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Biogenetic Interrelationships of Ergot Alkaloids[1]

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The biogenetic interconversions of clavine alkaloids have been studied by the use of clavine-C^{14} and clavine-H^3 alkaloids labeled biosynthetically in saprophytic culture, and four different ergot strains in saprophytic cultures. Agroclavine was shown to be oxidized irreversibly to elymoclavine. The attack appears to be directly on the methyl group. Agroclavine, but not elymoclavine, was shown to be converted irreversibly to the following clavine alkaloids: festuclavine, pyroclavine, and setoclavine. Elymoclavine was shown to be the precursor of lysergol, isolysergol, penniclavine, and isopenniclavine. These conversion reactions are irreversible. Only three clavine alkaloids were found to be metabolized in ergot strain 47 A, viz., agroclavine, elymoclavine, and lysergene, all of which have a double bond in position 8. Chanoclavine was shown not to be a precursor of the clavine alkaloids. Evidences indicate that it was not a breakdown product of agroclavine or of any other clavine alkaloid derived from agroclavine. Lysergene was shown to be converted to lysergol, isolysergol, penniclavine, and isopenniclavine. No evidence was found suggesting that lysergene is a normal intermediate in the biosynthesis of these compounds from elymoclavine. Each stereoisomer was found to be biosynthesized as such and not to be the result of a chemical or biochemical isomerization process.

INTRODUCTION

During the past year, some aspects of the metabolism of alkaloids have been clarified. Thus, a biogenetic relation between the benzoisoquinoline-type and the morphine-type alkaloids of opium has been established in a tracer study (1) in which norlaudanosoline-C^{14} was found to be converted to morphine-C^{14}. *O*-Demethylation has also been recognized as a pattern in the biosynthesis of morphine from thebaine via codeine (2). Recent tracer experiments (3) have provided support for earlier hypotheses (4) claiming phenolic coupling as a biosynthetic route for the alkaloids of the Amaryllidaceae.

To us, the ergot alkaloid seemed to represent an unusually suitable group of alkaloids for the study of biogenetic interrelations of alkaloids. These alkaloids are structurally closely related, representing various stages of oxidation. Earlier investigation in our laboratories (5) had shown that clavine alkaloids become biosynthetically labeled from mevalonic acid-2-C^{14} with excellent incorporation of C^{14} and that, by the use of ergot strain 47A, several alkaloids could be labeled to a significant degree. The problem of conversion by contaminating microbes is completely avoided since the pure ergot is grown on sterilized media in culture flasks.

The metabolic relationships between the individual clavine alkaloids and between the clavine-type alkaloids and the lysergic acid-type alkaloids have not been understood. Lately, several suggestions on possible conversion paths have been put forth. Normally, agroclavine and/or elymoclavine constitute the principal alkaloids of clavine-alkaloid-producing strains (6–9). As a rule,

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they also represent the principal components of the clavine-alkaloid fraction present along with the peptide-type alkaloids in ergot strains both in saprophytic cultures (6, 10) and in natural sclerotia of ergot (6, 11). In several biogenetic schemes, agroclavine, elymoclavine, and chanoclavine have been assigned the role of intermediates on the pathway of lysergic acid. Recently, Abe has summarized his biogenetic speculations (6). He assumes the existence of a hypothetical precursor, lysergic aldehyde, which either may be oxidized to lysergic acid or reduced via elymoclavine to agroclavine. Chanoclavine, the only known ergot alkaloid with incomplete D ring, in Abe's scheme is considered to be a breakdown product of agroclavine. In other biogenetic schemes, it has been looked upon as a precursor of the ergoline nucleus (12) and, also, as a side-reaction product (13). Rochelmeyer (12) has suggested the following biogenetic pathway:

chanoclavine → agroclavine →

elymoclavine → lysergic acid

Also, other schemes for the interrelationships of the ergot alkaloids have been proposed (13, 14).

EXPERIMENTAL

Ergot Strains

Four different ergot strains, all used in saprophytic cultures, were employed in this investigation:

1. A *Claviceps* strain isolated from *Pennisetum typhoideum* Rich. and designated as 47 A (7). We found this strain to produce 16 different alkaloids. Agroclavine and elymoclavine were present as major alkaloids; further, penniclavine, isopenniclavine, setoclavine, isosetoclavine, chanoclavine (secaclavine), lysergol, festuclavine, lysergene, lysergic acid, and isolysergol, the latter isolated for the first time from nature. It further produced four unidentified alkaloids, three of which do not correspond to any known clavine alkaloids (15).

2. A strain of *Claviceps purpurea* (16) isolated by M. Abe, Takeda Chemical Industries, Osaka, and producing chanoclavine, agroclavine, pyroclavine, and festuclavine.

3. A strain of *Claviceps purpurea* isolated from *Phleum pratense* and designated as PRL 1578 (17), but having lost its original ability to synthesize peptide-type ergot alkaloids, was redesignated as PRL 1578-P.

4. A strain of *Claviceps* isolated from a Mexican corn ergot and, as a parasite on corn, producing festuclavine, pyroclavine, agroclavine, chanoclavine, elymoclavine, and dihydroelymoclavine (15). It yielded no detectable amounts of alkaloids in saprophytic culture.

Growth Media in Conversion Experiments

All ergot cultures were grown stationary in darkness at 25°. Strain 47 A was grown on 15 ml. of sterilized yeast extract (1%)–glucose (2%) medium in 50-ml. cotton-plugged Erlenmeyer flasks for the conversion experiments.

