THE CELL-FREE BIOSYNTHESIS OF CLAVINE ALKALOIDS

BY

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CHAPTER I

INTRODUCTION

Review of Literature

The drug ergot is a mixture of the classical ergot alkaloids prepared from the sclerotial stage of a filamentous fungus. Tulasne worked out the life history of the parasite in 1853 and named the organism Claviceps purpurea (Fries) Tul. (1). Cultures of Claviceps were reported as early as 1881 (2), but it was not until 1948, after many attempts had failed, that Abe reported unequivocal production of ergot alkaloids in saprophytic cultures (3). These cultures revealed a number of new compounds that are different from the classical ergot alkaloids which are derivatives of lysergic acid. These new compounds contain the ergoline ring that Jacobs and Gould described for lysergic acid (4), but they have a methyl or hydroxymethyl at position 17 rather than the carboxyl group of lysergic acid. Important structures are shown in Figure 1. The compounds have been called clavine alkaloids since Abe first named agroclavine in 1951 (5). In 1957 Hofmann et al. isolated chanoclavine which has the D ring of ergoline open (6). Stauffacher and Tschertter reported the natural occurrence of three chanoclavine isomers (7). The chanoclavines are usually grouped with the clavine alkaloids even though they are exceptions to the tetracyclic ergoline ring system. At this time more than twenty clavine alkaloids have been isolated from nature (8).

DIMETHYLALLYLTRYPTOPHAN

CLAVICIPITIC ACID

LYSERGIC ACID

8-METHYL ERGOLINE

CHANOCLAVINE I

ELYMOCLAVINE

AGROCLAVINE

Figure 1. Ergoline-Related Structures

Taber and Vining showed that added tryptophan stimulated alkaloid production (9) and verified tryptophan as a precursor of clavine alkaloids using ^{14}C -labelled tryptophan (10). After Gröger et al. reported the incorporation of labelled mevalonate in 1960 (11), Plieninger et al. reported the incorporation of isopentenyl pyrophosphate in 1961 (12). Such evidence suggests that carbons 7, 8, 9, 10, and 17 are derived from isopentenyl pyrophosphate (See Figure 2). This was verified by step-wise degradation experiments in 1960 by Birch et al. (13). In 1964 Baxter reported the incorporation of ^{14}C -labelled methionine (14) which indicates the N-methyl group is derived from methionine via transmethylation. Thus, the precursors of clavine alkaloids are tryptophan (minus the carboxyl carbon), isopentenyl pyrophosphate, and the methyl group of methionine as shown in Figure 2.

The chanoclavines were proposed as precursors of the ergoline alkaloids (15), but only chanoclavine I is incorporated into agroclavine (16). Numerous intermediates in addition to the chanoclavines have been postulated in the biosynthesis of clavine alkaloids, but most of these have been eliminated as possible intermediates through the work of Baxter (17,18), Floss (19,20,21), and Plieninger (12) and their co-workers. Two possible intermediates, dimethylallyltryptophan (22) and clavicipitic acid (23), have been isolated from Claviceps cultures. Plieninger et al. reported the incorporation of radioactive dimethylallyltryptophan (12). The

Figure 2. Precursors of the Clavine Alkaloids

steps from tryptophan and isopentenyl pyrophosphate to lysergic acid are a series of oxidation steps (24) and the following sequence has been proposed: tryptophan → dimethylallyltryptophan → chanoclavine I → agroclavine → elymoclavine → lysergic acid (25). Abe and his co-workers have continually insisted on the partial sequence elymoclavine → agroclavine rather than the reverse reaction (26). If chanoclavine I is a direct precursor of agroclavine, there are two cis-trans isomerizations about the double bond in dimethylallyltryptophan (27) and the hydrogens at position 9 and 10 are retained in these reactions (28,29). The mechanisms for these reactions are not known. The proposed sequence and isomerizations are shown in Figure 3.

Studies of clavine alkaloid biosynthesis in cell-free systems are conceivable, but none have been reported. The conversion of agroclavine to elymoclavine occurs rapidly in vivo but could not be shown to occur in cell-free preparations (30). Hydroxylation at position 8 or 9 occurs via the enzyme peroxidase in enzyme extracts from Claviceps (31) as well as homogenates of higher plants (32). In 1966 Voigt and Bornschein reported partial biosynthesis of clavine alkaloids with broken cell preparations (33), but they have never reported synthesis in cell-free preparations.

