FERMENTATION PRODUCTION OF ERGOT ALKALOIDS

A thesis presented in fulfilment of the requirements for the degree of Ph.D of the University of London

by

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This work is dedicated to my father,
Charles Atwell and my grandfather,
George Chidgey, both of whom have
helped me considerably during the
past three years.

ABSTRACT

A 60 litre batch fermentation process for the production of ergot alkaloids (principally dihydroergosine) has been developed for the first time for a strain of the ergot fungus Sphacelia sorghi, originally isolated from Nigerian Sorghum, using a sucrose/asparagine/inorganic salts medium. A viscous glucan, which accumulated during the first four days of the fermentation, subsequently autolysed, restoring low viscosity, improving aeration and facilitating alkaloid yields of ~500 µg/ml. Maximum alkaloid yield was associated with swollen lipid-filled hyphae and the production of microspores.

Mixed culture of S. sorghi with an apparently non-alkaloid producing strain of Claviceps purpurea successfully exploited the superior glucanase activity of C. purpurea but the alkaloid yield was no better than that of a pure appropriate strain of S. sorghi. Further investigation of the inability of the C. purpurea strain to biosynthesise indole alkaloids revealed that parasitic tissue of the fungus could form 4-dimethylallyltryptophan, the first step of the ergoline biosynthetic pathway, but apparently progressed no further.

A novel alkaloid, 9'hydroxydihydroergosine, has been produced by S. sorghi, following directed biosynthesis consequent on the acceptance of the proline analogue 4-hydroxyproline by the multienzyme complex which forms the cyclic tripeptide moiety of ergot alkaloids. Since alkaloid production occurs after growth the toxicity of hydroxyproline could be avoided by addition as alkaloid synthesis commences. The leucine component of dihydroergosine could not be replaced by isoleucine or nor-leucine.

The uniqueness of 9'hydroxydihydroergosine amongst the few microbial secondary metabolites in which a hydroxyprolyl component can be recognised has been emphasised by the failure, both of an ergotamine-producing strain of C. purpurea and verruculogen-producing Penicillium simplicissimum and Aspergillus fumigatus, to yield hydroxyprolyl analogues.

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1. INTRODUCTION

Ergot has been described as "of the nature of a treasure-chest to pharmacologists" (Moir, 1932) and by Stoll (1965) as a "treasure house for drugs". The chemical diversity of the ergot alkaloids corresponds to the diversity of their biological properties. Historically, the study of these drugs served as a gateway to nineteenth and twentieth century pharmacology. The ergot fungus (Claviceps purpurea) particularly attacks rye (Secale cereale), and the mycelium replaces the host ovary initially with a soft white sphacelial tissue. Further growth of the parasite involves the differentiation to plectenchymatic sclerotial tissue which gives rise to the characteristic ergot sclerotium. The sclerotium resembles a horn or spur, consequently the name ergot originated from the French word "argot" meaning a cock's spur. Rye was first cultivated in southern Europe at the beginning of the Christian era and became the chief cereal in Russia, eastern and central Europe. Following epidemics of ergot, peasant communities were liable to be afflicted with St. Anthony's fire, a disorder later recognised as a form of ergotism. Records of ergot poisoning only date back to the early middle ages but several authors have argued that ergotism must have occurred in Ancient civilisations, in which it may also have been used as a drug. Hippokrates 400-357BC recommended for hastening childbirth, cooking coarsely powdered barley flour in water and drinking the infusion. Kobert (1889) argued that since this could hardly influence childbirth, it must be assumed that the barley had been ergotised. Kobert, to reinforce his argument, cited Hippokrates' many other prescriptions for various uterine applications, all of which contained barley flour. It was not until the 18th century that ergot sclerotia were actually recognised as the cause of ergotism, and also that sclerotia were fungal tissue and not diseased rye grains (Von Münchausen, 1764). Despite knowing the cause of ergotism, sporadic outbreaks still occur. The most recent reported outbreaks have been in Russia 1926-27, Pont-Saint Escrit, France, 1951 and in 1978 a less severe outbreak in Ethiopia (King, 1979). The first record of the use of ergot as a drug was in 1582 by Adam Lonicer. Tanret (1875) was first to isolate a crystalline alkaloid preparation "ergotinine crystallisee", and in 1918, Stoll isolated ergotamine, the

first chemically pure homogeneous alkaloid which exhibits the vasopressor properties of ergot. In 1935, ergometrine was characterised simultaneously in U.S.A., Germany and England thereby accounting for the oxytocic properties of ergot and extending the therapeutic value of this poisonous fungus. Thirty-one species of the genus Claviceps, which parasitise approximately 200 different host plants, have been described. From the ergots of these species approximately 50 alkaloids have been isolated and chemically characterised. The host range, and main alkaloids produced by some of the more important Claviceps spp. are summarised in Table 1.

The ergot alkaloids are indole derivatives, mostly based on the tetracyclic ergoline ring system (Fig 1), and are grouped according to their structure into the clavine alkaloids (Fig 2), and the lysergic acid alkaloids (Fig 3). The composition of the cyclic tripeptide side-chains of some of the latter is summarised in Table 2. The lysergic acid derivatives having the carboxyl function equatorial to the β position are pharmacologically active, whereas derivatives of iso-lysergic acid, having the carboxyl function in the ∝-position, possess only weak pharmacological activity. The clavine alkaloids (Table 3), include both Δ 8,9 and Δ 9,10 types with methyl or hydroxymethyl groups as the substituents at the C-8 position of the ergoline nucleus. The clavine alkaloids have negligible vasopressor or oxytocic activity but some e.g. agroclavine exert a potent central effect in animals.

Pharmacologists have been greatly interested in ergot alkaloids due to their multiple actions, which include effects on uterine and vascular smooth muscle, and for their use as a tool for studying the sympathetic nervous system. The pharmacological properties, poisonous effects and therapeutic uses of some of the ergot alkaloids are described in Table 4, (Mantle 1975). More recently discovered alkaloids and synthetic derivatives have been shown to inhibit prolactin release, also to stimulate the dopaminergic receptors and have been utilised effectively in the treatment of parkinsonism. The pharmacology of ergot alkaloids and related compounds has recently been reviewed (Berde and Schilde, 1978).

Ergot alkaloids were thought to occur only in species of Claviceps until 1960, when lysergic acid amide, isolysergic acid and chanoclavine were isolated from the seeds of Rivea corymbosa and Ipomoea tricolor,

ERGOT FUNGUS	HOST PLANT	PRINCIPAL ALKALOIDS
C. PURPUREA	CEREALS (RYE, WHEAT, BARLEY)	LYSERGIC ACID DERIVATIVES (ERGOTAMINE, ERGOTOXINE, ERGOMETRINE, ERGOSINE)
C. PASPALI	PASPALUM SPP	D-LYSERGIC ACID AND HYDROXYETHYLAMIDE
C. FUSIFORMIS	MILLET (PENNESETUM TYPHOIDES)	CLAVINE ALKALOIDS OF AGROCLAVINE ELYMOCLAVINE
C. GIGANTEA	MAIZE	DIHYDROCLAVINE ALKALOIDS OF FESTUCLAVINE, PYROCLAVINE AND DIHYDROELYMOCLAVINE
SPHACELIA SORGHI	SORGHUM VULGARE	DIHYDROERGOSINE DIHYDROELYMOCLAVINE FESTUCLAVINE PYROCLAVINE

TABLE 1 : Host Plants and Principal Alkaloids of the Main Ergot Fungi

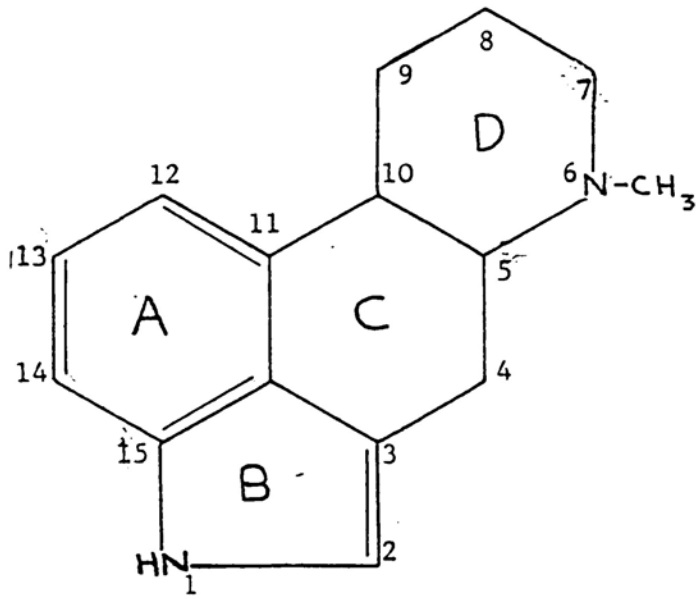
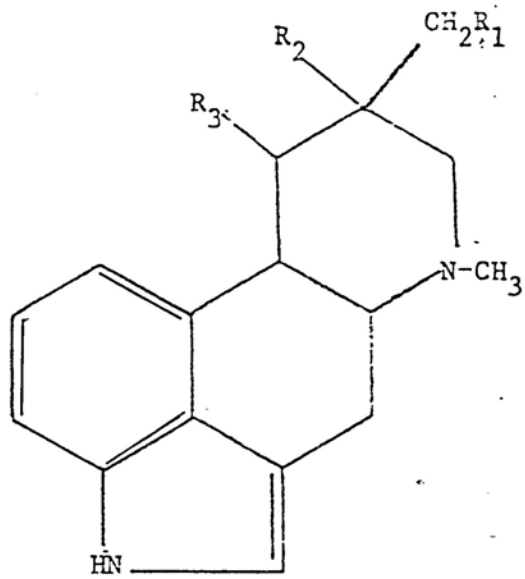
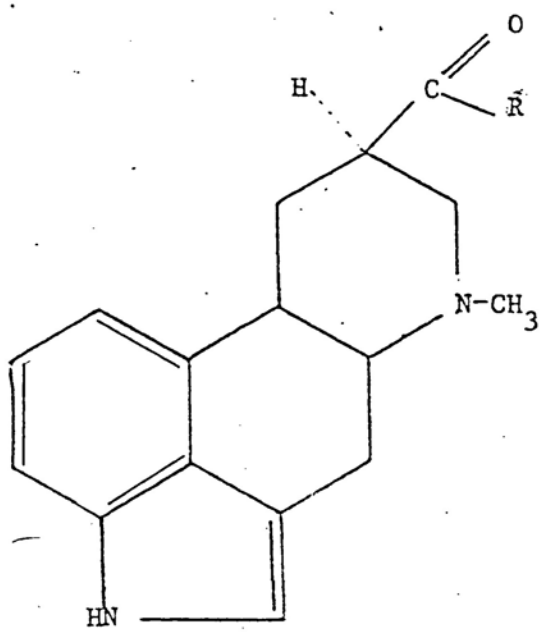


Fig. 1 Ergoline Ring System



R_1, R_2, R_3 = H or OH

Fig. 2 General formula of the clavine alkaloids



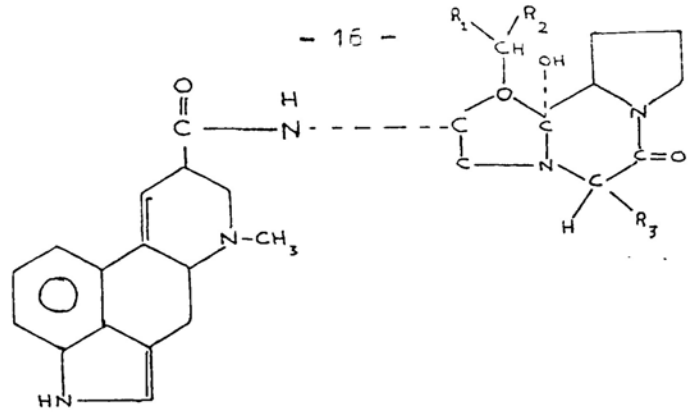
where R=H (lysergic acid)

R= NH_2 (lysergic acid amide)

R= residue of an amino alcohol or a cyclol tripeptide

Stereo-isomerism at C-8 gives isolysergic acid and the usual series of derivatives.

Fig. 3 General formula of the lysergic acid alkaloids



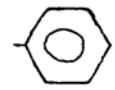
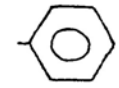
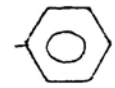
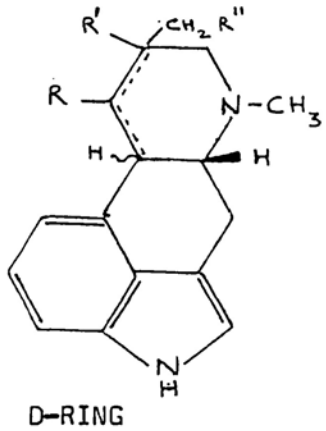
	R_1	R_2	R_3	
ERGOTAMINE	H	H	CH_2 	ALA (PRO)PHE
ERGOCRISTINE	CH_3	CH_3	CH_2 	VAL (PRO)PHE
ERGOSTINE	H	CH_3	CH_2 	Abu (PRO)PHE
ERGOSINE	H	H	$CH_2CH(CH_3)_2$	ALA (PRO)LEU
ERGOCRYPTINE	CH_3	CH_3	$CH_2CH(CH_3)_2$	VAL (PRO)LEU
ERGOVALINE	H	H	$CH(CH_3)_2$	ALA (PRO)VAL
ERGOCORNINE	CH_3	CH_3	$CH(CH_3)_2$	VAL (PRO)VAL

TABLE 2 : Structure of Peptide Ergot Alkaloids (from Rehacek, 1974)



D-RING

	Δ	R	R'	R''
AGROCLAVINE	8–9	H	NONE	H
ELYMOCLAVINE	8–9	H	NONE	OH
MOLLICLAVINE	8–9	OH	NONE	OH
LYSERGENE	8–17 and 9–10	H	NONE	NONE
LYSERGINE	9–10	H	H	H
ISOLYSERGINE[a]	9–10	H	H	H
LYSERGOL	9–10	H	H	OH
ISOLYSERGOL[a]	9–10	H	H	OH
SETOCLAVINE	9–10	H	OH	H
ISOSETOCLAVINE[a]	9–10	H	OH	H
PENNICLAVINE	9–10	H	OH	OH
ISOPENNICLAVINE[a]	9–10	H	OH	OH
FESTUCLAVINE	NONE	H_2	H	H
PYROCLAVINE[a]	NONE	H_2	H	H
COSTACLAVINE	NONE	H_2	H	H
FUMIGACLAVINE A	NONE	CH_3COO	H	H
FUMIGACLAVINE B	NONE	OH	H	CH_3

a C–17 projects to the rear as in lysergic acid

CHANOCLAVINE I

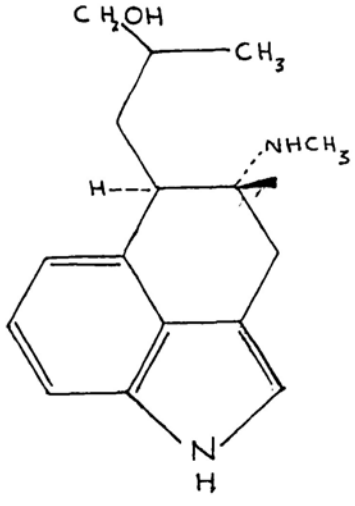


TABLE 3 : Structure of Clavine Alkaloids

both members of the Convolvulaceae, the "morning glory" family (Hofmann and Tscherter, 1960). Following this ergot alkaloids were isolated from Argyreai, another Convolvulaceous plant, and also from fungi other than Claviceps spp. Festuclavine and two new alkaloids Fumigaclavine A and Fumigaclavine B were isolated from Aspergillus fumigatus and the latter alkaloid also from Rhizopus arrhizus (Spilsbury and Wilkinson, 1961). Costaclavine was isolated from Penicillium chermesium, (Agurell, 1964). Chanoclavine I, agroclavine, elymoclavine, ergometrine and ergometrinine have been isolated from the Claviceps related fungi Balansia epichloe and Balansia strangulans. Cattle grazed on grasses infected by these fungi developed "ergot like" symptoms (Porter et al,1978).

Due to their immense pharmacological importance, it was desirable to produce large quantities of ergot alkaloids economically. Commercially desirable alkaloids can be obtained in three ways;

1. By isolation from Claviceps purpurea grown on cereal hosts.
2. By extraction of submerged fermentations of C. paspali and C. purpurea (Table 5).
3. By synthetic derivatisation of lysergic acid.

Although the parasitic mode accounts for 95% of world production of ergot alkaloids, it has many disadvantages. Alkaloid production is inextricably linked to cereal agriculture, is dependent on the weather and is also quantitatively variable. In 1936, Von Bekesy experienced this variability, when, using a newly developed inoculation technique, he found his first crop to be alkaloid free! The problems and optimisation of parasitic culture are discussed by Rochelmeyer (1950), Hecht (1955) and Von Bekesy (1956). Partial synthesis from lysergic acid is the predominant route for the commercial production of all medicinally used ergolines with the exception of the peptide alkaloids. Lysergic acid is produced by fermentation of C. paspali. Total synthesis is not economical. Saprophytic cultivation of ergot to give high yields of pharmacologically useful peptide alkaloids, therefore became a desirable objective.

The first in vitro production of ergot alkaloids was demonstrated by Abe (1951). Subsequently, many other workers reported in vitro production of ergot alkaloids, most of which were of the clavine type. This work is reviewed by Abe and Yamatodoni (1964). One of

ALKALOID	PHARMACOLOGICAL SPECTRUM	POISONOUS EFFECTS	THERAPEUTIC USES
ERGOTAMINE GP.) ERGOTOXINE GP.)	VASOCONSTRICTION UTERINE CONTRACTION CENTRAL NERVOUS STIMULATION ADRENERGIC BLOCKING SEROTONIN ANTAGONISM	PERIPHERAL VASCULAR DISTURBANCE FOETAL DISTRESS AGALACTIA SYMPATHETIC EXCITATION	MIGRAINE RELEIF (ERGOTAMINE) INHIBITION OF LACTATION (ERGOCRYPTINE)
ERGOMETRINE	UTERINE CONTRACTION SEROTONIN ANTAGONISM	FOETAL DISTRESS	AS AN OXYTOCIC AT THE END OF 2ND STAGE OF CHILDBIRTH CONTROL OF POST-PARTUM HAEMORRHAGE AND AN AID TO UTERINE INVOLUTION
D-LYSERGIC ACID HYDROXYETHYLAMIDE	CENTRAL NERVOUS STIMULATION UTERINE CONTRACTION	CENTRAL EXCITATION	NONE
D-LYSERGIC ACID AMIDE	CENTRAL NERVOUS STIMULATION	HALLUCINATIONS	NONE
D-LYSERGIC ACID DIETHYLAMIDE	CENTRAL NERVOUS STIMULATION SEROTONIN ANTAGONISM	HALLUCINATIONS	SOME PSYCHOSES
I-METHYL-D-LYSERGIC ACID BUTANOLAMIDE	SEROTONIN ANTAGONISM	NAUSEA	MIGRAINE PROPHYLAXIS ASPECTS OF CARCINOID SYNDROME
AGROCLAVINE) ELYMOCLAVINE)	CENTRAL NERVOUS STIMULATION	CENTRAL EXCITATION AGALACTIA INFERTILITY	NONE
DIHYDROERGOSINE) DIHYDROCLAVINES)	WEAK	NONE	NONE
D-6-METHYLCYANOMETHYL-ERGOLINE	HYPOTHALMIC STIMULATION	NONE	NONE

TABLE 4 : Pharmacological Properties, Poisonous Effects and Therapeutic Uses of some Ergot Alkaloids

ALKALOID	COMPANY [a.]			
	BIOCHEMIE	FARMITALIA	RICHTER	SANDOZ
ERGOCORNINE	+	+	+	+
ERGOCRISTINE	+	+	+	+
ERGOCRYPTINE	+	+	+	+
ERGOTAMINE		+	+	
ERGOMETRINE				
Δ 9,10 LYSERGIC ACID		+		
Δ 8-9 LYSERGIC ACID (PASPALIC ACID)	+			+

a. BIOCHEMIE GMbH KUNDL/TIROL, AUSTRIA
FARMITALIA S.p.A., MILANO, ITALY
GEDEON RICHTER, BUDAPEST, HUNGARY
SANDOZ-WANDER AG, BASEL, SWITZERLAND

TABLE 5 : Industrial Submerged Production of Ergot Alkaloids

these fungi, Claviceps fusiformis produces high yields of alkaloid, mainly agroclavine, in surface culture, but also produces a β 1 - 3 glucan. Consequently when grown in submerged culture the medium becomes extremely viscous and both growth and alkaloid production are impaired. Banks et al (1974) however, selected a variant from C. fusiformis which parasitises millet in Senegal. This strain differed in that it accumulated glucan during the first few days in submerged culture, which it subsequently hydrolysed through the action of a β-1,3 glucanase and a β-glucosidase, (Dickerson et al, 1970). These enzymes appeared coincident with the formation of septate, swollen hyphal fragments rich in triglyceride oil. Alkaloid yields of 4 mg/ml were attained in large-scale batch fermentation in a sucrose/ammonium sulphate/mineral salts medium at pH 5 (Fig 4). Half-replacement culture increased the yield to 6 mg/ml. High alkaloid titres were associated with a sclerotial type of growth.

Perhaps one of the biggest breakthroughs in the saprophytic culture of ergot fungi came when Arcamone et al (1961) reported a method for the large-scale production of lysergic acid ∝-hydroxy-ethylamide by Claviceps paspali. This pharmacologically uninteresting alkaloid is normally produced in trace amounts in parasitic culture, but an industrial fermentation process was developed in which the fungus yielded alkaloid equivalent to half of the dry weight of the mycelium. Following this, other workers reported successful C. paspali fermentations (Pacifici et al, 1962, Gröger and Tyler, 1963, Kobel et al,1964). The C. paspali strain used by Kobel et al produced Δ 8,9 lysergic acid, in which the double bond could be rearranged when treated with dilute alkali, to give Δ 9,10 lysergic acid. Castagnoli and Mantle (1966) also succeeded in isolating Δ 8,9 lysergic acid from submerged cultures of C. purpurea. The course of a typical fermentation of C. paspali is shown in Fig 5. All C. paspali strains, with the exception of that of Kobel et al (1964), which produced high yields of alkaloid did not produce conidia and were purple-pigmented. In parasitic culture C. paspali produces abundant conidia and the sclerotia are light brown in colour. These high-alkaloid producing strains were unstable and Kobel (1969) reported a progressive decrease in alkaloid production from 1940 mg/l to 20 mg/l over 10 successive routine subcultures. The decrease was concomitant with an increase in the proportion of white fluffy colonies to the

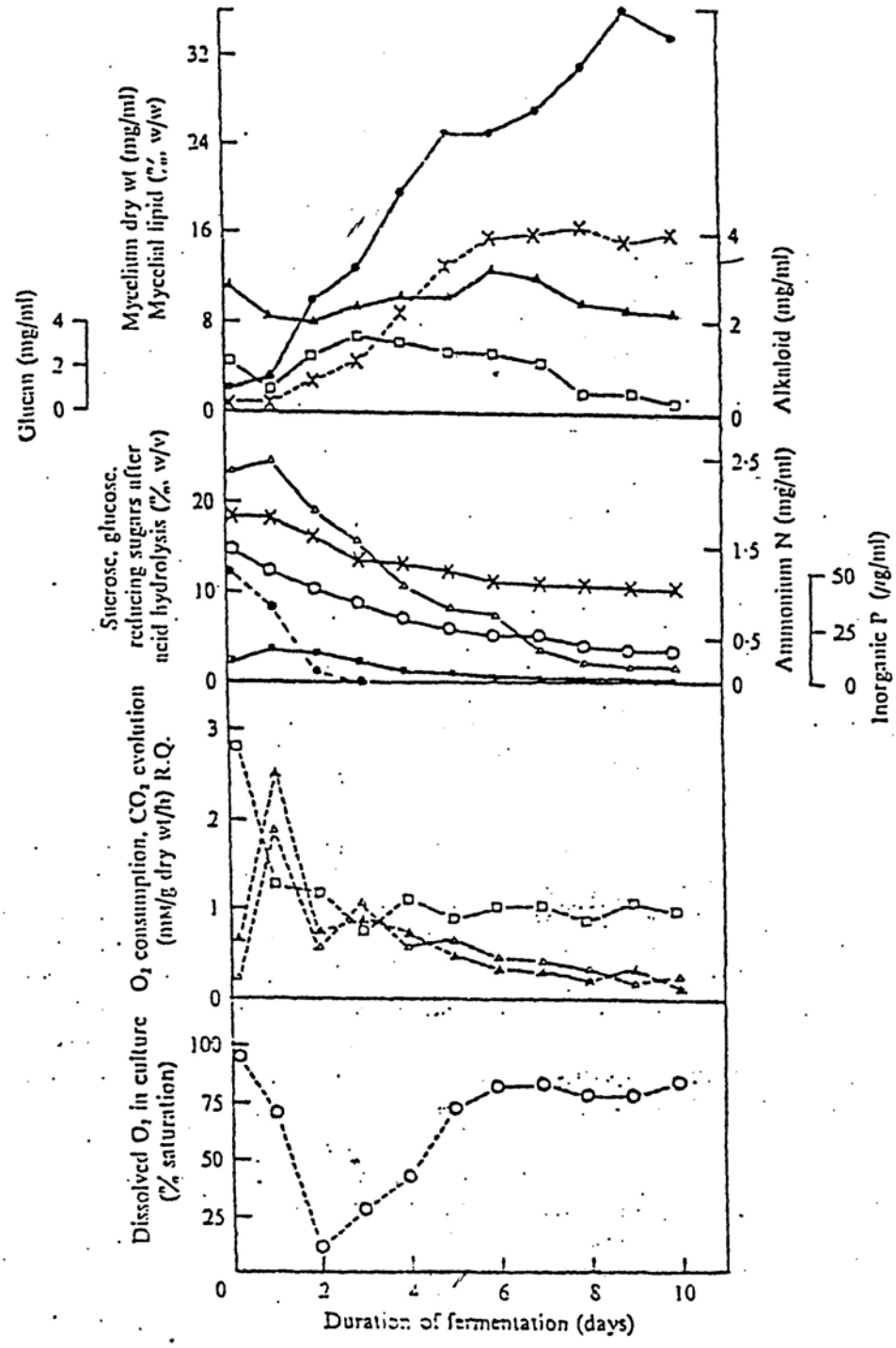


Fig. 4 Course of Clavicaps fusiformis (strain 139/2/1G) fermentation in a 400 l fermenter. ● —— ● , Mycelium; □—— □ , glucose; x - - - x, alkaloid; ▲—— ▲, mycelial lipid; ○—— ○ , sucrose; ■—— ■, ammonium N; ● - - - ● , inorganic P; x ——— x, reducing sugar; □ - - - □ , R.Q.; ▲ - - - ▲, qCO_2; △ - - - △, qO_2; ○ - - - ○, dissolved oxygen.

usual purple pigmented ones when a dilute suspension of hyphal fragments was plated on agar.

The demonstration that the origin of the ergoline ring system was from tryptophan and mevalonic acid suggested that precursor feeding might increase the alkaloid yield. Arcamone et al (1961) tested various amino acids and found that only DL-tryptophan caused a substantial increase in alkaloid production when added at the beginning of the fermentation. Pacifici et al (1963) also found this effect. However, Tyler (1961) found that addition of L-tryptophan caused a decrease in alkaloid production.

The first successful laboratory scale production of the highly desirable peptide alkaloids was described by Tonolo (1966). The strain used was typical in that it did not produce spores, and through continuous re-selection had lost its pathogenicity. Alkaloid production, mainly ergotamine, on mannitol 20% / peptone 3%, was of the order of 1 mg/ml. Amici et al (1966) reported similar yields on sucrose 30% / ammonium citrate / mineral salts medium. Detailed descriptions of the progress of fermentation in shake flask and in 800 l stirred fermenters are given by Amici et al (1967) (Fig 6) and Arcamone et al (1970). The maximum alkaloid yield was obtained in 10-12 days, of which more than 90% was located in the mycelium (Amici et al, 1967). Alkaloid production was associated only with a plectenchymatic sclerotial type of growth (Mantle and Tonolo, 1968). These sclerotial-like cells are, in submerged culture, swollen to about three times the size of normal sphacelial hyphae, and are filled with triglyceride oil, of which the main fatty acid component is ricinoleic acid (Mantle et al, 1969). Kobel and Sanglier (1978) also describe this type of growth to be a prerequisite for ergotoxine alkaloid production in their C. purpurea strain. No alkaloid was produced when the fungus exhibited any other morphology.

