MICROBIOLOGICAL CONVERSIONS IN THE CLAVINE ALKALOIDS

by

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CHAPTER I

INTRODUCTION

The building units of the ergoline moiety of the clavine-type ergot alkaloids have been shown to be tryptophan, mevalonic acid or a biological equivalent (9,44) and methionine (11).

The metabolic intermediates between the building units and the tetracyclic alkaloids have been the subject matter of much recent research. The interrelationships which exist between the different ergot alkaloids have been elucidated to a certain extent (1,7,9,20,21,25,27,41). Numerous culture methods and techniques have been tried by different researchers in an attempt to find the *in vivo* biogenetic pathway leading to the formation of the ergoline alkaloids, and the biogenetic relationship between the pharmacologically inactive clavine-type alkaloids and the pharmacologically active and medicinally important lysergic acid derivatives. Of these methods one may cite: the use of different strains of *Claviceps*, both parasitically and saprophytically (2,8,24,27,29, 42); the use of molds (1,12,40); of mushrooms (13); of leaves of higher plants (23); of plant homogenates (38); of liver homogenates (17); and of pure enzymes

(30,34), to effect structural transformations in these alkaloids.

Bacteria are being increasingly utilized to effect chemical transformations which would otherwise be difficult and costly to accomplish by purely chemical means (31,32,36,43). It was thought appropriate therefore to use this microbiological tool to study the effect of certain bacterial cultures on chanoclavine and lysergol, recently found in quantity in an Ipomoea seed (5), and on agroclavine and elymoclavine, all of which belong to the ergoline or clavine-type alkaloids of ergot.

It was believed that such transformations may: shed light on metabolic intermediates in the biosynthetic pathway of these alkaloids, particularly on the much disputed role of chanoclavine in the biosynthesis of the tetracyclic clavine-type alkaloids (1,20,25); help find a source of an enzyme or enzymes which can effect such transformations; or make it possible to transform these alkaloids into lysergic acid or its derivatives or into more immediate precursors of these.

Three bacterial cultures were used in this investigation: Streptococcus faecalis, Pseudomonas aeroginosa and Acetobacter aceti.

Streptococcus faecalis although known as one of the "reducing bacteria" is also known to effect

oxidative reactions (43). The other two species are known to be oxidizing bacteria and therefore they may be able to effect, for example, the oxidation of lysergol to lysergic acid or to oxidatively close the open ring in chanoclavine.

CHAPTER II

MATERIALS AND METHODS

This chapter includes materials, methods and composition of media and reagents used in this study. The isolation and purification of chanoclavine and lysergol, which is also a part of this study, is given separately in Chapter III.

Species used in the microbiological transformation studies

A. *Streptococcus faecalis* Andrewes and Horder, 1906, strain 179. This strain was isolated from a stool culture at the American University Hospital in Beirut in 1952. It is an *alpha*-hemolytic strain.*

Streptococcus faecalis, strain 148. This strain was isolated from a throat culture at the American University Hospital in Beirut in 1951. It is non-hemolytic.*

B. *Acetobacter aceti* (Beijerinck) Beijerinck, 1900, NCTC strain 1345. This was obtained as a lyophilized culture from the American Type Culture Collection, No. 8303.

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* This strain was obtained through the courtesy of Dr. Robert Matossian, Department of Bacteriology and Virology School of Medicine, American University of Beirut, Beirut, Lebanon.

C. Pseudomonas aeroginosa (Schroeter) Migula, 1900, ATCC strain P7. This was obtained as a lyophilized culture from the American Type Culture Collection, No. 10197.

Culture media used for the cultivation of the microorganisms

A. Streptococcus faecalis, strain 179 and strain 148 were grown and maintained in two different media:

1. Cooked Meat Medium (16)

Beef Heart	454 gm.
Proteose Peptone 'Difco'	20 gm.
Bacto-Dextrose 'Difco'	2 gm.
Sodium Chloride	5 gm.
Distilled water	1000 ml.

Separate ten ml. portions of the medium, containing the suspended beef heart, were placed in 30-ml. screw capped vials and sterilized at 121° C. for 15 minutes.

2. Tryptose - Phosphate Medium (18)

Bacto-Tryptose 'Difco'	20 gm.
Bacto-Dextrose 'Difco'	2 gm.
Sodium Chloride	5 gm.
Disodium Phosphate	2.5 gm.
Distilled Water	1000 ml.

Separate ten ml. portions of the medium were placed in screw capped tubes and sterilized at 121° C. for 15 minutes. The final pH of the medium was 7.2 - 7.3 .

Streptococcus faecalis, strain 179, was also grown in the following experimental medium:

3. Yeast - Glucose Medium

Brewer's Yeast Extract 'Mann'	5 gm.
Glucose	20 gm.
Distilled Water	1000 ml.

Separate 100 ml. portions of the medium were placed in 500-ml. Erlenmeyer flasks, and sterilized at 121° C. for 20 minutes.

For restoring the alpha-hemolytic property (15) of Streptococcus faecalis, strain 179, it was subcultured from the Cooked Meat Medium into Avery's Medium (No.4) by three consecutive passages in the latter, and incubating each for 24 hours at 37° C. The culture from the third passage was again subcultured into the Cooked Meat Medium for 24 hours at 37°C. and was then stored at room temperature. To test the alpha-hemolytic character of Streptococcus faecalis, strain 179, the culture from the third passage through Avery's Medium was plated on Blood Agar (No.5) and incubated at 37° C. for 24 hours. A greenish clear zone appeared around the small, convex, round, smooth-edged, glistening, opaque colonies.

4. Avery's Medium (28)

Beef Extract 'Difco'	3 gm.
Sodium Chloride	5 gm.
Peptone 'Difco'	10 gm.
Distilled Water	1000 ml.

Five ml. portions were placed in cotton-plugged tubes, and sterilized at 121° C. for 15 minutes. To each tube, one drop of sterile defibrinated rabbit blood was added, aseptically. The tubes were incubated at 37° C. for 48 hours to test for sterility.

5. Blood Agar (28)

Beef Extract 'Difco'	3 gm.
Sodium Chloride	5 gm.
Peptone 'Difco'	10 gm.
Agar 'BDH'	20 gm.
Distilled Water	1000 ml.

Forty two ml. of the hot Blood Agar was measured into a sterile 125-ml. Erlenmeyer flask and left to cool down to 45° C. To this was added three ml. of sterile defibrinated rabbit blood. Fifteen ml. of the mixture was transferred aseptically into each of three sterile petri dishes. The dishes were then placed in the refrigerator until used.

B. Acetobacter aceti, NCTC strain 1345, was subcultured from a lyophilized culture dated June 29, 1966, into the following experimental medium:

6. Primary Inoculum Medium

Brewer's Yeast Extract 'Mann'	5 gm.
Glucose	5 gm.
Distilled Water	1000 ml.

Separate ten ml. portions of the medium were placed in 100-ml. Erlenmeyer flasks and sterilized at 121° C. for 20 minutes. The organism was grown at 30° C. for 48 hours. Good growth took place and a heavy sediment appeared. This is the Primary Inoculum Culture. This culture was stored in the refrigerator as a stock culture. Five ml. of the Primary Inoculum Culture was transferred separately to each one of the 500-ml. Erlenmeyer flasks containing 100 ml. of the Yeast - Glucose Medium. The flasks were placed on a reciprocating shaker, and shaken at 100 oscillations per minute and at 26° - 30° C. for 48 hours. Very abundant growth was observed at the end of this period.

For the maintenance of Acetobacter aceti, the organism was subcultured from the Primary Inoculum Medium into the following experimental slant culture:

7. Malt-Agar

Malt Extract 'Difco'	30 gm.
Agar 'BDH'	15 gm.
Distilled Water	1000 ml.

The final pH of this medium was 5.5 . Growth was slow and slight on this medium.

C. Pseudomonas aeroginosa, ATCC strain P7, was subcultured from a lyophilized culture dated

August 25, 1964, into the Primary Inoculum Medium (No. 6) and grown for 48 hours at 30° C. It was maintained on Malt-Agar (No. 7) slants.

The experimental medium used for the biotransformation reactions had the following composition:

8. Biotransformation Medium

Corn Steep Liquor Solids 'Staley'	2.5	gm.
KH_2PO_4	0.6	gm.
$MgSO_4 \cdot 7H_2O$	0.25	gm.
Glucose	5	gm.
Distilled Water	1000	ml.

Separate 50 ml. portions of the medium were placed in 500-ml. Erlenmeyer flasks and sterilized at 121° C. for 20 minutes.

Alkaloids and alkaloidal mixtures used in the biotransformations

A. Total Alkaloid Extract from Claviceps purpurea, strain 47 A.*

Claviceps purpurea, strain 47 A, was inoculated from an agar slant into the following Shake-Culture Medium:

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* This strain was originally isolated from Pennisetum typhoideum ergot by V.E.Tyler,Jr., and obtained from French Equatorial Africa by A.E.Schwarting (39).

9. Shake - Culture Medium (14)

Mannitol	50	gm.
KH_2PO_4	1	gm.
$MgSO_4 \cdot 7H_2O$	0.3	gm.
$FeSO_4 \cdot 7H_2O$	0.013	gm.
$ZnSO_4 \cdot 7H_2O$	0.004	gm.
$(NH_4)_2SO_4$	3.668	gm.
Succinic Acid	5.4	gm.
Distilled Water	1000	ml.

The pH of the medium was adjusted to 5.2 with a 20% freshly prepared aqueous solution of potassium hydroxide (about 22 ml. was used per 1000 ml. of medium).

Separate 100 ml. portions of the medium were placed in 500-ml. Erlenmeyer flasks and sterilized at 121° C. for 20 minutes. The flasks were placed on an Eberbach rotary shaker, rotating at about 200 revolutions per minute. When sufficient growth was obtained, an inoculum was transferred to a Sucrose-Low Phosphate Medium (14) of the following composition:

10. Sucrose - Low Phosphate Medium

Sucrose	50	gm.
Mannitol	50	gm.
KH_2PO_4	0.1	gm.
$MgSO_4 \cdot 7H_2O$	0.3	gm.
$FeSO_4 \cdot 7H_2O$	0.013	gm.
$ZnSO_4 \cdot 7H_2O$	0.004	gm.
$(NH_4)_2SO_4$	3.668	gm.
Succinic Acid	5.4	gm.
Distilled Water	1000	ml.

The pH of the medium was adjusted to 5.2 with a 20% freshly prepared aqueous solution of potassium hydroxide (about 22 ml. was used per 1000 ml. of medium). One liter of the medium was placed in a 10-liter Roux bottle and sterilized at 121° C. for 20 minutes. This surface culture was incubated at 26° C. for 40 days.

The medium was filtered, made alkaline to pH 10 with dilute ammonium hydroxide solution and the total alkaloids extracted with seven successive portions of methylene chloride in a VirTis Extracto-Matic extraction apparatus. The methylene chloride extract was treated with anhydrous sodium sulfate, filtered and the filtrate evaporated to a small volume under vacuum, in a flash evaporator at 30° C. This extract is known to contain the following alkaloids (6): agroclavine, elymoclavine, penniclavine, isopenniclavine, setoclavine, isosetoclavine, chanoclavine, lysergol, isolysergol, festuclavine, lysergene, lysergic acid and four unknown alkaloids.

The extract will be referred to as Total 47 A extract.

B. The chromatographically pure alkaloids: elymoclavine and agroclavine. These were obtained from Koch - Light Laboratories Ltd., England.

C. Pure chanoclavine-I, m.p. 213° - 214° C. and

lysergol, m.p. 248° - 249° C. These two alkaloids were obtained from an Ipomoea seed, known as Kaladana in Pakistan (5). See Chapter III.

Chromatographic Methods

A. Thin-layer Chromatography

1. System AED (22)

Adsorbent: Silica Gel G (Merck).

Solvent :		
	Ethyl acetate	5 ml.
	Acetone	5 ml.
	Dimethylformamide	1 ml.

The solvent took 20-30 minutes to travel 10 cm.

2. System BPW

Adsorbent: Silica Gel G (Merck).