Figure 3. The Proposed Clavine Alkaloid Biosynthetic Sequence

Statement of Problem

The ergoline ring is the common structural unit of ergot alkaloids. The precursors of the ring system are tryptophan (minus the carboxyl carbon), isopentenyl pyrophosphate, and the methyl group of methionine via transmethylation. Numerous intermediates in the biogenesis of the ergoline ring have been postulated, but only two compounds, dimethylallyltryptophan and clavicipitic acid, have shown any promise as actual intermediates of the pathway. Thus, in spite of much work, the biosynthetic pathway remains obscure.

An alternative to intuitively deducing the intermediates of the pathway is to isolate some or all of the enzymes involved in the pathway. Since none of the intermediates are known for clavine alkaloids biosynthesis, all of the enzymes of the pathway must be extracted in a cell-free system. A cell-free preparation isolated from Claviceps purpurea PRL 1980 which is capable of synthesizing clavine alkaloids from the known precursors is the subject of this study.

CHAPTER II

EXPERIMENTAL

Materials

The materials used in this study were obtained from the following sources; all those not listed were purchased as reagent grade from commonly available commercial sources and all materials were used without further purification.

Claviceps purpurea PRL 1980 was generously given by Dr. W. A. Taber, Department of Microbiology, Texas A. & M. University, College Station, Texas; 3-methyl-3-buten-1-ol, Chemicals Samples Co., Columbus, Ohio; L-tryptophan, Fisher Scientific Co.; L-methionine, Nutritional Biochemical Corp.; isopentenyl pyrophosphate, Mann Laboratories, New York, New York; 2,5-diphenyloxazole, Beckman Instruments, Inc., Fullerton, California; radioactive compounds, New England Nuclear Corp., Boston, Massachusetts; adenosine triphosphate, liver concentrate, thiamine pyrophosphate, flavine mononucleotide, pyridoxal phosphate, nicotinamide adenine dinucleotide phosphate, flavine adenine dinucleotide (Grade III), nicotinamide adenine dinucleotide (Grade III), cytochrome c (Type III), coenzyme - A (Grade I-L), and D-lysergic Acid, Sigma Chemical Co., St. Louis, Missouri; agroclavine, K & K Laboratories; Silica Gel G, Warner-Chilcott Laboratory Instruments Division, Richmond, California; p-dimethylaminobenzaldehyde, Mallinckrodt Chemical Works, St. Louis, Missouri; Kodax Medical X-Ray Film (SB - 54), Eastman

Kodax Co., Rochester, New York; GAF Liquadol Developer and Ansco Liquifix and Hardener, General Aniline and Film Corp.; paspalic acid was given by H. Kobel, Sandoz A. G., Basel, Switzerland; dimethylallyltryptophan was kindly supplied by H. G. Floss, Department of Medicinal Chemistry, Purdue University, Lafayette, Indiana; and setoclavine was generously supplied by A. Hofmann, Sandoz A. G., Basel, Switzerland.

Procedure

Preparation of Isopentenyl Pyrophosphate

Isopentenyl pyrophosphate was synthesized from isopropenol essentially according to the method of Foote and Wold (34). Their procedure was followed to the lithium salt of isopentenyl phosphate. The acid form of isopentenyl phosphate was obtained using a Dowex 50-8X, 50-100 mesh, H^+ form, cation exchange column. The eluate was lyophilized and mixed with an equimolar portion of tributylamine. Using the method of Michelson (35) for making nucleotide pyrophosphates, tributylammonium isopentenyl phosphate was dried with alternate washes with dioxane and benzene. Toluene could not be used as it reacted with the product. All other procedures were as given by Foote and Wold. Extreme care must be taken that all solvents are absolutely dry.

Growth and Preparation of Cells

Claviceps purpurea PRL 1980 was maintained in 125 ml. Erlenmeyer flasks containing 50 ml. of basal medium (36). Transfers were made every 5 or 6 days. Subcultures for inoculation into alkaloid-producing medium were grown for 4 days.

The mannitol-succinate-salts medium of Taber and Vining (9) was employed for alkaloid production. The subcultures above were used to inoculate 2-liter Erlenmeyer flasks containing 500 ml. of medium. The flasks were wrapped with brown paper and were placed on a New Brunswick Scientific Corp. rotary shaker for aeration. The cultures were grown for 7 to 10 days at 24°C. with continuous shaking at 110 rpm. The cells were harvested by filtration and washed three times with 100 ml. ice cold 0.2 M potassium phosphate buffer, pH 6.5. The cells were resuspended in an equal volume of cold buffer solution.