One of the main problems of high alkaloid yielding strains is degeneration. After nine successive transfers on agar of an ergotoxine-producing C. purpurea strain alkaloid production decreased to only 1% of its original value with a corresponding increase from 0% to 82% in the proportion of white cotton-like colonies to purple colonies (Kobel and Sanglier, 1978). Kobel (1969) described a similar degeneration problem in C. paspali. This instability has been attributed

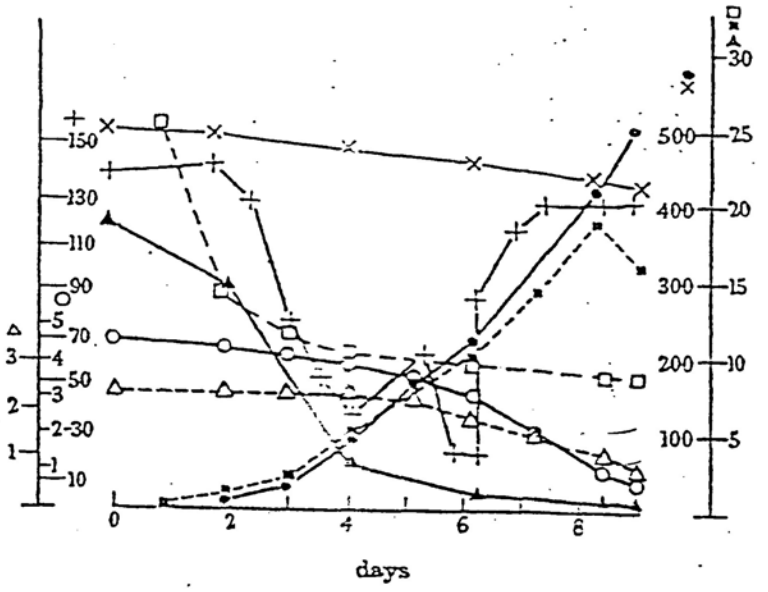


Fig. 5 Course of a typical LAD fermentation by Claviceps paspali, strain F-550, in a 500 l. stirred fermenter in medium B. △, Succinic acid, g%; O, mannitol, g%; □, Q_{O2}; ■, dry weight, mg/ml.; ▲, inorganic phosphate, mg P%; ●, LAD, g/ml.; x, ammonia, mg N%; +, dissolved oxygen per cent saturation at 1. atm over-pressure of air.

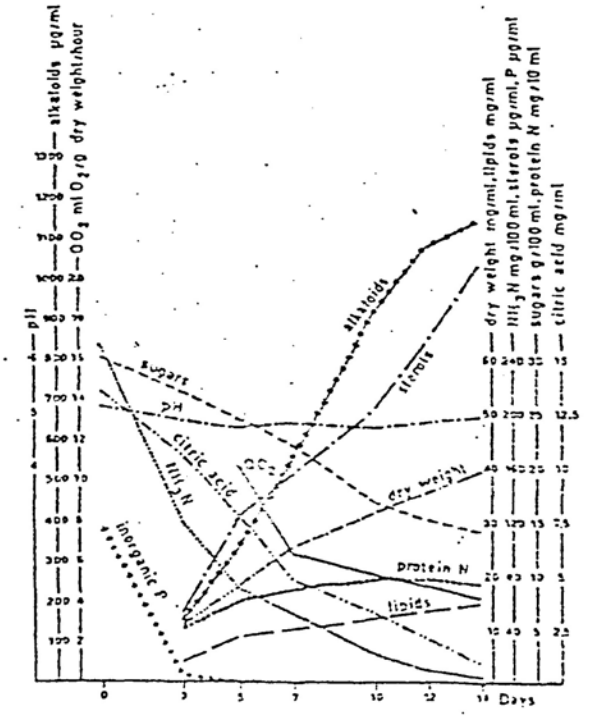


Fig. 6 Course of fermentation on medium T25 in submerged culture of Claviceps purpurea, strain 275 FI.

to heterokaryosis, evidence having been presented for a relationship between the production of alkaloid and heterokaryosis (Amici et al, 1969). There are still a lot of difficulties experienced in large-scale fermentation of peptide alkaloids. Some of these problems are summarised as follows (Kobel and Sanglier, 1978):

1. The bulk of the peptide alkaloids is located in the cells and is not excreted into the medium. No satisfactory method of promoting excretion has been found.
2. The long cultivation period of two to three weeks, and the absence of any antibiotic formation by Claviceps spp. requires very high standards of operation to preserve sterility.
3. The sensitivity of the organism to shear forces and the necessity for high oxygen tension requires a well-balanced system of aeration and stirring.
4. The fungus is affected by small alterations in the medium composition, especially when anti-foams must be added. Most antifoams cause considerable loss in alkaloid yield.
5. Strains yielding high titres of alkaloid are generally difficult to preserve.

From a survey of the literature (some examples of which are shown in Figs 4, 5 and 6), it seems that C. paspali, C. fusiformis and C. purpurea fermentations are similar in the following respects;

1. In submerged cultivation of these fungi, the carbohydrate, organic acid, nitrogen and phosphate constituents are rapidly utilised with the concomitant accumulation of lipids and sterols in the mycelium. Ergot alkaloid fermentations are phosphate-limited, and high levels of phosphate have been found to inhibit alkaloid formation (De Waart and Taber, 1960, Mary et al, 1965, Taber, 1967, Robbers et al 1972, 1978). Rapid uptake of phosphate is correlated with high alkaloid production.

2. The course of fermentation in C. paspali and C. purpurea can be separated into two distinct phases, a rapid growth phase and an alkaloid-producing phase. However in C. fusiformis the distinction is less marked.

3. In contrast to C. fusiformis, no glucan was detected throughout the C. paspali and C. purpurea fermentations, C. purpurea produces a potent β-glucanase after 2-3 days growth (Dickerson et al, 1970) so allowing no accumulation of glucan. The glucan accumulated by day 2

of the C. fusiformis fermentations has a pronounced effect on dissolved O_2 in the culture, and alkaloid yields are low, unless the glucan becomes hydrolysed through the action of a β-glucanase, where-upon dissolved O_2 concentration returns to near saturation as in the C. paspali and C. purpurea fermentations.

4. The form and concentration of the carbon source is an important factor in alkaloid production. Alkaloids are generally produced on carbohydrates which are not immediately utilisable e.g. sucrose or mannitel. Sucrose has first to be degraded by a β-fructofuranisidose (Dickerson et al, 1970) Glucose is not generally used as a sole carbon source since it tends to suppress alkaloid synthesis (Drew and Demain, 1977). The carbohydrate constituent also has an important effect on the osmolarity of the medium. A medium with a high osmotic pressure is required for alkaloid synthesis (Arcamone et al, 1961, Tonolo 1966, Amici et al 1964, Puc and Socic, 1977).

5. All three organisms will not utilise nitrate nitrogen. The best nitrogen sources were ammonium salts or amino acids.

6. Addition of tryptophan to the medium has a stimulatory effect on alkaloid production by several Claviceps spp. (Arcamone et al, 1961, Pacifici et al, 1963, Bu'lock and Barr, 1968, 1979, Szczyrbak 1972, Robbers et al, 1972) and an inhibitory effect on others (Gröger and Tyler, 1963, Kelleher, 1969). The effect of tryptophan described by Bu'lock and Barr (1968, 1979) appears to be incorrectly attributed to C. purpurea, since the ergot fungus which they used produced only clavine alkaloids and had been earlier referred to as C. fusiformis (Waiblinger and Gröger, 1972). The stimulatory effect of tryptophan is maximal if it is added within the first 12 hours of fermentation (Robbers et al, 1978). Vining (1970) considers that tryptophan stimulates alkaloid production through increased activity of the enzyme-system synthesising alkaloid. Exogenous tryptophan removes the inhibition of alkaloid production by inorganic phosphate (Robbers et al, 1972).

7. The high-alkaloid yielding strains of C. paspali and C. purpurea are non-sporulating. Pazoutova et al (1977) reported an inversely proportional relationship between conidial formation and alkaloid production in Claviceps purpurea, strain 129, which produces large quantities of clavine alkaloids. However, the alkaloids produced by this strain are of the clavine type and were produced while the mycelium

was sporing, both features more characteristic of *C. fusiformis* than *C. purpurea*.

8. Alkaloid production is associated with swollen, roughly isodiametric, "sclerotial-like" cells, which are filled with lipid. This type of growth has been described as prerequisite for alkaloid production in *C. purpurea* (Mantle and Tonolo, 1968). Vorisek *et al* (1974) also reported a positive correlation of alkaloid production with the synthesis of lipids. In *C. fusiformis* however, a certain amount of alkaloid can also be synthesised by sphacelial tissue, indicating that alkaloid synthesis by this species is not as closely restricted to sclerotial cells as has been demonstrated in *C. purpurea*.

Other ergot fungi could be sources of potentially useful ergot alkaloids, for example, *Claviceps gigantea*, which produces a mixture of dihydroclavine alkaloids (Agurell *et al*, 1963), and *Sphacelia sorghi* which produces large amounts of dihydroergosine (Mantle and Waight, 1968), in addition to chanoclavine, dihydroclymoclavine, festuclavine, pyroclavine and several other uncharacterised minor alkaloids. Alkaloid production exceeding 0·5 mg/ml *in vitro* by a strain of *S. sorghi* isolated from ergots of *Sorghum vulgare*, indigenous to Nigeria, has been achieved on a sucrose/asparagine/mineral salts medium (Mantle, 1973). *S. sorghi* produces a glucan, which as with early studies of *C. fusiformis*, has prevented successful submerged fermentation. Dihydroergosine has weak pharmacological activity (Berde and Schild, 1978) and is a relative stable substance. It could be desirable as a source of dihydrolysergic acid, the preferred substrate for the synthesis of D-6-methyl-8-cyanomethylergoline which is an effective anti-implantation agent in rats and mice, in the absence of the central toxicity of many other alkaloids (Semonsky and Kucharczyk, 1969, Mantle and Finn, 1971).

With the achievement of successful fermentation production of certain ergot alkaloids, biosynthetic studies which had previously been carried out while the fungus was parasitising the host plant, became much easier in pure culture. An understanding of the biosynthesis of ergot alkaloids and regulation of the biosynthetic pathways would hopefully, in turn, lead to optimisation of fermentation processes for these pharmacologically important substances.

Following the structure elucidation of lysergic acid (Jacobs and

Craig, 1934) several theoretical hypotheses on its biogenesis were published. Among these, Mothes et al (1958) proposed the condensation of tryptophan with an isoprenoid C-5 unit and then demonstrated that tryptophan - β - ^{14}C injected into the internodes of ergot-infected rye plants was incorporated into ergometrine and peptide alkaloids. However, Suhadolnick et al (1958), using the same technique obtained extremely low incorporations of tryptophan - 7a - ^{14}C and, in contrast to Mothes et al, concluded that tryptophan was not an ergoline precursor. Mothes and Weygand (1959), carried out the first radioactive feeding experiments with saprophytic cultures of Claviceps strain SD 58, and obtained incorporations of 10 - 39% of tryptophan - β - ^{14}C into elymoclavine. This clear evidence for the incorporation of tryptophan has since been confirmed by many research groups, and tryptophan is now universally accepted as a precursor of the ergoline nucleus, into which it is incorporated with only the loss of the carboxyl group (Floss et al, 1964).

Evidence for involvement of the second postulated precursor came with the demonstration that mevalonic acid is efficiently incorporated into the ergoline ring system. Efficiency of incorporation, 9-23% (Gröger et al, 1959), strongly suggested a direct precursor role. Baxter et al (1962), showed that the N-methyl group originated from L-methionine. Incorporation of L-methionine- (methyl ^{14}C, ^{3}H) into alkaloids was without change in the $^{3}H/^{14}C$ ratio, suggesting that the methyl group is introduced as an intact unit.

Assembly of the ergoline ring system from the established precursors required decarboxylation, N-methylation and isoprenylation of the tryptophan moiety. The problem then was, which step came first? Tryptamine and N_{in} - methyltryptamine were not incorporated (Baxter et al, 1961; Floss and Gröger, 1963), indicating that decarboxylation was not the first step. N - methyltryptophan was apparently incorporated into elymoclavine, but this was later shown to be due to demethylation to tryptophan before incorporation. These studies made it highly probable that isoprenylation was the first step. The site of this proposed isoprenylation, the C-4 position of tryptophan, is fairly unreactive, therefore many workers investigated the possible means of its activation (Baxter et al, 1960; Pleininger et al, 1961; Floss and Mothes, 1963; Floss et al, 1965). It was concluded that prior activation of the C-4 position was unlikely, and therefore that direct

isoprenylation of tryptophan to 4-dimethylallytryptophan (DMAT) occurred, (Fig 7). DMAT was found to be incorporated into clavine, and peptide alkaloids (Weygand et al, 1964), and in some Claviceps spp. it was 5-10 times more efficient as a precursor than tryptophan (Plieninger et al, 1964; Agurell 1966). Later, DMAT was isolated from ergot cultures in which alkaloid synthesis had been inhibited by either the exclusion of oxygen (Robbers and Floss, 1968), or by the addition of ethionine, a methionine antagonist that inhibits N-methylation (Agurell and Lindgren, 1968). The final piece of conclusive evidence came when an enzyme catalysing the formation of DMAT from L-tryptophan and α,ρ-dimethyl allylpyrophosphate was isolated from Claviceps fusiformis, (Heinstein et al, 1971).

The next step involves formation of the obligate intermediate chanoclavine-I. This alkaloid is oxidised to the corresponding aldehyde, which in turn is converted to the tetracyclic alkaloid, agroclavine. Four isomers of chanoclavine have been isolated, but only chanoclavine I acts as the precursor of agroclavine. Further oxidation steps yield elymoclavine and lysergic acid (Fig 7).

The apparently "normal" labelling of the tetracyclic alkaloids, starting from mevalonic -2-^{14}C in the trans-carbon of the isoprene moiety is found to be the consequence of two isomerisations at the allylic double bond. One cis-trans isomerisation takes place between 4-dimethylallytryptophan and chanoclavine I (Patchlatko et al, 1975) and the second between chanoclavine I and agroclavine. It was also found that neither N-nor-6,7-secoagroclavine or 6,7-secoagroclavine were alkaloid precursors indicating that the first methyl hydroxylation takes place before the closure of ring C. Considering these results, and that isomerisation occurs between 4DMAT and chanoclavine I, it may be speculated that 4-(Z-4'-hydroxy-3 -methyl-2'-butenyl)-tryptophan (Z-OH-DMAT Fig 8)is a true intermediate in the biosynthesis of chanoclavine I. Plieninger et al (1971) synthesised E-OH-DMAT and Z-OH-DMAT, and found that they were more efficiently incorporated into elymoclavine than agroclavine. Other groups have also obtained this result. Maier and Gröger (1978) observed in a strain of Claviceps paspali that on feeding with ^{14}C OH DMAT, that chanoclavine I was not labelled but that lysergic acid became radioactive via elymoclavine. It was concluded that E-OH-DMAT is an unnatural substrate and is treated by the organism as if the OH-group was absent. Z-OH-DMAT is converted to

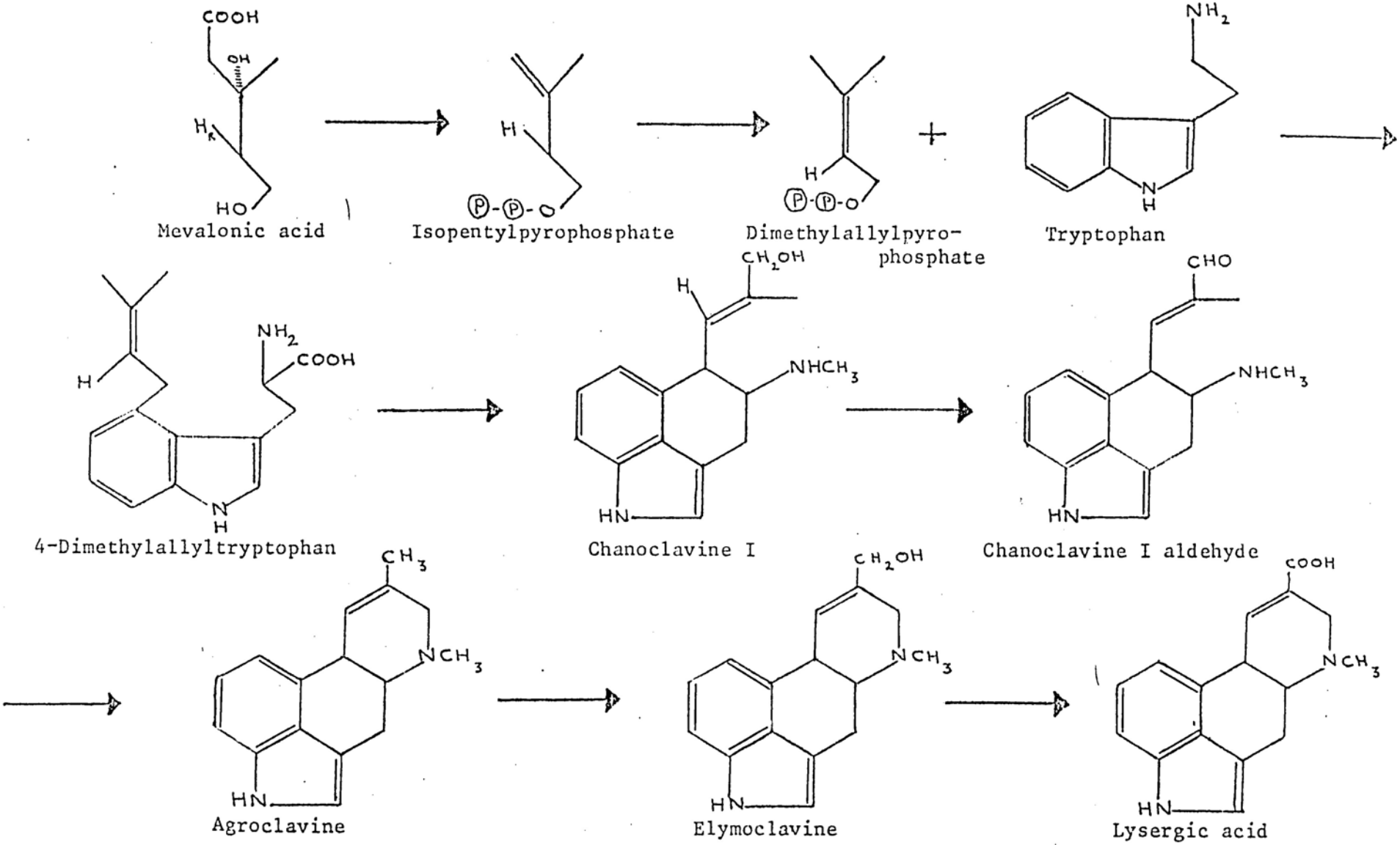


Fig. 7 Biogenesis of the Ergoline Ring System

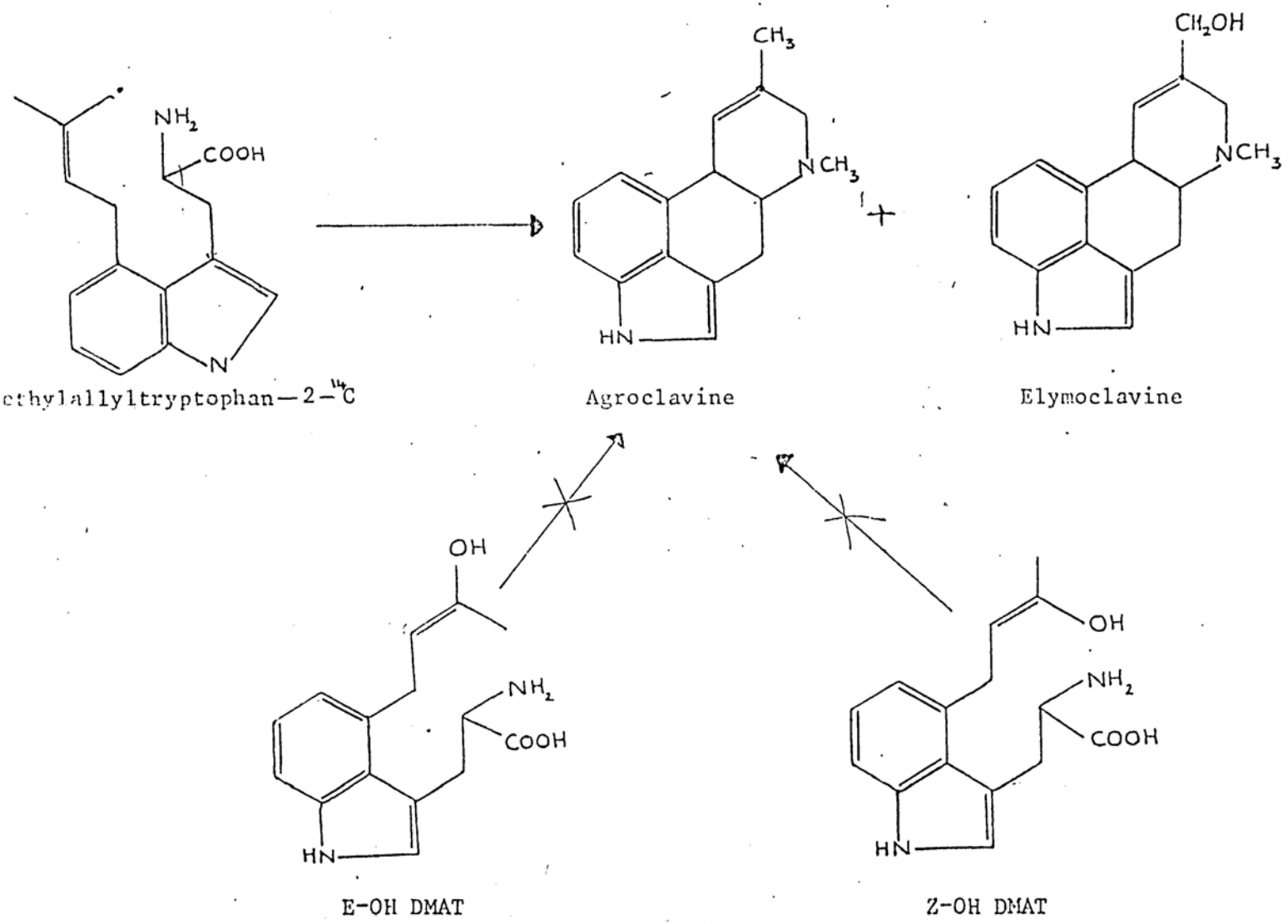


Fig. 8 Incorporation of DMA-Tryptophan into ergolines

E-OH-DMAT before incorporation, and is not an obligatory intermediate in ergoline biosynthesis. However, Petroski and Keller (1977, 1978) then reported the synthesis of 4-(E-4′-hydroxy-3′-methyl-but-2′-enyl) tryptophan (OH-DMAT) from iso-pentylpyrophosphate and L-tryptophan using a cell free extract of Claviceps paspali. OH-DMAT has also been found in culture filtrates of Claviceps purpurea, PRL 1980, fermentations (Anderson and Saini, 1974). Petroski and Keller (1978) proposed that, for the following reasons OHDMAT should be considered a natural intermediate; (i) it has been shown to be incorporated into both elymoclavine and lysergic acid amide; (ii) it has been shown to be produced enzymatically from known precursors by an alkaloid producing isolate of C. paspali and (iii) it occurs in culture filtrates of Claviceps fermentations though which stereoisomer is not known. They proposed a plausible mechanism for its incorporation into lysergic acid alkaloids (Fig 9).

Several studies on the formation of ring D in the ergoline nucleus have yielded interesting results. Chanoclavine-I-aldehyde was found to be a more efficient precursor of the tetracyclic ergolines than chanoclavine I, suggesting its participation as an intermediate between chanoclavine I and agroclavine. Also in connection with D ring formation Acklin et al (1975) investigated the role of paliclavine, the allylisomer of chanoclavine, in biosynthesis. No incorporation into tetracyclic clavines, nor into the isomeric pair paspalic acid and lysergic acid occurred. Clearly, the mechanism of D-ring formation is not yet fully understood.

Many other results concerning reactions in the main biogenetic pathway chanoclavine I → agroclavine → elymoclavine → lysergic acid are cited in the literature. The most recent detailed review is by Floss (1976). The hydroxylation of the methyl group of agroclavine to give elymoclavine is a specific reaction of the Claviceps species (Vining, 1970), and requires the presence of peroxidase or oxygen transferase, and of cytochrome P-450. Jindra et al (1968) found that the oxygen of the hydroxyl group originates from molecular oxygen. It has also been found that conversion of the clavine alkaloids into lysergic acid derivatives passes through paspalic acid. Some of the biogenetic relationships among ergot alkaloids are shown in Fig 10.