Abe's ergot strain was grown on a medium consisting of 40 ml. of a sterilized solution of mannitol 50.0 g., KH_2PO_4 1.0 g., $MgSO_4.7H_2O$ 0.3 g., $FeSO_4.7H_2O$ 13 mg., $ZnSO_4.7H_2O$ 4 mg., succinic acid 6.8 g., distilled water 1000 ml., ammonia to pH 5.2. The other strains were grown on 40 ml. of the yeast extract–glucose medium in 125-ml. flasks.

Inoculation of Ergot Cultures

An 8- to 14-day-old yeast extract–glucose culture was homogenized for 20 sec. in a sterile Waring blendor and, depending on the amount of medium to be inoculated, from 0.1 to 0.5 ml. was used as inoculum.

Biosynthetic Labeling of Clavine Alkaloids

Twenty microcuries (μc.) of mevalonic acid-2-C^{14} (in the form of dibenzylethylenediamine salt, Tracerlab Inc. and New England Nuclear Corp.) was added aseptically as a sterile solution to a 12- to 14-day-old yeast extract–glucose (1%) culture previously fed 20 mg. L-tryptophan. After 2–3 weeks, the incorporation of radiolabel into the alkaloidal fraction amounted to 5–7% of the added amount of radioactivity.

In order to obtain some of the minor alkaloids with sufficiently high activity so as to permit conversion experiments, tritiated DL-tryptophan was used as it is much less expensive than mevalonic acid-2-C^{14}. A sterile solution of 25 μc. DL-tryptophan-U-H^3 (Volk Radiochemical Corporation) and 5 mg. of unlabeled L-tryptophan were added to each of 20 cultures of strain 47 A growing on 40 ml. yeast extract–glucose.

Extraction, Separation and Purification of Labeled Alkaloids

The mixture of biosynthetically labeled alkaloids, extracted as described previously (5), was

streaked on formamide-treated Whatman 3-MM paper (20 × 55 cm.) and chromatographed for 3.5 hr. with benzene–pyridine (6:1) as solvent (FBP) (15). In this solvent system the alkaloids produced by strain 47 A have the following R_A values ($R_{Agroclavine} = 100$): chanoclavine 5, penniclavine 23, elymoclavine 34, lysergol 37, isopenniclavine 47, isolysergol 67, festuclavine 77, setoclavine 93, agroclavine 100, isosetoclavine 105, lysergene 121, X-1 20, X-2 17, X-3 12, and X-4 3. Of these alkaloids, lysergol, isolysergol, and, particularly, X-1 to X-4, festuclavine, isosetoclavine, and lysergene were present only in trace quantities. Pyroclavine has in this system R_A value 87. The radioactive alkaloids were detected on the developed chromatograms and extracted individually as described earlier (15).

The single alkaloids were purified further by rechromatography on formamide-treated paper with chloroform–pyridine (6:1) (FCP) as solvent (chanoclavine), in system FBP for 6–8 hr. (isolysergol, isopenniclavine, penniclavine), in butanol–acetic acid–water (4:1:1) (BAW), and then in FBP (elymoclavine, lysergol, agroclavine, setoclavine).

The mixture of tritiated alkaloids was first separated in system FBP. The unknown alkaloids X-1, X-2, X-3, and X-4 were extracted together and were then separated in system FCP (15) and rechromatographed in BAW.

The alkaloids were extracted from the cultures used in the conversion experiment and from the blanks as described previously (5) except that the separated mycelium was soaked twice for an hour in 5 ml. of distilled water containing a drop of acetic acid. These washings were combined with the liquid nutrient medium, alkalinized, and then extracted with chloroform.

Except for an aliquot that was counted to determine the recovery of radioactivity, the total alkaloidal mixture from a conversion experiment was spotted on an area of 4 × 28 mm. on formamide-treated Whatman 3-MM paper (20 × 55 cm.) and then developed with benzene–pyridine (6:1) as solvent. After evaporation of solvent and formamide, a 38-mm. wide strip containing the separated alkaloids was cut out and run through a chromatogram scanner.

Quantitative Determinations of Alkaloids

After scanning, the individual alkaloids on the chromatographic strip were determined spectrophotometrically (Bausch and Lomb Spectronic 20) according to the method of Pöhm and Fuchs (18). The absorbance was measured at 375 mμ in the case of setoclavine, at 400 mμ for penniclavine and isopenniclavine, and at 550 mμ for the remaining alkaloids. Standard curves were prepared with isopenniclavine (also standard curve for penniclavine), isosetoclavine (standard curve for setoclavine), chanoclavine, elymoclavine, and agroclavine (also standard curve for remaining alkaloids). Due to exposure of the chromatograms to heat and air during the drying process, the quantities of each alkaloid are approximate rather than absolute.

Chemical Synthesis of Clavine Alkaloids

In order to obtain lysergol-C^{14}, isolysergol-C^{14}, and lysergene-C^{14} in amounts sufficient for conversion experiments, these alkaloids were prepared according to the method of Schreier (19) by heating biosynthetically labeled elymoclavine-C^{14} (0.5 mg.) with potassium hydroxide (25 mg.) in methanol (0.3 ml.) to 135–140° for 2 hr. under nitrogen in a sealed glass tube. The reaction mixture was extracted with chloroform and separated in system FBP (15). Lysergol, isolysergol, and lysergene were then eluted individually.

Festuclavine-C^{14} was prepared by hydrogenation of agroclavine-C^{14} (0.3 mg.) dissolved in 2 ml. of absolute ethanol over platinic oxide catalyst (1 mg.) at normal temperature and pressure (19). The radioactive reaction mixture extracted with chloroform was separated by chromatography in system FBP (15).

