenated alkaloids were, however, present in the alkaloid extracts. One such compound (present in var. Minnesota as a major component) appears to be formed at a rate comparable with coniine. These compounds could well prove to be the unsaturated, intermediate, allylic oxidation products of γ-coniceine proposed by Leete and Adityachaudhury (1967). Alternatively they may prove to be diastereomers of the generally accepted naturally occurring forms of conhydrine and pseudoconhydrine. As little is known of the alkaloids of fresh young plants of either of these varieties of *Conium* and the accepted stereochemistry of these compounds is based on work carried out on dried plant material, the composition of the latter may well represent the thermodynamically preferred compounds whereas kinetically other isomers may actually be the first formed *do novo*. The gas–liquid partition chromatography procedure used here would be expected to resolve the diastereomeric form of both conhydrine and pseudoconhydrine, as well as unsaturated conhydrine or pseudoconhydrine from their saturated counter parts (*cf.* the separation of γ-coniceine from coniine).

Interestingly, reticuline of both stereo forms [(*R*)-(−) and (*S*)-(+)] is synthesized in the opium poppy and the predominant isomer synthesized by the seedling is different from that synthesized by the mature plants (Battersby *et al.*, 1965; Martin *et al.*, 1967).

Identification and Possible Role of Compound D. A striking observation from the thin-layer chromatography autoradiograms of the radioactive alkaloid extracts (Figures 1 and 2) was the early appearing ^{14}C area labeled compound D, whose disappearance correlated well with the recovery of radioactivity from the gas–liquid partition chromatography continuous counting of the same extracts (Table II, last column). This together with the pattern of early increase of radioactivity and its decrease, while the radioactivity of the other alkaloids were increasing (Figure 3) gave this compound the earmarks of a natural intermediate in the biosynthesis of the *Conium* alkaloids. The fact that a compound with the same chromatographic and electrophoretic properties was also found in *Sedum sarmentosum* (a plant from a family totally unrelated to *Conium maculatum* but producing similar propylpiperidine alkaloids), gave additional support to this hypothe-

sis. Finally, the incorporation of ^{14}C from compound D compared with acetate in the feeding experiment was good by currently accepted standards and appears even better if one takes into account that a maximum expected theoretical incorporation of only 25% into γ-coniceine would be expected, as three-quarters of the ^{14}C of D was going to non-alkaloidal fractions (Figure 3). Thus it was thought that the elucidation of the structure of compound D would throw a new light on the problem of biosynthesis of some (if not all of) the piperidine alkaloids. The elucidation of the structure of this unusual compound is the subject of the paper immediately following (Koleoso *et al.*, 1969) where the question of its role in the biosynthesis of these alkaloids is discussed.

Other Possible Intermediates. The behavior of D on electrophoresis and its apparent hydrolysis products early suggested it was possibly a lactam, the most plausible of which, to be intermediates prior to γ-coniceine, were valerolactam, and 3-ketoindolizidine (*via* the reduced compound, δ-coniceine). As can be seen from Table I, they proved to be clearly different from D. Their hydrolysis products were almost identical with the exception of ion-exchange behavior. Finally, in biosynthesis experiments with $^{14}CO_2$, no radioactivity was detectable, corresponding to valerolactam, 3-ketoindolizidine, or indolizidine (δ-coniceine).

Though several reports indicate that cadaverine can serve as a precursor of piperidine alkaloids (Leete, 1958; Clarke and Mann, 1959; Cromwell and Roberts, 1964), the analysis of the "aqueous phase" of the plant extracts prior to alkaloid extraction, after 1-hr exposure to $^{14}CO_2$ (biosynthesis I) failed to reveal even a trace of cadaverine even though γ-coniceine in the extract was labeled. Together with Cromwell and Roberts (1964) failure to isolate from *Conium* plants, the enzyme diamine oxidase which converts cadaverine into the semialdehyde, or lysine decarboxylase which converts lysine into cadaverine, this finding suggests that cadaverine is not a natural intermediate in the biosynthesis of *Conium* alkaloids. In fact the only reported occurrence of cadaverine outside of certain lily blossoms (Smith and Meeuse, 1966) and necrotic peas grown in high saline conditions (Prikhod'ko and Klyshev, 1964, 1965) has been in bacterially contaminated material (McKee, 1962). The question may be posed then as to whether cadaverine is a *normal* plant product at all? The relevance of diamine oxidases to alkaloid biosynthesis is examined by Spenser (1968) and the hazards of ascribing a function to isolated enzyme (activity) is discussed by Davis (1954). Through the highly reactive Δ^1-piperideine, the second best precursor fed by Cromwell and Roberts,[10] has not been found in *Conium*, its acetylated dimer, ammodendrine, together with the trimers isotripiperideine and aldotripiperideine, have been isolated from *Coelidium fourcadei* (Arndt and Du Plessis, 1968). The presence of radioactive Δ^1-piperideine-2-carboxylic acid, the best precursor incorporated to date (Cromwell and Roberts, 1964) in the extracts, remains to be demonstrated. Studies are currently under way to determine whether this latter compound or cadaverine are normal products of plant metabolism.