Cell-Free Studies

For cell-free studies the cells were homogenized in cold buffer solution with a Virtiss "45" homogenizer at maximum speed for 30 seconds. The unbroken cells and cell debris were centrifuged down at 3100 x g for 15 minutes at 0°C. and discarded. The supernatant, which will be referred to as the original homogenate, was centrifuged at 100,000 x g for 30 minutes at 0°C. The pellet was resuspended in 10 ml. phosphate buffer with a Potter-Elvehjem homogenizer. The supernatant was further fractionated with

ammonium sulfate at 0°C. The fractions were subjected to exhaustive dialysis at 5°C. A total of 16 liters in three changes of 0.2 M potassium phosphate buffer, pH 6.5, was used.

Incubations were carried out in 50 ml. Erlenmeyer flasks at 24°C. for 12 hours. The flasks were continuously shaken with a Dubnoff Metabolic Shaking Incubator at the lowest speed. Incubation was stopped by the addition of 1 ml. concentrated ammonium hydroxide followed by extraction with five 30 ml. portions of chloroform. The alkaloids were in the chloroform fraction and were concentrated to dryness. The alkaloids were taken up in 2 ml. of chloroform and spotted on thin-layer chromatography plates. The plates were prepared by spreading a slurry of Silica Gel G over glass plates with a Desaga/Brinkmann spreader at a thickness of 0.5 mm. The plates were air dried and then activated in an oven at 110°C. for 30 minutes.

Identification of Clavine Alkaloids

Six different solvents were used to identify the alkaloids: ethyl acetate: acetone: dimethylformamide:: 5:5:1 (37); chloroform: butanol:: 2:1 with an ammonia atmosphere (7); methyl acetate: isopropanol: ammonia:: 45:35:20 (38); chloroform: diethylamine:: 9:1 (39); chloroform: methanol: ammonia:: 80:20:0.2 (39); and cyclohexane: chloroform: methanol:: 5:3:2 (37). After the chromatograms were developed the solvent was removed under vacuum and the plates were sprayed with van Urk reagent which produces a blue

color reaction with ergot alkaloids and other indole derivatives (37). The alkaloids were identified using appropriate standard samples. Agroclavine was obtained from K & K Laboratories while elymoclavine, chanoclavine I and chanoclavine II were purified by thin-layer chromatography and identified in at least three solvents by comparison with R_f values in the literature.

Miscellaneous Procedures

When radioactive tryptophan was used the chromatograms were covered with X-ray film in the dark. After a 10 day exposure to the radiation, the autoradiograms were developed for 30 seconds in GAF Liquidol developer and fixed in Ansco Liquifix and Hardener. The black spots on the autoradiograms correspond to radioactive compounds on the chromatograms. Some chromatograms were not sprayed, except for the standard alkaloids, and areas on the chromatogram that correspond to standard alkaloids and to black spots on the autoradiograms were scraped from the chromatogram and dissolved in toluene containing 0.5% 2,5-diphenyloxazole. Each of these samples was counted in a Beckman Liquid Scintillation System.

Total alkaloid samples were determined spectrophotometrically in a Beckman DB Spectrophotometer according to the method of Taber and Vining (40). Total alkaloid concentration was determined as agroclavine.

Total protein was determined as the trichloroacetic acid precipitate according to the method of Anderson et al. (41).

CHAPTER III

RESULTS AND DISCUSSION

Whole Cell Studies

Growth studies were undertaken to acquaint the author with the culturing of Claviceps and to study the dynamics of alkaloid production. Taber and Vining (41) worked out the nutritional requirements for alkaloid production in small cultures of Claviceps purpurea PRL 1980. Alkaloid production and growth for this strain are shown in Figure 4. Since large quantities of cells were anticipated for cell-free studies, their medium was tested for alkaloid production in larger cultures. Alkaloids were produced in 50 ml., 500 ml., and 10 liter cultures.