Introduction of Labeled Alkaloids

Each single labeled alkaloid was dissolved in 5.00 ml. of 0.25% aqueous succinic acid. From the 5.00 ml. of filtered radioactive solution, 0.50 ml. was used to determine the amount of disintegrations/min./ml. Aliquots of the remaining solution were introduced under the mycelial pad of the growing ergot by means of a syringe equipped with a sterilized hypodermic syringe adapter, filter HA (Millipore Filter Corp.) and a hypodermic needle 3½ in. long.

An aliquot of each solution was also introduced into a flask containing sterile medium alone (blank).

Determinations of Radioactivity

Determinations of radioactivity of isolated alkaloids were carried out with a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc.), and absolute counting rates were calculated according to the internal standard method (20). The radioactivities of the tritiated alkaloids were not determined as absolute counting rates. Determinations of radioactivity on paper chromatograms were made with a "Forro Radiochroma-

tograph" (The Forro Scientific Co.) chromatogram scanner (20). The scanning procedure was also used to determine the purity of the introduced labeled alkaloid.

In Tables I–III, listings of <200, <300, etc., d./min. do not necessarily mean that there actually was any activity present. It represents an estimate of the possible amount of radioactivity that could remain undetected under those conditions.

RESULTS

The results obtained are shown in Tables I–III and in Figs. 1 and 2.

DISCUSSION

Conversion of Agroclavine

In a preliminary communication (21), we showed the role of agroclavine as a precursor of elymoclavine, penniclavine, and isopenniclavine, and this conclusion was confirmed by Expts. XI and XII (Table I). It had been noted that the concentration of elymoclavine usually was lower in old cultures (40 days) of strain 47 A than in young ones, and that, instead, the amount of penniclavine had increased. Voigt and Wichmann (10) used this finding, together with the finding that the redox potential of cultures increased with age, as a basis for their conclusion that elymoclavine serves as a precursor of penniclavine. Under our experimental conditions, the alkaloid production started 10–14 days after the inoculation of the 47 A culture and reached a maximum after 4–5 weeks.

In Expt. XI, agroclavine-C^{14} was introduced into 23-day-old cultures of 47 A, which were harvested first after a total growth time of 39, 46, and 46 days, respectively. The yields as well as radioactivity of elymoclavine were also small in these cultures compared with the relatively large amounts and activities of penniclavine and isopenniclavine. In Expt. XII, agroclavine-C^{14} was introduced into 12-day-old cultures of 47 A and, although the exposure time was shorter than in Expt. XI, only a minute amount of radioactivity remained in the extracted agroclavine in Expt. XII. One is tempted, therefore, to draw the conclusion that a young culture of 47 A has great ability to oxidize agroclavine but lesser ability to oxidize elymoclavine, whereas the reverse holds true in the case of old cultures of 47 A. The biosynthesis of setoclavine-C^{14} from agroclavine-C^{14} was best demonstrated by the use of the degenerated non-alkaloid-producing strain PRL 1578-P (Table III). In nearly all experiments, festuclavine was formed only in trace amounts, but a significant incorporation from agroclavine-C^{14} occurred in Expt. XI. The biogenesis of this alkaloid from agroclavine, as well as of its C-8 stereoisomer, pyroclavine, was better demonstrated (Table III) with Abe's strain, which produces both of these alkaloids. Also, the conversion of agroclavine-C^{14} to pyroclavine was demonstrated with good result in our Mexican non-alkaloid-producing strain (Table III).

Agroclavine-C^{14} was found to be the precursor of the following alkaloids (Tables I, III): elymoclavine-C^{14}, penniclavine-C^{14}, isopenniclavine-C^{14}, setoclavine-C^{14}, lysergol-C^{14}, isolysergol-C^{14}, pyroclavine-C^{14}, and festuclavine-C^{14}. The amounts of isosetoclavine and lysergene in a culture were not sufficient to study the biogenesis of these alkaloids. However, by analogy with setoclavine, a biogenesis of isosetoclavine from agroclavine is evident.

In the subsequent experiments, XXVII and XXVIII, it was found that lysergene-C^{14} was converted to lysergol-C^{14}, isolysergol-C^{14}, penniclavine-C^{14}, and isopenniclavine-C^{14}. Consequently, the question arose whether lysergene is an intermediate in the biosynthesis of these compounds from elymoclavine. The amount of lysergene normally present in the cultures was too small to permit any studies on its biogenesis and, therefore, an attempt was made to detect its origin by a dilution of the metabolic pool of this alkaloid. For this purpose non-labeled lysergene was introduced into cultures fed agroclavine-C^{14} (XII) and elymoclavine-C^{14} (XIV), respectively. This approach was taken in the hope that any radioactivity passing through the diluted lysergene pool would accumulate there. However, in either experiment the total activity of this alkaloid was no greater than that of the same alkaloid obtained from a culture to which no lysergene had been