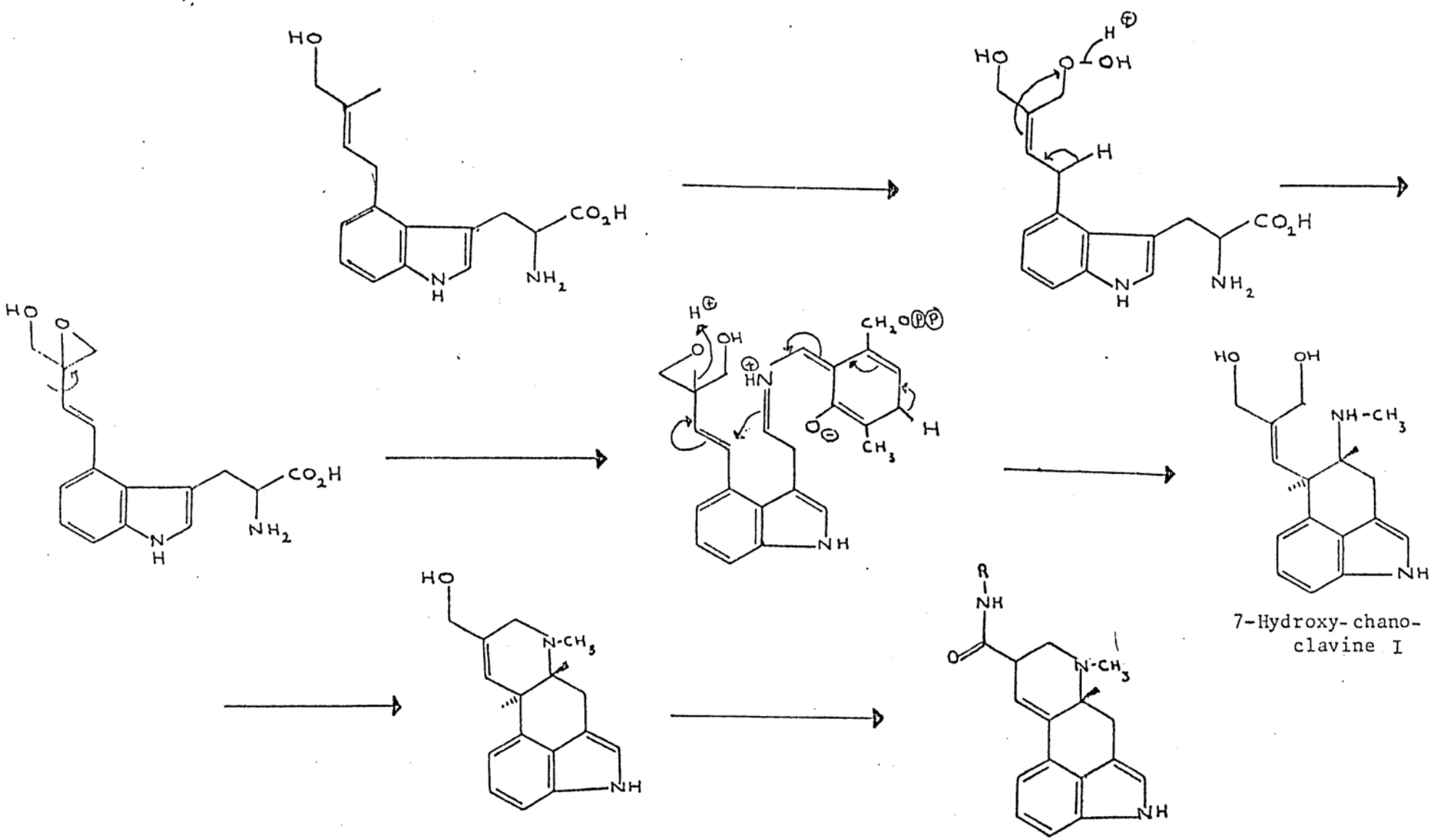


Fig. 9 Proposal for conversion of OH-DMAT to lysergic acid derivatives via hydroxy-chanoclavine I

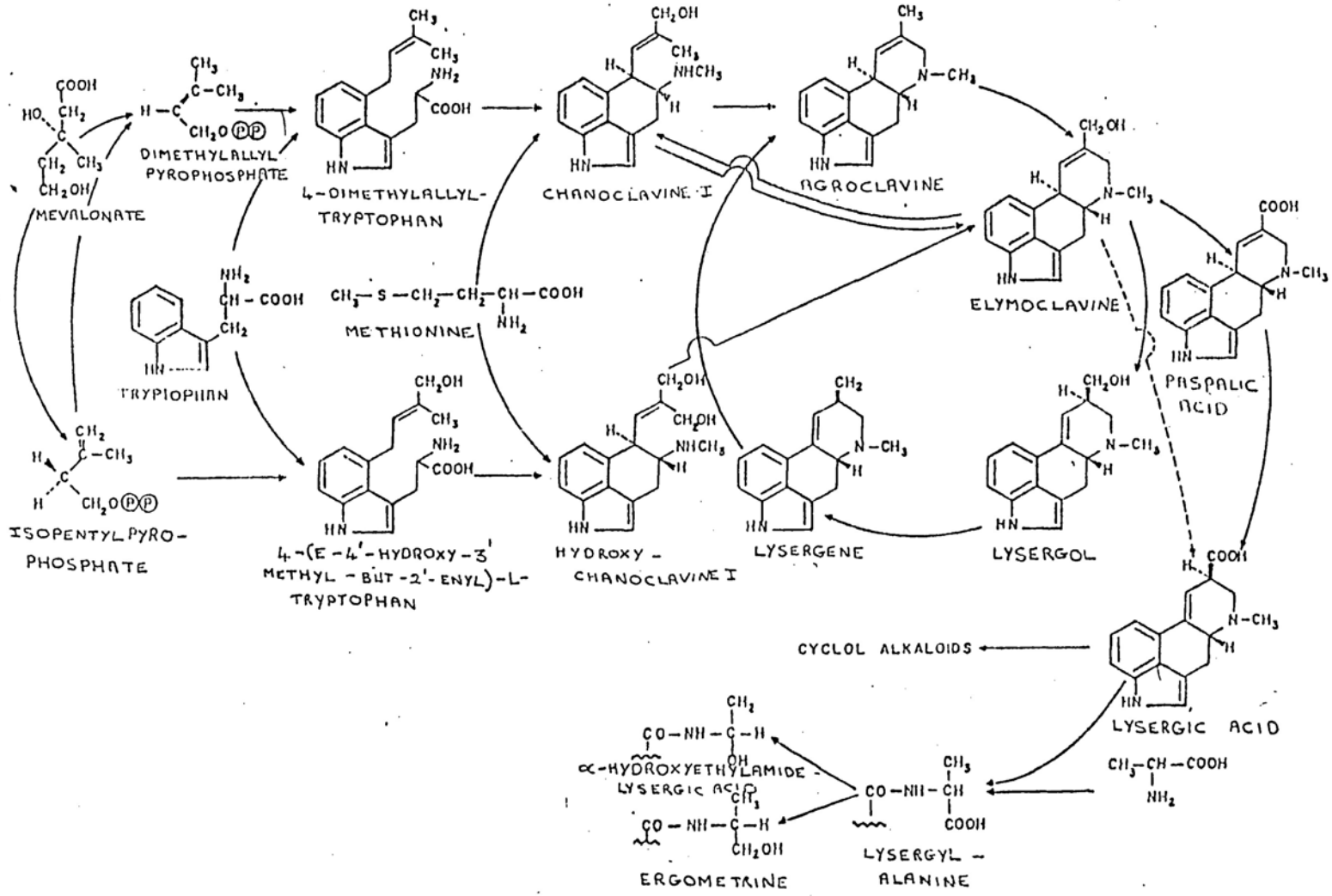


Fig. 10 Some biogenetic relationships among ergot alkaloids. (Rehacek, 1980)

The second part of the problem of ergot alkaloid biosynthesis is that of formation of lysergic acid and its derivatives. The detailed sequence of steps involved in the conversion of elymoclavine to lysergic acid amides has not yet been fully elucidated. ^{14}C - labelled $\Delta^{8,9}$ lysergic acid, the naturally occurring paspalic acid, was found to be incorporated into lysergic acid amides with an efficiency approaching that of elymoclavine, and this is regarded as proof that double bond isomerisation takes place at the oxidation stage of the acid, (Fig 11). Further derivatisation of lysergic acid to the corresponding amides requires activation of the carboxyl group, most likely in the form of a coenzyme A thiolester. Gröger et al (1972) demonstrated that cell-free extracts of Claviceps purpurea can catalyse the formation of lysergyl-coenzyme A. This evidence can be taken to indicate that activation takes place at the lysergic acid stage. A slightly different route has been found to operate in the formation of the dihydrolysergic acid peptide alkaloid, dihydroergosine in Sphacelia sorghi (Barrow et al, 1974). A feeding experiment with double-labelled mevalonate - (2-^{14}C-5-^{3}H) showed that one hydrogen from C-5 mevalonate was retained in this alkaloid, excluding the intermediacy of $\Delta^{9,10}$ lysergic acid derivatives. Further experiments showed good incorporation of festuclavine, dihydroelymoclavine and dihydrolysergic acid but poor incorporation of agroclavine into dihydroergosine. This indicates that in this instance the entire sequence from the clavines to the peptide alkaloids takes place with the absence of the double bond in ring D. The reduction of the allylic double bond must therefore take place early in the biosynthetic sequence.

The formation of the peptide moiety of ergot alkaloids has received a lot of attention over the past years, but it is still not well understood. Feeding experiments revealed that phenylalanine, proline, valine, leucine, alanine and lysergic acid are specifically incorporated into the appropriate parts of the corresponding peptide alkaloids. Available evidence also suggests that the α-hydroxy amino acid part originates from the corresponding amino acid, hydroxylation occurring in the peptide chain. Several workers have attempted to elucidate the mode of assembly of the peptide alkaloids. Various experiments feeding potential dipeptide and tripeptide intermediates indicated that they were hydrolysed prior to incorporation into alkaloid. For example, Gröger and Johne (1972) fed radioactive

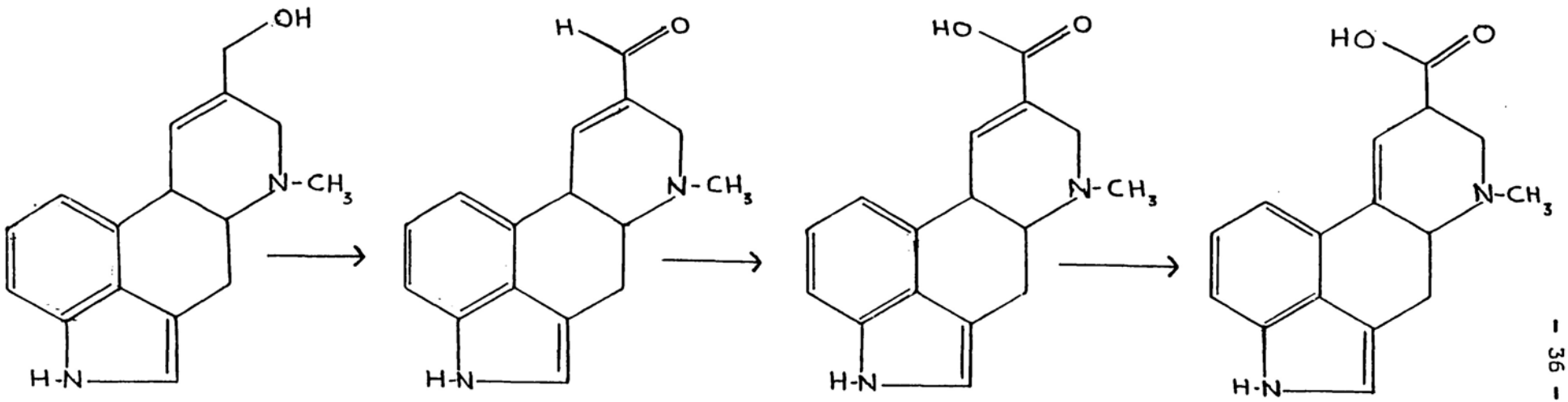


Fig. 11 Double bond isomerisation

L-valyl-L-proline, L-valyl-L-proline lactam, L-leucine-L-proline lactam and found that they were not selectively incorporated into either ergocornine or ergokryptine but into both of these alkaloids. Such an isotope distribution could only have resulted from a cleavage of the precursors. Radioactivity was also found in the protein fraction and amino acid pool (Gröger et al, 1974). Other experiments have also demonstrated that tripeptides were cleaved before incorporation. Floss et al (1971) fed valine, valine precursors, lysergylvaline or isolysergylvaline and always found a higher specific radioactivity in the hydroxyvaline part of the tripeptide moiety of ergocornine than in the valine part. This can be interpreted as indicating that the peptide moiety of ergocornine is built up starting from the proline rather than the lysergic acid end. In this way the valine which remains as valine in the product would be introduced earlier and would thus be diluted by more non-labelled pools than the valine which is eventually transformed into hydroxyvaline.

Abe et al (1970) isolated diketopiperazines from ergot cultures, in particular L-leucyl-D-proline lactam. The time course of their appearance suggested a connection with alkaloid formation. The available evidence then excluded all the plausible free intermediates in the formation of the peptide chain of these alkaloids. This led Floss et al (1974) to hypothesise that the assembly of the peptide chain takes place on the surface of a multi-enzyme complex (Fig 12), not unlike that catalysing the formation of peptide antibiotics in bacteria. Hitherto, all experiments have failed to detect a still hypothetical "cyclol-synthetase-complex" in Claviceps. One approach would be the use of protoplasts as the starting material for cell-free preparations. Stahl et al (1977) detected some enzymes of the aromatic pathway as well as dimethylallyltransferase and chanoclavine-I-cyclase in protoplasts from different Claviceps strains. Robbers et al (1979) and Keller et al (1980) have reported the synthesis of alkaloids by protoplasts of Claviceps. Keller et al showed that alkaloid synthesis by C. purpurea protoplasts occurred only in those containing large vacuoles and relatively large lipid inclusions. This form resembles the plectenchymatic type of growth previously described in intact mycelium as a prerequisite for alkaloid production (Mantle, 1968). Keller et al's results showed different incorporation

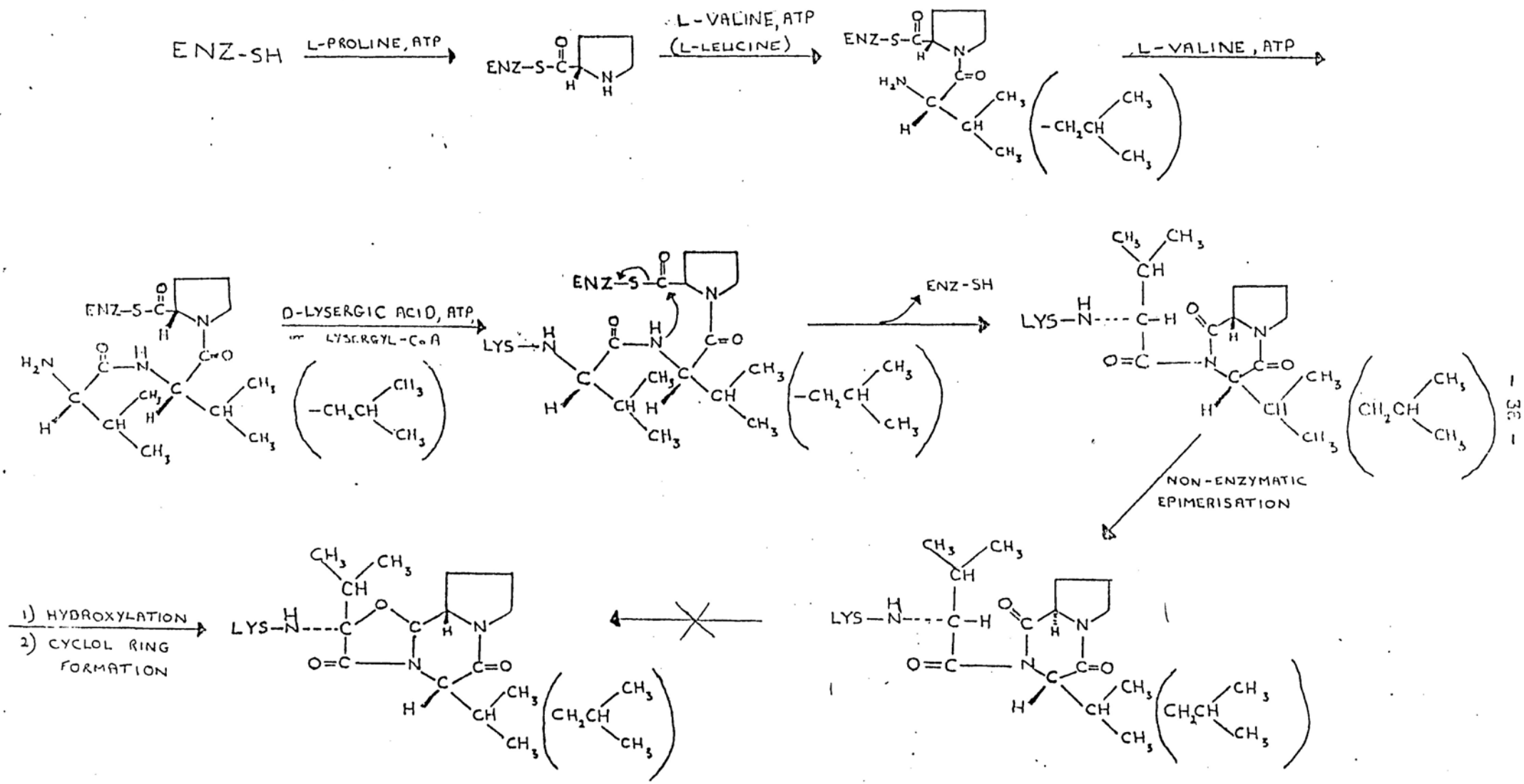


Fig. 12 Hypothetical formation of peptide ergot alkaloids on a multienzyme complex. (Floss, 1976)

rates of [^{14}C] phenylalanine, [^{14}C] alanine and [^{14}C] proline into ergotamine suggesting that these compounds are subject to isotope dilution by the amino acids of the internal pool. Of all the precursors of ergotamine fed, only D-lysergic acid stimulated alkaloid production and they proposed therefore that the concentration of D-lysergic acid is the rate-limiting factor in peptide ergot alkaloid peptide synthesis. They also found that addition of the ergoline precursors, tryptophan and methionine, led to a decrease in ergotamine synthesis both in the presence and absence of D-lysergic acid. This inhibitory effect must therefore take place at the stage of peptide synthesis and not ergoline synthesis. Methionine was the amino acid of lowest concentration in the cell, and these results may indicate it having a regulatory role in control of biosynthesis. Their results suggest that ergotamine synthesis in protoplasts and mycelium of C. purpurea is dependent on the actual concentrations of the free precursor constituents in the cellular pool. Achievement in preparation of a homogeneous vacuole preparation from C. purpurea protoplasts will hopefully provide a cell-free system for ergot alkaloid synthesis.

Peptide antibiotic synthesis is known also to occur on multienzyme complexes, and that these enzymes have rather broad specificities. Consequently structurally similar amino acids were able to replace certain of the amino acid residues of the peptide chain during the antibiotic synthesis (Katz and Demain, 1977). Using this fact and that peptide alkaloid biosynthesis is also predicted to occur on a multienzyme complex, it was demonstrated that amino acids of the peptide sidechain of ergot alkaloids could be replaced by some of their analogues (Table 6) (Beacco et al, 1977). With the same strain and the same analogue, the incorporation is higher in the mutant requiring for growth that very amino acid that will be replaced in the alkaloid (Table 6). These results are in agreement with those of Keller et al (1980) in that the biosynthesis of the peptide moiety of the ergot alkaloids appears to be controlled by the relative concentration of amino acids in the internal pool. Using this means of redirecting biosynthesis, new and possibly pharmacologically interesting alkaloids could become available. Floss et al (1980) have also used this technique to try and clarify the mechanism of α-hydroxy-α-amino acid formation in peptide alkaloid synthesis.

Strain	Main Alkaloid Produced	Amino Acid or Analogue added to the medium	Amount of the Alkaloid containing the added aminoacid or analogue (% of the total)
S40 phe-	ergocristine	p-chlorophenylalanine	63
S40	ergocristine	p-chlorophenylalanine	35
S40 phe-	ergocristine	p-fluorophenylalanine	66
S40	ergocristine	p-fluorophenylalanine	35
275 FI	ergotamine	p-fluorophenylalanine	30
B21	ergocornine ergocryptine	L-norvaline	59
B21	ergocornine ergocryptine	L-norleucine	23
B21 leu-	ergocornine ergocryptine	L-alfa aminobutyric acid	15
B21 leu-	ergocornine ergocryptine	5,5,5-trifluoroleucine	21
B21 leu-	ergocornine ergocryptine	Beta hydroxy leucine	11

TABLE 6 : Amino Acid Analogues Incorporation Observed with Various Strains of C. Purpurea.

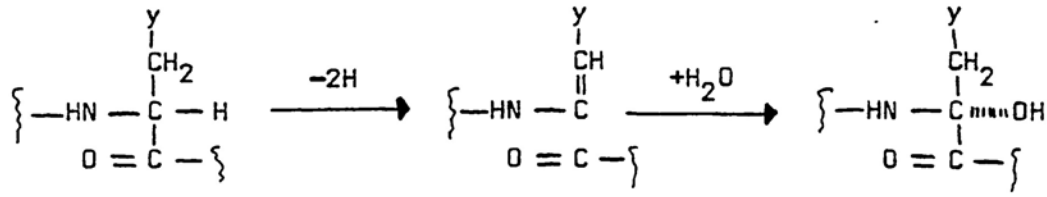
The three possible mechanisms are shown in Fig 13. They found when 2-amino butyric acid was added to a culture of an ergotamine producing strain of C. purpurea, the resultant alkaloid mixture contained ergocristine in addition to ergotamine. (R,S)-2-amino [3-^{13}C, 3-D_2] butyric acid was fed to C. purpurea and the resultant ergocristine isolated by preparative TLC. Mass spectrometric analysis showed peaks corresponding to fragment ions at $^{m}/_{e}$ 328 (peptide fragment-H) and m/e 300 (peptide fragment-H-CO). These were isotopically shifted 3 mass units, as compared with the fragmentation pattern of ergotamine and related peptide ergot alkaloids. Fragment ions m/e 244 (prolylphenylalanyl lactam) and m/e 267 (lysergic acid amide) were unchanged. This pattern indicates the presence of the label exclusively in the ∝-hydroxy-∝-amino acid portion of the molecule. The predominance of P+3 species containing ^{13}C and two atoms of deuterium clearly indicates that both hydrogens of the methylene group of 2-aminobutyrate have been retained during the transformation into the ∝-hydroxy-∝-amino butyric acid moiety of ergocristine. The results indicate that the formation of the ∝-hydroxy-∝-amino acid moiety does not proceed via a 2,3 dehydro amino acid intermediate. Anderson et al (1979) demonstrated that the alkaloid enzyme system of an ergotamine producing C. purpurea could accept dihydrolysergic acid in place of lysergic acid with the resultant production of dihydroergotamine.

Only three enzymes of alkaloid biosynthesis have been isolated:

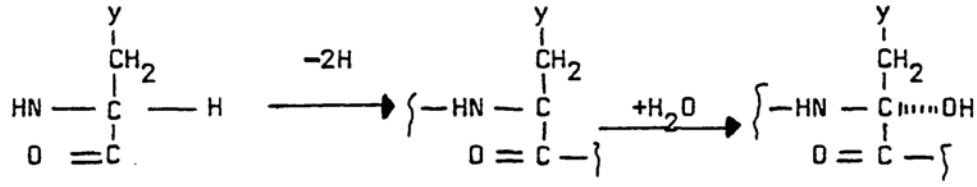
(i) Dimethylallyltryptophan synthetase. This was isolated by Heinstein et al (1971) and catalyses the first pathway-specific reaction of ergoline biosynthesis, that of the conversion of L-tryptophan and dimethylallylpyrophosphate to dimethylallyltryptophan. The enzyme is inhibited by agroclavine and elymoclavine, which, for the organism used (C. fusiformis), are end products of the pathway. This inhibition is uncompetitive with either substrate and occurs at alkaloid concentrations reached in a typical fermentation. DMAT synthetase activity increases approximately one day before the appearance of alkaloid and decreases dramatically towards the end of the fermentation, raising the possibility that end-product repression may be a factor controlling the activity of this enzyme.

(ii) Chanoclavine-I-cyclase which catalyses the conversion of chanoclavine I into the tetra-cyclic ergolines agroclavine and

(i) Dehydrogenation to the 2,3 dehydro amino acid followed by addition of water.



(ii) Dehydrogenation to the amino acid followed by hydration.



(iii) Replacement of H- with OH by a direct hydroxylation.

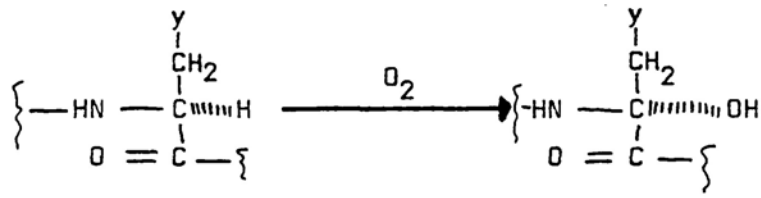


Fig. 13 Three possible mechanisms for the conversion of the amino acid into the hydroxy amino acid moiety of peptide ergot alkaloids.

elymoclavine. Three research groups have isolated this enzyme (Ongunlana et al, 1970, Gröger and Sajdl, 1972 and Heinstein et al unpublished results). Chanoclavine-I-cyclase also converts chanoclavine-I-aldehyde into agroclavine (Erge et al, 1973). The time course of the appearance and decline of this enzyme in cultures closely resembles that of dimethylallyltryptophan synthetase, and is also inhibited by elymoclavine as well as lysergic acid.

(iii) Agroclavine hydroxylase. Hsu and Anderson (1970) isolated a mixed function oxidase requiring a NADPH or NADPH-generating system which catalysed the conversion of agroclavine to elymoclavine. The reaction was dependent on cytochrome P-450.

The physiology and regulation of alkaloid biosynthesis has, due to its importance, commanded a great deal of interest. Probably one of the most important aspects of the study of regulation of ergot alkaloid biosynthesis is the role of tryptophan. Tryptophan is not only the direct precursor of ergot alkaloids, but is also involved in the induction and depression of the enzymes which synthesise alkaloids. It also represses the enzymes and enzyme forming systems of its own synthesis. Addition of tryptophan in the early stages of a fermentation can enhance the alkaloid yield. The two other precursors of the ergoline ring system, mevalonic acid and methionine are apparently never limiting, and are unable to induce alkaloid biosynthesis.

Teuscher (1964) showed that Claviceps mycelium can actively transport L-tryptophan from the medium, and that there is a correlation between the ability of a strain to produce alkaloid and its ability actively to transport L-tryptophan; only alkaloid-producing strains were found to take up this amino acid. The role of tryptophan in regulating alkaloid synthesis does not appear to be clear-cut in some alkaloid-producing strains. Analysis of several alkaloid producing Claviceps strains showed no consistent relationship between tryptophan accumulation and alkaloid production. Other Claviceps strains showed no increase in alkaloid production when supplemented with tryptophan (Gröger and Tyler 1963, Kelleher, 1969).

The stimulation of alkaloid biosynthesis by tryptophan in some strains is thought to be caused by a "training phenomenon" involving some type of inducer action. This hypothesis was reinforced when it

was found that certain non-metabolised analogues of tryptophan also stimulated alkaloid production. Thiotryptophan is a more effective stimulator of alkaloid synthesis than tryptophan. It was concluded therefore that ergot alkaloid production was an inducible process which can be activated by tryptophan (Floss and Mothes 1964). Vining (1970) suggested that L-tryptophan increases alkaloid production by promoting the development of a more active enzyme system. Since maximum enzyme activity is obtained if Claviceps is exposed to L-tryptophan during the initial of a fermentation, it is possible that only mycelium formed in the presence of excess tryptophan is conditioned to a high capacity for alkaloid synthesis at a later stage of development. This loading of the internal tryptophan pool with an excess of tryptophan is likely to affect the regulatory systems in the cell. Bu'lock and Powell (1965) consider that excessive accumulation of such primary metabolites induces the enzymes for secondary biosynthetic pathways. During the trophophase, absence of this induced enzyme activity, in the presence of tryptophan is attributed to catabolite repression, (Horowitz 1965, Marshall et al, 1968). Vining (1970) suggests that a part of the enzyme system required for alkaloid formation is tryptophan inducible and is synthesised during the trophophase.

Exogenous tryptophan is also capable of removing the inhibition of alkaloid production by phosphate. The effect of phosphate is to depress tryptophan biosynthesis (Robbers et al, 1972) and to activate alkaloid splitting enzymes (Robbers et al, 1978). Inorganic phosphate, which at high concentrations completely inhibits alkaloid formation, also represses dimethylallyltryptophan synthetase (Krupinski et al, 1976) and chanoclavine-I-cyclase (Erge et al, 1973). It appears therefore that tryptophan plays a role which goes beyond its precursor function in ergot alkaloid biosynthesis.

The production medium for a successful ergot alkaloid fermentation must contain a carbohydrate and an organic acid of the tricarboxylic acid cycle, or a compound metabolically related to it, (Arcamone et al, 1961, Amici et al 1967, 1969). This is illustrated by a high ergotamine yielding strain of C. purpurea, from which two different sub-strains were obtained. Neither produced alkaloid and had an impaired capacity either to utilise sucrose or to utilise

citric acid (Amici et al, 1967),

Parallel with alkaloid accumulation, a large amount of lipid, 30-40% of the dry weight, is produced. This large production of lipid and its correlation with that of alkaloids, has stimulated study of the metabolic factors which might influence the former. It has been suggested, following results obtained in the study of lipid accumulation in Candida (Whiteworth and Ratledge, 1975) and Rhodotorula (Brady and Chambliss, 1967), that micro-organisms which accumulate large amounts of lipids lack some control mechanisms. These might include a deficiency in phosphofructokinase and the enzymes of the pentose-phosphate cycle might be largely derepressed. Phospho-fructokinase activity was calculated for alkaloid producing strains of C. purpurea and C. paspali and compared with that in a strain of Saccharomyces cerevisiae which does not accumulate lipids (Spalla et al, 1978). The results expressed as nmol of substrate converted per min per mg protein, were 0·5, 1·6 and 120 for C. purpurea, C. paspali and S. cerevisiae respectively. The low phosphofructo-kinase activity found in these Claviceps would preclude any significant functioning of the Embden-Myerhof cycle. In four different strains of C. purpurea (Amici et al 1967, 1969) which produce peptide alkaloids, the molecules of sugar utilised (expressed as glucose) for each mole-cule of lipid produced (expressed as palmitic acid) are, respectively 7·4, 3·6, 6·7 and 16. If this sugar was utilised through the Embden-Myerhof-Krebs-cycle pathway, 15, 7, 13 and 32 molecules of NADPH for each molecule of lipid produced would have been respectively formed. Since for the synthesis of one molecule of lipid, 14 molecules of NADPH are needed, it may be concluded that at least in the first three cases the sugar would have been entirely consumed for the formation of the coenzyme and there would be nothing left for the build up of the skeleton of the lipids and for the formation of other primary as well as secondary metabolites. The NADPH therefore has to be formed through a pathway more efficient than the Embden-Myerhof Krebs cycle, for example the pentose phosphate cycle.

Rehacek (1980) reports that the carbohydrate metabolism proceeds almost identically in alkaloid productive and non-productive Claviceps cultures up to the oxidative decarboxylation of pyruvate to acetyl-coenzyme A. The activity of the key enzymes of the TCA cycle is 1-2 times higher than that of the glycolytic and hexomonophosphate cycle

enzymes, indicating that the TCA cycle is here the most effective mechanism for carbohydrate degradation. With citrate synthetase, a negligible activity has been found in a productive Claviceps culture and 3 to 5 times higher activity in a strain with a very low alkaloid content. Accumulation of citrate suppresses the citrate synthetase activity and activates acetylcoenzyme A carboxylase (Atkinson 1968). During alkaloid synthesis acetylcoenzyme A enters the TCA cycle to a limited degree and then takes an alternative pathway to the synthesis of fatty acids (Che-chang et al, 1967). The lipid content in the mycelium increases almost parallel to the activity of acetylcoenzyme A carboxylase and the rate of accumulation of alkaloid (Rehacek et al, 1971, El-sayed and Rehacek, 1978).