Most of the alkaloids in whole cultures are in the mother liquor which is not used in cell-free preparations. These alkaloids were isolated, purified, and identified for use as reference alkaloids. Hsu and Anderson (42) have shown that agroclavine represents 55% of the total alkaloids in Claviceps purpurea PRL 1980. Elymoclavine, chanoclavine I, and chanoclavine II represent 35%, 1.3%, and 1.8% of the total alkaloids, respectively. Anderson (43) has reported lesser amounts of molliclavine, festuclavine, penniclavine, lysergic acid, and five unknown compounds which are van Urk sensitive for this strain. Using the chromatography solvents listed earlier, unknown compounds d and e (43) have been identified as chanoclavine II and chanoclavine I,

respectively. Compound a has been identified as paspalic acid using an authentic sample of paspalic acid furnished by H. Kobel. A. Hofmann supplied an authentic sample of setoclavine which was used to verify setoclavine in extracts of this strain. Isosetoclavine and isopenniclavine were isolated from these cultures and identified in at least three solvents from R_f values in the literature. Table I shows the identification of compounds d and e, isosetoclavine, and isopenniclavine.

Table I

Chromatographic Identification of Four Clavine Alkaloids

The alkaloids were isolated from the mother liquor of whole cell cultures. The solvents are: MCA - chloroform: methanol: ammonia:: 80:20:0.2; DC - chloroform: diethylamine:: 9:1; EAD - ethyl acetate: acetone: dimethylformamide:: 5:5:1; CB - chloroform: butanol:: 2:1 with an ammonia atmosphere; and MIA - methyl acetate: isopropanol: ammonia:: 45:35:20.

Alkaloid	$R_f \times 100$				
	MCA	DC	EAD	CB	MIA
Chanoclavine I	12	11	10	51	76
Unknown e	11	11	10	50	76
Chanoclavine II	10	7	5	38	68
Unknown d	10	8	5	37	68
Isoetoclavine (Literature)	68	38	86	-	-
Isoetoclavine	66	37	85	-	-
Isopenniclavine (Literature)	56	10	77	-	-
Isopenniclavine	55	10	80	-	-

Intracellular alkaloid concentration was measured throughout alkaloid production as a monitor of production dynamics.

Figure 5 shows that the intracellular alkaloid concentration is at a maximum from day 7 until day 10. The rate of alkaloid production is proportional to the slope of the alkaloid production curve in Figure 4 and in Figure 5. Since the rate of alkaloid production is proportional to enzymatic activity and is at a maximum on days 6 and 7, the cells were harvested for cell-free preparations on day 7.

Cell-Free Studies

To obtain cell-free preparations three methods of rupturing the cells were used: sonication, grinding with sand, and blending. All of these methods caused rupture of the cells, and all produced cell-free preparations with some enzymatic activity. The specific activity of homogenates ground with sand was slightly higher than for homogenates by the other methods, but good homogenates were laborious to prepare for large cultures. Since the manipulation time of the homogenates is important in obtaining active enzyme preparations, blending the cells with a Virtiss "45" homogenizer at high speed for a short time was chosen as the method of preparation. This method of rupturing the cells was convenient for both small and large cultures and good enzymatic activity was still present.

The pH of homogenates of alkaloid producing cells that have been washed with distilled water is between 6.2 and 6.6. On

Figure 4. Growth and Alkaloid Production. The cells were grown and the total alkaloid and total protein were measured as described in the text.

Figure 5. Intracellular Alkaloid Concentration. The cells were grown in the dark at 24°C. and were filtered and washed with 0.2 M potassium phosphate buffer, pH 6.5. After blending the cells for 2 minutes in a Virtiss "45" homogenizer at high speed, the homogenate was filtered and washed three times with buffer solution and three times with distilled water. The filtrate, including the washings, were lyophilized and the total alkaloid concentration was measured as described in the text.

Figure 6. The Effect of pH on Cell-Free Alkaloid Production. The cells were grown for 10 days in the dark at 24°C. The original homogenate was prepared as described in the text and divided into seven fractions. Incubations were in 0.2 M potassium phosphate buffer at the given pH values for 24 hours. The pH was adjusted from 3.1 with concentrated potassium hydroxide. Each flask contained 5 microcuries in 0.23 micromoles D,L-tryptophan, 10 micromoles isopentenyl pyrophosphate, 10 micromoles methionine, and 10 micromoles adenosine triphosphate. The total volume of each flask was 10 ml. and the pH was unchanged at the end of incubation.

this basis 0.2 M potassium phosphate buffer, pH 6.2, was used for the initial cell-free preparations. The effect pH on cell-free alkaloid production is shown in Figure 6. Maximum production occurred in media of pH 6.5 and 7.0 and 0.2 M potassium phosphate buffer, pH 6.5, was used in subsequent experiments.