TABLE I

DISTRIBUTION OF RADIOACTIVITY AMONG ALKALOIDS RECOVERED FROM EACH 47 A CULTURE AND BLANK

Introduced labeled alkaloid	Exposed to (time)	Recovery of radioactivity	Radioactivity of isolated alkaloids (amounts of alkaloids in μg.)							
			Agroclavine	Elymoclavine	Lysergol	Penniclavine	Isopenniclavine	Setoclavine	Isolysergol	Festuclavine
Agroclavine—Experiment XI										
d./min.		%								
(84,000)	Blank (23 days)	93.8	73,900 (125)	<200	<200	<200	<200	<900	<200	<200
(84,000)	Medium + 47 A (16 days)	60.8	19,200 (110)	3,100 (45)	500 (−)	11,400 (65)	3,400 (35)	3,400 (30)	500 (10)	800 (5)
(84,000)	Medium + 47 A (23 days)	50.3	14,500 (100)	1,300 (20)	2,000 (15)	9,100 (70)	2,900 (30)	4,200 (30)	1,400 (10)	1,100 (10)
(84,000)	Medium + 47 A (23 days)	44.4	12,700 (115)	1,500 (20)	1,300 (10)	7,900 (55)	1,700 (15)	2,600 (25)	1,000 (10)	500 (5)
Experiment XII										
(57,500)	Blank (15 days)	87.0	43,000 (45)	<200	<500	<500	<200	<200	<200	<200
(115,000)	Medium + 47A (15 days)	35.4	2,000 (95)	18,100 (120)	(40)	7,300 (75)	3,500 (45)	1,200 (15)	2,200 (25)	<200
(115,000) + 250 μg. lysergene	Medium + 47 A (15 days)	61.5	2,000 (150)	55,300 (475)	(75)	4,000 (70)	1,700 (25)	900 (30)	1,800 (30)	800 (10)
Elymoclavine—Experiment XIII										
(129,000)	Blank (21 days)	91.5	<200	118,000 (175)	<1,000	<1,000	<200	<200	<200	
(214,000)	Medium + 47 A (21 days)	47.9	<200 (125)	85,000 (275)	(20)	11,000 (45)	3,800 (10)	<200 (20)	3,200 (10)	
Experiment XIV										
(26,500)	Blank	84.9	<200	21,200 (240)	<500	<500	<200	<200	<200	
(53,000) + 250 μg. lysergene	Medium + 47 A (15 days)	69.3	<200 (280)	22,200 (325)	(50)	8,250 (85)	2,400 (25)	<200 (20)	1,850 (30)	<200 (10)
(53,000)	Medium + 47 A (15 days)	49.4	<200 (120)	11,200 (210)	(40)	9,000 (75)	2,700 (20)	<200 (15)	2,000 (20)	<200 (8)

TABLE I—*Continued*

Introduced labeled alkaloid	Exposed to (time)	Recovery of radioactivity	Radioactivity of isolated alkaloids (amounts of alkaloids in μg.)							
			Agroclavine	Elymoclavine	Lysergol	Penniclavine	Isopenniclavine	Setoclavine	Isolysergol	Festuclavine
Penniclavine—Experiment XV										
(26,500)	Blank (17 days)	78.0	<200	<400	<200	17,200 (35)	<200	<200	<200	<200
(51,000)	Medium + 47 A (17 days)	52.6	<200 (45)	<400 (225)	<200 (30)	24,000 (75)	<200 (20)	<200 (15)	<200 (10)	<200 (0)
(51,000)	Medium + 47 A (17 days)	56.5	<200 (75)	<400 (195)	<200 (20)	22,000 (90)	<200 (25)	<200 (10)	<200 (10)	<200 (0)
Experiment XVI										
d./min.		%								
(25,000)	Blank (20 days)	85.7	<200	<500	<200	17,600 (30)	<500	<200	<200	<200
(50,000)	Medium + 47 A (20 days)	55.6	<200 (75)	<500 (145)	<200 (20)	26,000 (65)	<500 (15)	<200 (10)	<200 (15)	<200 (0)
Isopenniclavine—Experiment XVII										
(32,400)	Blank (22 days)	81.8	<200	<200	<500	<200	25,400 (45)	<200	<200	<200
(51,100)	Medium + 47 A (22 days)	53.1	<200 (45)	<200 (105)	<500 (55)	<200 (40)	25,100 (65)	<200 (20)	<200 (50)	<200 (10)
Setoclavine—Experiment XVIII										
(21,300)	Blank (16 days)	73.0	<300	<200	<200	<200	<200	13,400 (20)	<200	<200
(35,500)	Medium + 47 A (16 days)	59.8	<300 (55)	<200 (155)	<200 (25)	<200 (35)	<200 (15)	18,700 (35)	<200 (5)	<200 (0)
Lysergol—Experiment XIX (synthetic lysergol)										
(9,300)	Blank (25 days)	47.0	<200	<500	4,000 (10)	<200	<200	<200	<200	<200
(18,600)	Medium + 47 A (25 days)	22.0	<200 (110)	<500 (0)	4,000 (65)	<200 (45)	<200 (10)	<200 (10)	<200 (20)	<200 (0)
(18,600)	Medium + 47 A (25 days)	25.8	<200 (165)	<500 (5)	4,500 (70)	<200 (45)	<200 (15)	<200 (20)	<200 (30)	<200 (0)