In contrast to the active growth phase, in which the TCA cycle is the main amphibolic pathway, the alkaloid production phase is characterised by the cells gaining energy and intermediates for their synthetic processes mainly through the glyoxylate pathway (Rehacek and Sajdl, 1979). The TCA cycle and the glyoxylate cycle have many reactions in common and in eukaryotes the two cycles are physically separated. Most of the enzymes of the glyoxylate cycle are located in organelles called glyoxysomes, and those of the TCA cycle are in the mitochondria. Consequently, Rehacek (1980) suggested that there might possibly be participation of both glyoxysomes and mitochondria in the regulation of alkaloid biosynthesis.

Spalla et al (1978) proposed a hypothesis, (summarised below), concerning the main mechanisms regulating the production of alkaloids by Claviceps spp.,

"The exhaustion of phosphate from the culture medium at first causes a marked slowing down or complete halt of growth. This in turn, would cause accumulation of inducers and precursors of alkaloids: tryptophan, amino acids which constitute the sidechain of the peptide alkaloids, acetate, phosphoenolpyruvate etc. and appearance of the inducible enzymes needed for secondary metabolism.

The following conditions have to be observed for performance of the strictly correlated lipid and alkaloid syntheses: (1) sugar consumption must continue even if growth has stopped; (2) considerable amounts of NADPH should be produced; (3) the 2 and 3 C carbon atom intermediates, precursors of lipids and alkaloids must be produced

and accumulated.

The lack of phosphofructokinase might satisfy points 1 and 2. In the absence of this glycolysis-regulating enzyme, pyridine nucleotides and citric acid cannot exert their inhibitory effect and sugar consumption takes place beyond the normal regulation pattern. The production of high amounts of NADPH might be secured by activity of the pentose cycle in the absence of phosphofructokinase.

The presence in the medium of an organic acid, such as citric or succinic acid which can be oxidised through the citric or isocitric acid cycles without the intervention of phosphate, would cause a saturation of the enzymes of the cycles with the consequence that accumulation occurs at the entry of the cycles themselves (in particular of acetyl coA and phosphoenolpyruvate).

The accumulation of alkaloids, lipids and sterols would therefore be the result of a concerted mechanism; on the one hand there is the phosphate depletion which stops growth and causes induction of the enzymes or on the other hand, there is the presence of other kinds of controls partly of genetic origin (e.g. absence of phosphofructokinase) and partly due to the particular composition of the culture medium (e.g. presence of a metabolisable organic acid). This complex mechanism for regulation of alkaloid production could explain some of the difficulties encountered in developing and establishing a successful fermentation process.

Alkaloids can also regulate their own biosynthesis. End-product clavine alkaloids (agroclavine or elymoclavine) have been shown to inhibit the first pathway-specific enzyme DMAT synthetase from Claviceps SD58, in vitro, at a concentration of 3mM (a concentration similar to the maximum alkaloid yield in submerged culture of this strain). Elymoclavine also inhibits chanoclavine-I-cyclase and anthranilate synthetase. End-product regulation by elymoclavine has been most effectively demonstrated in protoplasts of Claviceps fusiformis (Cheng et al, 1980). Removal of the cell wall seems at least to remove a permeability barrier to elymoclavine.

The main objectives of this work were to achieve successful submerged fermentation of Sphacelia sorghi for production of alkaloid; to gain a better understanding of the apparently complex array of

alkaloid products and to explore the biosynthesis of dihydroergosine and some aspects of the control of alkaloid biosynthesis in this organism.

2. MATERIALS AND METHODS

2.1 Origin of Strains

Sphacelia sorghi AB_2 was selected from *S. sorghi* used by P. G. Mantle (1973), and was originally isolated from Nigerian Sorghum.

Claviceps purpurea:

29/4 Ergotoxine-producing strain obtained from L. Nisbet (1975).

12_2 - strain same as used by Corbett *et al* (1974).

AG14, 117/2/SF Derivatives of strain used by Tonolo (1966).

Aspergillus fumigatus isolated from soil.

Penicillium simplicissimum isolated from New Zealand soil (Day, 1980).

2.2 Maintenance of Cultures

Cultures were maintained by subculturing every three weeks on Medium T agar slopes (Section 2.3). *S. sorghi* and *P. simplicissimum* were incubated at 27°, *C. purpurea* at 24° and *A. fumigatus* at 37°.

2.3 Medium Composition

(i) Medium T. Sucrose, 100g.; L. Asparagine, 10g.; $Ca(NO_3)_2 . 4H_2O$, 1g.; KH_2PO_4, 0·25g.; $MgSO_4 . 7H_2O$, 0·25g.; KCl, 0·125g.; $FeSO_4 . 7H_2O$, 0·033g.; $ZnSO_4 . 7H_2O$, 0·027g.; Yeast extract, 0·1g.; L-cysteine hydrochloride, 0·01 g.; Distilled H_2O to 1 litre, pH adjusted to pH 5·2 with NaOH and sterilised at 15 p.s.i/15 mins. Solid medium contained 1·5% agar.

(ii) Modified Medium T. Sucrose, 150g.; L-Asparagine, 15g.; KH_2PO_4, 0·25g.; $ZnSO_4 . 7H_2O$, 0·027g.; $FeSO_4 . 7H_2O$, 0·033g.; $MgSO_4 . 7H_2O$, 0·25g.; distilled water to 1 litre. pH adjusted to pH 5·5 with NaOH and sterilised at 15 p.s.i/15 mins.

(iii) Medium TS. Sucrose, 100g.; L-Asparagine, 10g.; KH_2PO_4, 0·5g.; $MgSO_4 . 7H_2O$, 0·3g.; $FeSO_4 . 7H_2O$, 0·007g.; $ZnSO_4 . 7H_2O$, 0·006g.; Yeast extract, 0·1g.; distilled water to 1 litre. pH adjusted to pH 5·2 with NaOH and sterilised at 15 p.s.i/15 mins.

(iv) Medium T (Asp. or Glu.). As for Medium T, except 10g. L-Asparagine was replaced by 20g of L-Aspartic acid or 20g L-Glutamic acid.

(v) Medium T (Fructose - Glucose). As for Medium T except 100g. sucrose was replaced by 50g. Glucose and 50g. Fructose.

(vi) Medium B. Sucrose, 200g.; $(NH_4)_2SO_4$, 11·8g.; KH_2PO_4, 0·5g.; $MgSO_4 . 7H_2O$, 0·5g.; $FeSO_4 . 7H_2O$, 0·033g.; $ZnSO_4 . 7H_2O$, 0·108g.;

distilled water to 1 litre. pH adjusted to pH 5•2 with NaOH. 2% $CaCO_3$ was added before sterilisation.

(vii) Medium T25. Sucrose, 300g.; Citric acid, 15g.; $Ca(NO_3)_2 \cdot 4H_2O$, 1g.; KH_2PO_4, 0•25g.; KCl, 0•125g.; $MgSO_4 \cdot 7H_2O$, 0•25g.; $FeSO_4 \cdot 7H_2O$, 0•007g.; $ZnSO_4 \cdot 7H_2O$, 0•006g.; Yeast extract, 0•1g.; distilled water to 1 litre. pH adjusted to pH 5•2 with NaOH and sterilised at 15 p.s.i/15 mins.

(viii) Amino Nitrogen Media. Sucrose, 100g.; L-Asparagine, 5g. or L-Aspartic acid, 10g.; KH_2PO_4, 0•25g.; $MgSO_4 \cdot 7H_2O$, 0•25.; KCl, 0•125g.; $FeSO_4 \cdot 7H_2O$, 0•033g.; $ZnSO_4 \cdot 7H_2O$, 0•027g. pH adjusted to pH 5•2 with NaOH and sterilised at 15 p.s.i/15 mins. Solid medium contained 1•5% agar.

(ix) Czapek Dox./ Yeast Extract Broth. 35g. Czapek Dox (Difco) + 5g. Yeast Extract (Difco); distilled water to 1 litre. Sterilisation at 15 p.s.i/20 mins.

(x) Sterilisation of Medium Supplements.

Amino Acids:-

Proline, Leucine; These were batched with the medium before autoclaving.

Tryptophan, Parafluorophenylalanine, 4-Aza-leucine; Aqueous solution sterilised by millipore filtration (and added to the medium after autoclaving).

Hydroxy-L-proline; Solution sterilised at 5p.s.i/20 mins.

Proline Analogues; Aqueous solutions sterilised by millipore filtration.

Radioactive Amino Acids; Supplied sterile by the Radiochemical Centre, Amersham.

Nystatin; Nystatin was a) dissolved in a small volume of methanol before incorporation into medium, or b) dissolved in water and added to a somewhat concentrated medium so that the normal composition was attained after Nystatin addition. In either case the initial nystatin solution was sterilised by millipore filtration prior to adding to the medium.

DL-Ethionine. Solution sterilised at 5p.s.i/20 mins.

2.4 Membranes

Membranes (Technicon-Cuprophan 933-0225-01) were cut to required size and boiled for 30 minutes in two changes of distilled water. Each membrane was placed between two sheets of filter paper and stacked in glass petri dishes. The stack was saturated with distilled

water before sterilising (15p.s.i/20 mins).

2.5 β-1,3 - Glucanase

The glucanase had been prepared from Basidiomycete QM806 by C. Pollard, Biochemistry Dept., Imperial College, and had hydrolysing activity of 350 μg glucan/mg. protein/min.

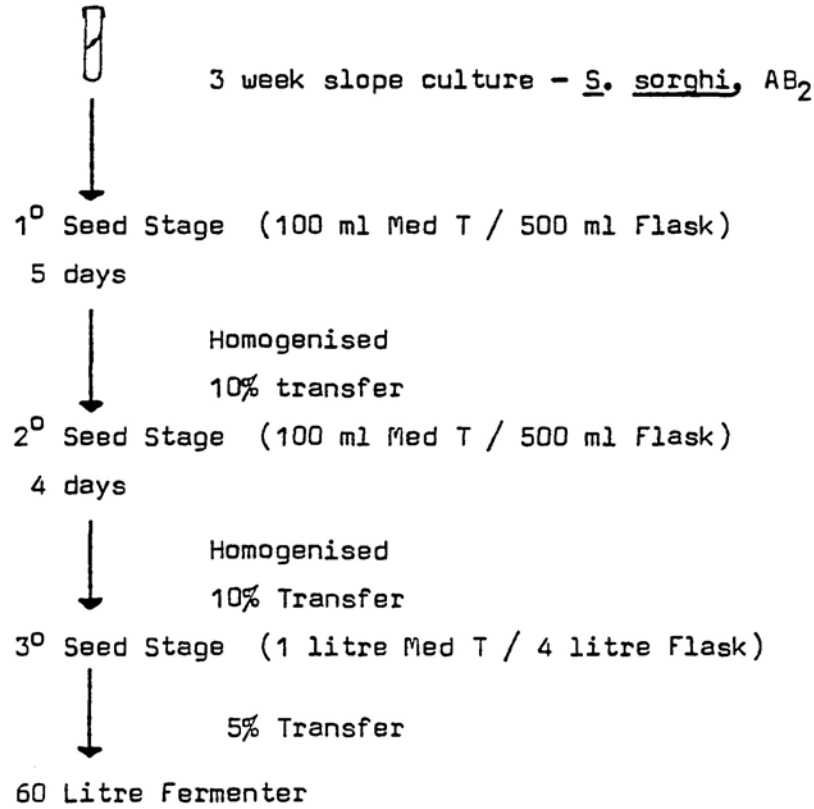
2.6 Flask Fermentation

(i) Surface Culture. A spore/mycelial suspension was prepared from a 3 week old slope culture and used to inoculate a flask of Medium T (100 ml Med T / 500 ml Erlenmeyer) and incubated at 27°.

(ii) Submerged Culture. A seed flask of Medium T was inoculated as for surface culture, but was incubated on a rotary shaker (200 r.p.m./ 10 cm eccentric throw) at 27°. After 7 days the seed stage was homogenised and a 10%(v/v) transfer made into Medium T production stage flasks and incubated as before.

2.7 60 Litre Fermentations

(i) Fermentation Seed Stages



Fermentation Temperature maintained at 27°.

(ii) FERMENTER DIMENSIONS - SIXTY LITRE (60L) BRUNSWICK FERMENTERS

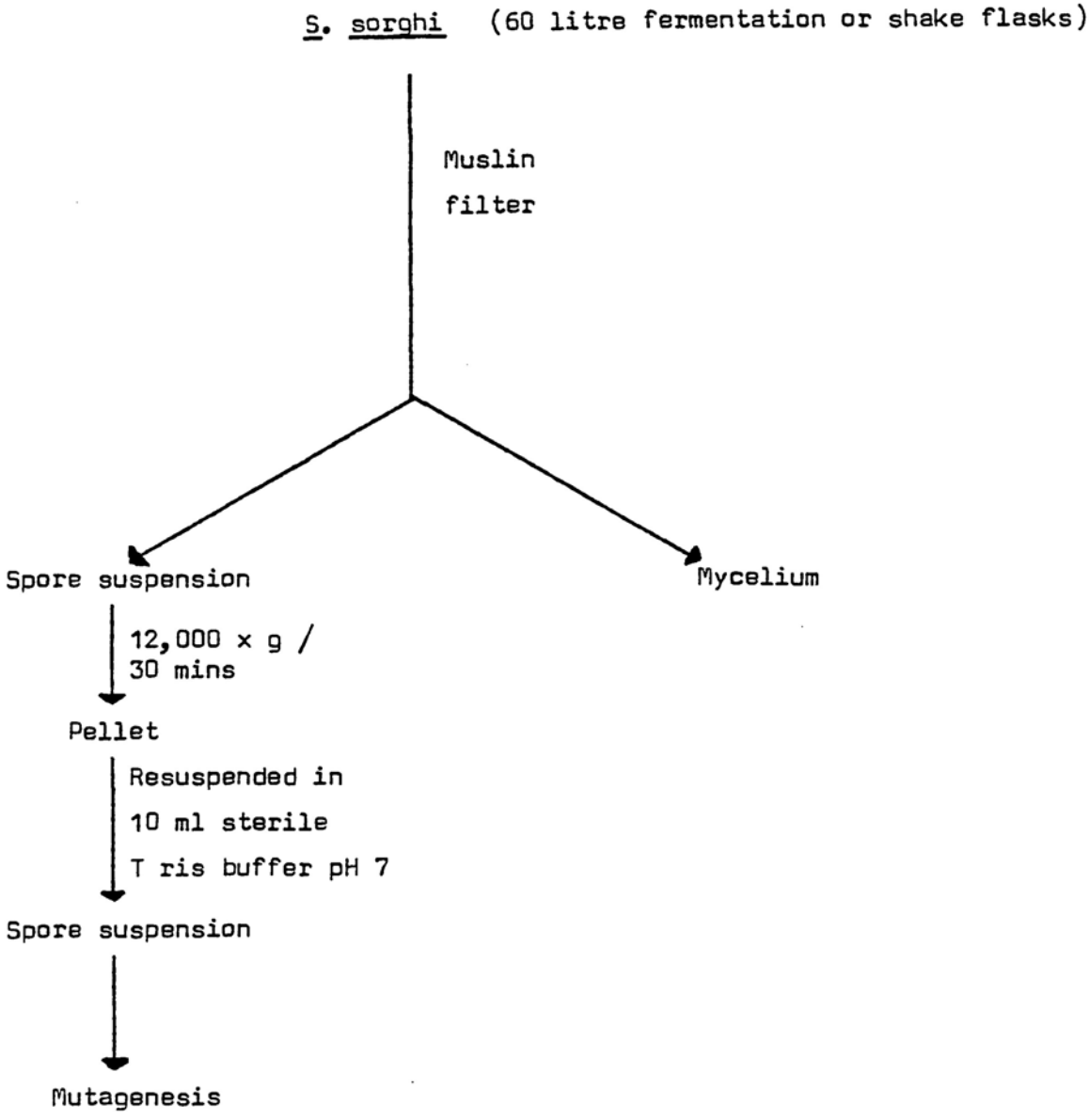
DIMENSION	
LIQUID HEIGHT (cm)	48•0
INTERNAL TANK DIAMETER (cm)	40•0
H/D RATIO	1•2
EFFECTIVE IMPELLER DIAMETER (cm)	15•0
NUMBER OF IMPELLERS	1
$^{d}/_{D}$ RATIO	0•375
NO. OF IMPELLER BLADES	4
IMPELLER BLADE DIMENSIONS (cm)	5x5
LIQUID SURFACE - TOP IMPELLER	32
BOTTOM IMPELLER - DISHED END (cm)	16•0
SPARGER - BOTTOM IMPELLER DISTANCE (cm)	7•0
BAFFLE WIDTH (cm)	4•0
BAFFLE - WALL GAP (cm)	0•7
DEPTH OF DISHED END	9•0

(iii) OPERATION PARAMETERS

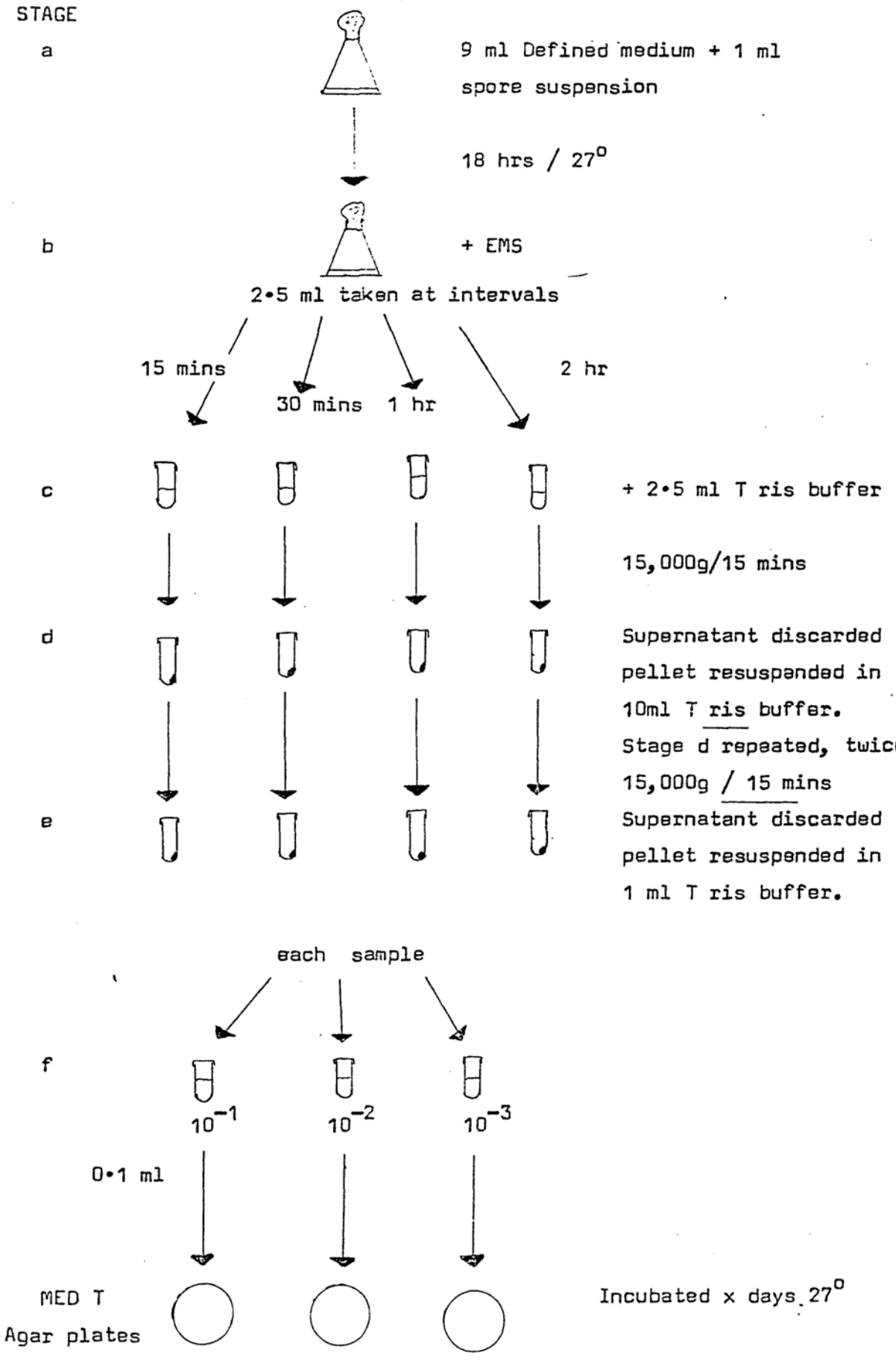
PARAMETER		
TOTAL CAPACITY (l) (approx. vol.)	100	
TOTAL WORKING VOLUME (l)	60	
MEDIUM VOLUME (l)	54	
INOCULUM LEVEL (l)	3	
POSITIVE GAUGE PRESSURE (p.s.i)	15	
SHAFT SPEED (r.p.m)	187	
AIR FLOW RATE (l/min)	30	0-24 hrs
	60	24hrs - 13 days
TEMPERATURE (C)	27	

2.8 Mutagenesis

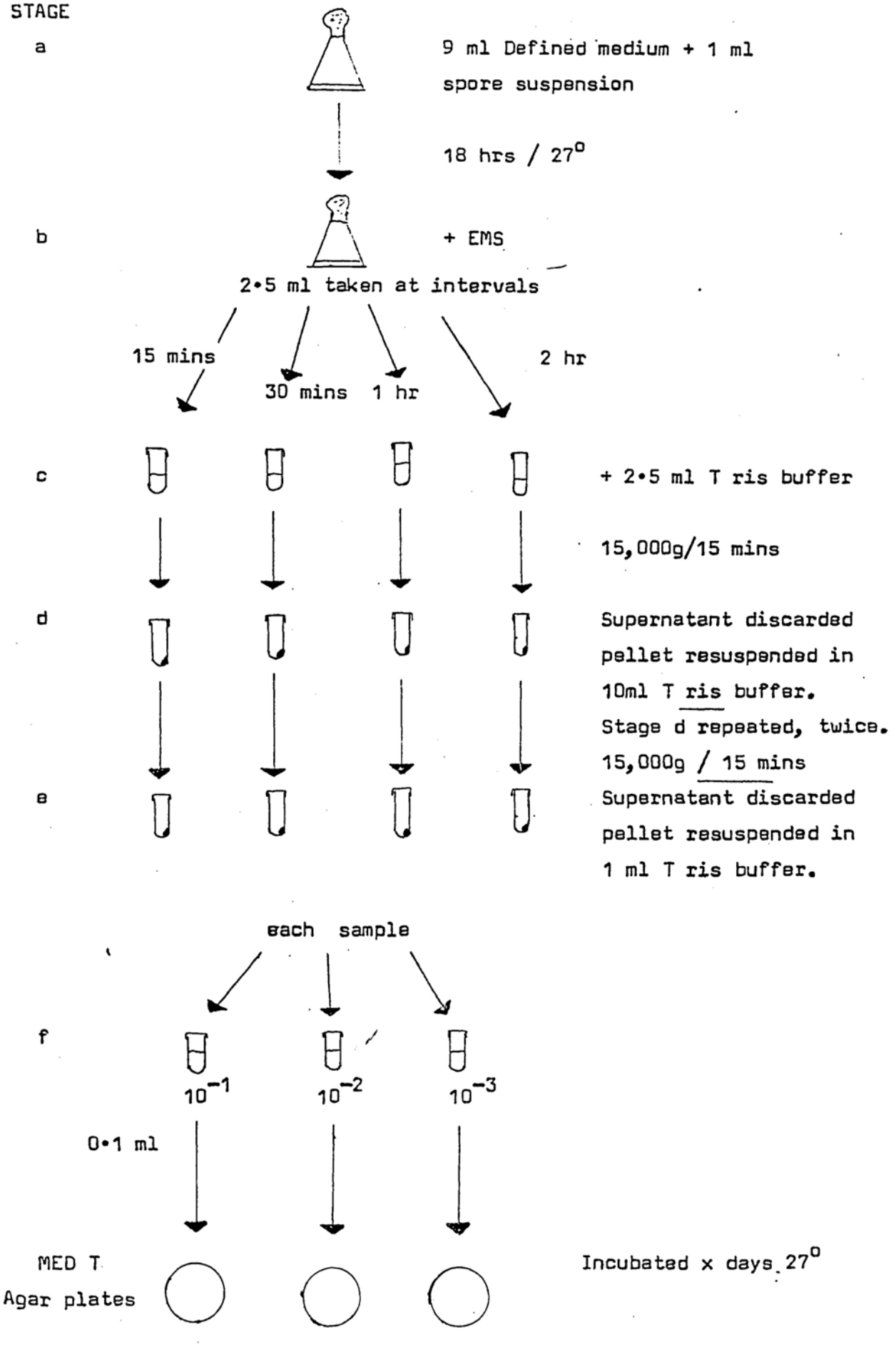
(i) Preparation of *S. sorghi* spore suspension.



(ii) Ethyl Methane Sulphonate Mutagenesis



(ii) Ethyl Methane Sulphonate Mutagenesis



2.9 Analytical Procedures

1. Alkaloid

(a) Colorimetric Assay. Suitably diluted culture filtrate was mixed with twice its volume of Van Urk Reagent (p - dimethylaminobenzaldehyde, 0·625g.; 65% v/v H_2SO_4, 500 ml; 5% $FeCl_3 6H_2O$, 0·5ml). The resultant blue colour was allowed to develop for 10 minutes after which the absorbance was measured at 570nm in an Eel Colorimeter. Absorbance was compared with that of standard solutions of ergotamine in the range 0 - 100 µg/ml.

(b) Extraction of Basic Alkaloids from Culture Filtrate.

Culture filtrate was adjusted to pH 8-9 with 0·88 SG NH_4OH and extracted twice with an equal volume of diethyl ether. The diethyl ether was taken to dryness on a rotary evaporator.

(c) Isolation of Amphoteric Alkaloids from Culture Filtrate.

After removal of basic alkaloids, the culture filtrate was adjusted to pH 4-5 with 6N HCl. Amphoteric alkaloids were removed from the acidic filtrate by ion-exchange chromatography, as for amino acids (Section 2.9.6).

(d) Alkaloid Extraction of Sclerotia and Mycelium.

Freeze-dried mycelium or powdered sclerotia (8 parts by weight) and sodium hydrogen carbonate (1 part) were mixed to a paste with water. This mixture was shaken with diethyl ether for 1 hour (x3) to extract basic alkaloids.

Amphoteric alkaloids were extracted by treating the fungal tissue with methanol.

(e) Separation of Constituent Alkaloids by Thin-Layer Chromatography (TLC).

(i) Sphacelia sorghi Alkaloids.

The components of an alkaloid mixture were resolved on plates of silica gel G_{254} (0·25 mm thickness) using Chloroform/Methanol/Ammonia (95:7:5) as the developing solvent. The alkaloids were tentatively located by their quenching of the fluorescent dye (254 nm) and confirmed by spraying the chromatogram with Ehrlichs Reagent (3% p-dimethyl-amino-benzaldehyde in concentrated HCl). Alkaloids were seen as blue spots.

(ii) Amphoteric Alkaloids.

Chromatograms were developed twice in Chloroform/Methanol/Ammonia (80:20:6) to resolve amphoteric alkaloids having an R_f value of about 0·1.

(iii) Claviceps purpurea Alkaloids.

C. purpurea alkaloids were resolved by developing in Ethyl acetate/Dimethylformamide/Ethanol (13:1•9:0•1) (McLaughlin et al, 1964). Detection was partly by fluorescence under UV light (350 nm), and confirmed by spraying with Ehrlichs reagent.

In (i) - (iii), where possible, chromatograms included reference alkaloids.

(iv) Preparative Chromatography.

Preparative plates (Kieselgel 60 GF_{254}) were prepared to 1mm thickness. Alkaloids were recovered by removing the silica from appropriate regions and eluting it with methanol.

2. Mycelial Dry Weight

Mycelium was washed with distilled water, transferred to a dried, weighed filter paper and dried to constant weight at 60°.

3. Glucan

Filtrate was mixed with an equal volume of ethanol and the precipitated glucan collected on a glass rod. This was transferred to dried, weighed filter paper and dried to constant weight at 60°.

4. Estimation of Inorganic Phosphate

Reagents: Reducing Reagent: 1-amino-2-napthol-4-sulphonic acid (0•2g), sodium bisulphite (1•2g), sodium sulphite (1•2g) and distilled water (104 ml). Acid Molybdate Solution: $(NH_4)_6\ MO_7\ O_{24}.\ 4H_2O$ (1•25g) in 2•5N HCl (100 ml).