Identification of Alkaloids Synthesized in Cell-Free Preparations

A small amount of alkaloids (less than 0.1% conversion from tryptophan) was produced in crude homogenates as shown in Figure 7. To improve incorporation these homogenates were fractionated. The homogenate was centrifuged at 100,000 x g and the supernatant was further fractionated with ammonium sulfate. Figure 8 shows that significant alkaloid biosynthesis occurs only in the 60-80% ammonium sulfate fraction. The four alkaloids which are most abundant in whole cell cultures are identified from this cell-free preparation in Figure 8. Agroclavine is clearly identified in 8a and 8b; elymoclavine in 8b, 8c and 8d; and chanoclavine I and chanoclavine II in 8b, 8c, 8d, and 8e. Quantitative data verifying the 60-80% ammonium sulfate fraction as the biosynthetically active fraction is given in Table II. To further check the identity of these alkaloids, the requirements for the known precursors was tested. Figure 9 and the data in Table III verify the requirement of isopentenyl pyrophosphate and methionine for the production of chanoclavine I and chanoclavine II. In sample 3 of Figure 9, it

Figure 7. Alkaloid Production in the Crude Homogenate and Its Fractions. Cells were grown and prepared as described in the text. Sample 1 is the 60-80% ammonium sulfate fraction while sample 2 is the crude homogenate. The solvents are: EAD - ethyl acetate:dimethylformamide:acetone; CB - chloroform:butanol with an ammonia atmosphere; and MIA - methyl acetate:isopropanol:ammonia. Incubations included: 5 microcuries in 0.23 micromoles D,L-tryptophan; 5 micromoles isopentenyl pyrophosphate; 5 micromoles methionine; 1 micromole magnesium ion; 5 micromoles adenosine triphosphate; and 1 milligram liver concentrate in a total volume of 10 ml. Incubation time: 12 hours.

Figure 8. Identification of Clavine Alkaloids in Cell-Free Preparations. Cells were grown for 7 days in the dark at 24°C. The homogenate and its fractions were prepared as described in the text. The fractions were incubated for 18 hours at 24°C. in 0.2 M phosphate buffer, pH 6.5, containing: 5 microcuries in 0.23 micromoles D,L-tryptophan; 5 micromoles isopentenyl pyrophosphate; 5 micromoles L-methionine; 5 micromoles adenosine triphosphate; 1 milligram liver concentrate; and 1 micromole magnesium ion. The alkaloids were extracted as described in the text and divided into five fractions for chromatography. The solvents are: 8a - chloroform:diethylamine; 8b - cyclohexane:chloroform:methanol; 8c - chloroform:butanol with an ammonia atmosphere; 8d - chloroform:methanol:ammonia; and 8e - methyl acetate:isopropanol:ammonia. The standard alkaloids are the blue spots on the yellow chromatograms and were visualized with van Urk reagent. The black spots on the dark blue autoradiogram represent radioactive spots on the chromatogram. Agroclavine and elymoclavine were spotted on top of sample 1 in 8a, 8b, 8c, 8d and 8e. Chanoclavine I and chanoclavine II were spotted separately on 8a, 8b, 8c, and 8d and on top of sample 1 in 8e. The letters represent: A - agroclavine; E - elymoclavine; C-I - chanoclavine I; C-II - chanoclavine II; L - lysergic acid; and T - tryptophan. The numbers refer to the samples: 1. 60-80% ammonium sulfate fraction; 2. 40-60% ammonium sulfate fraction; 3. 0-40% ammonium sulfate fraction; 4. ultracentrifuge supernatant - dialyzed; 5. ultracentrifuge supernatant - not dialyzed; 6. ultracentrifuge pellet; 7. original homogenate; and 8. broken cells. Total volume in each flask was 7 ml.

Figure 8a

Figure 8b

Figure 8c

Figure 8d

Figure 8e

Table II

Cell-Free Alkaloid Biosynthesis

Cells were grown and prepared as described in the text. Incubation mixtures included: 5 microcuries in 0.23 micromoles D,L-tryptophan; 5 micromoles isopentenyl pyrophosphate; 5 micromoles L-methionine; 5 micromoles adenosine triphosphate; 1 micromole magnesium ion; and 2 milligrams liver concentrate. Sample 7 contained 5 micromoles of each of the following instead of liver concentrate: nicotinamide adenine dinucleotide, cytochrome c, pyridoxal phosphate, and nicotinamide adenine dinucleotide phosphate. Incubations were for 20 hours at 24°C. in a total volume of 6 ml. The alkaloids were separated and counted as described in the text.