[10] One might question whether it was Δ^1-piperidine, in fact, which was fed as it was not characterized by Cromwell and Roberts (1964) or in the original synthesis (Jakoby and Fredricks, 1959) where only a color test was applied and no yield reported. It is difficult to understand Cromwell and Roberts' failure to give more significance to the phenomenal incorporation of ^{14}C (39%) from Δ^1-piperideine-2-carboxylic acid.

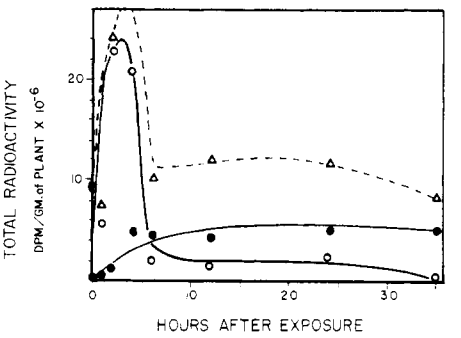


FIGURE 3: Time course of distribution of ^{14}C in total alkaloid fraction, between compound D and γ-coniceine. Data obtained from biosynthesis I (Table IIA). Values for compound D were taken as the ^{14}C unrecovered from the gas–liquid partition chromatography expressed as disintegrations per minute per gram of fresh plant.

References

Ahrens, F. B. (1902), *Ber. 35*, 1330.
Alworth, W. L., and Rapoport, H. (1965), *Arch. Biochem. Biophys. 112*, 45.
Arndt, R. R., and DuPlessis, L. M. (1968), *J. S. Afr. Chem. Inst. 21*, 54.
Battersby, A. R., Evans, G. W., Martin, R. O., Warren, Jr., M. E., and Rapoport, H. (1965), *Tetrahedron Letters*, 1275.
Blaschke, G., Parker, H. I., and Rapoport, H. (1967), *J. Am. Chem. Soc. 89*, 1540.
Clarke, A. J., and Mann, P. J. G. (1959), *Biochem. J. 71*, 596.
Cromwell, B. T. (1956), *Biochem. J. 64*, 259.
Cromwell, B. T., and Roberts, M. F. (1964), *Phytochemistry 3*, 369.
Davis, B. (1954), *Advan. Enzymol. 16*, 247.
Dietrich, S. M. C., and Martin, R. O. (1968), *J. Am. Chem. Soc. 90*, 1921.
Fairbairn, J. W., and Ali, A. A. E. R. (1968a), *Phytochemistry 7*, 1593.
Fairbairn, J. W., and Ali, A. A. E. R. (1968b), *Phytochemistry 7*, 1599.
Fairbairn, J. W., and Challen, S. B. (1959), *Biochem. J. 72*, 556.
Fairbairn, J. W., and Suwal, P. N. (1961), *Phytochemistry 1*, 38.
Galinovsky, F., and Mulley, H. (1948), *Monatsh. Chem. 79*, 426.
Grundon, M. F., and Reynolds, B. E. (1964), *J. Chem. Soc.*, 2445.
Gupta, R. N. (1968), *Lloydia 31*, 318.
Gupta, R. N., and Spenser, I. D. (1968), *Chem. Commun.*, 85.
Hansen, D. L., and Bush, E. T. (1967), *Anal. Biochem. 18*, 320.
Jakoby, W. B., and Fredricks, J. (1959), *J. Biol. Chem. 234*, 2145.
King, J. A., Hoffman, V., and McMillan, F. M. (1951), *J. Am. Chem. Soc. 73*, 3165.
Koleoso, O., Dietrich, S. M. C., and Martin, R. O. (1969), *Biochemistry 8*, 4172.