Solvent :		
	n-Butanol	4 ml.
	Pyridine	1 ml.
	Water	5 ml.

The solvent consisting of the supernatant liquid took 85-90 minutes to travel 10 cm.

3. System CM (5)

Adsorbent: Silica Gel G (Merck).

Solvent :		
	Chloroform	17 ml.
	Methanol	3 ml.

The solvent took 30-35 minutes to travel 10 cm.

4. System CMA (25)

Adsorbent: Silica Gel G (Merck).

Solvent :		
	Chloroform	9 ml.
	Methanol	1 ml.

This was saturated with 25% ammonium hydroxide solution and the chamber was equilibrated with a 15% solution of ammonium hydroxide.

The solvent took 40-45 minutes to travel 10 cm.

In all cases, glass plates 20 X 20 cm. and 10 X 20 cm. were coated with Silica Gel G (Merck) made into a slurry with water in the ratio of 1:2, using a Camag applicator. For routine, qualitative work, the silica gel was spread to a thickness of 0.3 mm.; for preparative purposes, to a thickness of 0.5 - 1 mm. The plates were left for 5 minutes at room temperature, then placed in a drying oven at 110° C. for 30 minutes. The plates were then stored in a drying cabinet over calcium chloride.

The chromatographic chambers were left to equilibrate with the freshly prepared solvent overnight.

B. Paper Chromatography

5. System FCP (4)

Whatmann No. 1 or 3 MM paper was impregnated with a mixture of methanol:formamide (3:1). The impregnated sheets were hung for 15 minutes in the air, to allow the methanol to evaporate. They were used as flow chromatograms.

Solvent :	Chloroform	6 ml.
	Pyridine	1 ml.

C. Detection of the alkaloids

In both thin-layer and paper chromatography, the chromatograms, after drying, were first viewed under UV - light and the fluorescent spots marked on the chromatogram. For visualization, the chromatograms were sprayed with van Urk's reagent. Composition of van Urk's reagent as modified by Stahl and Kaldewey (33):

van Urk's Spray Reagent

p-Dimethylaminobenzaldehyde	1 gm.
HCl 25%	50 ml.
Alcohol 95%	50 ml.

The chromatograms were left to dry under a hood. The development and the change in the color of the spots were noted.

CHAPTER III

ISOLATION AND PURIFICATION OF CHANOCLAVINE AND LYSERGOL FROM KALADANA SEEDS.

Abou-Chaar (4) and Abou-Chaar and Digenis (5) found that an Ipomoea seed from Pakistan known locally as Kaladana (See Fig. 1), contained appreciable quantities of chanoclavine and lysergol, in addition to small quantities of elymoclavine, penniclavine, isopenniclavine and setoclavine (4).

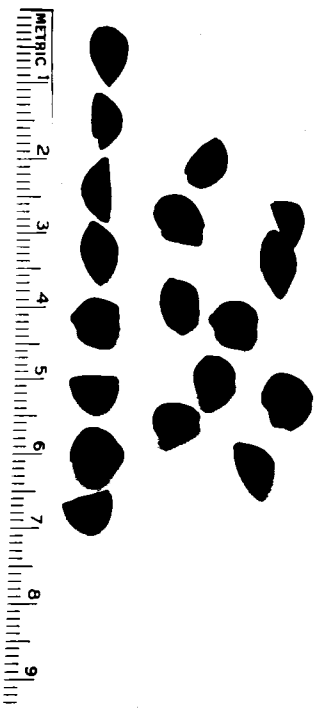

Figure 1.- Kaladana seeds (5).

It was felt that these seeds would be a good source for obtaining chanoclavine and lysergol in quantity. However, Abou-Chaar and Digenis (5) had worked with two - to ten - gram samples of the seed. It was therefore necessary to develop a method for isolating these two alkaloids in quantity from a much larger sample of seed.

The following procedure was developed for the isolation and purification of chanoclavine and lysergol from a 500 gm. sample of Kaladana seeds from Pakistan.*

A. Defatting of the seeds. Fat Content

A 500 gm. sample of Kaladana seeds was ground to a 40 mesh powder in a Wiley drug mill. The ground seed was defatted in a Soxhtlet apparatus with petroleum benzin, boiling point 30° - 40° C.(BDH-Analar grade), for 6 hours. The thimbles containing the powder were dried at 45° C. in a circulating hot air drying oven for 7 hours. The dried defatted powder weighed 436.8 gm.

Fat content. The petroleum benzin extracts were transferred quantitatively to an evaporating dish, and the petroleum benzin allowed to evaporate under the

* Purchased from Messrs. Ali Gohar & Co., P.O.Box 3405, Serai Road, Karachi, through the courtesy of Mr. G. Paraskevas.

hood, at room temperature. The dish was next placed in a drying oven at 105° C. for one hour and dried to constant weight, then stored in a desiccator over calcium chloride.

The residual petroleum benzin extractive weighed 55.23 gm. This represents a fat content of 11.05%, calculated on the basis of the air dry seed.

B. Extraction of the Total Alkaloids. Total Alkaloid Content

The defatted powdered seed, weighing 436.8 gm., was divided into two equal halves, and each half placed in a 2-liter Erlenmeyer flask containing a mixture of one liter of anhydrous ether (A.R. Fisher, Cat.No. E-138) and 40 ml. of dilute ammonium hydroxide solution. The two flasks were shaken intermittently for 30 minutes, stoppered and left to stand overnight in the dark. All subsequent operations were carried under subdued light.

The content of each flask was transferred to a separate 1000-ml. cylindrical separatory funnel. The content of each funnel was percolated with anhydrous ether until the percolate showed absence of alkaloids when tested with van Urk's reagent. The powder in each funnel required about five liters of anhydrous ether in addition to the quantity originally mixed with it.

The ether extracts were combined and evaporated to dryness in a flash evaporator, under vacuum at room temperature. The oily, brownish residue was next washed out quantitatively from the flask with 350 ml. of a 1% aqueous tartaric acid solution. The latter was kept in the refrigerator overnight. The tartaric acid solution containing the alkaloids was shaken twice with 50 ml. of anhydrous ether. The ether washings were discarded. The acidic solution was rendered alkaline to pH 10 with 35 gm. of solid anhydrous sodium bicarbonate, and extracted ten times with 300 ml. portions of methylene chloride (Technical grade, Eastman Kodak). The methylene chloride extracts were pooled, dried with anhydrous sodium sulfate, filtered, and evaporated to dryness in a flash evaporator under vacuum at 30° C.

The crude alkaloid mixture weighed 2.32 gm. indicating a total alkaloid content of 0.53 per cent, calculated on the basis of the defatted seed.

C. Fractionation of the Crude Alkaloid Mixture

The crude alkaloid mixture in the flask, weighing 2.32 gm., was shaken with 25 ml. of methyl alcohol (BDH-Analar grade). The methyl alcohol dissolved the trace alkaloids and the chanoclavine, leaving the bulk of the lysergol as an amorphous precipitate. The supernatant methanol extract was removed with a pasteur

pipette and reserved. The residue was washed with two 3 ml. portions of cold methanol. The washings were added to the reserved methanol extract. The residue, which was almost pure lysergol, was recrystallized from methanol. This lysergol had been fully characterized by Abou-Chaar and Digenis (5). The mother liquors form the crystallization of the lysergol were added to the reserved methanol extract and the whole was evaporated to dryness under vacuum at 30° C.

This residue which was further dried under nitrogen, weighed 1.89 gm. This was named Fraction A.

Fraction A was taken up in 200 ml. of chloroform (BDH-Analar grade), centrifuged, and the supernatant liquid carefully removed and kept in the refrigerator for further fractionation. The residue, Fraction B dried under nitrogen, weighed 0.19 gm. This fraction was dark brown in color and was completely soluble in methanol.

The crude alkaloid mixture, the chloroformic extract of Fraction A, and Fraction B were chromatographed in System AED (No.1). See Fig. 2.

D. Column Chromatography of the Chloroformic Extract of Fraction A

As seen from Fig. 2, the chloroformic extract of Fraction A contained most of the chanoclavine. This

was now chromatographed on an alumina column as follows:

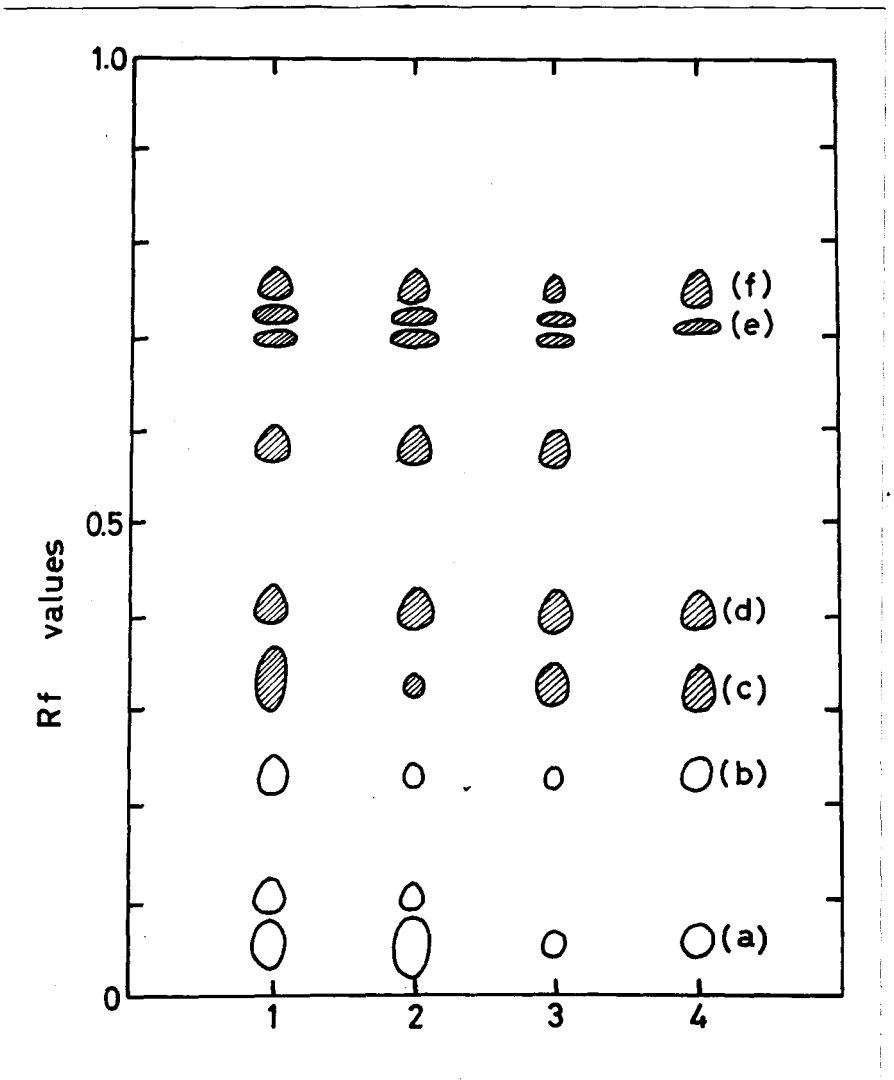

Figure 2.- Chromatogram in System AED (No.1,p.12). 1. crude alkaloidal mixture, 2. chloroformic extract of fraction A, 3. Fraction B, 4. known alkaloids: (a) chanoclavine, (b) elymoclavine, (c) lysergol, (d) penniclavine, (e) isopenniclavine, (f) setoclavine.

Two hundred and fifty grams of aluminum oxide standardized according to Brockmann (Merck,1097) was made into a slurry with chloroform, and transferred to a glass column measuring 2 X 100 cm. and fitted with a stopcock and a pledget of glass wool at its base, taking care to evacuate all air from the column.

The chloroformic extract of Fraction A, measuring 200 ml., was poured on the column and elution continued with chloroform (BDH-Analar grade). The first 300 ml. of eluate was alkaloid free when tested on chromatographic paper with van Urk's reagent. From here on, the eluates were collected in test tubes in 5 or 10-ml. portions. Five microliters from every fourth tube was spotted on a thin-layer plate and the plate developed in System AED (No.1) to check on the presence and kind of alkaloid in the eluate. See Table I.