Ammonium Sulfate Fraction	Agroclavine	Elymoclavine c.p.m./flask	Chanclavine I
0-30% (I)	480	200	360
30-60% (II)	120	150	220
60-80% (III)	1360	320	1030
I and II	650	240	450
I and III	360	170	260
II and III	1150	240	520
7.	770	190	380

appears that chanoclavine I is synthesized in the absence of adenosine triphosphate; however liver concentrate, which is a mixture of the cofactors that are found in liver tissue, was in the sample. It is likely that liver concentrate contains appreciable amounts of adenosine triphosphate.

Having found the enzymes of clavine alkaloid biosynthesis to be in the 60-80% ammonium sulfate fraction, a study of the

Figure 9. The Requirements for Alkaloid Production. The cells were grown and prepared as described in the text. The alkaloids were extracted as described in the text and the solvent was methyl acetate: isopropanol: ammonia. The numbers represent the fractions: 1. complete system; 2. liver concentrate is replaced with 5 micromoles of nicotinamide adenine dinucleotide, flavine mononucleotide, cytochrome c, flavine adenine dinucleotide, coenzyme A, and thiamine pyrophosphate; 3. adenosine triphosphate omitted; 4. isopentenyl pyrophosphate omitted; and 5. methionine omitted. The complete system constituted: 5 microcuries in 0.23 micromoles D,L-tryptophan; 5 micromoles isopentenyl pyrophosphate; 5 micromoles methionine; 1 milligram adenosine triphosphate; 1 milligram liver concentrate; and 1 micromole magnesium ion. Incubations were for 20 hours in a total volume of 9 ml.

C-I O
C-II O
C O
B O

1 2 3 4 5

Table III

Cell-Free Clavine Alkaloid Biosynthesis

The cells were grown for 7 days and prepared as described in the text. Incubation flasks contained 5 microcuries in 0.23 micromoles D,L-tryptophan, 5 micromoles isopentenyl pyrophosphate, 5 micromoles L-methionine, 1 micromole magnesium ion, and 2 milligrams liver concentrate in a total of 7 ml. Incubation time: 20 hours. The following samples omitted: 2 - isopentenyl pyrophosphate; 3 - methionine; 4 - adenosine triphosphate; and 5 - liver concentrate. Sample 6 is a boiled enzyme preparation.

Compound	1	2	3	4	5	6
			c.p.m./flask			
chanoclavines	3920	270	320	350	200	150

cofactor requirements of the pathway was undertaken. Several combinations of cofactors have failed to promote alkaloid synthesis and one of these is indicated by sample 2 of Figure 9. The addition of individual cofactors to liver concentrate does not stimulate alkaloid synthesis as shown in Figure 10. Some of these cofactors decrease activity. Thus, the correct combination or correct concentration of cofactors has not been found. It is possible that one or more of the enzymes of the pathway has been inhibited in the combinations that have been tried.

Unidentified Products in Cell-Free Preparations

In addition to the four clavine alkaloids that have been identified in these cell-free preparations, several derivatives of tryptophan which may be intermediates or products of the pathway are produced in these enzyme systems. For example, the spot between

Figure 10. Cofactor Requirements for Alkaloid Production. The cells were grown and prepared as described in the text. The 60-80% ammonium sulfate fraction was prepared and divided into eight fractions. The fractions contained: 10 microcuries in 0.45 micromoles D,L-tryptophan; 5 micromoles isopentenyl pyrophosphate; 5 micromoles methionine; 5 micromoles adenosine triphosphate; 1 micromole magnesium ion; and 2 milligrams liver concentrate in a total of 8 ml. The fractions had 5 micromoles of the following cofactor added: 1. nicotinamide adenine dinucleotide; 2. Pyridoxal phosphate; 3. cytochrome c; 4. flavine mononucleotide; 5. flavine adenine dinucleotide; 6. thiamine pyrophosphate; 7. coenzyme A; and 8. no additions. The solvent was methyl acetate: isopropanal: ammonia:: 45:35:20 and the incubation time was 12 hours. The total volume was 8 ml.