TABLE I—*Continued*

Introduced labeled alkaloid	Exposed to (time)	Recovery of radioactivity	Radioactivity of isolated alkaloids (amounts of alkaloids in μg.)							
			Agro-clavine	Elymo-clavine	Lysergol	Penni-clavine	Iso-penni-clavine	Seto-clavine	Iso-lysergol	Festu-clavine
Experiment XX										
(19,200)	Blank (12 days)	70.1	<200	<500	12,000 (20)	<200	<200	<200	<200	<200
(45,500)	Medium + 47 A (12 days)	56.0	<200 (55)	<500 (65)	23,400 (70)	<200 (75)	<200 (25)	<200 (15)	<200 (25)	<200 (0)
Isolysergol—Experiment XXI (synthetic isolysergol)										
(30,000)	Blank (12 days)	78.0	<200	<200	<200	<200	<200	<200	21,700 (25)	<500
(48,500)	Medium + 47 A (12 days)	68.3	<200 (45)	<200 (175)	<200 (40)	<200 (45)	<200 (20)	<200 (15)	29,500 (45)	<500 (0)
(48,500)	Medium + 47 A (12 days)	68.2	<200 (55)	<200 (295)	<200 (50)	<200 (50)	<200 (15)	<200 (15)	30,000 (55)	<500 (5)
Experiment XXII										
d./min.		%								
(24,000)	Blank (19 days)	72.7	<200	<200	<200	<200	<200	<200	16,850 (20)	<500
(40,000)	Medium + 47 A (19 days)	53.0	<200 (55)	<200 (125)	<200 (50)	<200 (50)	<200 (20)	<200 (10)	19,700 (35)	<500 (0)
Festuclavine—Experiment XXIII										
(17,900)	Blank (12 days)	94.7	<200	<200	<200	<200	<200	<200	<200	16,300 (20)
(35,800)	Medium + 47 A (12 days)	63.5	<200 (120)	<200 (95)	<200 (55)	<200 (45)	<200 (20)	<200 (10)	<200 (35)	21,000 (35)
Experiment XXIV (synthetic festuclavine)										
(34,000)	Blank (18 days)	81.6	<200	<200	<200	<200	<200	<200	<200	27,800 (25)
(54,300)	Medium + 47 A (18 days)	69.3	<200 (150)	<200 (340)	<200 (25)	<200 (75)	<200 (30)	<200 (40)	<200 (20)	36,400 (65)
(72,800)	Medium + 47 A (18 days)	63.3	<200 (75)	<200 (100)	<200 (20)	<200 (65)	<200 (25)	<200 (10)	<200 (5)	42,100 (35)

TABLE I—*Continued*

Introduced labeled alkaloid	Exposed to (time)	Recovery of radioactivity	Radioactivity of isolated alkaloids (amounts of alkaloids in μg.)							
			Agroclavine	Elymoclavine	Lysergol	Penniclavine	Isopenniclavine	Setoclavine	Isolysergol	Festuclavine
Chanoclavine—Experiment XXV										
										Chanoclavine
(16,500)	Blank (18 days)	60.7	<200	<200	<200	<200	<200	<200	<200	7,100 (25)
(27,500)	Medium + 47 A (18 days)	39.0	<200 (130)	<200 (170)	<200 (25)	<200 (60)	<200 (25)	<200 (15)	<200 (20)	6,700 (40)
Experiment XXVI										
(37,000)[a]	Medium + 47 A (26 days)	39.8	<200 (150)	<300 (25)	<200 (35)	<200 (75)	<200 (20)	<200 (35)	<200 (20)	13,600
Lysergene—Experiment XXVII (synthetic lysergene)										
										Lysergene
(11,200)	Blank (26 days)	34.8	<200	<200	<200	<300	<200	<300	<200	2,700 (10)
(18,750)	Medium + 47 A (26 days)	33.5	<200 (65)	<300 (155)	650 (30)	4,100 (65)	650 (25)	400 (15)	400 (20)	300 (0)
Experiment XXVIII (synthetic lysergene)										
d./min.		%								
(37,200)	Blank (24 days)	41.0	<200	<200	<200	<200	<200	<200	<200	14,000 (35)
(51,300)	Medium + 47 A (24 days)	15.6	<300 (150)	<400 (20)	1,000 (55)	1,900 (40)	1,700 (15)	1,000 (20)	1,000 (30)	500 (5)

[a] No blank experiment carried out.

added. From the above findings, it seems unlikely that lysergene is a normal intermediate in the biosynthesis of lysergol, isolysergol, penniclavine, and isopenniclavine from elymoclavine but may rather represent an alkaloid that can be metabolized by strain 47 A.

The low R_A value of chanoclavine in system FBP as well as the minute amount of it present in a culture made the biogenesis of this alkaloid difficult to investigate, since usually a small amount of activity remained at the origin of the chromatogram where the alkaloidal mixture had been spotted. However, in one experiment with agroclavine-C^{14} (Expt. XI, lysergene-added culture), no activity was present on the scan at the origin, nor was any activity present on the scan corresponding to chanoclavine. Agroclavine or any other clavine alkaloids derived from it, therefore, seem not to be precursors of chanoclavine. Chanoclavine

TABLE II

DISTRIBUTION OF RADIOACTIVITY AMONG ALKALOIDS RECOVERED FROM EACH 47 A CULTURE AND BLANK

Introduced labeled alkaloid	Tritium-labeled alkaloids		Radioactivity of isolated alkaloids (amounts of alkaloids in μg.)							
	Exposed to (time)	Recovery of radioactivity	Agro-clavine	Elymo-clavine	Lysergol	Penni-clavine	Isopenni-clavine	Seto-clavine	Isoly-sergol	X-1
X-1-H³—Experiment XXIX										
counts/min.		%								
(3,600)	Blank (12 days)	73.2	<200	<200	<200	<200	<200	<200	<200	2,300 (15)
(7,200)	Medium + 47 A (12 days)	66.2	<200 (95)	<300 (125)	<200 (30)	<200 (60)	<200 (15)	<200 (25)	<200 (25)	4,000 (35)
X-2-H³—Experiment XXX										
										X-2
(11,500)[a]	Medium + 47 A (20 days)	35.0	<200 (120)	<200 (160)	<200 (25)	<200 (40)	<200 (20)	<200 (10)	<200 (15)	3,500 (15)
X-4-H³—Experiment XXXI										
										X-4
(8,500)[a]	Medium + 47 A (20 days)	37.6	<200 (160)	<200 (190)	<200 (50)	<200 (55)	<200 (30)	<200 (25)	<200 (25)	3,700 (20)

[a] No blank experiment carried out.