To 1 ml of culture filtrate was added 1 ml of acid molybdate solution and 0•1 ml reducing reagent and the volume made up to 10 ml with distilled water. After 10 minutes the resultant blue colour was measured at 660 nm. Phosphate concentration was calculated from a standard curve in the range 0-2 µmoles. (Fiske and Subbarow, 1925).

5. Estimation of Reducing Sugars (Schoorl Procedure)

Reagents: Fehling's A solution. Copper Sulphate pentahydrate (69•3g), distilled water to 1 litre. Fehling's B solution. Sodium potassium tartrate (346g), sodium hydroxide (142g), distilled water to 1 litre. 2% Starch Solution. Starch (2g) suspended in distilled water (10 ml) and boiling distilled water (90 ml). This mixture boiled for 2 minutes then cooled.

Fehling's A (5 ml) and Fehling's B (5 ml) were mixed and 2%

starch solution (20 ml) added. After boiling gently for 3 minutes, the solution was cooled to 20°C. 4N H_2SO_4 (10 ml) and 30% KI solution (5 ml) were added and the mixture titrated with 0·05N sodium thiosulphate using 2% starch solution as indicator.

6. Mycelial Amino Acids

Amino acids were extracted from mycelium with 2M perchloric acid / 18 hours, after which the mycelium was separated from the supernatant. The supernatant was cooled, adjusted to pH 5 with chilled saturated KOH, and the precipitated $KClO_4$ removed by filtration. The supernatant was then applied to a column of Zeocarb 225 (H^+) cation exchange resin. The amino acids were eluted from the resin with 2N NH_4OH and the eluate taken to dryness on a rotary evaporator at 60°.

The amino acids were qualitatively determined by high-voltage electrophoresis in pH 3·5 buffer. (0·5% Pyridine / 5% acetic acid / 10 mM EDTA).

7. Detection of Proline and Hydroxyproline

Freeze-dried culture filtrate was dissolved in a little distilled water and amino acids separated by TLC using n-Butanol/Acetic Acid/Water (4:1:5). The presence of proline and hydroxyproline was detected by dipping the TLC plate in a solution containing 100 mg of Isatin, 50 ml of n-Butanol and 5 ml glacial acetic acid, then heating it at 40°C/5 minutes. Proline gives an intense blue colour (sensitivity 0·2 μg) and hydroxyproline gives a pale blue colour (sensitivity 0·8 μg).

2.10 Autoradiography of ^{14}C Alkaloids

Prefogged (Laskey and Mills, 1975) Kodak BB5 film, or, when extra sensitivity was required Kodak Omat X film was used for autoradiography of ^{14}C alkaloids. The autoradiograms were incubated at -70° for 2-7 days, as appropriate.

2.11 Preparation of ^{14}C Agroclavine

^{14}C Agroclavine was prepared by feeding Claviceps fusiformis cultures with ^{14}C Na mevalonate (prepared from ^{14}C mevalonic acid lactone by evaporating the benzene carrier and neutralising with the appropriate amount of NaOH.) ^{14}C agroclavine was isolated from the resultant alkaloid mixture by preparative TLC. It was further purified by dissolving in chloroform and reprecipitating by adding

low boiling point petroleum ether (30-40°). The precipitate was separated by filtration and redissolved in the minimum volume of high boiling point petroleum ether (100-120°) in a bath of boiling water. On slow cooling colourless crystals of agroclavine were obtained. The ^{14}C agroclavine crystals were dissolved in dilute HCl and adjusted to pH5 with dilute NaOH. This solution was sterilised by millipore filtration before adding to S. sorghi cultures.

2.12 Agroclavine

Agroclavine was produced by Claviceps fusiformis and having been separated from other alkaloids by preparative TLC was chromatographically homogeneous. It was formulated as for ^{14}C agroclavine, for addition to S. sorghi cultures.

2.13 Cell-free Preparation of S. sorghi

A mycelial mat (7g wet wt) from a 14 day old surface culture flask of S. sorghi which was positive for alkaloid (200µg/ml) was washed with 0·01M tris/HCl buffer (pH 8). The washed mycelium was mixed with sand and cardice and ground in a mortar with 10 ml of the same buffer containing 0·02M each of diethyldithiocarbamate, thioglycolic acid and mercaptoethanol. The resultant slurry was centrifuged at 10,000 x g for 30 minutes at 6°C. The supernatant was dispensed into an Erlenmeyer flask to which ^{14}C-methylene tryptophan or ^{14}C-dimethylallyltryptophan was added. Incubation was at 27° and after 6 hours the cocktail was freeze-dried, and then extracted for amphoteric alkaloids with methanol.

2.14 Preparation of Protoplasts

(i) Fungal Protoplasts

Mycelium (0·5g wet weight) from 8-9 day old shake cultures, which were positive for alkaloid, was harvested by filtration through a sintered glass funnel and washed with 0·7M KCl. The mycelium was suspended in 6ml of 0·7M KCl and 0·67ml of liquid β-glucuronidase/arylsulfatase obtained from Helix pomatia (Boehringer Mannheim Biochemicals or Sigma). This mixture was incubated at 30° with gentle shaking (100 rpm) for 6 hours, after which, a drop was examined under the microscope for protoplasts. SDS (sodium lauryl sulphate) was then added to this drop in order to confirm or otherwise the diagnosis of protoplasts. A preparation deemed to be rich in protoplasts was

centrifuged at 3000 rpm for 10 minutes, and the residue washed twice with 0•7M KCl. The protoplasts were finally resuspended in 1ml 0•7M KCl.

(ii) Yeast Protoplasts

An 18 hour culture (50 ml) of Saccharomyces cerevisiae was harvested, centrifuged and washed in distilled water. The pellet was resuspended in 20ml Sorbitol (1M) / EDTA (25mM EDTA) to which 0•9 ml of mercaptoethanol was added. This was incubated at 30°C/20 minutes with gentle shaking, after which it was centrifuged and washed twice in sorbitol 0•8M. The pellet was resuspended in 20ml Sorbitol 1•2M-/ citrate 0•1M/-EDTA 0•01M, pH 5•8. Glucuronidase (0•2ml) was added, and the solution incubated at 30°C for 2 hours.

2.15 Addition of Hydroxy-L-proline to Fungal Protoplasts

The protoplast suspension, obtained from 0•5g (wet wt.) mycelium, was added to 10 ml of 0•7M KCl and 1μ Ci of ^{14}C-hydroxy-L-proline. After 48 hours agitated (100 rpm) incubation at 27° the suspension was centrifuged at 12,000 x g for 10 minutes and the supernatant assayed for alkaloid.

2.16 Dinitrophenol Derivitisation of Amino Acid

10 milligrams of amino acid was mixed with 20 mg of sodium hydrogen carbonate and then dissolved in 0•4 ml water at 40°. To this 18•5 mg of FDNB (1-fluoro-2,4-dinitrobenzene) was added and the mixture stirred for 1 hour. Unreacted FDNB was removed by extraction of the solution with diethyl ether. To the aqueous layer 0•03 ml of conc. HCl was added, causing crystallisation of the DNP derivative of the amino acid.

The DNP-amino acid was separated from other material by preparative TLC on silica gel using Butanol/acetic acid/water (4:1:1) as the developing solvent.

2.17 High-voltage Electrophoresis (H.V.E)

H.V.E. of amino acids was carried out on preparative chromatography paper (Watman, 3MM) in pH 3•5 buffer (0•5% Pyridine, 5% acetic acid). Electrophoretograms were run for 2 hours at 3kV. Amino acids were detected by staining the electrophoretograms with a ninhydrin solution (3% ninhydrin in 15ml Cadmium acetate and 85 ml acetone). Amino acids stained pink.

2.18 High-Pressure Liquid Chromatography

For HPLC, a Jobin Yvon Chromatospec prep 100 (Table 7) was used, the column of which was packed with silica TLC Kieselgel (60H, 15 m). Elution of alkaloid from the column was the Freon TF (trichlorotrifluoroethane) / Methanol / Ammonia was monitored spectrophotometrically at 230 nm.

PARAMETER	
VOID VOLUME	400 ml
COLUMN DIAMETER	8 cm
COLUMN DEPTH	15 cm
PRESSURE	
- COLUMN	5 BAR
- RESERVOIR	4 BAR
FLOW RATE	15ml/minute

Table 7 : Operating Parameters of Jobin Yvon Chromatospec Prep. 100.

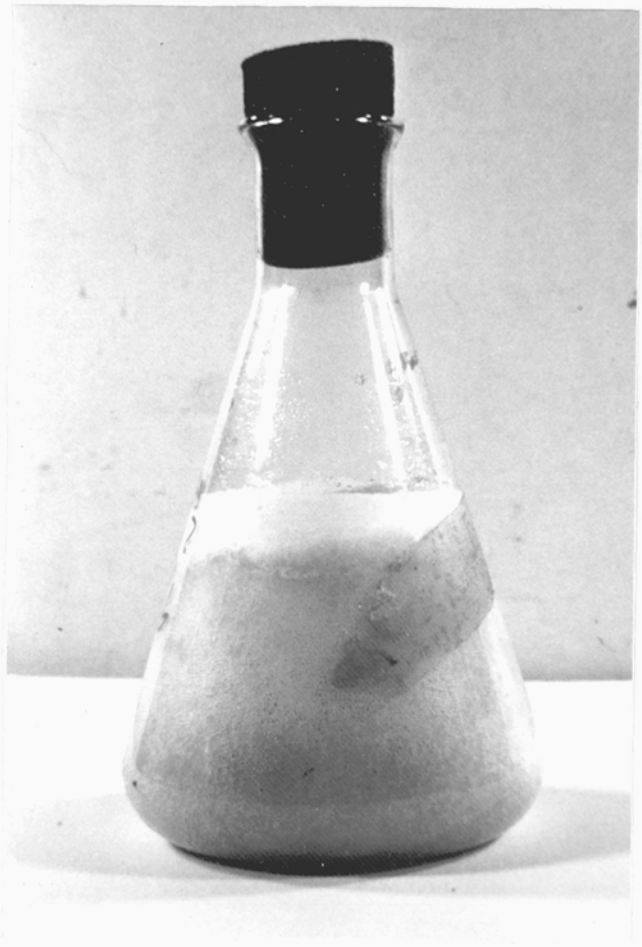
3. SPHACELIA SORGHI

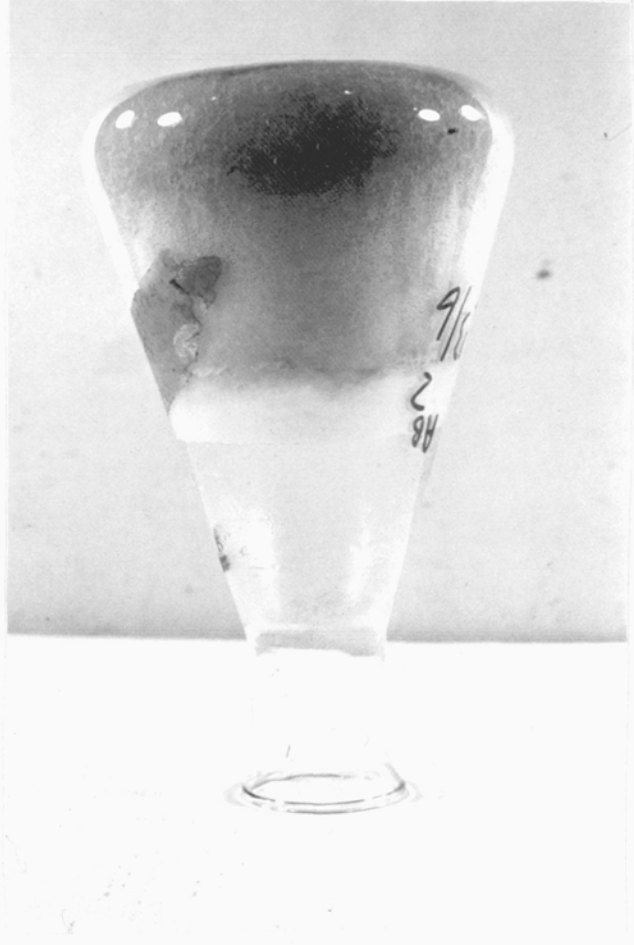
3.1 (i) Cultivation of Sphacelia Sorghi

Since S. sorghi produces glucan in liquid cultures, thereby causing the culture filtrate to become viscous, alkaloid biosynthesis is inhibited in submerged fermentations. It seemed possible the glucan problem might be overcome if a β-glucanase capable of breaking down S. sorghi glucan was introduced into the fermentation system. The β-glucanase could be added either in the form of a pure enzyme, or in the form of another organism which produces and secretes the enzyme into the medium in an active form. These two proposals were tested. Addition of Basidiomycete QM806 β-glucanase (10 mg / 100 ml S. sorghi culture) caused complete breakdown of S. sorghi glucan within 18 hours, but disadvantages of this method are the time, and expense of isolating the enzyme. To test the feasibility of mixed culture, Claviceps purpurea (strain 29/4), which is very efficient at producing a β-glucanase in submerged culture but does not produce alkaloids under the conditions used in this experiment, was added to cultures of S. sorghi. Since glucanase production by C. purpurea is optimum at about 10 days (Shaw, 1979), 10% transfer of whole culture of this age was made to 7 day submerged cultures of S. sorghi (strain AB_2). Control flask cultures became so viscous (Fig 14) that they failed, as usual, to produce alkaloid, but in contrast, the flasks to which C. purpurea had been added became much less viscous, and S. sorghi alkaloids were produced.

Following this preliminary experiment several strains of C. purpurea were screened qualitatively by the above technique to compare their ability to breakdown S. sorghi glucan. Strain 12_2 was found to be the best and was therefore used subsequently.

The effect on S. sorghi of the amount and timing of the addition of C. purpurea was investigated (Fig 15). Unexpectedly, the control flasks of S. sorghi, which had not received C. purpurea, produced alkaloid, giving a final yield comparable with that previously obtained in static liquid cultures. Flasks of S. sorghi to which C. purpurea was added also produced alkaloid, but generally, did not attain the yield of the control flasks. It is notable that there was considerable variation in the alkaloid yields of individual flasks. Addition of 2% C. purpurea at 5 days (Fig 15) apparently gave enhanced alkaloid yield.





Upturned
Flask

Fig 14 Glucan Production by S. sorghi in shake flask culture

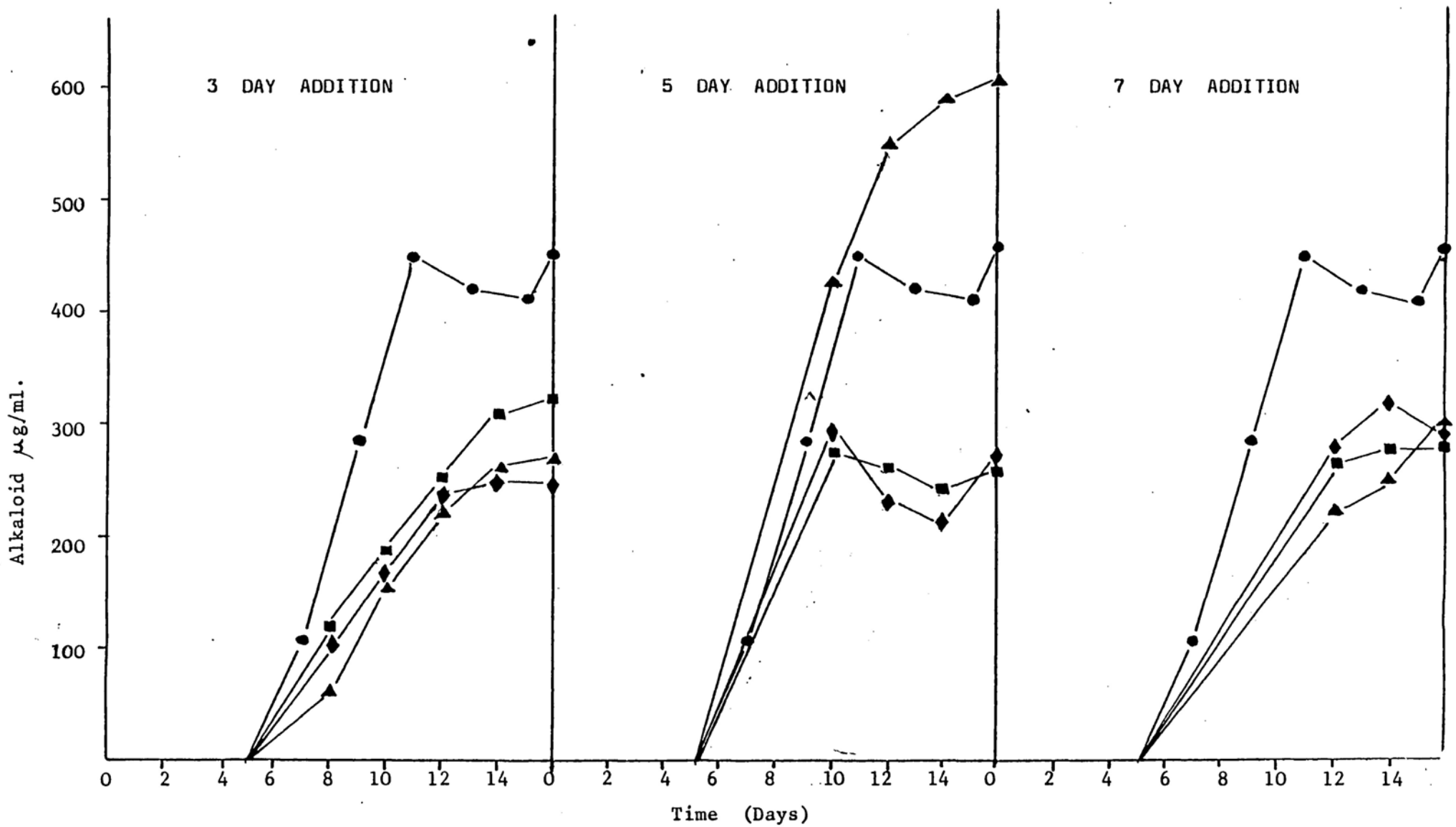


Fig 15 Effect on Alkaloid Production by S. sorghi of time and level of addition of C. purpurea

● – ● control, ▲ – ▲ , 2% v/v, ■ – ■ 5%, ◆ – ◆ , 10%.

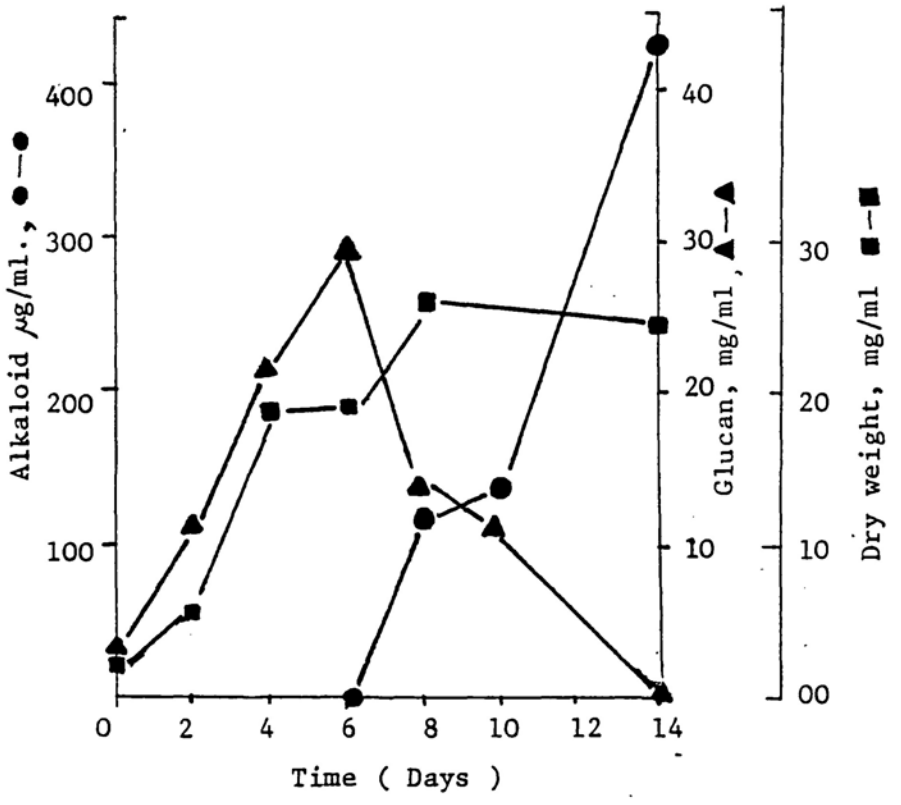
However, it is possible that this treatment group just happens to consist of five of the highest yielding flasks randomly selected. The novel production of alkaloid in submerged cultures displayed in this experiment, was associated with a spontaneous decline in glucan concentration during the course of the fermentation. From qualitative estimations of culture viscosity, it was not possible to be confident that the C. purpurea glucanase had significantly affected the rate of S. sorghi glucan autohydrolysis. Indeed, if there is any effect, it is the suppression of alkaloid production.

Two subisolates of S. sorghi AB_2 (one a glucanase producer and the other apparently not) were grown in a seed stage for 7 days, homogenised and a 10% transfer inoculated into production stage flasks. Flasks were sampled every two days for analysis of glucan, alkaloid and dry weight.

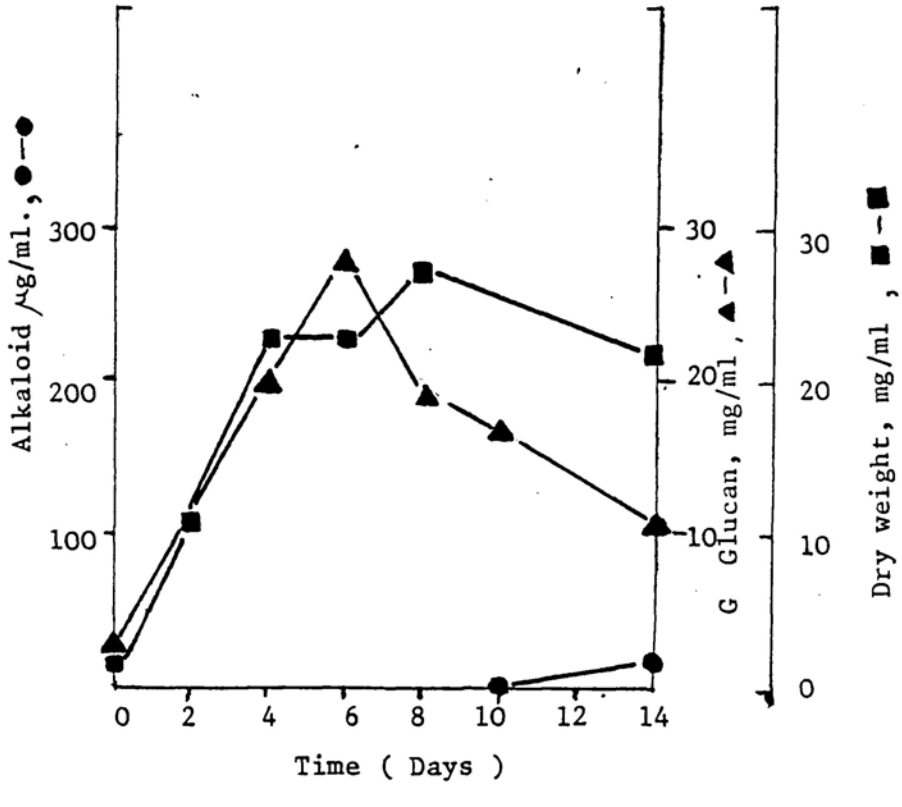
Unexpectedly, the sub-isolate which had hitherto apparently failed to autolyse glucan produced early in the fermentation, degraded most of its glucan by the end of the fourteen day experimental period (Fig 16). At this stage a trace of alkaloid was detected. In contrast, the other sub-isolate autolysed glucan more rapidly after the sixth day of fermentation coincident with which typical alkaloid production (400 µg/ml) occurred. It appears therefore, that there is a point in S. sorghi fermentations where glucan must be reduced below a certain concentration otherwise alkaloid production does not take place. It seems likely that this effect is on gas transfer efficiency, but if adequate aeration is restored too late in the fermentation, the mycelium may have entered a senescent phase and consequently be unable to respond by synthesising alkaloid. Further experiments used the early glucanase-producing variant.

(ii) Selection of a Suitable Production Medium for S. sorghi Alkaloids

Several media known to support alkaloid biosynthesis in other ergot fungi, were tested for their effect on alkaloid and glucan production in submerged culture by S. sorghi (Table 8). The seed stage was grown in Medium T and a 10% $^{v}/_{v}$ transfer was used to inoculate the various production media. Medium T gave the best yield of alkaloid, and most of the glucan synthesised early in the fermentation had autolysed within 11 days. Two of the modifications of



(a)



(b)

Fig 16 Alkaloid, glucan and dry weight production by S. sorghi, AB_2 in shake-flask culture

(a) early-glucanase producer

(b) late-glucanase producer

MEDIUM	ALKALOID (µg / ml)	GLUCAN (mg/ml)
MED T	355	4•4
MOD MED T	230	40
MED TS	140	41
MED T - ASP	0	0
MED T - GLU	TRACE	38
FRUCTOSE - GLUCOSE MED T	0	21
MED B SUCROSE 100g/l	0	37
200g/l	0	49
300g/l	0	13•4
MED T25 SUCROSE 100g/l	0	) Whole Culture Foamed and Solidified
200g/l	0	)
300g/l	0	)

RESULTS FROM 11 DAY OLD SHAKE CULTURES (MEAN OF TWO FLASKS)

TABLE 8 : Effect of Various Production Media on Alkaloid Production by S. sorghi, strain AB_2

medium T also supported alkaloid production even though glucan concentration remained fairly high. In most other media glucan accumulated and no alkaloid could be detected. However, where aspartic acid had been substituted for asparagine in medium T, there was no evidence of glucan throughout the fermentation. In a further experiment similar results were obtained when the seed stage and production stage were of the same medium composition.

(iii) Large Scale Fermentations

A 60 litre fermentation was carried out using S. sorghi, AB_2 (Fig 17). During the fermentation the fungus grew forming pellets which were less than 3 mm in diameter. Sporulation was not particularly evident. Rapid uptake of phosphate, and synthesis of glucan were distinct early events, and, as expected from shake flask experiments, alkaloid was later produced as glucan autolysed. Alkaloid concentration increased linearly from day 6, coincident with complete sucrose utilisation and a reduction in glucan concentration to almost zero. The final yield of alkaloid (0·5 mg/ml) was of the same order as that normally achieved in static culture. S. sorghi was maintained by subculturing every 3 weeks and after approximately 6 months AB_2 was used for another 60 litre fermentation (Fig 18). Uptake of phosphate was less rapid than before but maximum glucan concentration was increased by over 40%. During the first three days when the culture viscosity had not yet been increased by mycelial growth and glucan, profuse sporulation, predominantly macrospores, occurred. Most of these spores appeared to germinate during subsequent days giving rise to a pelleted growth form similar to that in the previous fermentation. Alkaloid production was delayed until after most of the glucan had been autolysed, but, although the fermentation was somewhat protracted, sucrose concentration did not reach zero. The alkaloid yield (0·35 mg/ml) was not as high as that previously attained.

It is conceivable that interpolation of a sporulation phase within this second fermentation reflected a change in the fungus which had been selected during repeated sub-culturing.