Eluates of fractions 3 and 4 were pooled, as well as those of fractions 5 and 6. All fractions were dried with anhydrous sodium sulfate, filtered through a sintered glass funnel, grade C (coarse) and evaporated to dryness under vacuum at 30° C. The residues were dissolved separately in methanol, transferred to small brown vials and the methanol solutions evaporated to dryness under nitrogen; then stored in a desiccator over calcium chloride.

The combined residue from tubes 63-150 (See Table I)

Table I.- Eluates from the Chloroformic Extract of Fraction A on the Alumina Column and their Alkaloidal Content

Fractions	Solvents	Eluates (Tube No.)	Alkaloids present & their R_f-values in System AED+
1	Chloroform	1 - 12*	Lysergol (+) R_f 0.33, Fluorescent alkaloids 1,2,3 (+++) R_f 0.58, 0.70, 0.75, respectively.
2	Chloroform 99.5 Methanol 0.5	13 - 62*	Non-fluorescent alkaloid 'X' (+++) R_f 0.1, Elymoclavine (++) R_f 0.23 Lysergol (+) R_f 0.33 Fluorescent alkaloids 1,2,3 (traces).
3	Chloroform 99 Methanol 1	63 - 81*	Chanoclavine (+++) R_f 0.04 Non-fluorescent alkaloid 'X' (traces). Lysergol & Elymoclavine (traces).
4	Chloroform 98 Methanol 2	82 - 150**	Chanoclavine (+++) R_f 0.04
5	Chloroform 97 Methanol 3	151-170**	Penniclavine (+) R_f 0.41 Isopenniclavine (traces) R_f 0.72
6	Methanol	171-180**	Penniclavine (+++) R_f 0.41 Isopenniclavine (++) R_f 0.72

+ The relative concentration of each alkaloid in the different fractions, as determined visually from the thin-layer plates, is indicated below by (+), (++), or (+++).

* 5-ml. eluates.

** 10-ml. eluates.

contained the bulk of the chanoclavine. This was dissolved in 50 ml. of a 1% phosphoric acid solution, the solution made alkaline to pH 7.2 with about 5 ml. of dilute ammonium hydroxide solution, and extracted with four 50 ml. portions of chloroform. The chloroform solution extracted most of the non-fluorescent alkaloid 'X', lysergol, and elymoclavine, in addition to a small quantity of chanoclavine. The aqueous layer was made more strongly alkaline, to pH 10-11, with dilute ammonium hydroxide solution and extracted with seven 40 ml. portions of methylene chloride. The methylene chloride extract contained mainly chanoclavine with only slight traces of the other alkaloids extracted into the chloroform layer. The methylene chloride extract was dried with anhydrous sodium sulfate, filtered and the filtrate evaporated to dryness under vacuum at 30° C. The alkaloid residue was dissolved in 40 ml. of hot acetone (BDH-Analar grade). The acetone solution was filtered through a sintered glass funnel, grade C, reduced in volume under vacuum until crystallization was induced, and was then placed in the refrigerator overnight.

The first crop of crystals weighing 35.8 mg., melted at 205° - 210° C. This was recrystallized twice from acetone. The melting point of the recrystallized chanoclavine, as determined on the Fisher-Johns micro-

melting point apparatus was 213° - 214° C. with decomposition, similar to that reported in the literature (25).

To test the purity of the chanoclavine, it was chromatographed in the following Systems: AED (No.1), CMA (No.4) and FCP (No.5). In all these systems it showed only one spot on the chromatogram. This spot was non-fluorescent and when sprayed with van Urk's reagent, it developed a violet color which changed to blue on standing.

The ultraviolet spectrum* of chanoclavine as determined in absolute alcohol, showed three absorption peaks at : 225,284 and 293 mμ, and a trough at 245 mμ, all of which correspond to those given in the literature (26,35). See Fig. 3.

The infra-red spectrum** of chanoclavine was determined in chloroform. See Fig. 4.

The melting point and chromatographic behavior of this isolated chanoclavine confirm its identity as chanoclavine-I (25).

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* All UV-spectra were determined with a Perkin-Elmer 202 Automatic Spectrophotometer.

** All IR-spectra were determined with a Perkin-Elmer Infracord Spectrophotometer.

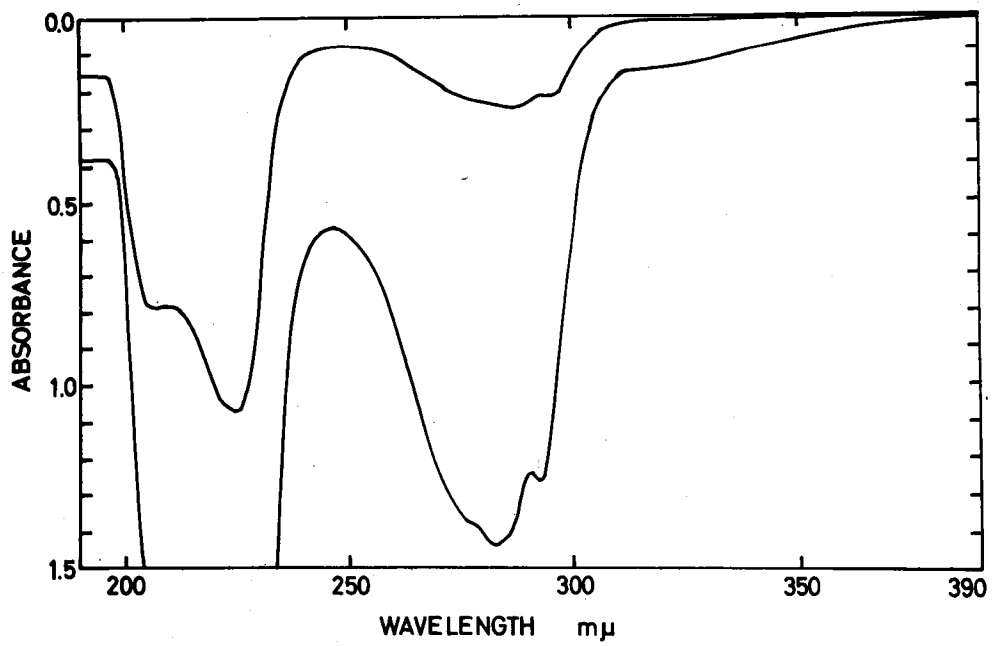

Figure 3.- UV - spectrum of chanoclavine.

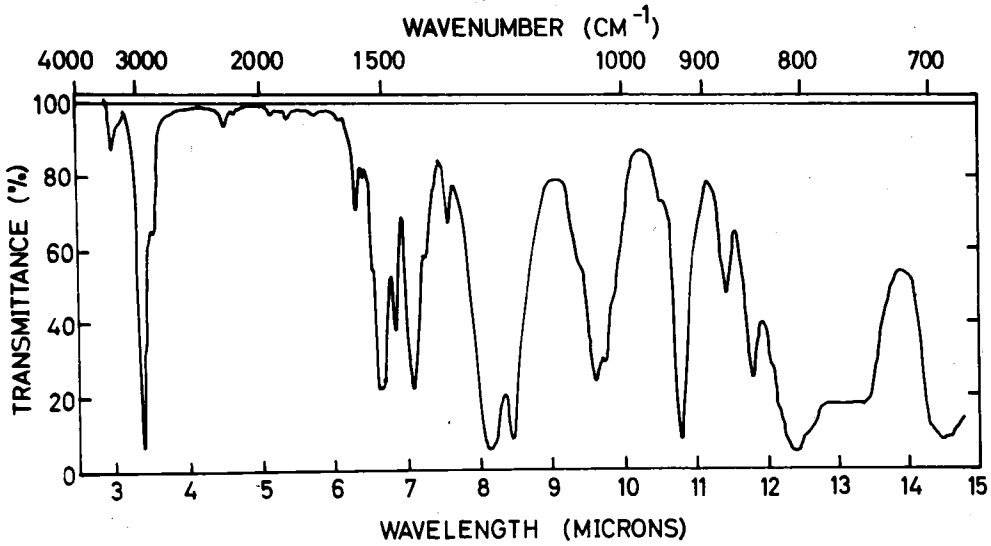

Figure 4.- IR - spectrum of chanoclavine.

Extraction and purification of Unknown alkaloid 'X'

During the routine qualitative chromatography of the crude alkaloid extract of the seed, a non-fluorescent, van Urk positive spot, just above chanoclavine in System AED was noted (See Table I). The bulk of this substance appeared in Fraction 2 (tubes 13-62) (Table I).

The dried residue remaining after the evaporation of the eluate in tubes 13-62, as previously described, was dissolved in three ml. of chloroform and streaked quantitatively on three 20 X 20 cm. preparatory Silica Gel G plates. The plates were developed in System AED (No.1). The band with R_f 0.1 was scraped into a 25-ml. glass stoppered flask and eluted with a mixture of equal volumes of methanol and methylene chloride. The eluate was filtered through a sintered glass funnel, grade M (medium) and reduced in volume to three ml., after which it was again streaked quantitatively on two Whatmann 3MM chromatographic papers. The papers were developed in System FCF (No.5) as flow chromatograms for 6 hours. The band having R_f 0.36 was cut out and eluted with 2% ammoniacal alcohol (3) (consisting of a mixture of 2 ml. dilute ammonium hydroxide solution plus 98 ml. alcohol 95%) for 24 hours. This eluate was evaporated to dryness under vacuum, leaving 0.5 ml. of formamide with the residue. This was taken up in

10 ml. of 1% phosphoric acid solution. The acidic solution was made alkaline to pH 10 with dilute ammonium hydroxide solution and extracted with four, 10 ml. portions of chloroform. The separated chloroform was evaporated to dryness under vacuum at 30° C., further dried under nitrogen, and stored in a desiccator over calcium chloride.

During the chromatography and elution of this alkaloid, the ever-presence of lysergol as a contaminant was noted. It was further shown that unknown alkaloid 'X' was very unstable in acidic solutions and decomposed to a compound that behaved chromatographically the same as lysergol (38).

Unknown alkaloid 'X', is non-fluorescent in UV light, develops a purple color with changes to blue on standing, when sprayed with van Urk's reagent. It is soluble in chloroform and insoluble in methanol.

Its ultraviolet spectrum was taken in absolute alcohol. See Fig. 5.

The chromatographic behavior of unknown alkaloid 'X' is given in Table II.

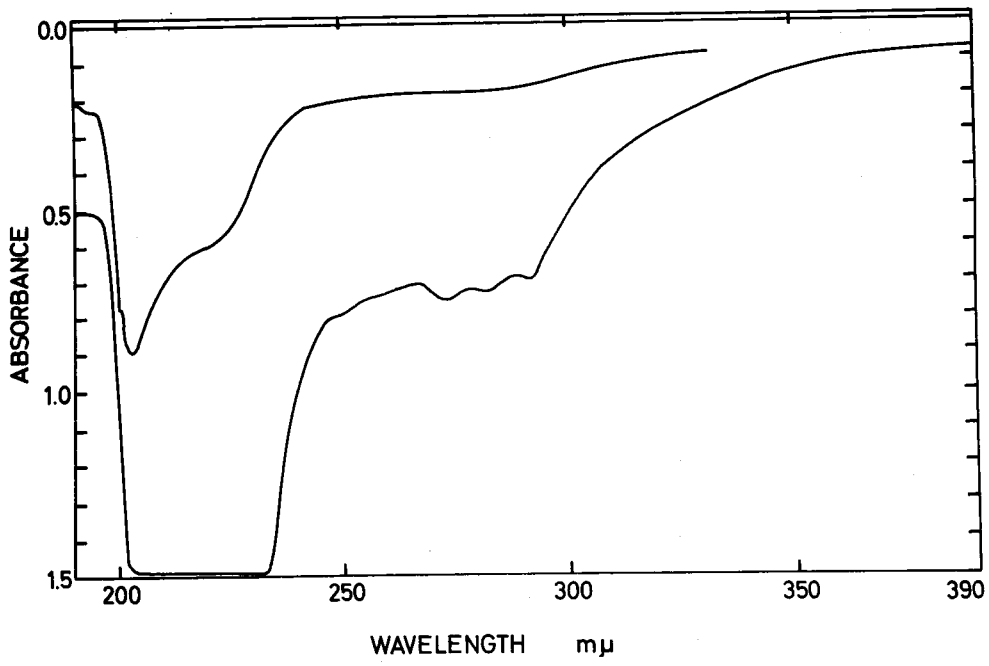

Figure 5.- UV - spectrum of unknown alkaloid 'X' from Kaladana seeds.