Leete, E. (1958), *J. Am. Chem. Soc. 80*, 4393.
Leete, E., and Adityachaudhury, N. (1967), *Phytochemistry 6*, 219.
Liebisch, H. W., Marekov, N., and Schütte, H. R. (1968), *Z. Naturforsh 23B*, 1116.
Liebman, A. A., Mundy, B. P., and Rapoport, H. (1967), *J. Am. Chem. Soc. 89*, 664.
Martin, R. O. (1968), *Anal. Chem. 40*, 1197.
Martin, R. O., Warren, M. E., and Rapoport, H. (1967), *Biochemistry 6*, 2355.
McKee, H. S. (1962), Nitrogen Metabolism in Plants, Oxford, Clarendon, p 402.
O'Donovan, D. G., and Keogh, M. F. (1968), *Tetrahedron Letters*, 265.
Prikhod'ko, L. S., and Klyshev, L. K. (1964), *Tr. Inst. Botan. Akad. Nauk Kaz. SSR 20*, 166.
Prikhod'ko, L. S., and Klyshev, L. K. (1965), *Chem. Abstr. 62*, 9474.
Rapoport, H., Stermitz, F. R., Baker, D. R. (1960), *J. Am. Chem. Soc. 82*, 2765.
Reiner, J. M. (1953), *Arch. Biochem. Biophys. 46*, 53.
Schöpf, C., Arm, H., and Braun, F. (1952), *Ber. 85*, 937.
Scott, P. T., Pearson, D. E., and Bucher, L. J. (1954), *J. Org. Chem. 19*, 1815.
Sharma, R. K., Khajuria, G. S., and Atal, C. K. (1965), *J. Chromatog. 19*, 433.
Smith, B. N., and Meeuse, B. J. D. (1966), *Plant Physiol. 41*, 343.
Smith, E. D., and Radford, R. D. (1961), *Anal. Chem. 33*, 1160.
Spenser, I. D. (1968), *Compr. Biochem. 20*, 231.
Stermitz, F. R., and Rapoport, H. (1961), *J. Am. Chem. Soc. 83*, 4045.
Waldi, D. (1964), *in* New Biochemical Separations, James, A. T., and Morris, L., Ed., London, Van Nostrand, p 157.
Zielke, H. R., Byerrum, R. U., O'Neal, R. M., Burns, L. C., and Koeppe, R. E. (1968), *J. Biol. Chem. 243*, 4757.
Zilversmit, D. B., Entenman, C., and Fishler, M. C. (1943), *J. Gen. Physiol. 26*, 325.

The Biosynthesis of Conium Alkaloids. Identification of a Novel Nonnitrogenous Base from *Conium maculatum* as 3-Formyl-4-hydroxy-2H-pyran*

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ABSTRACT: The rapidly turning over compound D, detected in the alkaloid extracts of *Conium maculatum*, *Sedum sarmentosum*, and *Punica grantum* during our studies of the kinetics of ^{14}C incorporation into the known propylpiperidine alkaloids, has been isolated by ion exchange and paper chromatography. Its behavior is consistent with that of a weak base ($pK = <0.1$) bearing no charged groups in the pH range 2–10.

The nuclear magnetic resonance spectrum showed an aldehyde proton at τ 0.55, two vinyl protons (conjugated) at τ 2.82 and 3.56, a pair of methylene protons at τ 5.38, and an exchangeable proton at τ 5.76. The high-resolution mass spectrum showed a molecular ion peak at 126 ($C_6H_6O_3$), an $(M - 17)^+$ at 109 ($C_6H_5O_2$), and an $(M - 29)$ at 97 ($C_5H_5O_2$) (base peak). Mass fragmentation after D_2O exchange and oxime formation confirmed the compound to be 3-formyl-4-hydroxy-2H-pyran, final choice among the alternate *ortho* and two *para* isomers being made on the basis of the ultraviolet absorption (λ_{max} 280 mμ (ϵ 5100)) as well as the nuclear magnetic resonance spectrum. Though this compound appears unrelated to the known propylpiperidine bases, its possible role in the formation of *N*-heterocycles is discussed.

Several biogenetic pathways have been proposed for the formation of propylpiperidine alkaloids of the *Conium* and *Sedum* type (Leete, 1968). Feeding experiments using either lysine-^{14}C or acetate-^{14}C have shown that both can serve as *precursors* of the *Conium* alkaloids. However no evidence has ever been obtained for the existence of any *intermediate*[1] prior to the already known alkaloids.

During the course of our studies on the biosynthesis of the alkaloids of *Conium maculatum* (poisonous hemlock) using the $^{14}CO_2$ kinetic approach, several unidentified, highly radioactive, minor bases were detected (Dietrich and Martin, 1968, 1969). The most intriguing of these was the earliest labeled one which contained more than 90% of the ^{14}C in the total alkaloid extract after only 1-hr photosynthesis in

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[1] The distinction proposed by (Davis, 1954) is made between the terms precursor as "any substance whether endogenous or exogenous that can be converted by an organism into some product" and *intermediate* as "a compound formed and converted by the organism into a product."