(iv) Single Spore Isolation of S. Sorghi

Macrospores of S. sorghi were found to be a mixture of uninucleate

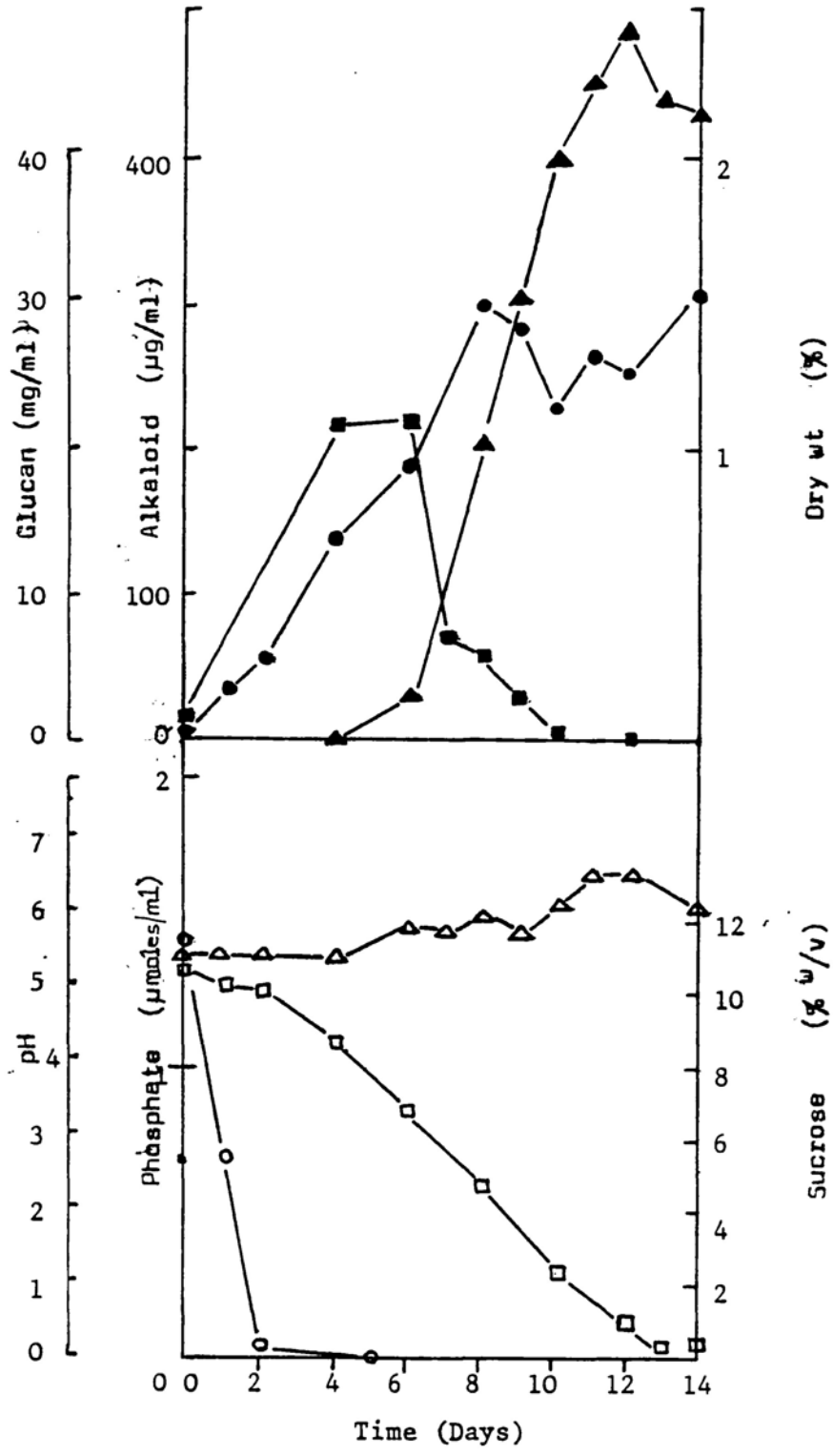


Fig 17 Course of S. sorghi AB_2 fermentation in a 60-litre stirred fermenter.

▲ - ▲, alkaloid; ■ - ■, glucan; ● - ●, dry weight; ○ - ○, phosphate; □ - □, sucrose; △ - △, pH.

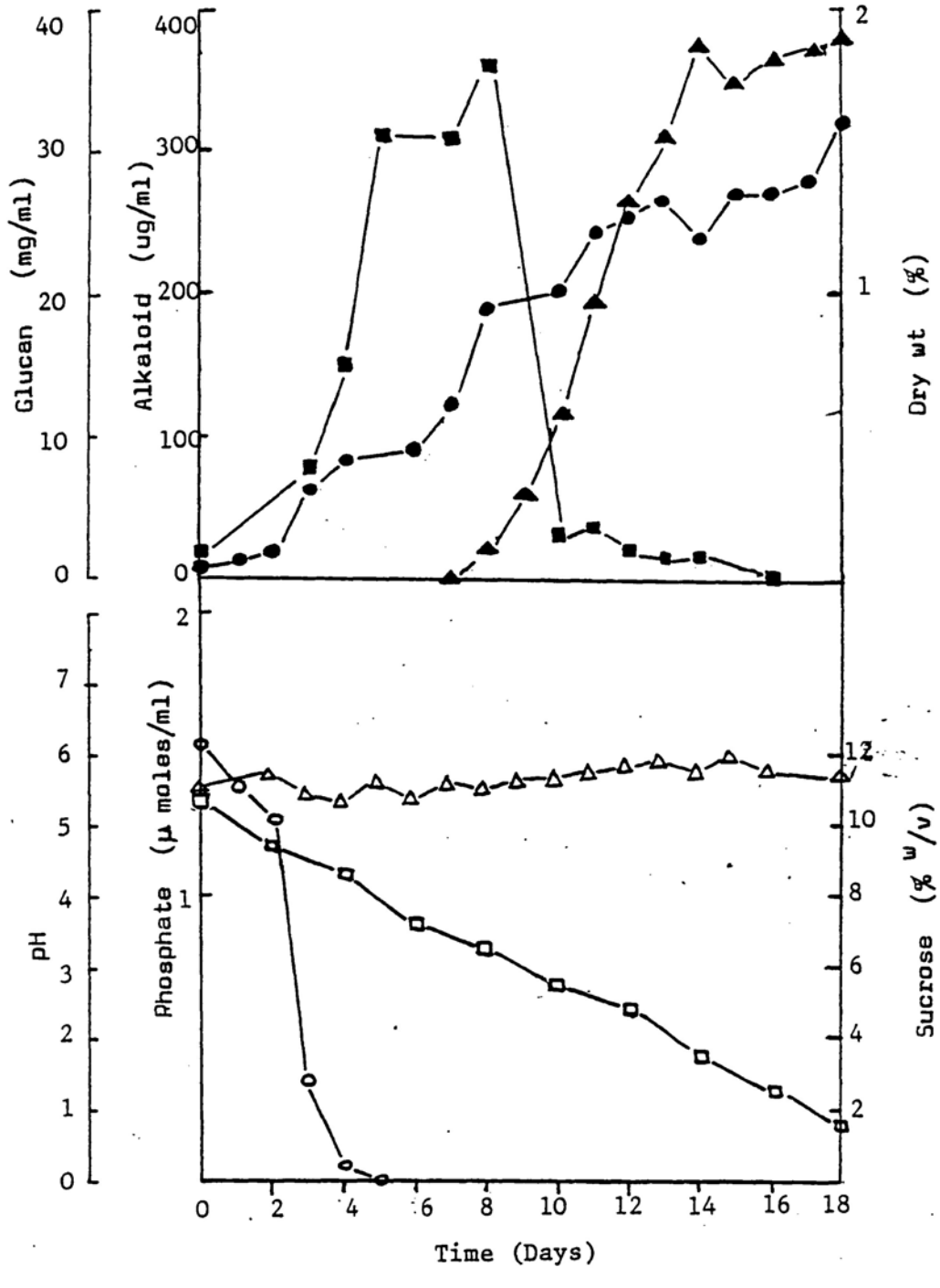


Fig 18 Course of a second fermentation of S. sorghi in a 60 litre stirred fermenter.
▲-▲, alkaloid; ■-■, glucan; ●-●, dry weight;
○-○, phosphate; □-□, sucrose; △-△, pH.

and multinucleate types. Consequently a homokaryon cannot be guaranteed simply by making a single macrospore isolate. To assess the validity of this conclusion with respect to stability of alkaloid production, a macrospore suspension was prepared from a high alkaloid producing isolate (600 µg/ml) of strain AB_2. The concentration of spores was such that well separated colonies arising from single spores were obtained. Fifty of these colonies were selected at random and subcultured direct to static liquid culture. After 21 days, the filtrates were assayed for alkaloid. Considerable variation in alkaloid yield occurred between isolates and, of the 50 tested, 34 produced only a trace of alkaloid, and the other 16 gave titres in the range 50-750 µg/ml (Fig 19). The morphology characteristic of low alkaloid production was a white, fluffy mat, whereas that of high production was cream-coloured and of a consistency similar to tripe! When a spore suspension was prepared from the highest alkaloid producing isolate, it unexpectedly contained predominantly microspores (which were all uninucleate). This switch to microsporulation was also exhibited by several of the other high alkaloid yielding single spore isolates. Consequently the next attempt at homokaryon selection was made using a microspore suspension. In contrast to macrospores, from which colonies became visible within 3 days, microspores took a week to produce visible colonies. Although the observation was not quantified, only a small proportion of microspores seemed to germinate. Twenty colonies arising from microspores were selected for assessment of alkaloid production as before. Twelve isolates gave alkaloid yields of approximately 450 µg/ml, 5 gave approximately 300 µg/ml and 3 produced only a trace.

3.2 Biosynthesis

(i) Incorporation of Radioactive (^{14}C) Amino Acids into S. Sorghi Alkaloids

The radioactive (^{14}C) amino acids (alanine, leucine, phenylalanine, proline and valine), permutations within which give rise to the various common peptide ergot alkaloid sidechains, were fed to S. sorghi surface cultures shortly after alkaloid was detected in the medium (13 days). Isoleucine, found in the peptide sidechain of β ergokryptine (Schlientz et al, 1967) and has been incorporated synthetically to form β-ergosine

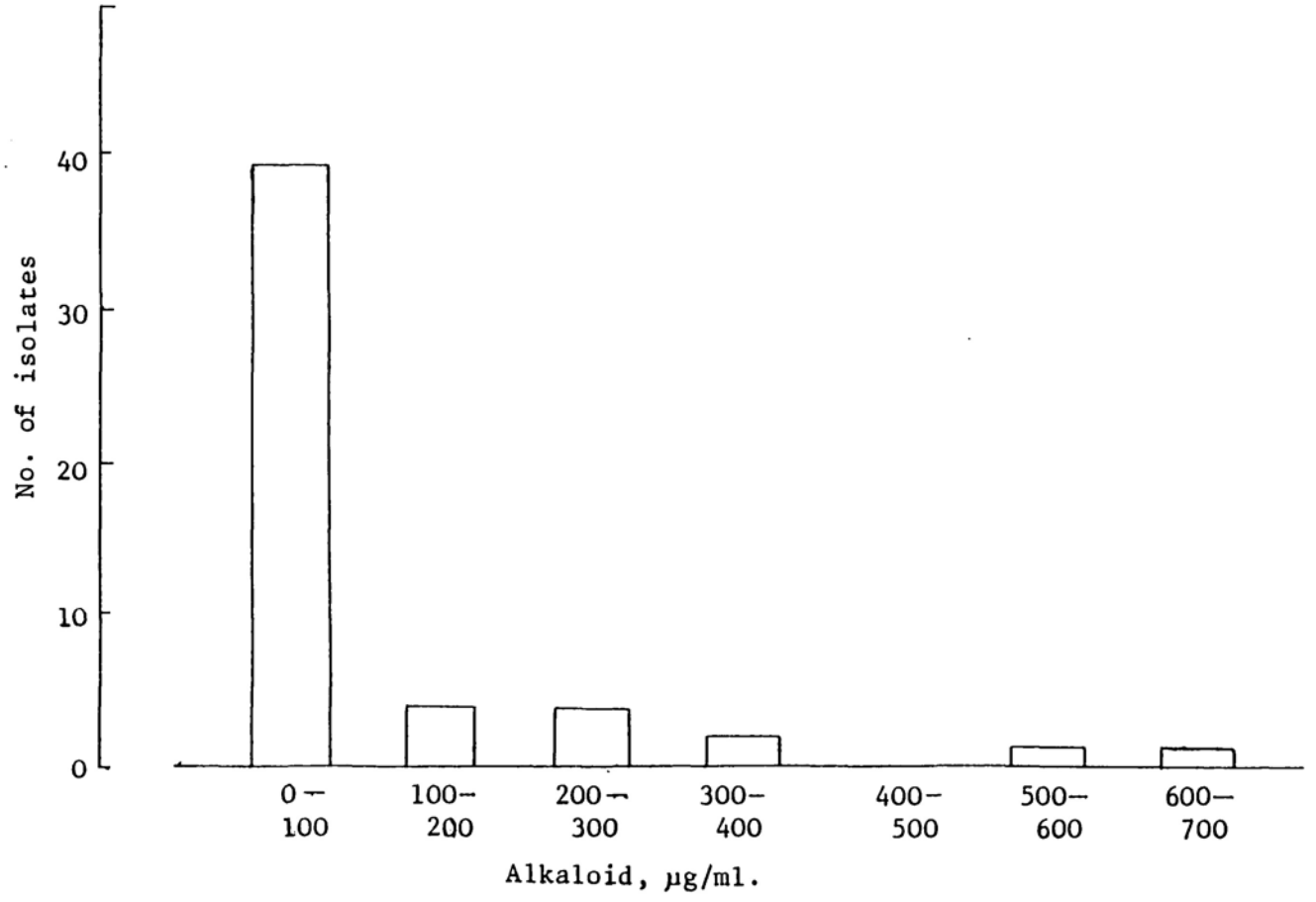


Fig 19 Alkaloid yield of single macrospore isolates.

(Stadler et al, 1977) was also fed. The purpose was to determine which of the uncharacterised alkaloids of S. sorghi are peptide-type and which amino acids constitute their sidechain.

At 21 days alkaloid in the filtrate was assayed, extracted and the extent of incorporation of each amino acid was calculated (Table 9). Autoradiography (Fig 20) showed the extent of incorporation of ^{14}C amino acids into peptide alkaloids. All the ^{14}C amino acids, with the exception of ^{14}C-isoleucine, were incorporated into alkaloid ^{14}C-leucine, ^{14}C-proline and ^{14}C-alanine were incorporated with the greatest efficiency. This was to be expected, since they are the amino acids which constitute the peptide sidechain of the principal alkaloid, dihydroergosine. Phenylalanine and valine were also incorporated into the dihydroergosine region of the chromatogram. This is assumed to be attributable to metabolism of these amino acids possibly to alanine or leucine respectively before incorporation into dihydroergosine. However the possibility that other peptide alkaloids co-chromatograph with dihydroergosine must be considered. Other radiolabelled alkaloids (Fig 20) have all incorporated leucine, proline and alanine and thus appear to have a peptide component. The simplest interpretation is that several peptide alkaloids contain leucine, proline and alanine, but if any of these radiolabelled amino acids are metabolised before incorporation, this interpretation would have to be varied.

(ii) Effect of the Addition of Hydroxy-L-Proline on Biosynthesis of S. sorghi Alkaloids

Hydroxy-L-proline (OH-Pro) was fed to S. sorghi cultures to determine whether it could be assimilated, and incorporated into the peptide sidechain of dihydroergosine, substituting for proline. A surface culture which was alkaloid positive at 14 days, was fed with 0·4 g of OH-Pro (4 mg/ml), and 7 days later the alkaloids were extracted from the culture filtrate. Chromatography of the alkaloid extract showed an increased proportion of alkaloid at R_f 0·15 (Fig 21). This might have been due to an enhanced yield of an alkaloid, normally found only in trace amounts, or, superimposition of a new alkaloid.

The experiment was repeated using 4 flasks. At harvest (21 days) the alkaloid titre of the pooled filtrates was 0·5 mg/ml. Preparative

^{14}C AMINO ACID FED 10μCi/FLASK	ALKALOID (μg/ml)	TOTAL C.P.M. IN ETHER EXTRACT OF FILTRATE FROM ONE FLASK	% INCORPORATION
LEUCINE	375	560,906	2•55
PROLINE	384	338,106	1•54
ALANINE	379	224,588	1•02
VALINE	310	126,129	0•57
PHENYLALANINE	289	83,294	0•379
ISOLEUCINE	350	0	0

TABLE 9 : Incorporation of ^{14}C Amino Acids into Alkaloid

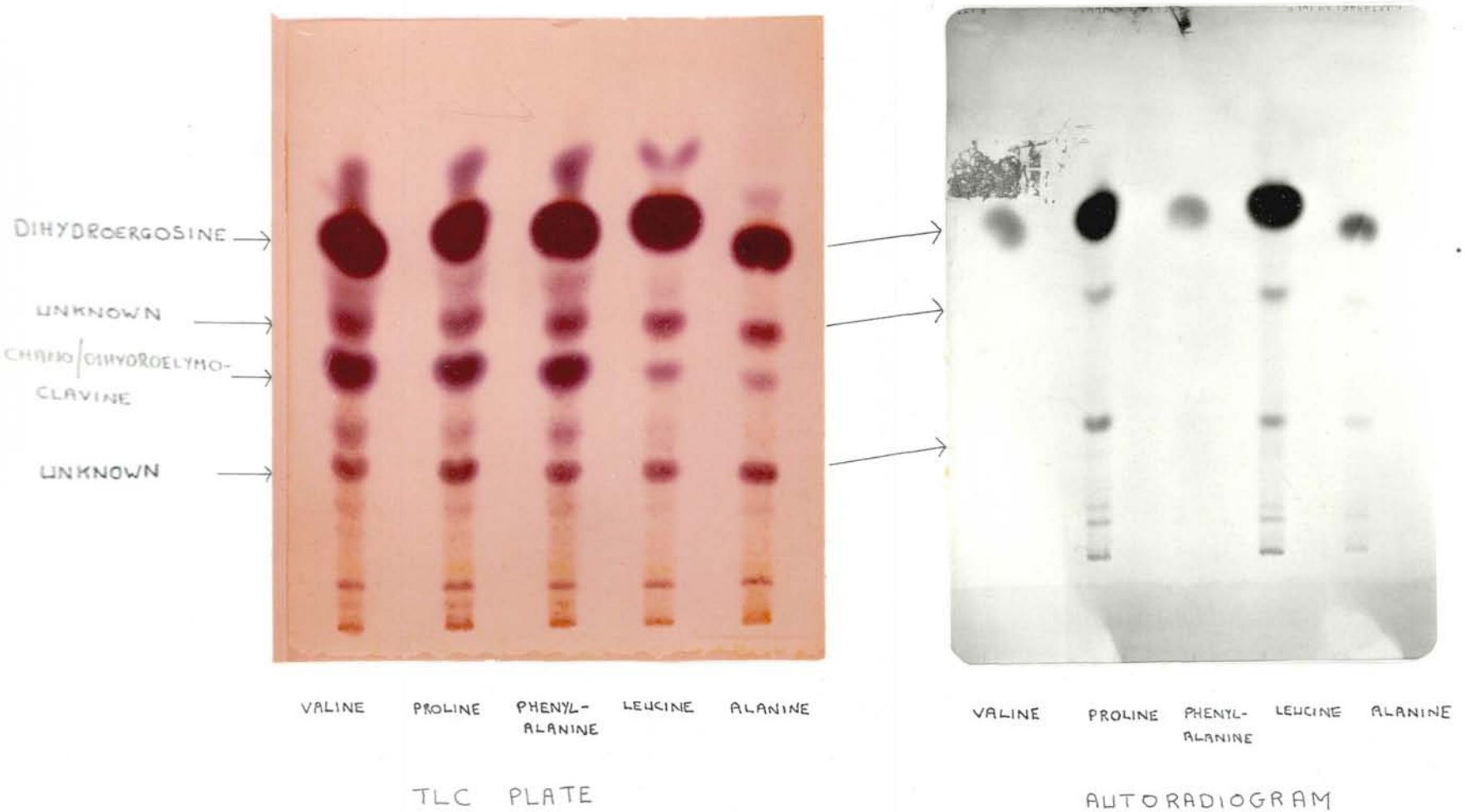


Fig 20 Incorporation of ^{14}C-amino acids into alkaloid by S. sorghi.

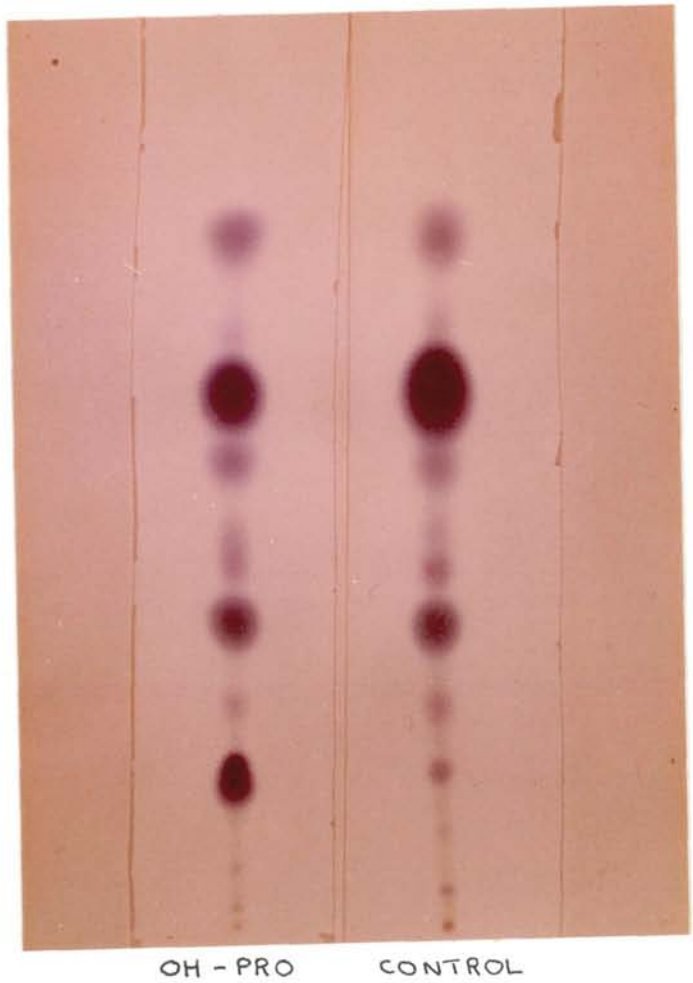


Fig 21 Effect of OH-Pro addition on spectrum of alkaloids produced by S. sorghi

TLC of the alkaloid extract yield 2·4 mg of the alkaloid at R_f 0·15 which was shown to be chromatographically homogeneous, and constituted approximately 5% of the total alkaloid. Field desorption mass spectrometry of the product showed a molecular ion (^{m}/e 565) which is 16 mass units heavier than that of dihydroergosine (^{m}/e 549). The electron impact mass spectrum of dihydroergosine also has important ions (^{m}/e 269 and ^{m}/e 154) which can be assigned (Vokoun and Rehacek, 1975) to structures a and b (Fig 22). The mass spectrum of the product of OH-Pro feeding showed an important ion also at ^{m}/e 269 (base peak), a weak ion at ^{m}/e 154 and an important ion at ^{m}/e 170. The latter is interpretated as 154 + 16, having the structure c (Fig 22). This data therefore suggests that hydroxyproline is incorporated to form a hydroxyprolyl analogue of dihydroergosine, 9^1-hydroxydihydroergosine (Fig 23). The continued presence of ^{m}/e 154, though diminished by conversion to ^{m}/e 170 may be attributed to one or both of the following:-

(a) a fragment of the ergoline nucleus (Fig 22, Vokoun and Rehacek, 1975) of 9^1 OH-dihydroergosine or

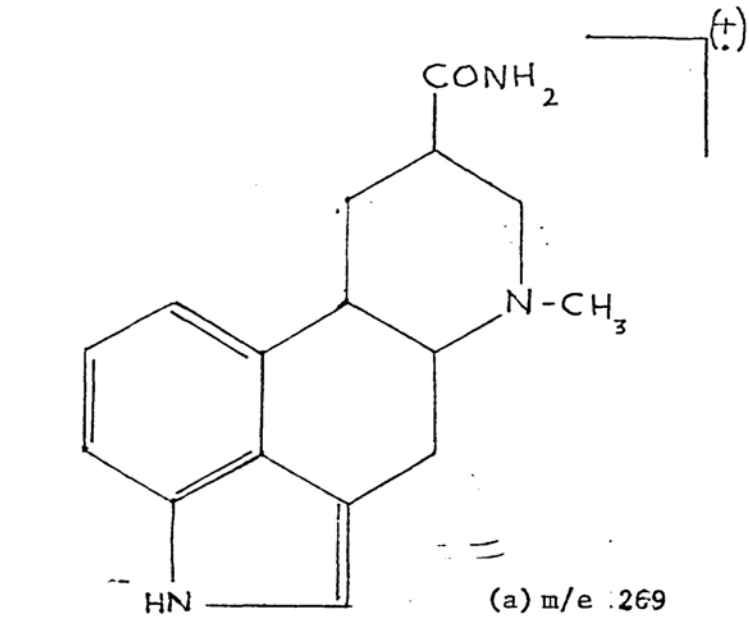
(b) the sidechain fragment (structure b, Fig 22) of the minor peptide alkaloid on which 9^1 OH-dihydroergosine superimposes and consequently from which had not been separated.

The incorporation of OH-Pro into dihydroergosine was confirmed by feeding 10 μCi of ^{14}C-OH-Pro to S. sorghi. The incorporation into alkaloid was 0·4%, and autoradiography showed that radioactivity was confined mainly to one region (Fig 24), that of OH-dihydroergosine. This is regarded as proof that the new alkaloid is OH-dihydroergosine.

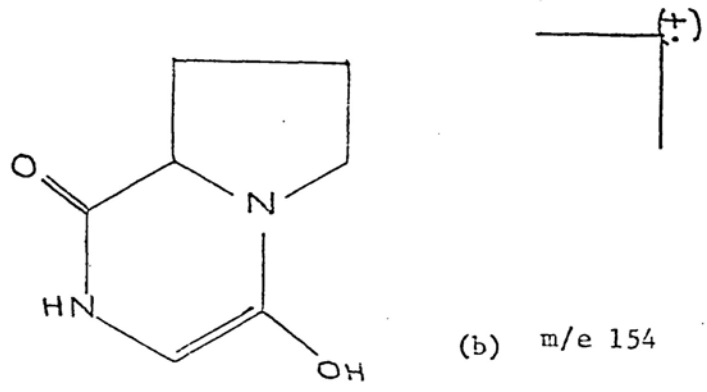
The effect of OH-Pro additions was tested at stages during the production of alkaloids by S. sorghi in surface and submerged cultures (Tables 10 and 11). No clear change in total alkaloid yield, or in the proportions of OH-dihydroergosine to other alkaloids occurred in response to OH-Pro concentrations over the range 0·01 - 10% ^{w}/v.

(iii) Effect of Hydroxy-L-Proline on Growth of S. Sorghi on Agar

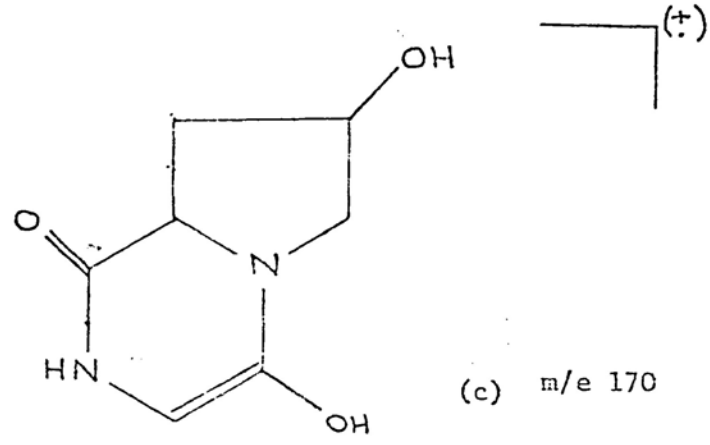
Sphacelia sorghi was inoculated at the centre of medium T agar plates which had been supplemented with OH-Pro in the range 0-4 mg/ml, final medium concentration. After 28 days incubation, an approximately linear growth response was evident at doses up to 1 mg/ml, further increases in OH-Pro concentration failed to inhibit completely the



(a) m/e 269



(b) m/e 154



(c) m/e 170

Fig 22 Some mass spectrometric fragments of dihydroergosine and OH-dihydroergosine

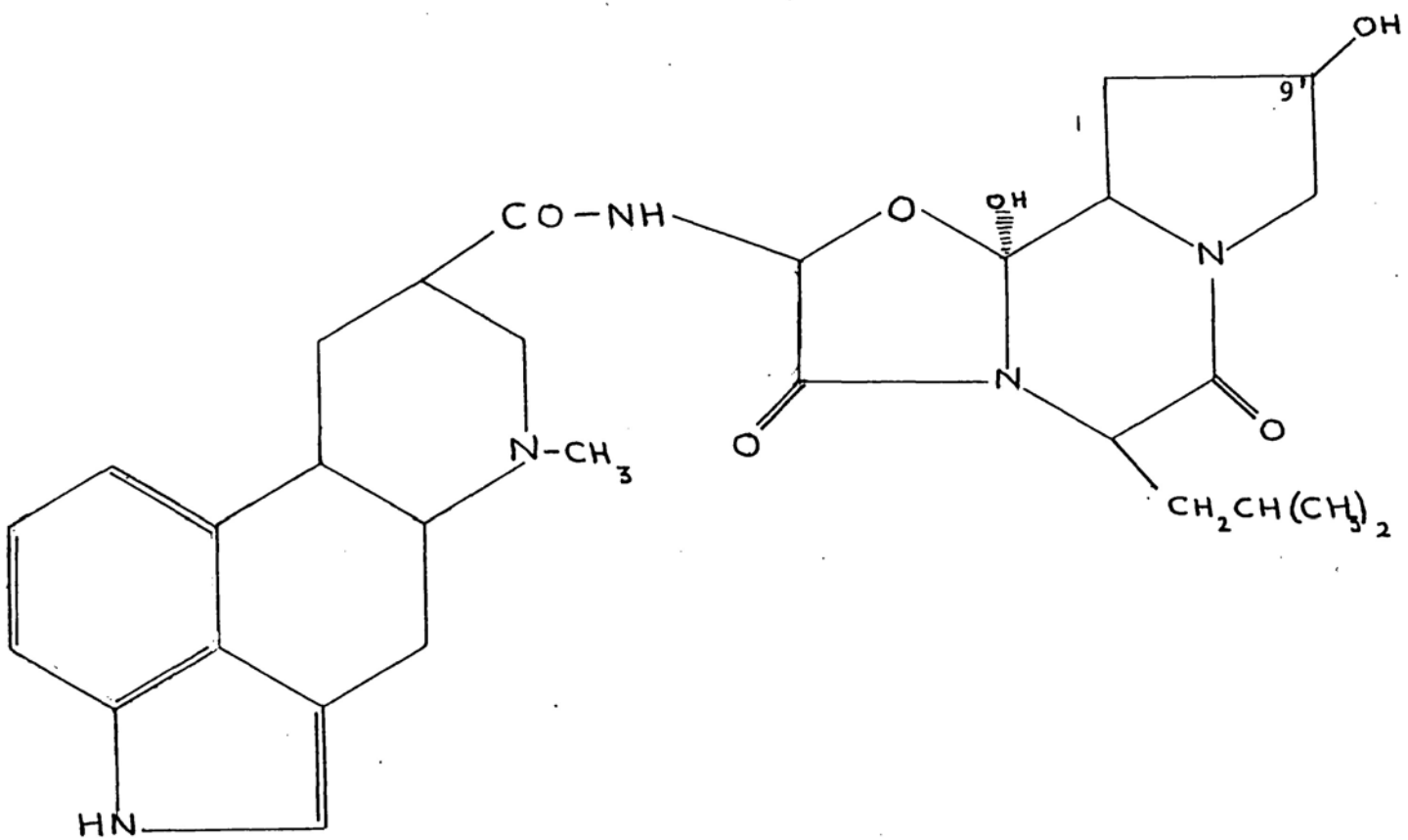


Fig 23 Structure of 9' hydroxy-dihydroergosine.