Table II.- Average R_f-values of Chanoclavine and of Unknown Alkaloid 'X' in Different Systems

Systems	Chanoclavine	Unknown Alkaloid 'X'
System AED (No. 1)	0.04 - 0.05	0.10 - 0.12
System CM (No.3)	0.08 - 0.09	0.12 - 0.14
System FCP (No.5)	0.24	0.36

CHAPTER IV

BIOTRANSFORMATION STUDIES WITH BACTERIA

As already indicated in Chapter I, three bacterial cultures were chosen for the biotransformation studies, namely: Acetobacter aceti, NCTC strain 1345; Pseudomonas aeroginosa, ATCC strain P7; and Streptococcus faecalis, strain 179, and strain 148.

The choice of these three microorganisms was based on literature reports (36,43) that they are able to effect oxidation reactions on certain substrates fed to them. Streptococcus faecalis is also known as a "reducing bacteria", five reducing enzymes having been isolated from one of its strains (19).

Eight major problems had to be solved before the actual biotransformation studies could be undertaken:

a. A medium which is suitable for the vegetative growth of the organism is not always suitable for the biotransformation reactions. Therefore, it was necessary to find for each organism a suitable culture medium for its vegetative growth and, in some cases, a different medium for the biotransformation studies.

b. The medium should not contain any ingredient which would later interfere with the test for the substrate or for its metabolites. For example, tryptophan

and other indoles, would give, like our substrates, a positive test with van Urk's reagent. Therefore media containing such substances could not be used for biotransformation studies.

c. It was necessary that the medium for the biotransformation studies possess an acidic pH so that it could solubilize the alkaloid base when this was added to it in a solid form, or prevent its precipitation when added as solution.

d. The optimum temperature for the biotransformation reactions had to be determined for each of the microorganisms, and then maintained during the incubation period.

e. The optimum incubation period had to be determined. A shorter period may be insufficient for the biotransformation to take place, while a longer period may result in the decomposition of the biotransformation products.

f. The technique of feeding the substrate to the culture is important, as also the maintenance of asepsis. The substrate was fed to the culture in two forms: as a solid and in solution.

g. A technique for controlling the aeration of the medium had to be developed: Acetobacter aceti and Pseudomonas aeroginosa are aerobic microorganisms, while Streptococcus faecalis is a microaerophilic or anaerobic organism.

h. Another very important consideration is the age of the culture at the time the substrate is to be fed. Bacterial cultures, unlike fungal cultures, show a very rapid growth rate; so that they need only a few hours, may be 8-24 hours, to reach their maximum metabolic rate of growth. Therefore, bacterial cultures of different ages were used in the hope of determining the optimum age at which they are best suited for the biotransformation studies.

Studies with Acetobacter aceti

A. Aerobic Incubations

1. Using Total 47 A Extract

Two 5-ml. portions of the Primary Inoculum Culture (p.8) were transferred separately to two 500-ml. Erlenmeyer flasks, each containing 100 ml. of the Yeast-Glucose Medium (No.3,p.6). The flasks were placed on a reciprocating shaker and shaken at 100 oscillations per minute and a temperature of 26° - 30° C. for 24 hours. Two 5-ml. portions of this bacterial culture were then transferred separately to a set of two 500-ml. Erlenmeyer flasks each containing 50 ml. of the Yeast-Glucose Medium,using sterile, graduated pipettes. A second set of two, 500-ml. Erlenmeyer flasks, each containing 50 ml. of the Yeast-Glucose Medium served

as controls. The two sets of flasks were placed on a reciprocating shaker, and shaken at 100 oscillations per minute and 26° - 30° C. for 24 hours.

Good growth was observed in the first set at the end of this incubation period, the second set remaining sterile. To each of the four flasks was now added, aseptically, one ml. of a 50% methanolic solution of the Total 47 A Extract containing 390 mcg. of the total alkaloids calculated as elymoclavine. The flasks were returned to the reciprocating shaker and maintained for several hours at the same conditions as above. Ten-ml. aliquots were withdrawn aseptically from each flask at intervals of 8, 16, 24, 48 and 72 hours, and in some cases more. The aliquots were then transferred separately to 50-ml. separatory funnels for the extraction of the alkaloids.

The content of each separatory funnel was made alkaline to pH 10 with dilute ammonium hydroxide solution and extracted with three 5-ml. portions of methylene chloride. The methylene chloride extracts were dried with anhydrous sodium sulfate, filtered, and evaporated to dryness under vacuum at 30° C. The residue was dissolved in one ml. of a mixture of equal volumes of methanol and methylene chloride. Five µl quantities of this extract were spotted on thin-layer chromatographic plates and developed in Systems AED and CM (p.12). Results are given in Table III.

2. Using Elymoclavine

The same procedure as outlined under Total 47 A Extract was used except that 3 ml. of a sterile* 0.03% solution of elymoclavine in 50% ethyl alcohol was added aseptically to each of the four flasks, each flask containing 60 ml. of the Yeast-Glucose Medium. Results are given in Table IV.

3. Using Agroclavine

The same procedure as outlined under Total 47 A Extract was used except that 3 ml. of a sterile* 0.03% solution of agroclavine in 50% ethyl alcohol was added aseptically to each of the four flasks, each containing 50 ml. of the Yeast-Glucose Medium. Results are given in Table IV.

4. Using Lysergol

The same procedure as outlined under Total 47 A Extract was used. The lysergol solution was prepared by dissolving one mg. of pure lysergol in 10 ml. of 50% ethyl alcohol and sterilized as for elymoclavine. One ml. of this solution was added aseptically to each of the four flasks, each flask containing 50 ml. of the Yeast-Glucose Medium. Results are given in Table IV.

---

*Fifteen mg. of the chromatographically pure alkaloid was dissolved in 50 ml. of a 50% ethyl alcohol solution, and sterilized by filtration through a sintered glass funnel, grade F (fine).

Table III.- Aerobic Incubations of Total 47 A Extract with Acetobacter aceti, NCTC strain 1345

Alkaloids added to culture	Incubation period (hours)	Alkaloids found in* : Controls	Incubations with organism
Total 47 A Extract	8	Chanoclavine (+) Elymoclavine (+++) Penniclavine (+) Lysergol (tr.)	Chanoclavine (+) Elymoclavine (+++) Penniclavine (+) Lysergol (tr.)
	16, 24, 48 and 72	Chanoclavine (+) Elymoclavine (+++) Penniclavine (+) Lysergol (tr)	Chanoclavine (+) Elymoclavine (+++) Penniclavine (++) Lysergol (-) Fluorescent Alk. X_1 (+), R_f 0 Non-fluorescent Alkaloids X_2 (+) R_f 0.70; X_3 (+) R_f 0.71

* The relative concentration of each alkaloid in the different aliquots, as determined visually from the thin-layer plates developed in Systems AED and CM, is indicated below as follows: (+++), (++), (+), tr.(trace), (-). R_f values quoted here are those determined in System AED (See Table I for other R_f values).

Table IV.- Aerobic Incubations of Elymoclavine, Agroclavine, and Lysergol with Acetobacter aceti, NCTC strain 1345

Alkaloids added to culture	Incubation period (hours)	Alkaloids found in* : Controls	Alkaloids found in* : Incubations with organism
Elymoclavine	8 - 72	Elymoclavine (+++)	Elymoclavine (+++)
	72 - 138	Elymoclavine (+++)	Elymoclavine (++)
Agroclavine	8 - 96	Agroclavine (+++)	Agroclavine (+++)
Lysergol	8	Lysergol (+++)	Lysergol (+++)
	16 - 72	Lysergol (+++)	Elymoclavine (tr.) Non-fluorescent Alkaloid X_4 (tr.) R_f 0.1 Lysergol (+++)
	96	Lysergol (+++)	Lysergol (++) Elymoclavine (tr.) Penniclavine (+) Isopenniclavine (tr.) Non-fluroscent Alkaloid X_4 (tr.) R_f 0.1

* See footnote under Table III.

Results of the aerobic incubations of Total 47 A extract, elymoclavine, agroclavine and lysergol with Acetobacter aceti:

It is clearly seen from Tables III and IV that, under aerobic conditions, lysergol was slowly metabolized to penniclavine by Acetobacter aceti.

Trace amounts of isopenniclavine, elymoclavine and of an unknown alkaloid X_4 appeared in the culture after 96 hours of incubation.

Elymoclavine began to decompose to a non-indole moiety after 72 hours of incubation.

B. Anaerobic or Microaerophilic Incubations

1. Using Total 47 A Extract

Five 1-ml. portions of the Primary Inoculum Culture were transferred separately to five 15-ml. screw-capped tubes, each containing 10 ml. of the Yeast-Glucose Medium. Another set of five tubes, each containing 10 ml. of the Yeast-Glucose Medium, served as controls. The two sets of tubes were placed in an incubator at 30° C. Good growth was observed only after 48 hours in the first set of tubes, the second set remained sterile.

To each of the 10 tubes was added 0.5 ml. of a 50% methanolic solution of the Total 47 A Extract containing 195 mcg. of the total alkaloids calculated

as elymoclavine. The tubes were then replaced in the incubator at 30° C.

Two tubes, one of each set, were removed at the following time intervals and the alkaloids extracted as outlined under Total 47 A Extract (p. 31): 8, 24, 48, 72 and 96 hours.

Chromatographic examination of the processed tube cultures in all of the tubes failed to show any qualitative or quantitative changes in either the controls or in the incubations with Acetobacter aceti under anaerobic or microaerophilic conditions.

No further anaerobic incubations of elymoclavine, agroclavine and lysergol with Acetobacter aceti were therefore attempted.

Studies with Pseudomonas aeroginosa

A. Aerobic Incubations

1. Using Total 47 A Extract

Two 5-ml. portions of the Primary Inoculum Culture (p.8) were transferred separately to a set of two 500-ml. Erlenmeyer flasks each containing 50 ml. of the Biotransformation Medium (No.8,p.9). A second set of two 500-ml. Erlenmeyer flasks, each containing 50 ml. of the Biotransformation Medium, served as controls. One drop of oleic acid (BDH) was added

aseptically to each of the four flasks as an antifoam agent. The flasks were then placed on a reciprocating shaker and shaken at 100 oscillations per minute and 26° - 30° C, for 24 hours.

Fairly good growth was observed in the first set of flasks at the end of this incubation period, the second set remaining sterile. To each of the four flasks was now added, aseptically, one ml. of a 50% methanolic solution of the Total 47 A Extract containing 390 mcg. of the total alkaloids calculated as elymoclavine. The flasks were returned to the reciprocating shaker and maintained for several hours at the same conditions as above. Ten-ml. aliquots were withdrawn aseptically from each flask at intervals of 8, 16, 24, 48 and 72 hours. The procedure for the extraction of these aliquots and for the chromatographic examination of their extracts was the same as that described under Total 47 A Extract (p.31).

Results of the incubation of Total 47 A Extract with Pseudomonas aeroginosa:

The chromatographic examination of the processed aliquots showed that the only alkaloid which underwent change was penniclavine. A three fold increase in the amount of penniclavine, over that found in the controls was observed after an incubation period of 16 hours. This increase remained constant after 72 hours

of incubation. In the meantime, agroclavine disappeared completely from the incubation aliquots, as compared to the controls.

2. Using Elymoclavine

The same procedure as outlined above was used except that 3 ml. of a sterile 0.03% solution of elymoclavine in 50% ethyl alcohol was added aseptically to each of the four flasks, each flask containing 60 ml. of the Biotransformation Medium.