TABLE III

DISTRIBUTION OF RADIOACTIVITY AMONG ALKALOIDS RECOVERED FROM EACH CULTURE OF ABE'S ERGOT STRAIN, MEXICAN ERGOT STRAIN AND STRAIN PRL 1578-P

Introduced labeled alkaloid	Exposed to (time)	Recovery of radio-activity	Radioactivity of isolated alkaloids (amounts of alkaloids in μg)			
			Agroclavine	Pyroclavine	Festu-clavine	Setoclavine
Agroclavine—Experiment XXXII						
d./min.		%				
(57,500)	Blank (15 days)	87.0	43,000 (45)	<500	<200	<500
(57,500)	Abe's strain + medium (15 days)	45.0	19,500 (175)	2,900 (15)	1,100 (10)	<500 (0)
(57,500)	Abe's strain + medium (15 days)	46.1	20,300 (160)	2,100 (20)	900 (10)	<500 (0)
Experiment XXXIII						
(115,000)	Medium + Mexican strain (15 days)	49.8	Approx. 35,000	Approx. 14,000	<500 (0)	<500 (0)
Experiment XXXIV						
(57,500)	Medium + PRL 1578-P (15 days)	47.3	9,100	<500	<500	11,200
(57,500)	Medium + PRL 1578-P (15 days)	44.1	12,700	<500	<500	7,500

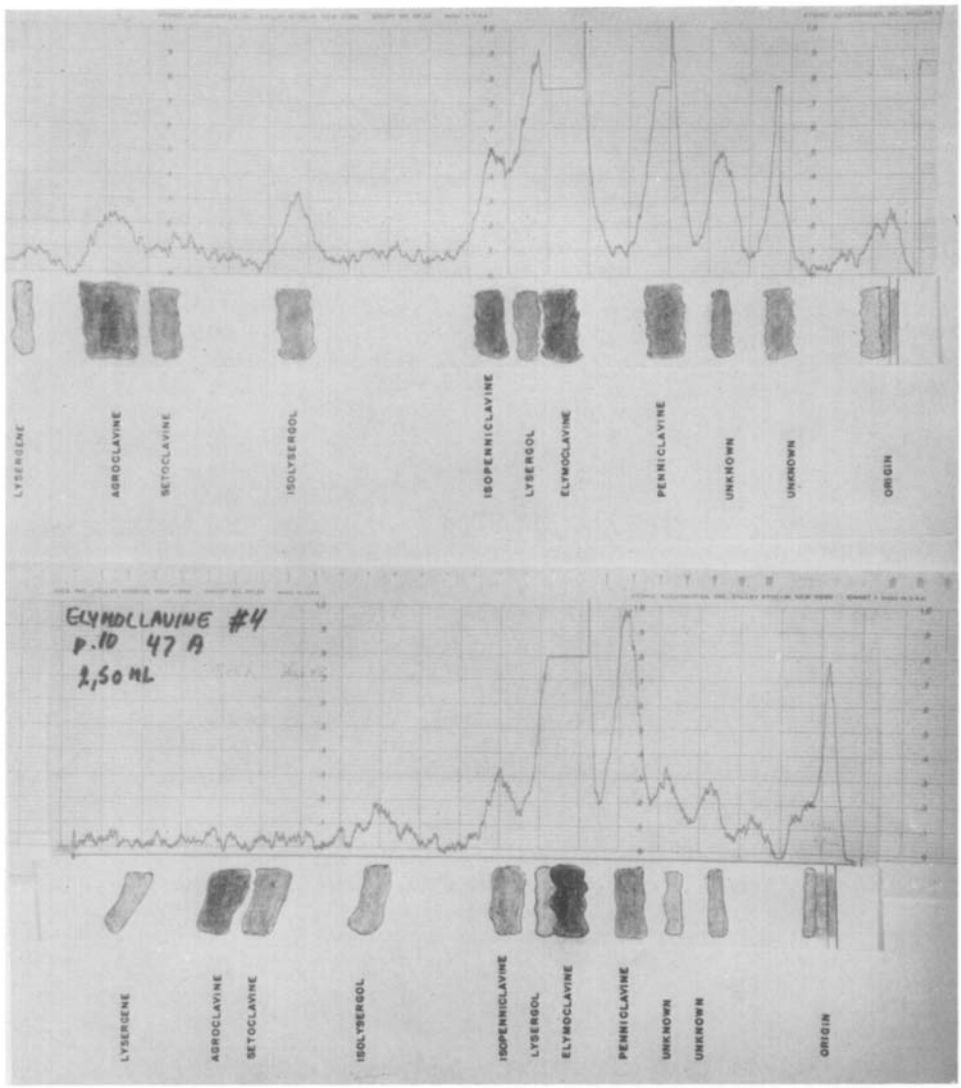

FIG. 1. Chromatogram scan of metabolized agroclavine-C^{14} (above) and elymoclavine-C^{14} (below) in ergot strain 47 A.

was found (XXV, XXVI) not to be a precursor of the clavine alkaloids.[2]

CONVERSION OF ELYMOCLAVINE

Preliminary experiments (21) had established elymoclavine as a precursor of penniclavine and isopenniclavine. These findings were verified in Expts. XIII and XIV. Furthermore, they proved that elymoclavine-C^{14} is the precursor of lysergol-C^{14} and isolysergol-C^{14}. Elymoclavine was found not to be the precursor of festuclavine, setoclavine, and agroclavine. In these experiments (XIII and XIV), it was difficult to determine the actual number of disintegrations/min. originating from elymoclavine-C^{14} and from lysergol-C^{14}. The activity of these two alkaloids therefore was counted as one. On the other hand, the existence of a shoulder and an extended slope

[2] R. M. Baxter (private communication) using Abe's strain has found that chanoclavine neither is a precursor nor a breakdown product of the tetracyclic clavine alkaloids. Using this strain, he likewise found the conversion of agroclavine to pyroclavine and festuclavine to be irreversible.