C-I O
C-II O
C O
B O

1 2 3 4 5

tryptophan and lysergic acid in sample 7 of Figure 8e is in the position to which dimethylallyltryptophan migrates in this solvent. If dimethylallyltryptophan is an intermediate of the pathway, it should be produced in these cell-free preparations. Various radioactive compounds in the regions of setoclavine, isosetoclavine, penniclavine, and isopenniclavine have been produced in some homogenates. These compounds are formed in cell-free preparations containing agroclavine and elymoclavine when the enzyme peroxidase is present (34). These preparations probably contain some peroxidase activity and any agroclavine and elymoclavine that is produced may be converted to the setoclavines and the penniclavines in the incubation mixture prior to isolation and identification of products. This would be most probable in crude homogenates. The unknown compounds b and c (43) occur in whole cultures and in the cell-free system. These compounds may represent products of the pathway although compound b is produced in most of the fractions of each homogenate. Since it does not require isopentenyl pyrophosphate or methionine it is probably a degradation product of tryptophan. Some of the pigments of Claviceps purpurea PRL 1980 are derived from tryptophan and migrate near the solvent front in solvent EAD of Figure 7. In crude homogenates these pigments are produced in large amounts as is indicated in Figure 7. Thus, the competing reactions for tryptophan in crude homogenates prevent the detection of clavine alkaloids. If agroclavine is produced it may be converted to penniclavine or isopenniclavine by peroxidase or to some other

product by enzymes present in crude cell-free preparations.

The Significance of Cell-Free Clavine Alkaloid Biosynthesis

At the present time all that is known about clavine alkaloid biosynthesis is: 1. the precursors are tryptophan, isopentenyl pyrophosphate, and methionine; 2. dimethylallyltryptophan and clavicipitic acid may be intermediates of the pathway; 3. there are two cis-trans isomerizations of unknown mechanism required in synthesizing agroclavine if chanoclavine I is a precursor of agroclavine; 4. peroxidase can hydroxylate agroclavine and elymoclavine in homogenates of Claviceps; and 5. many of the clavine alkaloids are interconvertible. The inability to deduce or isolate the intermediates of the pathway has stifled research in clavine alkaloid biosynthesis. A cell-free system capable of synthesizing clavine alkaloids from the precursors is a tool which can probe the details of the pathway and help answer the questions that have previously not yielded to experimental attack. It is probable that other workers have attempted the preparation of active cell-free systems and have sought other methods since there is little activity in crude homogenates. It is somewhat surprising that all of the enzymes of the pathway are in the 60-80% ammonium sulfate fraction. This means that the enzymes are of rather low molecular weight and are in the albumin fraction of protein in the cell rather than bound together in a complex or attached to an organelle. In a cell-free preparation small amounts of suspected intermediates can

be directly checked for incorporation without having to contend with the problem of transport of the molecule into the cells. In like manner, interconversions can be checked directly and the cofactor requirements for interconversion can be determined. Such experiments will relate to the biosynthetic sequence. The omission of methionine, adenosine triphosphate, magnesium ion, or liver concentrate should cause the accumulation of intermediates that precede their requirement in the pathway. The addition of inhibitors or flushing the system with nitrogen should prevent complete synthesis, and the intermediates of the pathway should accumulate. If these can be identified and synthesized in the laboratory the requirements for their conversion into agroclavine can be determined. The cell-free system may be improved so that liver concentrate can be replaced. These answers will eventually resolve the complete pathway into its correct biogenetic sequence and exact cofactor requirements for each reaction in the pathway. As the individual enzymes are isolated their mechanisms can be studied. Thus, the secrets of this long-known biosynthetic pathway are now open for inspection with cell-free preparations such as this cell-free system isolated from Claviceps purpurea PRL 1980.

CHAPTER IV

SUMMARY AND CONCLUSIONS

1. The precursors of clavine alkaloids are verified as tryptophan, isopentenyl pyrophosphate, and methionine.
2. The enzymes of clavine alkaloid biosynthesis are located in the 60-80% ammonium sulfate fraction of the ultracentrifuge supernatant of crude homogenates of alkaloid producing cells of Claviceps purpurea PRL 1980.
3. Agroclavine, elymoclavine, chanoclavine I, and chanoclavine II are produced in cell-free preparations containing the precursors listed above in 0.2 M potassium phosphate buffer, pH 6.5, containing magnesium ion, adenosine triphosphate, and liver concentrate.
4. It has not been possible to replace liver concentrate with a combination of cofactors.

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