Results of the incubation of Elymoclavine with Pseudomonas aeroginosa:

The chromatograms showed that elymoclavine, in an aqueous medium at pH 5.8 and up to 72 hours of aerobic incubations with Pseudomonas aeroginosa, was not metabolized or decomposed to any other substance. After 138 hours of aerobic incubation, however, about 0.1% decomposition of elymoclavine to non-indole compounds was observed.

B. Anaerobic or Microaerophilic Incubations

No anaerobic or microaerophilic incubation experiments were attempted with Pseudomonas aeroginosa because the latter, being an aerobe and a fastidious organism, did not grow sufficiently in the different media cited in Chapter II.

Studies with Streptococcus faecalis

A. Aerobic Incubations

1. Using Total 47 A Extract

Two one-ml. portions of a culture of Streptococcus faecalis, strain 179, in the Cooked Meat Medium (No. 1,p.5), were transferred separately to a set of two 500-ml. Erlenmeyer flasks, each containing 50 ml. of the Yeast-Glucose Medium. A second set of two, 500-ml. Erlenmeyer flasks, each containing 50 ml. of the Yeast-Glucose Medium served as controls. The two sets of flasks were then placed on a reciprocating shaker, and shaken at 100 oscillations per minute and 26° - 30° C. for 24 hours. Good growth was observed in the first set at the end of this incubation period, the second set remaining sterile.

The amount of Total 47 A Extract added to the four flasks, the incubation periods and the procedure for the extraction of the alkaloids, were the same as under Total 47 A Extract (p.31).

Results of the incubation of Total 47 A Extract with Streptococcus faecalis:

The chromatographic examination of the processed aliquots showed that under aerobic conditions Streptococcus faecalis, strain 179, did not metabolize

the mixture of alkaloids it was fed, not even when the pure alkaloids elymoclavine, agroclavine and lysergol were fed, as outlined under Acetobacter aceti.

B. Anaerobic or Microaerophilic Incubations

Alkaloid 'A'

1. Using Total 47 A Extract

To two 5-ml. sterile test tubes, was added 0.5 ml. of a 50% methanolic solution of the Total 47 A Extract (p.36). These test tubes were placed on a water-bath at 45° C. until the solvent evaporated. To one, was added 0.5 ml. of a 24-hour old culture of Streptococcus faecalis, strain 179, in the Cooked Meat Medium (p.5), and to the other 0.5 ml. of sterile Cooked Meat Medium. These two tubes were left at summer room temperature 26° - 30° C. for 18 hours. After this period of incubation, the contents of both tubes were processed as described before.

Results of the incubations of Total 47 A Extract with Streptococcus faecalis:

The chromatographic examination showed that the spot corresponding to chanoclavine in the control alkaloids had disappeared completely. It was replaced by a van Urk positive, non-fluorescent spot just above the spot of chanoclavine, in System AED and CM. All the

other alkaloids in the control as well as in the incubation tube were unchanged.

2. Using Chanoclavine

A solution of chanoclavine was prepared by dissolving 2 mg. of pure chanoclavine in 10 ml. of methanol. To each of ten 5-ml. sterile tubes, in two sets, 0.1 ml. of the chanoclavine solution was added. One ml. of a 24-hour culture of Streptococcus faecalis, strain 179, in a Cooked Meat Medium was added to each tube in one set. To each tube of the second set was added 1 ml. of the sterile Cooked Meat Medium. The two sets of tubes were incubated at summer room temperatures 26^{o} - 30^{o} C.

One tube of each set was withdrawn at 2.5, 4, 6, 8 and 16 hour intervals and the alkaloids extracted separately from each tube as previously described for Total 47 A Extract (p.31).

Results of the incubations of chanoclavine with Streptococcus faecalis:

The chromatographic analysis showed that, after only 2.5 hours, 80% of the chanoclavine was converted to an indole derivative, Alkaloid 'A', which behaved like a clavine alkaloid. Complete quantitative conversion of chanoclavine to Alkaloid 'A' by Streptococcus faecalis, strain 179, took 16-24 hours of incubation

at 26° - 30° C.

Further experiments were carried out, in the hope of identifying Alkaloid 'A'.

Six mg. of pure chanoclavine were dissolved in 1 ml. of methanol and this was added to 2 ml. of a 24-hour culture of Streptococcus faecalis, strain 179, in Cooked Meat Medium in a 5-ml. sterile test tube and was incubated at 26° - 30° C. The metabolism of chanoclavine was followed at 2-hour intervals by removing aseptically, with a capillary tube, about 2 µl of the culture medium and spotting it directly on thin-layer plates, and then developing the plates in Systems AED and CM.

Complete conversion of chanoclavine to Alkaloid 'A' took 18 hours. No trace of unchanged chanoclavine was left. The culture medium was transferred to a 10 ml. separatory funnel, made alkaline to pH 10 with dilute ammonium hydroxide solution, and extracted with three five ml. portions of methylene chloride. The methylene chloride extracts were dried with anhydrous sodium sulfate, filtered and concentrated under vacuum to 1 ml. This amount was streaked quantitatively on three preparatory Silica Gel G plates and developed in System CM. The band corresponding to Alkaloid 'A' was scraped out, eluted with 25 ml. of a mixture of equal volumes of methanol and methylene chloride. This was filtered, and evaporated to dryness under vacuum at

room temperature.

The ultraviolet spectrum of Alkaloid 'A' was taken in methanol where it showed the following peaks: at 225, 274, 282 and 292 mμ.

The chromatographic behavior of Alkaloid 'A' was compared with chanoclavine in four different systems (See Table V). Like chanoclavine, Alkaloid 'A' was non-fluorescent, and gave with van Urk's reagent a purple color changing to dark blue on standing. It was soluble in methanol and insoluble in chloroform and in acetone.

Table V.- Average R_f-values of Chanoclavine and Alkaloid 'A' in Different Systems

Systems	Chanoclavine	Alkaloid 'A'
System AED (No. 1)	0.04 - 0.05	0.08 - 0.12
System BPW (No. 2)	0.30	0.38
System CM (No. 3)	0.08 - 0.09	0.18 - 0.20
System FCP (No. 5)	0.24	0.33 - 0.35

In an attempt to recrystallize Alkaloid 'A' from acetone, white, prismatic crystals were obtained. The m.p. of these crystals when taken on a Fisher-Johns micro-melting point apparatus came to be above 300° C. An ultraviolet spectrum of these crystals in methanol showed peaks at : 225, 282 and 288 mμ, different from those of Alkaloid 'A'. This substance was therefore called 'A_1' .

The conversion of Alkaloid 'A' to 'A_1' by crystallization from acetone, was shown to be irreversible when 'A_1' was recrystallized from benzene or methanol. Substance 'A_1' was insoluble in methanol but was partly soluble in chloroform.

It was therefore necessary to repeat the incubation with Steptococcus faecalis to obtain again a sample of Alakloid 'A'. As no chanoclavine was available, no further incubation studies were possible before a further supply of chanoclavine became available. Such a supply was obtained by extraction from Kaladana seeds (See Chapter III).

Further studies with Streptococcus faecalis

Alkaloid 'B'

The processing of Kaladana seeds lasted for a whole month. Once chanoclavine became available again, it was used in a series of incubations with two strains

of Streptococcus faecalis, strain 179 and strain 148.

In this study 15-ml. screw-capped tubes containing 10 ml. of sterile Tryptose-Phosphate Medium (No.2,p.5) or 30-ml. screw-capped vials, containing 10 ml. of sterile Cooked Meat Medium were used. The plan of these incubations is summerized in Table VI.

Surprisingly, chromatographic examination of the processed tubes and vials at the end of each incubation period, showed no change in the concentration of chanoclavine, not only in the controls but also in the incubation tubes. No other indole metabolite besides chanoclavine could be detected chromatographically.

Microscopic examination of the bacterial culture of strain 179 showed that its morphology had changed markedly. Its behavior with Gram's stain, and when plated on Blood Agar had changed and there was no alpha-hemolysis on the latter.

It was thought that the lack of the alpha-hemolytic power of Streptococcus faecalis, strain 179, was correlated in a way with the negative results obtained above. Therefore, the alpha-hemolytic property of the organism was restored to it by following the procedure described in Chapter II,p.6.

To obtain a conclusive answer as to whether or not the chanoclavine was converted to a different substance, a new batch of pure crystalline chanoclavine, weighing 12 mg., was incubated with 10 ml. of a 24-hour culture

Table VI.- Plan of the Series of Anaerobic or Micro-aerophilic Incubations of Chanoclavine with Streptococcus faecalis.

Medium	Age of Culture* (hours)	Incubation period (hours)	Strain	Form and quantity of added Chanoclavine, per tube or vial
Tryptose-Phosphate Medium	24 or 48	8,16,24,48	148 & 179	Solid ** 0.5 mg.
" "	8 or 16	2.5,4,8,16, 24	148 & 179	Solid ** 0.5 mg.
" "	24 or 48	8,16,24,48	148 & 179	Solution*** 0.1 or 0.3ml.
" "	8 or 16	2.5,4,8,16, 24	148 & 179	Solution*** 0.1 or 0.3ml.
Cooked Meat Medium	24 or 48	2.5,4,8,16, 24	148	Solid ** 0.5 mg.
" "	8 or 16	2.5,4,8,16, 24	148 & 179	Solid ** 0.5 mg.
" "	8,13,16 or 24	2.5,4,8,16, 24	179	Solution*** 0.1 or 0.3ml.

* Controls consisted of the same number of tubes or vials, and contained the same amount of media. All cultures were made in duplicate.

** Chromatographically pure chanoclavine, in crystal form, was added to the cultures and controls.

*** A sterile, 0.1% solution of chanoclavine in phosphate buffer, pH 4.0.

of Streptococcus faecalis, strain 179, for 24 hours at 30° C. The extraction of the culture was performed in the same manner as described before. When the extract was chromatographed in System CM, only one alkaloid spot was detected. The extract was further purified by chromatography on preparatory thin-layer plates in System CM, crystallized from chloroform and dried under vacuum at 30° C.

The purified alkaloid, called Alkaloid 'B', was chromatographed in the following systems, alone and mixed with pure chanoclavine-I :

Systems AED (No.1), BPW (No.2) and the following two systems,

System CB (35)

Adsorbent: Silica Gel G (Merck)

Solvent : Chloroform 20 ml.
n-Butanol 10 ml.

The chamber was equilibrated with ammonia from a concentrated ammonium hydroxide solution placed in a beaker in the chamber. The solvent front travelled 10 cm. in 75 minutes.

System IB

A strip of Whatmann Ion Exchange Cellulose Phosphate paper, grade P-20 (now known as P 81 Cellulose Phosphate Paper), measuring 10X58 cm. was used. It was eluted descendingly with a 0.2M $NaHCO_3$ / Na_2CO_3 buffer*, pH 9.2, for 6¼ hours at 21.5° C.

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* Long,Cyril. Biochemists' Handbook, Spon,London, 1961, p.36 ; through the courtesy of Miss Valentine Mihranian.

The solvent front travelled 42 cm.

The results are given in Table VII.

Table VII.- Average R_f-values of Chanoclavine and Alkaloid 'B' in Different Systems

System	Chanoclavine	Alkaloid 'B'
System AED (No. 1)	0.04	0.04
System BPW (No. 2)	0.30	0.23
System CB	0.74	0.50
System IB	0.23	0.20

The solubility of Alkaloid 'B' was similar to that of chanoclavine: it was soluble in methanol and in methylene chloride but was sparingly soluble in chloroform. It melted at 185° - 190° C. with decomposition at the latter temperature.

The ultraviolet spectrum of Alakloid 'B' determined in absolute alcohol, is shown in Figs. 6 and 7. Its infra red spectrum, determined in chlorofrom (Baker, 9180) is shown in Fig. 8. It is clearly seen from these spectra that Alkaloid 'B' is different from chanoclavine.