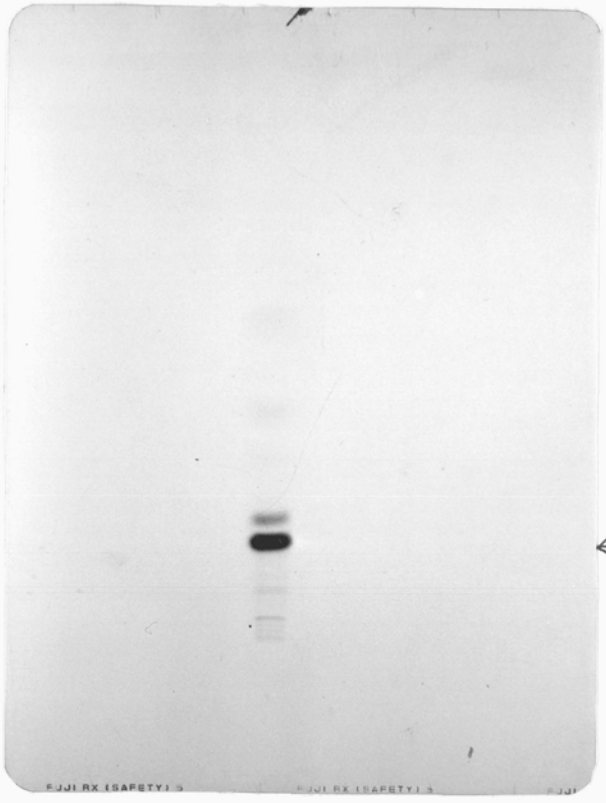


Fig 24 Incorporation of ^{14}C - OH - Pro into alkaloid by S. sorghi.

TIME OF ADDITION (DAYS)	HYDROXY-L-PROLINE ADDITION (g/100ml)		
	0•1	0•4	1
12	344 216	126 102	190 280
14	55 165	220 292	10 220
16	80 460	95 225	285 705
18	156 228	28 240	225 275

CONTROLS - 340, 128, 690 µg/ml

Alkaloid - µg/ml

TABLE 10 : Effect of OH-Pro on Alkaloid Production by *S. sorghi* in Surface Culture

TIME OF ADDITION (DAYS)	HYDROXY-L-PROLINE ADDED (g/100 ml)				
	0•01	0•02	0•1	0•2	0•5
7	92 116	66 34	80 88	46 98	70 84
9	88 96	75 87	41 95	68 84	74 78
11	78 92	48 84	39 71	66 50	59 63
13	55 67	85 59	46 78	80 36	90 82

CONTROLS - 42, 77, 79, 130 µg/ml

ALKALOID - µg/ml

TABLE 11 : Effect of OH-Pro on Alkaloid Production by S. sorghi in Submerged Culture

formation of colonies (Fig 25). The range of concentrations was therefore extended to 10 mg/ml, and again growth was not completely inhibited at this high concentration (Figs 26, 27). When mycelia grown on concentrations of OH-Pro were subcultured to unsupplemented medium, growth apparently returned to normal.

Another proline analogue, 3,4 dehydro-DL-proline, similarly inhibited growth of S. sorghi (Fig 28).

(iv) Effect of the Time of Addition of Various Concentrations of Hydroxy-L-Proline to Static Cultures of S. sorghi

Surface cultures of S. sorghi were fed at 5, 8 or 10 days with OH-Pro so as to give a final medium concentration in the range 2-500 µg/ml (the concentrations giving a marked linear dose response on agar medium, Section 3.2(iii)). The usual conditions of culture were varied in this experiment such that cultures were grown in 20 ml of medium T in 100 ml Erlenmeyer flasks. The culture filtrate was assayed at 21 days for alkaloid and OH-Pro. Hydroxyproline was not detected in any of the filtrates, indicating that it had all been taken up by the fungus from the medium. Nevertheless, the time or concentration of OH-Pro addition did not appear to have an effect on alkaloid production (Table 12). Since as usual there was wide variation between alkaloid yields of individual flasks, small changes in alkaloid production could not easily be recognised.

A subsequent experiment (Table 13) extended the range of concentrations of OH-Pro to 1500 µg/ml, and cultures were fed only at 5 days and 10 days. There was no effect on biomass accumulation, but, alkaloid production was significantly reduced by early treatment with the higher concentrations (500 µg/ml) of OH-Pro. Thin-layer chromatography of alkaloids extracted from each treatment group showed the presence of OH-dihydroergosine. There was no consistent evidence of dose response in the formation of this analogue (Fig 29), although the lowest concentration of OH-proline appeared to be the least efficient in redirecting alkaloid biosynthesis. It is concluded that addition of OH-Pro at any stage throughout most of the growth phase leads to the formation of the dihydroergosine analogue. This is consistent with the expectation that OH-Pro would not be involved in primary metabolism of S. sorghi. It is notable that when added at 5

CONCENTRATION (µg/ml)	TIME OF HYDROXY-PROLINE ADDITION (DAYS) 5	8	10
2	620	252	95
5	315	840	414
10	393	423	436
30	670	462	225
50	680	435	515
70	775	840	610
100	670	318	429
500	660	560	432

ALKALOID µg/ml

CONTROL 590 µg/ml

TABLE 12 : Effect of Time and Concentration of OH-Proline Addition on Alkaloid Production by S. sorghi

CONCENTRATION OF OH-Pro µg/ml	5 DAYS DRY WT (g)	5 DAYS ALKALOID (µg/ml)	10 DAYS DRY WT (g)	10 DAYS ALKALOID (µg/ml)
0	0•824	720	0•824	720
50	0•74	810	0•762	673
100	0•719	993	0•747	740
300	0•814	617	0•710	580
500	0•851	573	0•784	633
700	0•812	271	0•616	750
900	0•776	196	0•625	697
1100	0•837	377	0•71	667
1500	0•834	200	0•659	597

Mean of 3 flasks

TABLE 13 : Effect of addition of various concentrations of OH-Pro on alkaloid production by S. sorghi when added at 5 and/or 10 days.

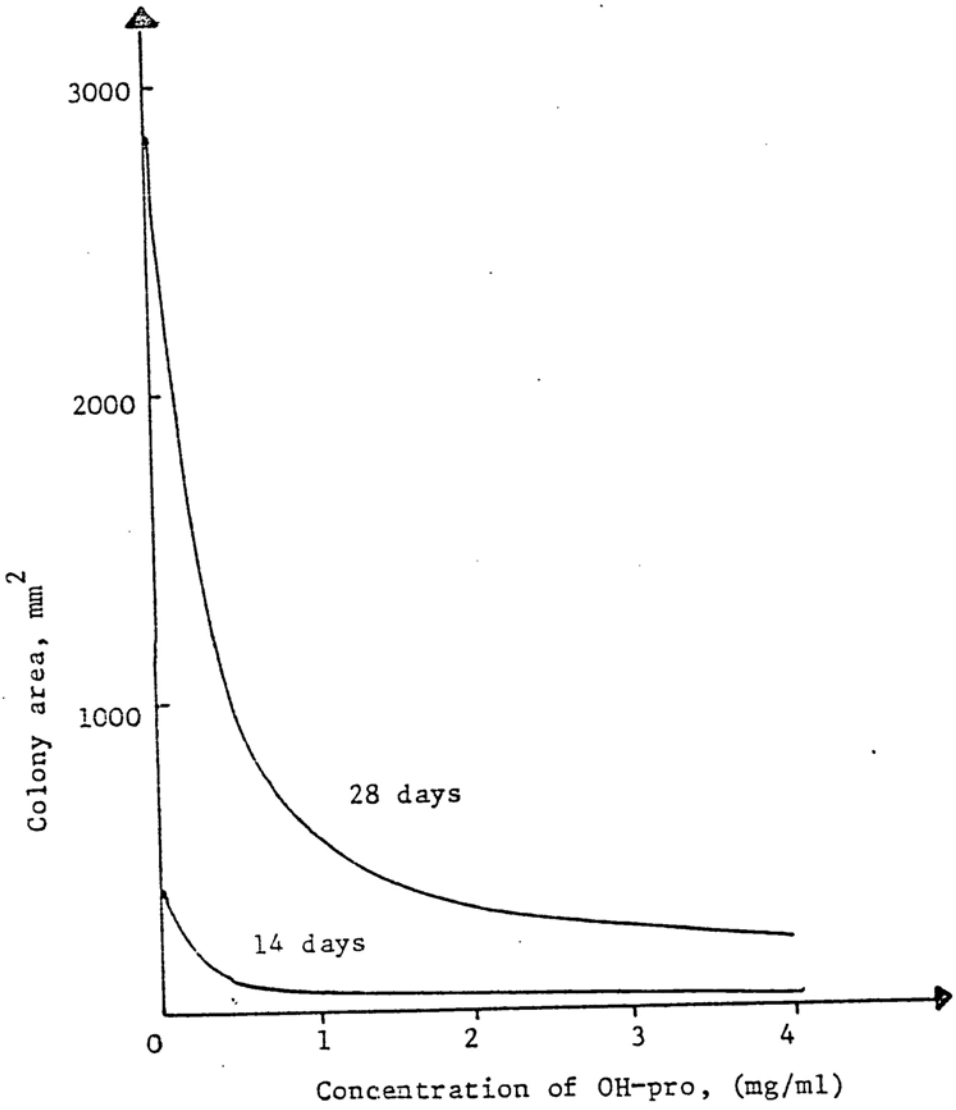


Fig 25 Inhibition of S. sorghi growth on agar by OH-pro (0-4 mg/ml)

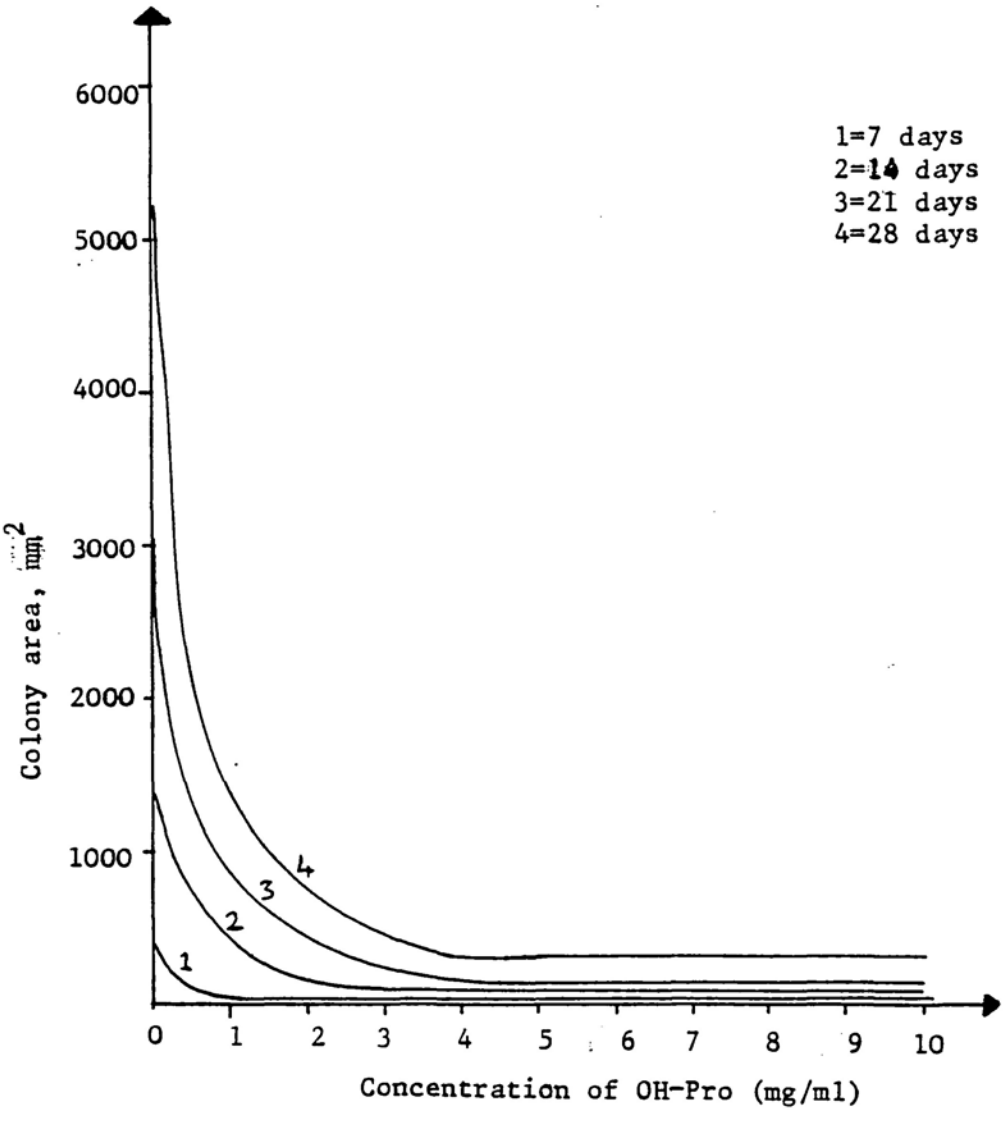


Fig 26 Inhibition of S. sorghi growth on agar by OH-pro (0-10mg/ml)



Fig 27 Effect of incorporation of OH-pro (0-10mg/ml) into agar on growth of S. sorghi.

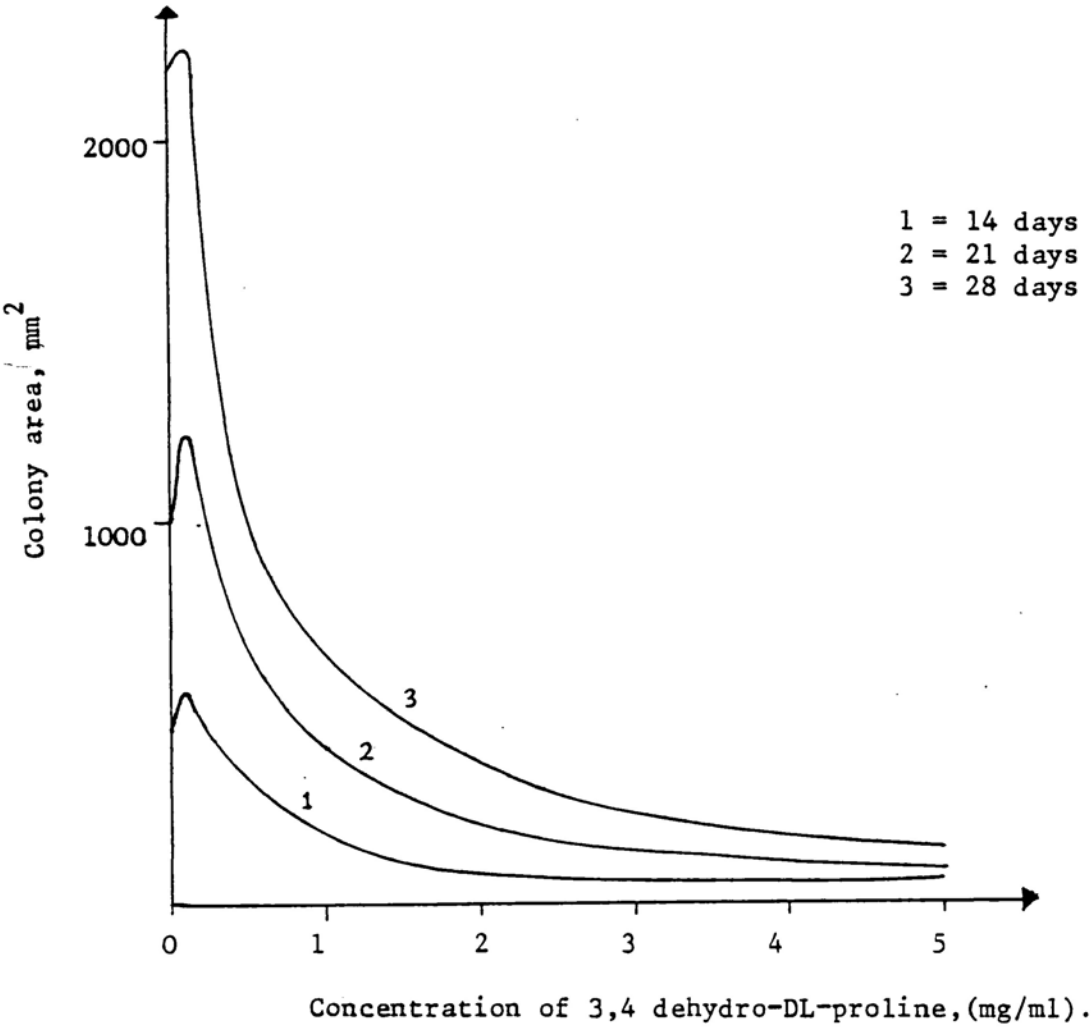


Fig 28 Effect of 3,4 dehydro-DL-proline on growth of S. sorghi on agar.

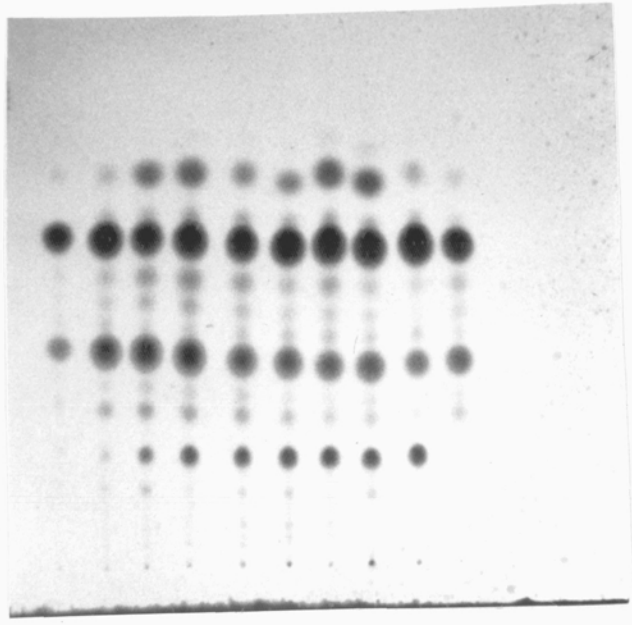


Fig 29 Effect of the addition of various concentrations of OH-pro on the production of OH-dihydroergosine.

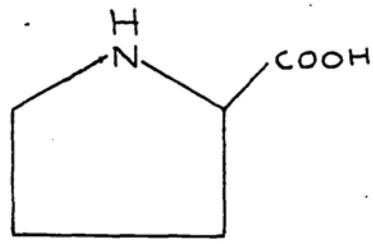
days, OH-Pro probably has to wait another 5 days before being involved in alkaloid biosynthesis. During this period most of the biomass is normally accumulated, yet concentrations of OH-pro which significantly suppress growth on agar, failed to affect dry matter accumulation in established surface cultures.

In a complementary experiment to confirm the location of a OH-pro analogue, 1 µCi of ^{14}C-OH-pro was added to cultures at 5, 8 or 10 days. Final alkaloid titres were approximately 400 µg/ml and autoradiography of these alkaloids showed radioactivity only in the OH-dihydroergosine region.

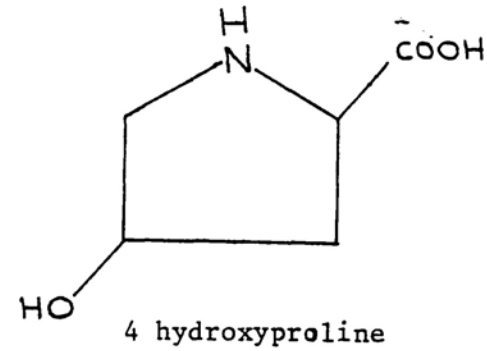
(v) Effect of Other Proline Analogues on Alkaloid Biosynthesis by S. sorghi

Several other proline analogues (Fig 30) were tested as before (20 ml Medium T / 100 ml Erlenmeyer flasks) for their ability to replace proline in dihydroergosine. At harvest (21 days) it appeared that alkaloid production had not been significantly affected by any of the analogues (Table 14). The standard alkaloid assay could not be achieved for the 3, 4 dehydro-DL-proline fed culture, as the filtrate reacted with Van Urk reagent to give a dark burgundy colour, which however, was not due to 3, 4 dehydro-DL-proline. The mycelial mats treated with 3, 4 dehydro-DL-proline, and thioproline were shrunken and malformed. With the former this might have been expected (expt 3.2(iii)). The alkaloids from each feeding were extracted and resolved by TLC. As expected both the allo- and cis- hydroxy-L-proline were taken up and incorporated into alkaloid, but hydroxy-D-proline remained in the medium and there was no evidence of incorporation into alkaloid. D-proline was also found in the medium at the end of the experiment. The cis-OH-dihydroergosine had a slightly lower R_f than allo-OH-dihydroergosine (Fig 31). No other changes in alkaloid spectrum suggesting incorporation of analogues were apparent, but, due to the complex mixture of S. sorghi alkaloids, it is possible that evidence for incorporation of trace amounts was masked.

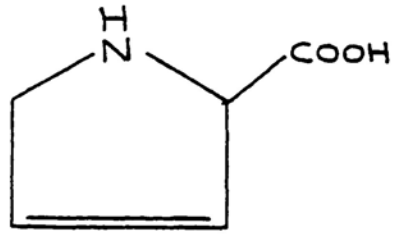
The leucine analogues, isoleucine and nor-leucine were unable to replace the leucine residue in dihydroergosine.



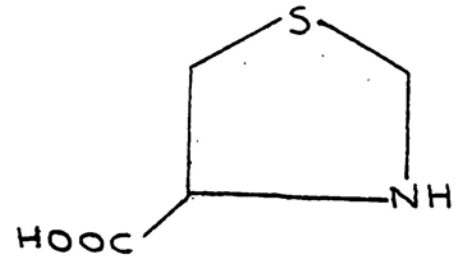
Proline



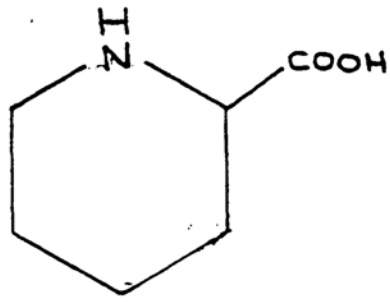
4 hydroxyproline



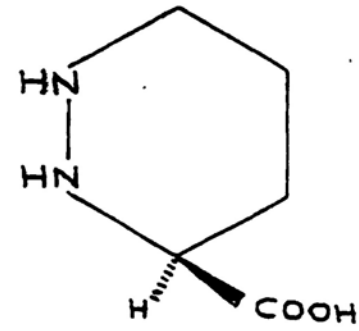
3,4 Dehydro DL proline



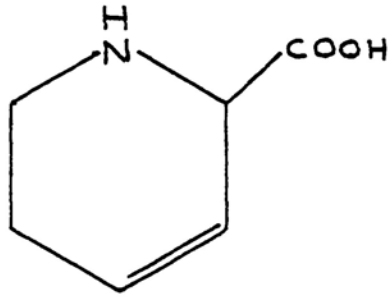
Thioproline



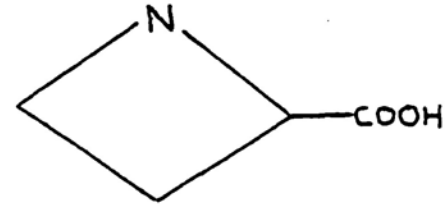
Pipecolic acid



S Piperazic acid



L 4,5 Dehydro-
pipecolic acid



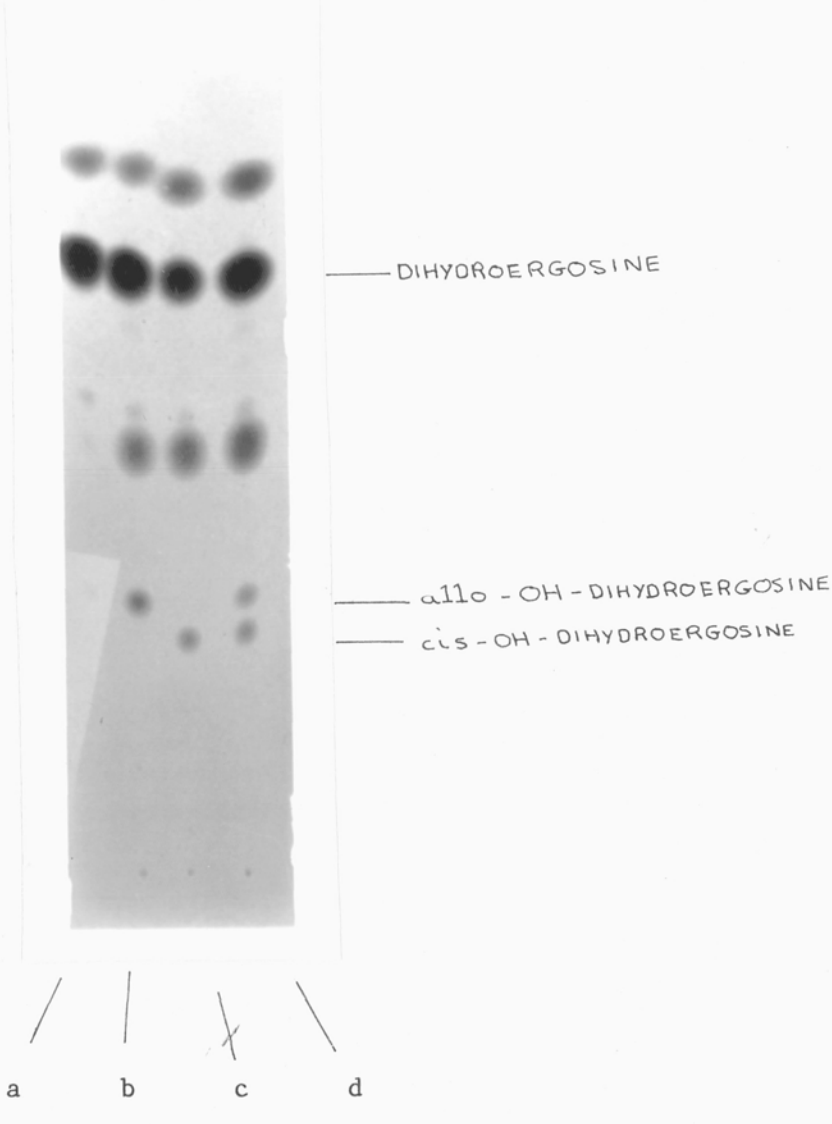
L Azetidine 4
carboxylic acid

Fig 30 Structure of some proline analogues

PROLINE ANALOGUE	CONCENTRATION IN MEDIUM µg/ml	ALKALOID µg/ml	INCORPORATION INTO ALKALOID
HYDROXY-L-PROLINE (ALLO-4-HYDROXY)	12•5	520 565	+
HYDROXY-L-PROLINE (CIS-4-HYDROXY)	12•5	516 710	+
D-PROLINE	12•5	378 530	-
HYDROXY-D-PROLINE (ALLO)	12•5	500 655	-
L-AZETIDINE-2-CARBOXYLIC ACID	6•25	518 575	-
3,4 DEHYDRO-DL-PROLINE	6•25	ASSAY MASKED BY INTERFERING COLOUR	-
DL-PIPECOLIC ACID	12•5	413 520	-
L-PIPECOLIC ACID	6•25	490 370	-
HYDROXY-L-PIPECOLIC ACID	6•25	- 515	-
L-BAIKAIN	6•25	373 327	-
THIOPROLINE	12•5	320 418	-
S-PIPERAZIC ACID	2•5	345 -	-
NONE CONTROL	-	555 505	-

Alkaloid titre at time of analogue addition (12 days) was $\sim$ 80 µg/ml

TABLE 14 : Effect of Proline Analogues on Alkaloid Production by *S. sorghi*



(a) No OH-pro addition
(b) allo-OH-pro addition
(c) cis-OH-pro addition
(d) mixture of b and c

Fig 31 Incorporation of cis-OH-proline and allo-OH-proline into S. sorghi alkaloids.