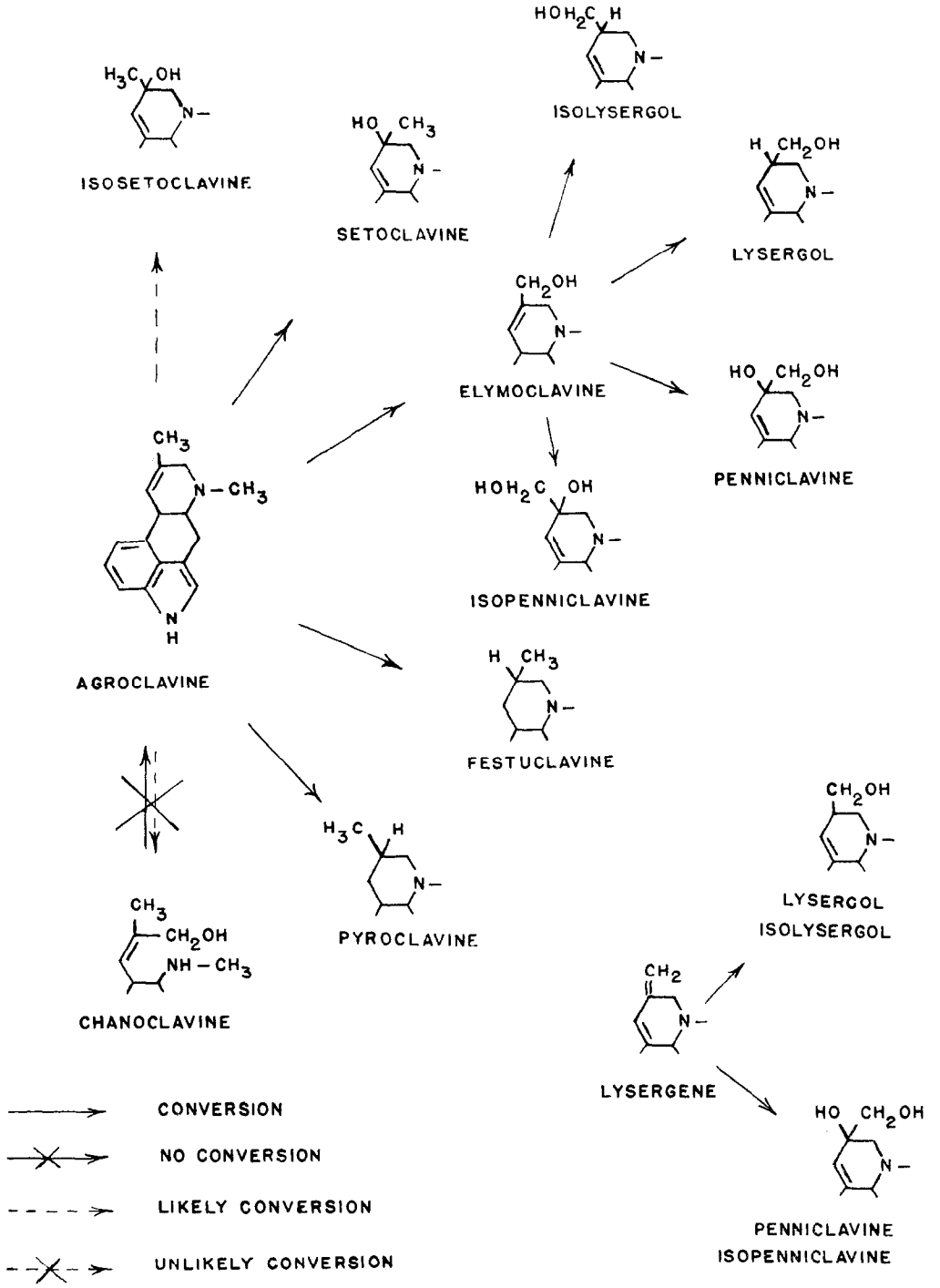

FIG. 2. Established biogenetic interrelationships among clavine alkaloids.

overlapping into the lysergol band on the "lysergol side" of the elymoclavine peak made it fully clear that lysergol-C^{14} was formed from elymoclavine-C^{14}.

The blank experiments all showed that no detectable conversion of one alkaloid to another takes place as a result of interaction with the medium or extraction procedure.

Oxidation of Agroclavine

Due to the presence of an 8,9-double bond in agroclavine, the well-established biochemical oxidation involving a dehydrogenation step followed by the addition of water cannot occur. However, assuming the involvement of two isomerization steps, as outlined below, this type of oxidation, although rather unlikely, could take place.

$$\text{agroclavine} \rightarrow \begin{matrix}\text{lysergine}\\ \text{isolysergine}\end{matrix} \rightarrow$$

$$\text{lysergene} \rightarrow \begin{matrix}\text{lysergol}\\ \text{isolysergol}\end{matrix} \rightarrow \text{elymoclavine}$$

Of the five theoretical intermediates suggested above, lysergine, lysergene, lysergol, and isolysergol (22, 15) have been reported to occur in nature. Abe *et al.* (22) also have considered a similar pathway, but in accordance with their earlier biogenetic theories (6), they proposed a reversed reaction sequence. Our experiments with lysergol-C^{14} (XIX, XX) and isolysergol (XII, XXII) ruled out a pathway involving the indicated isomerization steps. The oxidation of agroclavine to elymoclavine, therefore, seems to occur by a direct attack on the terminal methyl group.