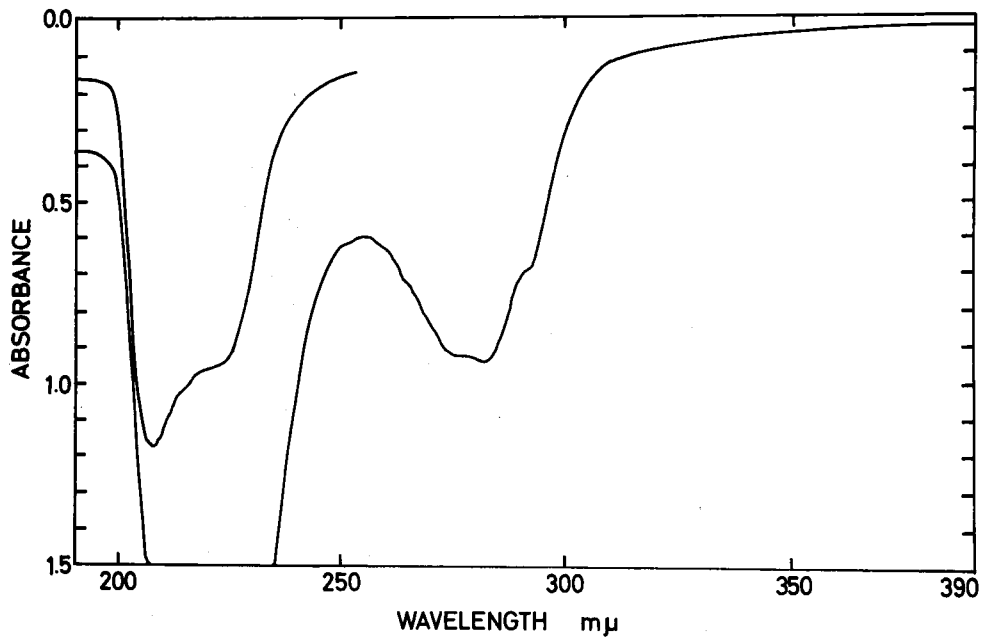


Figure 6.- UV - spectrum of Alkaloid 'B'.

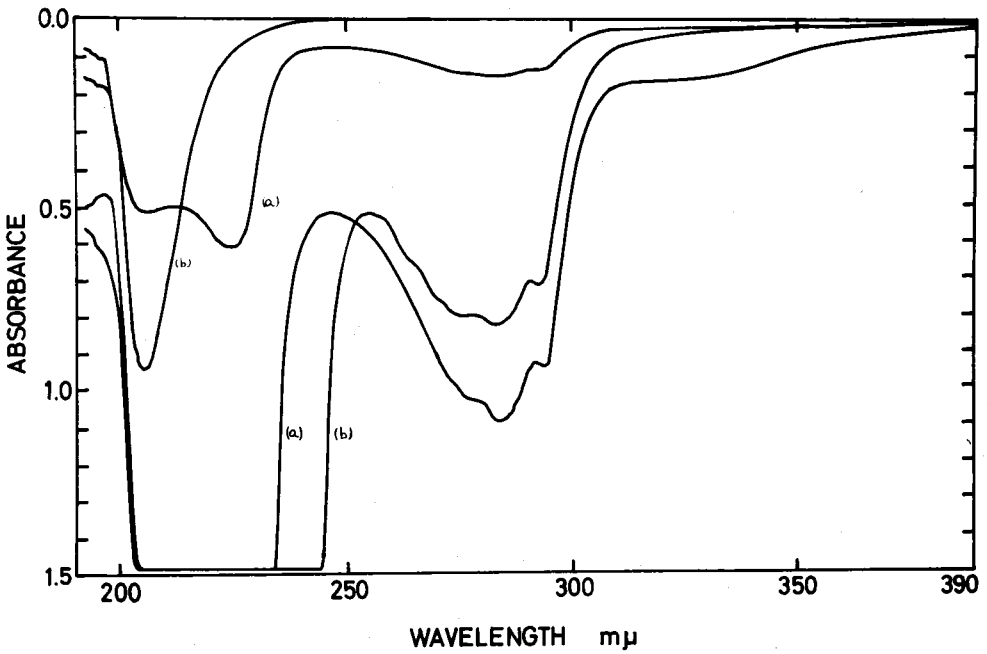


Figure 7.- UV - spectra : (a) chanoclavine,
(b) Alkaloid 'B'.

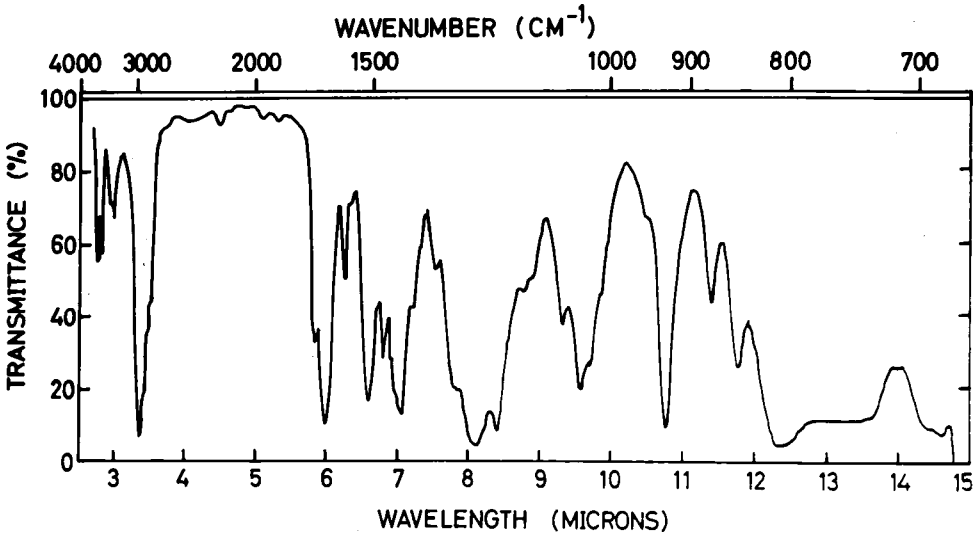


Figure 8.- IR - spectrum of Alkaloid 'B'.

CHAPTER V

DISCUSSION AND CONCLUSION

Abe and coworkers (1) using strains of Claviceps and other fungi proposed the following interrelationship among the clavine alkaloids (See Fig. 9).

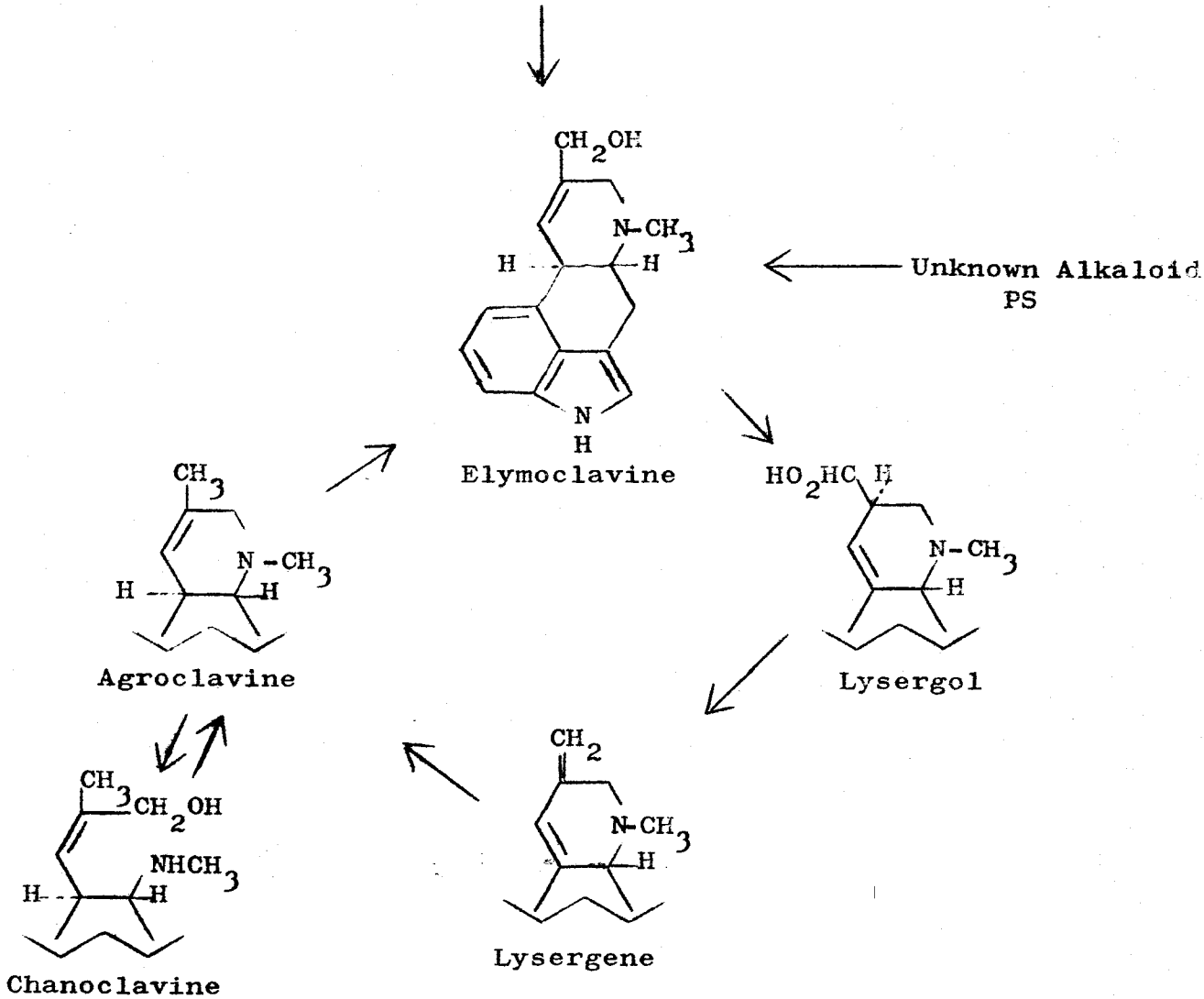


Figure 9.- Biosynthetic interrelationship among some ergoline-derivatives (1).

His proposed scheme shows elymoclavine to be the primary product in the cyclic biosynthetic route and the conversion of elymoclavine through lysergol and lysergene to agroclavine to be more common than the reverse process.

Agurell (9) and Baxter (10) do not agree with Abe's proposal but show that, in *in vivo* experiments, agroclavine and not elymoclavine is the precursor of the known tetracyclic clavine alkaloids. Figure 10 shows the scheme proposed by Agurell (9).

Most of the research done in this field was centered around agroclavine and elymoclavine. It was shown that agroclavine and elymoclavine can be easily 8-hydroxylated into seto-/isosetoclavine and penni-/isopenniclavine respectively by the use of a variety of tools e.g. molds (12,40), mushrooms (13), *Ipomoea* leaves (23);and that the responsible enzyme is a peroxidase or oxygen transferase and that the active hydroxyl is derived from molecular oxygen (7). Tissue homogenates from higher plants were also shown to effect the above hydroxylations (38).

The only known ergot alkaloids with incomplete D-ring, namely, chanoclavine-I, isochanoclavine-I and chanoclavine-II, were shown by some workers to be precursors to the tetracyclic clavine alkaloids (1,20), while others showed that it was not so (9).

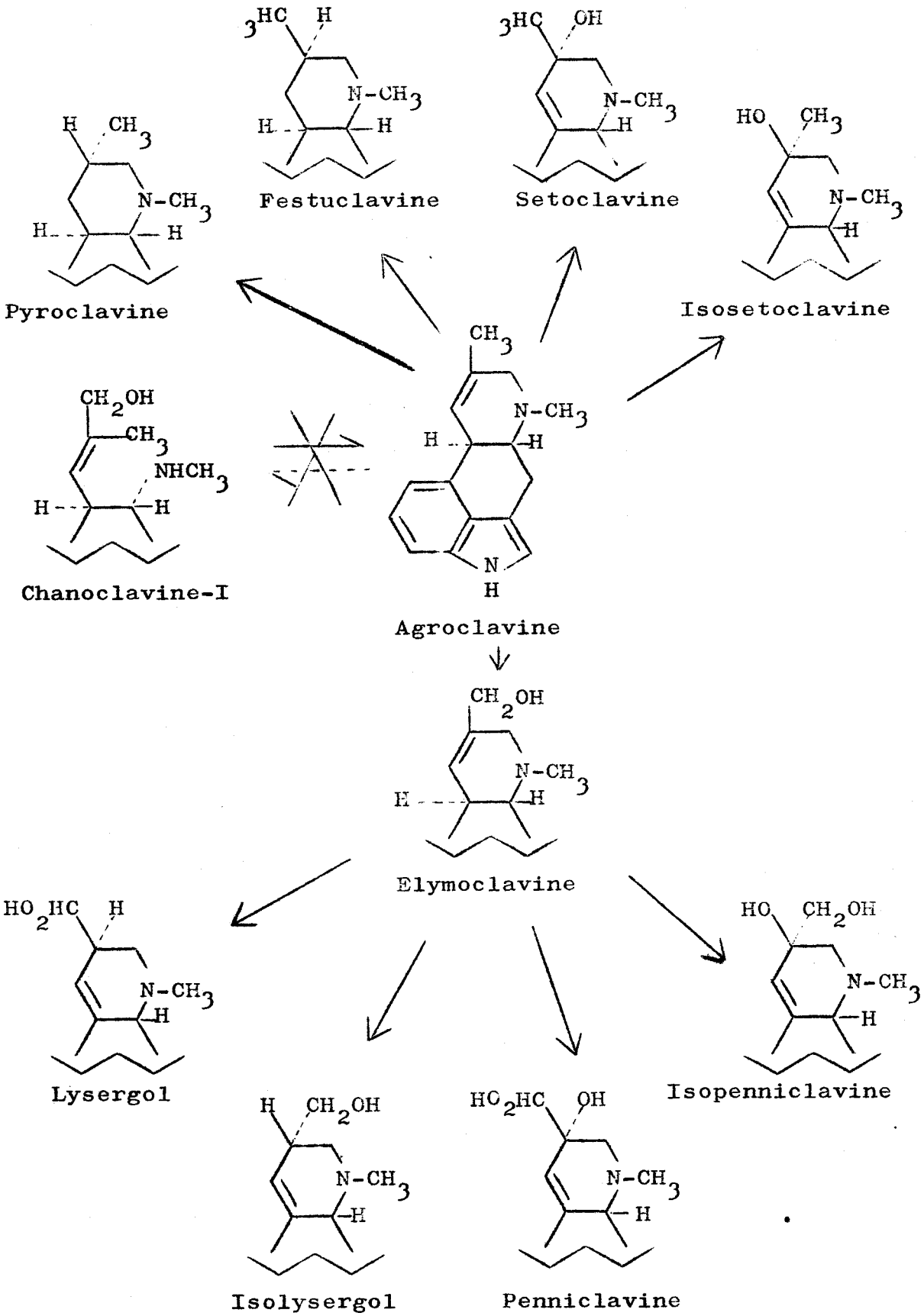


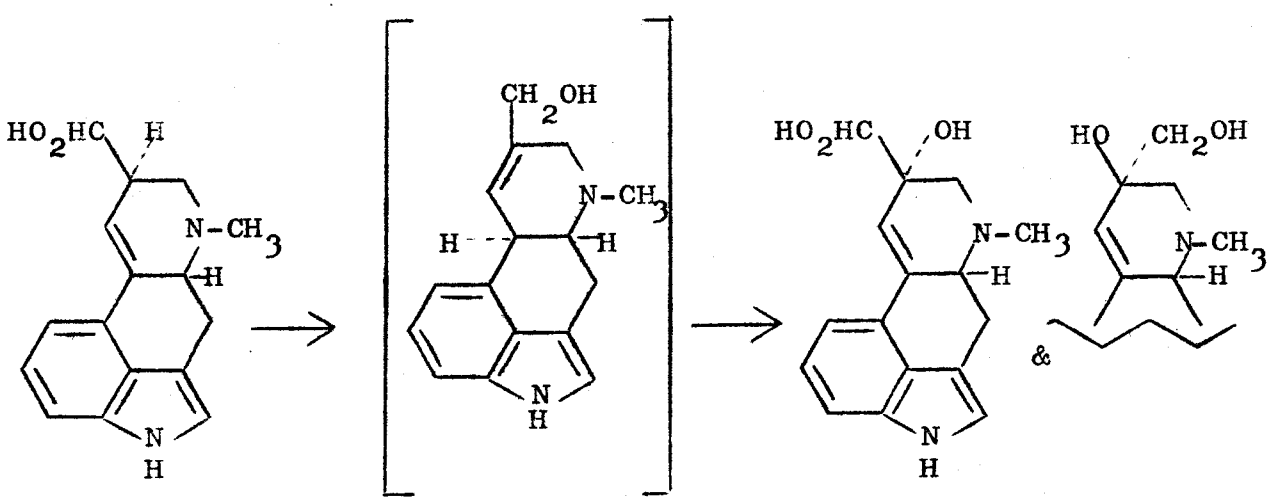
Figure 10.- Proposed biosynthetic interrelationship among clavine alkaloids according to Agurell (9).

Fehr et al.(20) and Gröger et al.(25) disproved the theory that isochanoclavine-I is the probable precursor of the tetracyclic ergoline alkaloids; instead they showed that radioactive chanoclavine-I and not isochanoclavine-I was the precursor of the isolated radioactive agroclavine. The conversion of chanoclavine-I to agroclavine is accompanied at some stage by a trans-cis isomerization of the double bond (20).

No intermediates have been isolated to-date between chanoclavine-I and the tetracyclic clavine alkaloid agroclavine.

Acetobacter aceti

In this investigation (p.36), the following biosynthetic sequence has been shown to exist in aerobic incubations with Acetobacter aceti (See Fig. 11).



Lysergol Elymoclavine Penniclavine Iso-penniclavine

Figure 11.- Biosynthetic sequence shown to occur in aerobic incubations with Acetobacter aceti.

While the conversion of lysergol to elymoclavine through lysergene and agroclavine has been shown to occur in Claviceps spp. and other fungi (1) (See Fig. 9), the conversion of elymoclavine to penniclavine is a simple hydroxylation at the 8-position with a shift of the 8,9 double bond to the 9,10-position and involves a peroxidase or an oxygen transferase as proposed by Agurell (7) (See Fig. 12).

As Acetobacter aceti is an "oxidizing bacteria", such a conversion can be expected. Further proof in support of the above mechanism is that, in anaerobic or under limited oxygen supply, Acetobacter aceti was unable to metabolize either lysergol or elymoclavine.

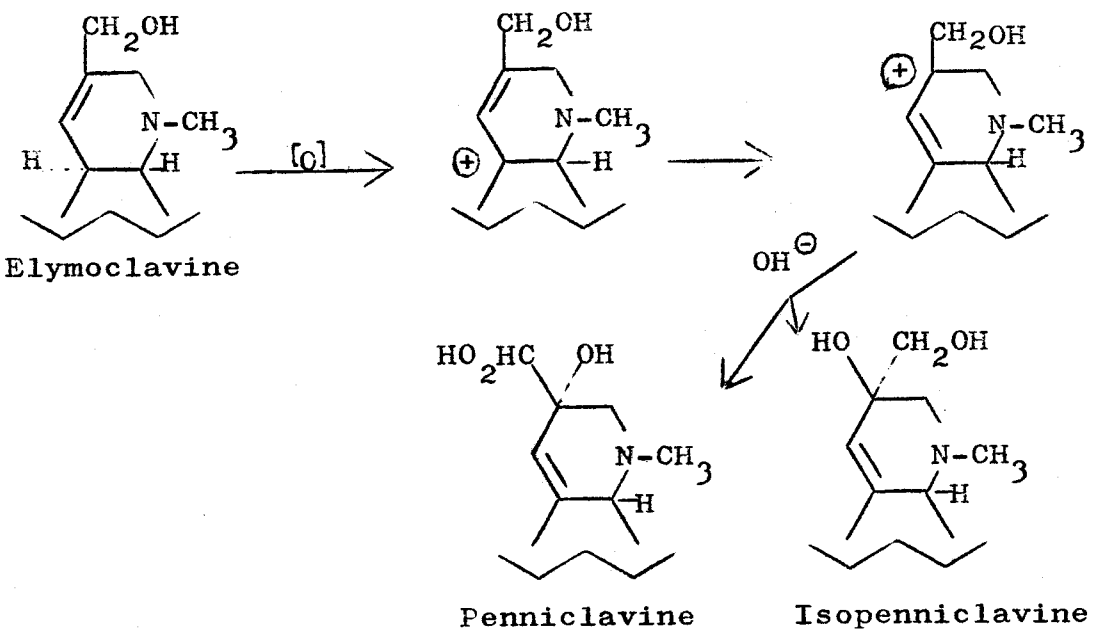


Figure 12.- Mechanism for the oxidative conversion of elymoclavine to its 8-hydroxy derivative, penni-/ isopenniclavine (7).

Pseudomonas aeroginosa

In the studies with Pseudomonas aeroginosa (p.38), the following interrelationship could be demonstrated (See Fig. 13).

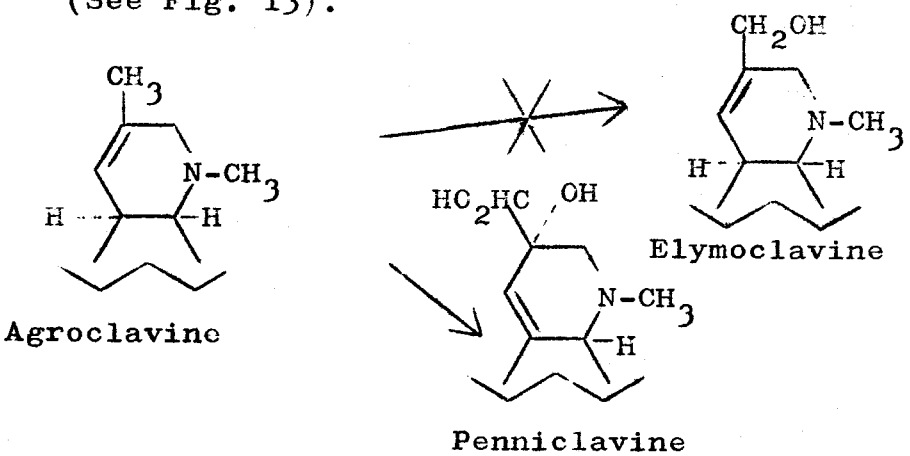


Figure 13.- Conversion of agroclavine to penniclavine in aerobic cultures by Pseudomonas aeroginosa.

The pathway from agroclavine to penniclavine has been shown by all researchers to be through the intermediate elymoclavine. Nevertheless, Pseudomonas aeroginosa, an "oxidizing bacteria", has been able to convert agroclavine directly to penniclavine in the presence of oxygen, but not in its absence, without passing through the intermediate elymoclavine. The following mechanism is proposed for the above conversion (See Fig. 14).

Although the proposed mechanism for the conversion of agroclavine goes through a known intermediate, setoclavine, the soundness of this proposal could not be confirmed by feeding setoclavine to Pseudomonas aeroginosa

and finding out whether or not it was converted to penniclavine, because it was not available in adequate amounts.