Conversion of Other Alkaloids

The experiments performed with penniclavine-C^{14} (XV, XVI), isopenniclavine-C^{14} (XVII), setoclavine-C^{14} (XVIII), lysergol-C^{14} (XIX, XX), isolysergol-C^{14} (XXI, XXII), and festuclavine-C^{14} (XXIII, XXIV), all showed that these compounds are virtually inert as far as the biosynthesis of the other clavine alkaloids of strain 47 A is concerned. Of course, the possibility exists that one or more of these clavine alkaloids might serve as precursors of lysergic acid.

The experiments also show that each stereoisomer of lysergol and isolysergol and of penniclavine and isopenniclavine is biosynthesized independently and does not represent the product of a chemical or biochemical isomerization process.

One might conceive of an initial hydration of the 8,9-double bond followed by a 9,10-dehydrogenation in the case of the oxidation of agroclavine to setoclavine and to isosetoclavine, and of elymoclavine to penniclavine and to isopenniclavine. These dihydrointermediates would then probably exist in the alkaloidal mixture to some extent. The unknown alkaloids (X-1, X-2, X-3 and X-4) of low R_f values in FBP might have been dihydro derivatives of alkaloids such as penniclavine, isopenniclavine, and setoclavine. Upon testing, X-1, X-2, and X-4 failed to transmit radiolabel to any other alkaloid. They cannot then be the possible dihydrointermediates and, in our opinion therefore, the oxidation of agroclavine to setoclavine and to isosetoclavine and of elymoclavine to penniclavine and to isopenniclavine probably does not involve dihydroalkaloids as intermediates.

Breakdown of Labeled Alkaloids

It was of importance to determine if the radiolabel would be reincorporated into new alkaloids from possible breakdown products of labeled alkaloids and, if so, how much of it. The possible occurrence of a rapid biochemical degradation of the introduced alkaloids followed by reincorporation of radioactivity would pose problems bearing on our biogenetic results. Consequently, this possibility was investigated both by a direct and an indirect approach.

The dried mycelia of the 47 A cultures used in Expt. XII were powdered and extracted with petroleum ether. The residue after evaporation of the petroleum ether (2.7 mg.) was counted in the scintillation spectrometer and found to contain only about 10 d./min. This figure, when compared with the originally introduced amount of 230,000 d./min., makes it clear that there occurs no significant, if any, reincorporation into alkaloids biosynthesized *de novo* from breakdown products. The lipidic fraction contains fats and large amounts of ergosterol. Consequently, no significant radioactivity could have passed from breakdown products through acetate, mevalonate or active "C_1", all of which are precursors of the ergot alkaloid nucleus, back into new alkaloids. Furthermore, the percentage recovery of introduced alkaloid is of similar magnitude whether the introduced alkaloid is converted to other clavine alkaloids or not.

CONCLUSIONS

The more the biogenetic interrelations of the clavine alkaloids were elucidated, the more evident became the prominent role of agroclavine in the biosynthesis of the clavine alkaloids. All 8-methylergoline alkaloids, viz., setoclavine, festuclavine, and pyroclavine, were rendered radioactive from feeding of agroclavine-C^{14} to the mold but not from feeding of elymoclavine-C^{14}. On the other hand, all ergot alkaloids possessing an 8-hydroxymethyl group, viz. lysergol, isolysergol, penniclavine, and isopenniclavine, became labeled both from fed agroclavine-C^{14} and from fed elymoclavine-C^{14}. The latter alkaloids, then, must be biosynthesized from agroclavine via elymoclavine. None of these reactions was found to be detectably reversible.

Our experimental findings indicate further the prerequisite of a double bond in position 8 if an alkaloid is to be metabolized. All tested alkaloids that are metabolized in strain 47 A, viz., agroclavine, elymoclavine, and lysergene, possess a double bond in this position. Only these alkaloids have this particular steric configuration at C-17. The finite place of lysergene in the biogenetic scheme of the clavine alkaloids remains undecided, and its fixation may require considerable experimental work.

The biogenetic pathways which we have established in this investigation for the clavine alkaloids by the use of strain 47 A, Abe's strain, strain PRL 1578-P, and a Mexican maize ergot strain are summarized in Fig. 2.

At the present, one can only speculate regarding the biochemical mechanism by which agroclavine and elymoclavine are converted to other clavine alkaloids. The formation of pyroclavine and festuclavine by the action of an agroclavine-hydrogenating enzyme seems very likely. The requirement for TPNH and oxygen in the microsomal oxidation (23) of foreign compounds in animals may be used in support of the view that peroxidases or oxygen transferases are involved. Animal studies on the drug metabolism of Methyprylon (24), which has some structural features in common with agroclavine, have revealed an oxidation of the dehydrogenated drug, and conceivably it may well be similar to the oxidation of agroclavine → elymoclavine → lysergic acid in ergot. A possible pathway in ergot for the biosynthesis of the peptide-type alkaloids involving peroxidases or oxygen transferases would then be:

agroclavine → elymoclavine → [lysergic aldehyde] → lysergic acid → peptide alkaloids

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