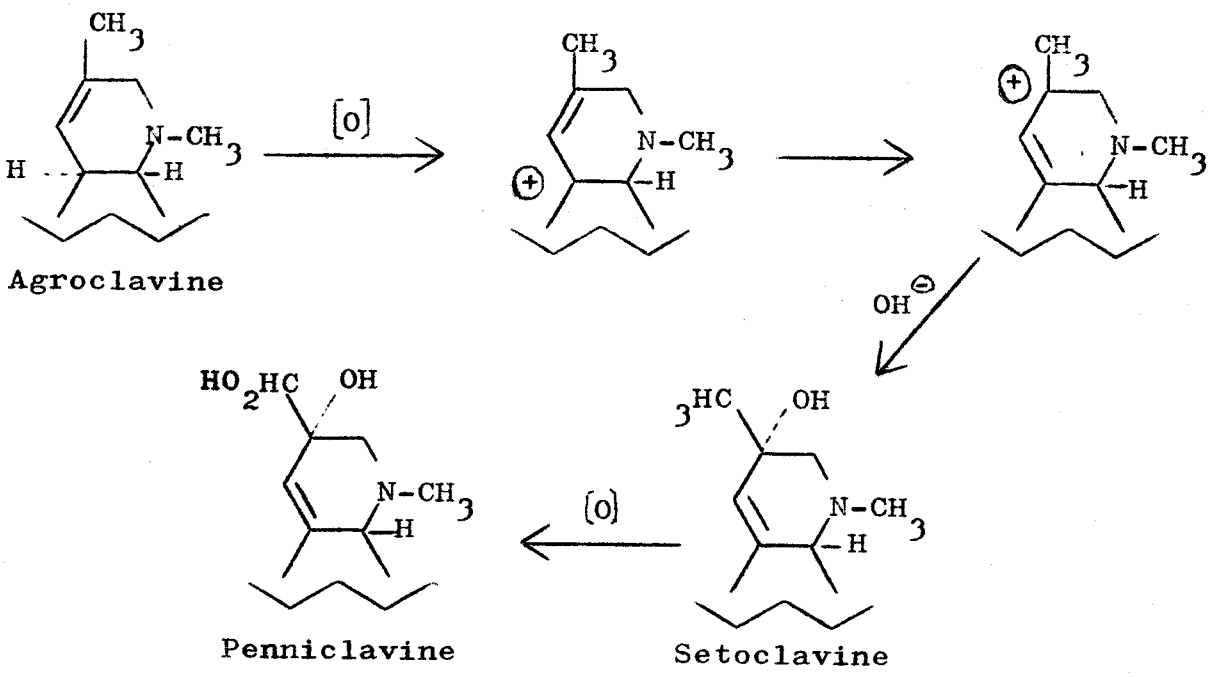


Figure 14.- A proposed mechanism for the conversion of agroclavine to penniclavine through the intermediate setoclavine.

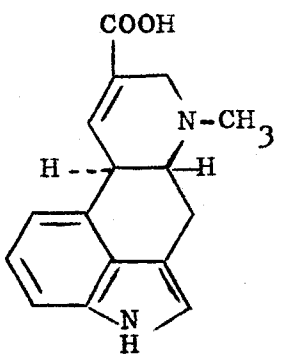
Streptococcus faecalis

In the studies with Streptococcus faecalis it was shown that chanoclavine-I was converted to an unstable clavine intermediate, Alkaloid 'A', which could have been a dihydroclavine alkaloid. The UV-spectrum showed a peak at 274 mμ which is characteristic of dihydroclavine alkaloids. Unfortunately this work could not be reproduced, probably due to a mutation in the enzyme

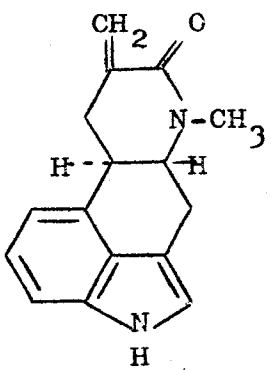
systems of Streptococcus faecalis.

When further studies with this supposed mutant of Streptococcus faecalis were carried out by feeding chanoclavine-I, a new clavine Alkaloid 'B', was isolated. The UV-spectrum of this compound was the same as that of chanoclavine except that the peak at 225 mμ and the trough at 245 mμ of chanoclavine were shifted to 208 and 255 mμ respectively in Alkaloid 'B'. The IR-spectrum of this compound showed the presence of a carbonyl group at 1670 cm^{-1} or 5.95 μ which is absent in the IR-spectrum of chanoclavine-I.

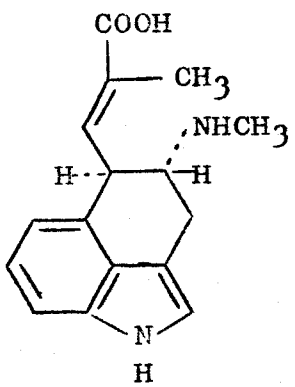
The chromatographic behavior of this compound on ion-exchange cellulose phosphate paper, showed that it was less basic than chanoclavine-I. Three probable structures could be proposed for Alkaloid 'B':



I



II



III

Compound I has a m.p. of 245-247° C., compound II has a m.p. higher than 300° C., while Alkaloid 'B' has a m.p. of 185° - 190° C. This rules out the possibility

of Alkaloid 'B' having structure I or II.

No literature reports, to the author's knowledge, shows the existence of a compound having structure III. The author tentatively assigns structure III to Alkaloid 'B' until further experimentation can prove or disprove it.

A very interesting correlation can result if Alkaloid 'B' has structure III. This alkaloid could be the missing link between chanoclavine-I and agroclavine. Figure 15, shows how Alkaloid 'B' could fit in the biosynthetic relationship between chanoclavine-I and agroclavine.

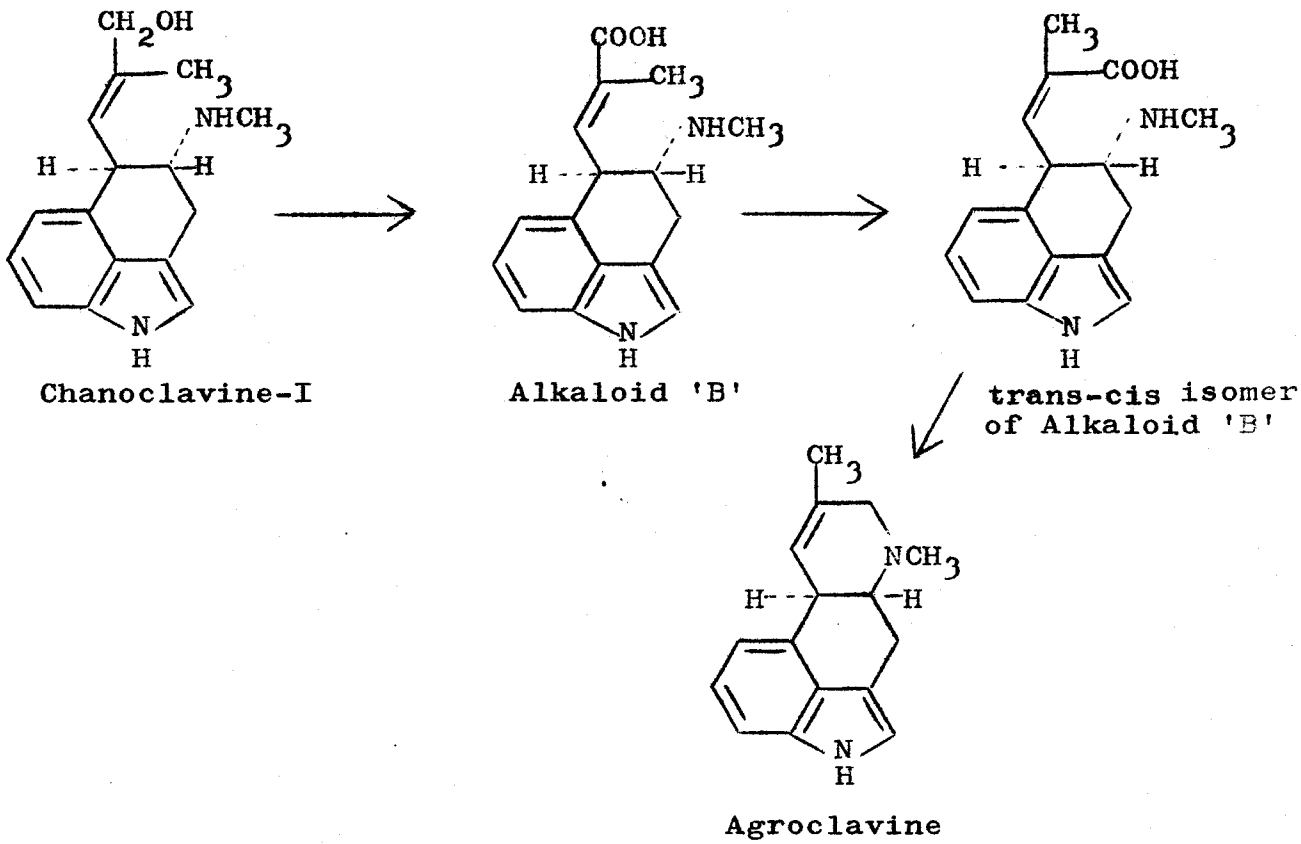
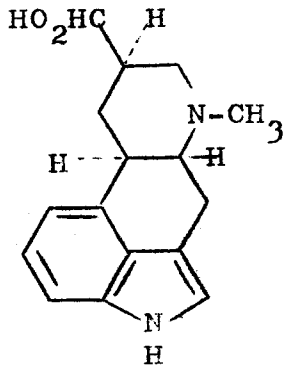


Figure 15.- Proposed biosynthetic pathway from chanoclavine-I to agroclavine.

The pathway proposed above, of chanoclavine-I passing through Alkaloid 'B' to agroclavine, is in accord with that shown by Fehr, Arigoni et al.(20). To be able to prove the above pathway, radioactive chanoclavine-I could be fed to Streptococcus faecalis and the radioactive Alkaloid 'B' isolated. The latter would then be fed to a Claviceps strain which produces agroclavine abundantly in saprophytic culture such as strain 15 B (2).

Kaladana seeds

In the work on Kaladana seeds, a new alkaloid 'X' was isolated and partially characterized. It was shown that alkaloid 'X' could be a dihydroclavine alkaloid, and that it was very unstable in acidic solutions, changing easily to lysergol. Chromatographic data show that alkaloid 'X' from Kaladana seeds could be dihydrolysergol of the following structure:



D - Dihydrolysergol-(I)

This compound was recently shown to occur naturally in ergot of maize (9).

Proof of the identity of this compound could not be obtained chromatographically because of the unavailability of known D-dihydrolysergol-(I).

CHAPTER VI

SUMMARY

In the course of this investigation a variety of techniques and analytical methods were used. Among these may be mentioned : microbiological culture methods; column chromatography; paper chromatography; ion-exchange paper chromatography; thin-layer chromatography; methods of isolation, purification and crystallization of compounds of natural origin; and the use of melting point, UV-light, and UV-and IR- spectroscopy for the characterisation of the isolated compounds.

1. A method was developed for the extraction and fractionation of the total alkaloids of Kaladana seed.

a. Chanoclavine, previously reported in this seed (4,5), was isolated in a pure form and shown to be Chanoclavine-I.

b. Lysergol was obtained in a high yield, thus confirming the first reported instance (4,5) of its occurrence in the seeds of higher plants.

c. A new alkaloid, Alkaloid 'X', was isolated and was tentatively identified as D-dihydrolysergol-I.

2. In the microbiological conversion studies with Acetobacter aceti, the following biosynthetic pathway

was shown to occur:

Lysergol ——→ (Elymoclavine) ——→ Penniclavine.

The conversion of lysergol to elymoclavine has precedence in the work of Abe (1), while that from elymoclavine to penniclavine is shown to occur through the oxidation of the allylic hydrogen at C-10 of elymoclavine, isomerization of the intermediate and its subsequent hydration to penniclavine (7). Support for this proposition comes from the fact that the above conversion occurs only under aerobic conditions, while it does not take place in the absence of oxygen.

3. It was shown that only under aerobic conditions was Pseudomonas aeroginosa able to convert agroclavine directly to penniclavine and not through elymoclavine as an intermediate. A mechanism is proposed which involves the oxidation of the allylic hydrogen at C-17 of agroclavine, isomerization of the intermediate, hydration to setoclavine and subsequent oxidation of the $-CH_3$ group to the $-CH_2OH$ group of penniclavine.

4. Streptococcus faecalis was shown to be able to metabolize chanoclavine-I to an unstable, dihyroclavine alkaloid, Alkaloid 'A'. A possible mutant of Streptococcus faecalis metabolized chanoclavine-I yet to another stable clavine Alkaloid 'B'.

5. A structure was tentatively assigned to Alkaloid 'B', in which the $-CH_2OH$ of chanoclavine has been oxidized to a carboxyl group.

6. The possible role of Alkaloid 'B' as an intermediate in the biosynthetic relationship between chanoclavine-I and agroclavine, is discussed.

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CURRICULUM VITAE

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In September, 1966, he was married to Miss Arsiné Der-Ohannessian.