(vi) Ethyl Methane Sulphonate (EMS) Mutagenesis Of S. sorghi

(a) Macrospore mutagenesis

A greater incorporation of amino acid analogue is obtained using an auxotrophic mutant for that particular amino acid, compared with a wild-type strain. (Beacco et al, 1978). Consequently auxotrophic mutants of S. sorghi, AB_2, for proline, and also for leucine were sought.

Spores withdrawn from a 60 litre fermentation (Section 3.1(iii)) were treated with a chemical mutagen, ethyl methane sulphonate (EMS). Several concentrations of EMS in the range 50-600 mM were tested to determine the extent of their lethal effect (Fig 32). The viable count of the original spore suspension was $3{\cdot}1 \times 10^5$ spores/ml.

Colonies arising from mutagenised spores were of two distinct types, either white and fluffy, or, cream-coloured and compact. A selection of these were tested for alkaloid production in surface culture. The mycelial mats from the white and fluffy colonies maintained this morphology in liquid medium but did not produce alkaloid, whereas, compact cream-coloured colonies produced mats which were buff coloured with a darker underside and synthesised a typical spectrum of alkaloids, up to 400 µg/ml.

Another spore suspension was prepared and treated with 300 mM EMS for 60 minutes, the treatment shown to give a 2-3 log kill (Fig 32) in the hope of isolating auxotrophic mutants for either proline, leucine or tryptophan. A filtration enrichment step was introduced between stages e and f (Section 2.8(ii)) to remove spores which germinated in the defined medium, and were therefore not auxotrophs. Unfortunately the culture became bacterially contaminated at some stage during the length of time and multiple filtrations required for non-auxotrophs to recover from mutagen treatment and germinate. Addition of aureomycin (200 µg/ml) failed to control this contamination. No further spores germinated after 102 hours. The viable count of the spore suspension was $1{\cdot}2 \times 10^7$ spores/ml. The total count at 102 hours was $4{\cdot}3 \times 10^5$ spores/ml, so from the expected kill it can be assumed that most of these are viable. It is possible that at least one of the required auxotrophs would have been isolated.

Surprisingly, few macrospores were found in subsequent fermentations or on agar cultured mycelia. The fungus had changed to producing mainly

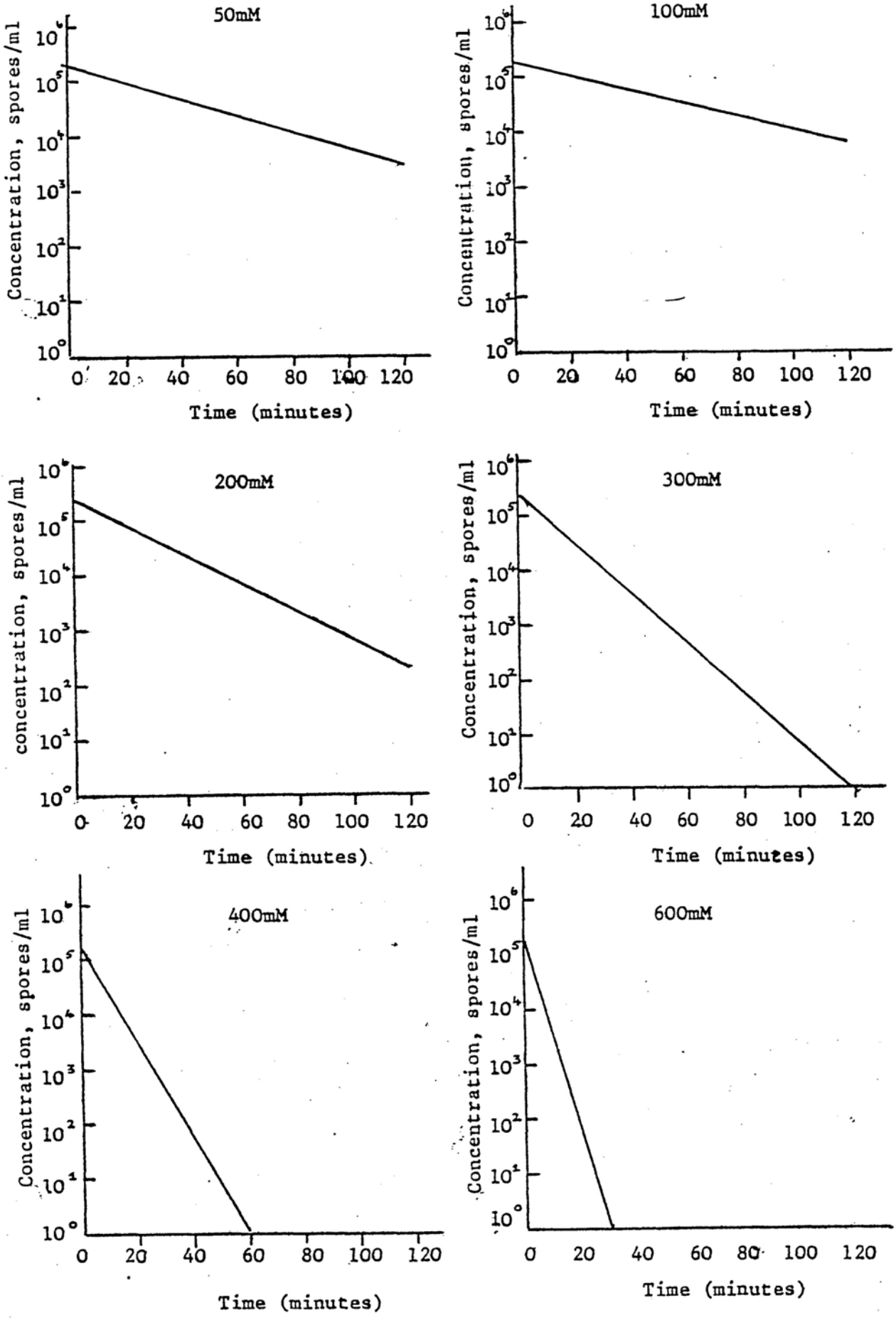


Fig 32 Lethal effect on S. sorghi of several concentrations of EMS.

microspores. This transition was apparent in all cultures of the organism. Induction of macrospore production was attempted as follows:-

(a) Varying the age of the seed stage from 4-7 days before transfer.
(b) Varying the amount of inoculum for the production stage in the range 2-20%.
(c) Omitting to homogenise the seed stage before transfer to the production stage.
(d) Changing the incubation temperature from 27°C to either 20°, 24°, 30° or 37°C during days 1-5 of the production stage.
(e) Adding $CaCl_3$ (0·5 - 2%) to the production stage.

None of these treatments yielded a significant number of macrospores.

(b) Microspore Mutagenesis

A microspore suspension was prepared which had a total count of $9·44 \times 10^7$ spores/ml, and a viable count of 1×10^6 spores/ml. Using the same procedure as for macrospore mutagenesis, the spores were treated with 300 mM EMS for 30 minutes. In the filtration enrichment step, however, there was no evidence of spore germination, even after 4 days incubation. Consequently, the spore suspension was not filtered. The total count of the final spore suspension before dilution and plating was $1·5 \times 10^6$ spores/ml. After 16 days incubation, colonies were present on only three (2×10^{-2} dilution and 1×10^{-4} dilution) of the 36 medium T agar plates inoculated (9×10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}). The viable count of the mutagenised spore suspension, as calculated from the 10^{-2} dilution counts was $5·4 \times 10^4$ spores/ml, giving confidence that there had been approximately a 2 log kill. It is assumed that the spurious result of only three plates supporting growth of S. sorghi could have been caused by traces of methanol from the glass spreader contaminating the agar plates. S. sorghi is particularly sensitive to traces of methanol in the medium, (Section 3.2(vii)). From the survivors, sixty isolates from the slower growing colonies on the 10^{-2} dilution plates were screened for proline and/or leucine auxotrophy. Isolates were subcultured onto defined medium, defined medium + 20 µg/ml leucine, and defined medium + 20 µg/ml proline. No auxotrophs were observed, but a much larger screening programme would be needed before being confident that no proline or leucine auxotrophs could be produced by this treatment.

At step C (Fig 33) fourteen isolates which were taken at random from the auxotrophy testing plates were used to inoculate surface culture flasks. For two other isolates, surface cultures were established, by subculturing from a colony grown on each of the three auxotrophy testing media (Fig 33). Agar cultures were also established in parallel. Alkaloid yields in the fourteen random isolates ranged from 0 - 600 µg/ml. In spite of isolates 1 and 2 being derived from single uninucleate microspores, thereby being homokaryotic, the same wide variation in alkaloid yield, previously experienced with S. sorghi was evident. However even this variation was not stable through one subculture (3 weeks) and there was no consistent influence of alkaloid production (steps D and E, Fig 33) of a period of growth on the special media at step C. The relative proportions of component alkaloids were similar in all alkaloid producing isolates.

(vii) Effect of Various Antagonists on Growth of S. sorghi

For testing the efficiency of mutagenesis, a concentration of antagonist was required which was just inhibitory to S. sorghi growth. Various antagonists were tested (Table 15). Parafluorophenylalanine inhibited growth at concentrations > 20 µg/ml, whereas, 4-aza-leucine did not inhibit growth at any of the concentrations used. It is apparent that even the lowest concentration of methanol (0·5% $^{v}/_{v}$) used in one test to incorporate nystatin into the medium, was inhibitory to the growth of S. sorghi.

(viii) Experiments with Protoplasts of S. sorghi

Protoplast preparations were made from 8-9 day old, alkaloid positive ($\sim$ 100 µg/ml) cultures of S. sorghi, using the method of Stahl et al (1977) described in Section 2. The time required for protoplast formation, 6 hours, was longer than that required in C. purpurea, 4 hours, (Stahl et al, 1977, Robbers et al, 1979). During the course of several S. sorghi protoplast preparations it was found that the Sigma supplied β-glucuronidase was more efficient than that supplied by Boehringer and consequently was used thereafter. The validity of this selection was confirmed using a model system for yeast protoplasts.

^{14}C-OH-Pro was fed to a protoplast preparation of S. sorghi and incubated with gentle shaking (100 rpm) at 27^{o} for 48 hours after which

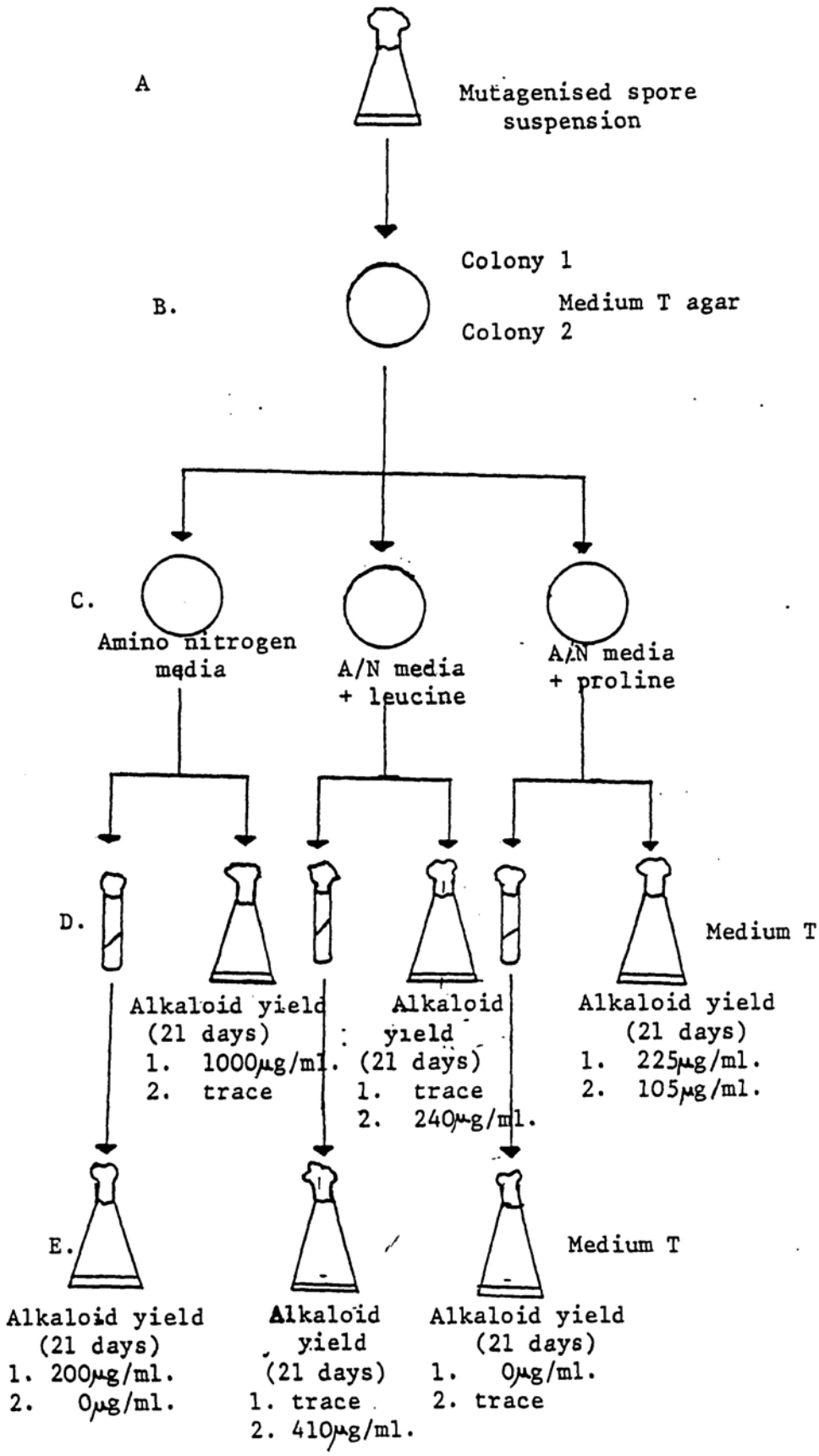


Fig 33 Selection of some survivors from microspore mutagenesis

MEDIUM CONCENTRATION OF ANTAGONIST (ug/ml)	ANTAGONIST			
	PARAFLUORO-PHENYLALANINE	4-AZA LEUCINE	NYSTATIN	NYSTATIN + METHANOL
5		+	+	-
10		+	+	-
20	+/-	+	+	-
50	-	+	+	-
70	-	+	+	-
100	-	+	+	-
200	-	+		

+ - Normal growth

- - No growth

+/- - Slight growth

TABLE 15 : Effect of Various Growth Antagonists on Growth of S. sorghi

the filtrate was assayed for alkaloid. Although a trace of alkaloid was detected, subsequent autoradiography of a chromatogram of the alkaloids revealed no regions of radioactivity. It is probable that the small amount of alkaloid in the incubate arose from leakage of alkaloid already present within the cells before protoplast preparation rather than from active alkaloid biosynthesis by the protoplasts. Consequently, it is concluded that these protoplasts were biosynthetically inactive with respect to cyclic tripeptide ergolines.

In similar biosynthetic studies in C. fusiformis alkaloids (Cheng et al, 1980), only protoplasts of a low density, that is those rich in lipid and containing numerous vacuoles, were found to synthesise elymoclavine. Since in present S. sorghi studies the protoplasts were of high density, this could explain why there was apparently no alkaloid synthesis.

(ix) Tryptophan Addition to S. Sorghi Cultures

Tryptophan was added to 8 day old shake-flask cultures which were producing alkaloid. The concentration used (0·5 mg/ml) had been previously shown to stimulate alkaloid production in C. fusiformis (C. Szczyrbak, 1972). The flasks were harvested at 14 days. The mean alkaloid titre of 5 replicate flasks was 283 µg/ml, slightly less than the controls (358 µg/ml), indicating that tryptophan apparently does not stimulate alkaloid production in S. sorghi.

(x) Effect of Ethionine on S. Sorghi Alkaloid Biosynthesis

Addition of ethionine, a methionine antagonist which blocks the N-methylation step in ergoline biosynthesis, at concentrations of 160, 300, 700 and 900 µg/ml to S. sorghi surface cultures which had just begun to synthesise alkaloid, failed to effect a block at the N-methylation step. The highest concentration added was 5 times greater than the effective concentration for blockage of alkaloid biosynthesis in C. fusiformis (G. Kilbee, private communication). It could be concluded that either S. sorghi alkaloid biosynthesis is very insensitive to ethionine, or that it cannot be blocked at all by this methionine antagonist. If the latter occurs doubt must be raised over participation of a normal biosynthetic route of the ergoline nucleus in S. sorghi.

(xi) Experiments with a Cell-Free Extract of S. Sorghi

A cell-free preparation was made from the compact cream-coloured areas of a 14 day old surface mat of S. sorghi, which was producing alkaloid (200 µg/ml). The cell-free extract was equally divided into two parts. To one was added ^{14}C-tryptophan and to the other ^{14}C-dimethylallyltryptophan and both were incubated at 27°C for 6 hours. Autoradiography of amphoteric and basic alkaloid extracts from each feeding revealed that ^{14}C-tryptophan had disappeared (possibly incorporated into protein), but had not apparently been incorporated into any component of the alkaloid biosynthetic pathway. All the ^{14}C-dimethylallyltryptophan appeared to be unchanged.

(xii) Effect of Agroclavine Addition on S. Sorghi Alkaloid Biosynthesis

Agroclavine was added to shake flask cultures of S. sorghi on day 8, when alkaloid was already being synthesised. On day 14, the alkaloid content of culture filtrate was assayed, the alkaloids extracted and examined chromatographically. Agroclavine was present in all cultures, in amounts approximately proportional to the concentration added. The typical spectrum of S. sorghi alkaloids also occurred, and there was no evidence that agroclavine had disturbed the normal biosynthesis of alkaloids. The only unusual feature was a substance, which had a slightly higher R_f value than dihydroergosine, and gave a green colour with Ehrlichs reagent. Assuming that no agroclavine was utilised by the fungus, a value equivalent to the amount of added agroclavine, was deducted from the total measured alkaloid titre of the culture filtrate. The titre, thus attributed to S. sorghi (Table 16) was similar in all treatments, and it was concluded that agroclavine does not repress the biosynthesis of S. sorghi alkaloids.

The validity of this conclusion was further explored in surface cultures in which maximum alkaloid biosynthesis could be expected. Various concentrations of agroclavine were added at several stages of growth, before (Day 9) and during (Day 11,12 and 14) alkaloid biosynthesis (Table 17). As in the previous experiment, addition of agroclavine at any stage failed to effect the production and spectrum of S. sorghi alkaloids. However, the mycelial mats in the agroclavine fed flasks were a greyish-green colour instead of the usual off-white colour. The intensity of this colour change increased with the concentration of

CONCENTRATION OF AGROCLAVINE ADDED (µg/ml)	ALKALOID TITRE (µg/ml)
0	350
10	279
50	342
100	353
200	355

CONTROL MEAN 10 FLASKS = 335 µg/ml

AGROCLAVINE MEAN 2 FLASKS

TABLE 16 : Effect of Agroclavine on Alkaloid Production by S. sorghi in Submerged Culture

TIME OF ADDITION	LEVEL OF AGROCLAVINE ADDED (µg/ml CULTURE) 50	100	200	250
9 DAYS	575	545	525	525
11 DAYS	440	485	245	410
12 DAYS	610	595	555	590
14 DAYS	530	465	510	590

CONTROL - 630 µg/ml

HARVESTED AT 21 DAYS Alkaloid = µg/ml

TABLE 17 : Effect of Agroclavine on Alkaloid Production by S. sorghi in surface culture

agroclavine (Fig 34).

^{14}C-agroclavine (0•39 µCi/mM), was fed to S. sorghi surface cultures on Day 12 when the alkaloid titre was 300 µg/ml. On day 16, when the alkaloid titre was 700 ug/ml the alkaloids were extracted from the culture filtrate. Autoradiography of a chromatogram of the alkaloids showed radioactivity only in the regions of agroclavine and the "green substance" (p. 102). This confirmed that agroclavine is not incorporated in the biosynthesis of S. sorghi alkaloids, and that the agroclavine had mostly remained stable during the experiment.

3.3 (i) Isolation of Minor Alkaloids of S. sorghi

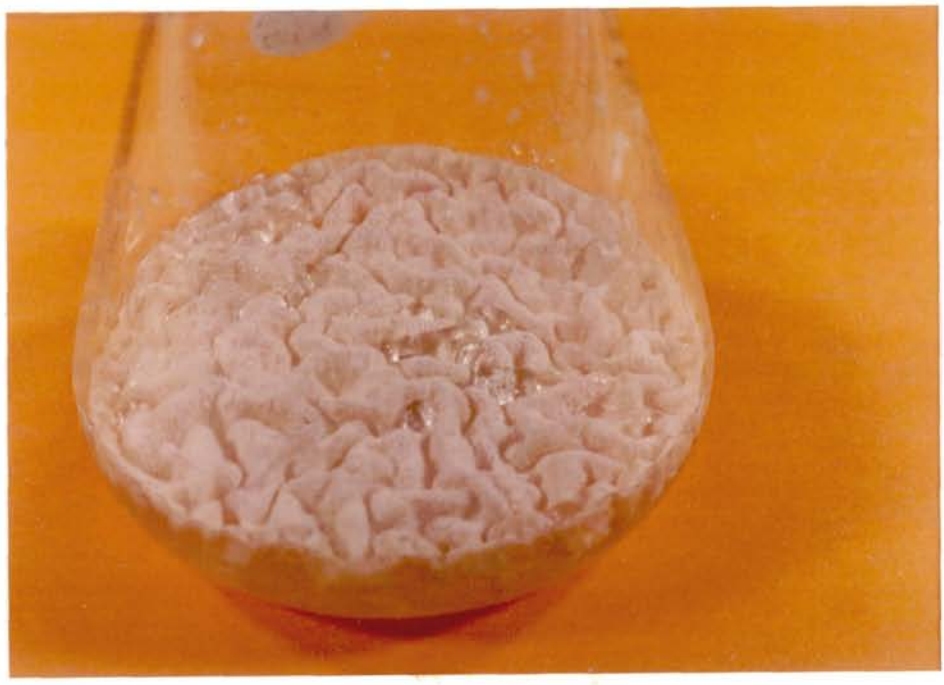
Alkaloids were produced by surface cultures of S. sorghi (150 flasks) which had been fed with OH-pro (15 g/l) on day 14. Approximately 8 litres of culture filtrate (pooled filtrate titre approximately 400 µg/ml) was extracted withdiethyl ether, and approximately 1•8 g of crude alkaloid mixture was obtained. Prior to attempts to isolate minor alkaloids from the complex mixture, several possible separation methods were explored.

(a) Adsorption Chromatography

Two types of column packing, alumina and silica were tested for their effectiveness in separating the alkaloid mixture. Alumina was prepared to grade II (50g alumina / 1•5ml distilled water) and then packed in a column. Silica was mixed to a slurry with distilled water, packed in a column and washed with chloroform. Crude alkaloid (0•1g) in chloroform was applied to the top of each column. Elution with chloroform / methanol (9:1) failed to effectively resolve the alkaloid mixture.

(b) Ion-Exchange Chromatography

Sephadex C25 (NH_4^+ form) was used for ion-exchange chromatography of alkaloids. The NH_4^+ form of the resin was generated using 2N ammonium hydroxide until the effluent was pH 11-12, after which it was washed with distilled water until the effluent was pH 8. The extracted alkaloids were dissolved in 1N H Cl, adjusted to neutrality with 2 N NH_4OH, and were applied to the top of the column. The column was again washed with distilled water. The alkaloids were eluted with 2N NH_4OH. The first alkaloid to be eluted was dihydroergosine. Subsequent fractions contained the full spectrum of S. sorghi alkaloids.



Control - no agroclavine addition



Agroclavine added - 250µg/ml

Fig 34 Surface culture of S. sorghi grown in presence of agroclavine

Surprisingly, the last fractions again contained only dihydroergosine.

(c) High-Pressure Liquid Chromatography

Initially, several solvents were tested in thin-layer chromatography for their ability to resolve the alkaloid mixture. In hexane, toluene, Freon TF (tri-chlorofluoroethane), chloroform, dichloromethane, diethyl ether, methylene cyanide and ethyl acetate, alkaloid remained at the origin. Methanol effected a degree of separation, 4 regions of alkaloid being observed (Fig 35). In ethanol, two regions of alkaloid which had not moved far from the origin were detected (Fig 35). Consequently, for economic reasons, Freon TF and ammoniacal methanol were selected for formulating an elution system for HPLC.

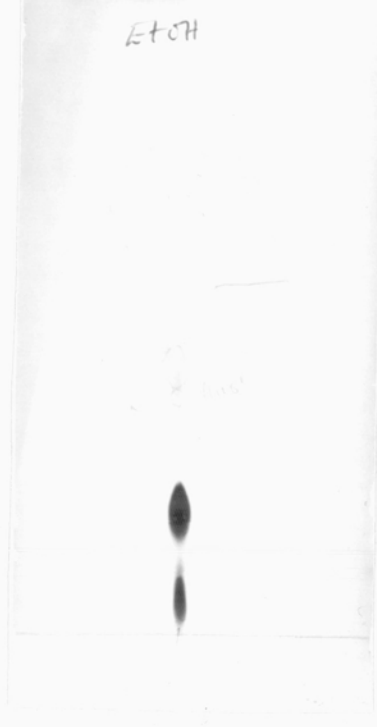
The crude alkaloid mixture (1•5g) in methanol was applied to the top of the column, which was followed by increasing concentrations of ammoniacal methanol (30 - 100%) in Freon, using a total of about 13 litres of solvent. Elution of alkaloid was monitored by following absorption at $\nearrow$ 230nm. The first peak recorded appeared immediately after the void volume, and all subsequent peaks followed on continuously, each peak overlapping considerably. Fractions, corresponding to the region of maximum absorbance of each peak, were collected and alkaloid from each was examined by TLC (Fig 36). The less polar alkaloids (Festuclavine, pyroclavine and dihydroergosine) tended to be eluted fairly efficiently in the earlier part of the elution profile. The other components, including the minor alkaloids, the purification of which was a prime objective of this separation experiment, were not separated from each other. This type of HPLC system did not therefore seem to be very efficient at separating the individual components of the complex mixture of S. sorghi alkaloids. It is possible however, that extensive research and development of solvent systems, supporting materials and reverse-phase chromatography could make this technique more effective.

(ii) Effect of Various Adsorbents and Solvent Systems on the TLC of S. Sorghi Alkaloids

The solvent system normally used for resolution of S. sorghi alkaloids ($CHCl_3$: MeOH : NH_4OH - 95:7:5 - Fig 37), does not sufficiently separate the individual alkaloids to facilitate easy preparative TLC. Consequently, a range of different adsorbents and solvent systems were tested to find a more efficient solvent system for resolving the



(a) Methanol



(b) Ethanol

Fig 35 TLC of S. sorghi Alkaloids by methanol and by ethanol

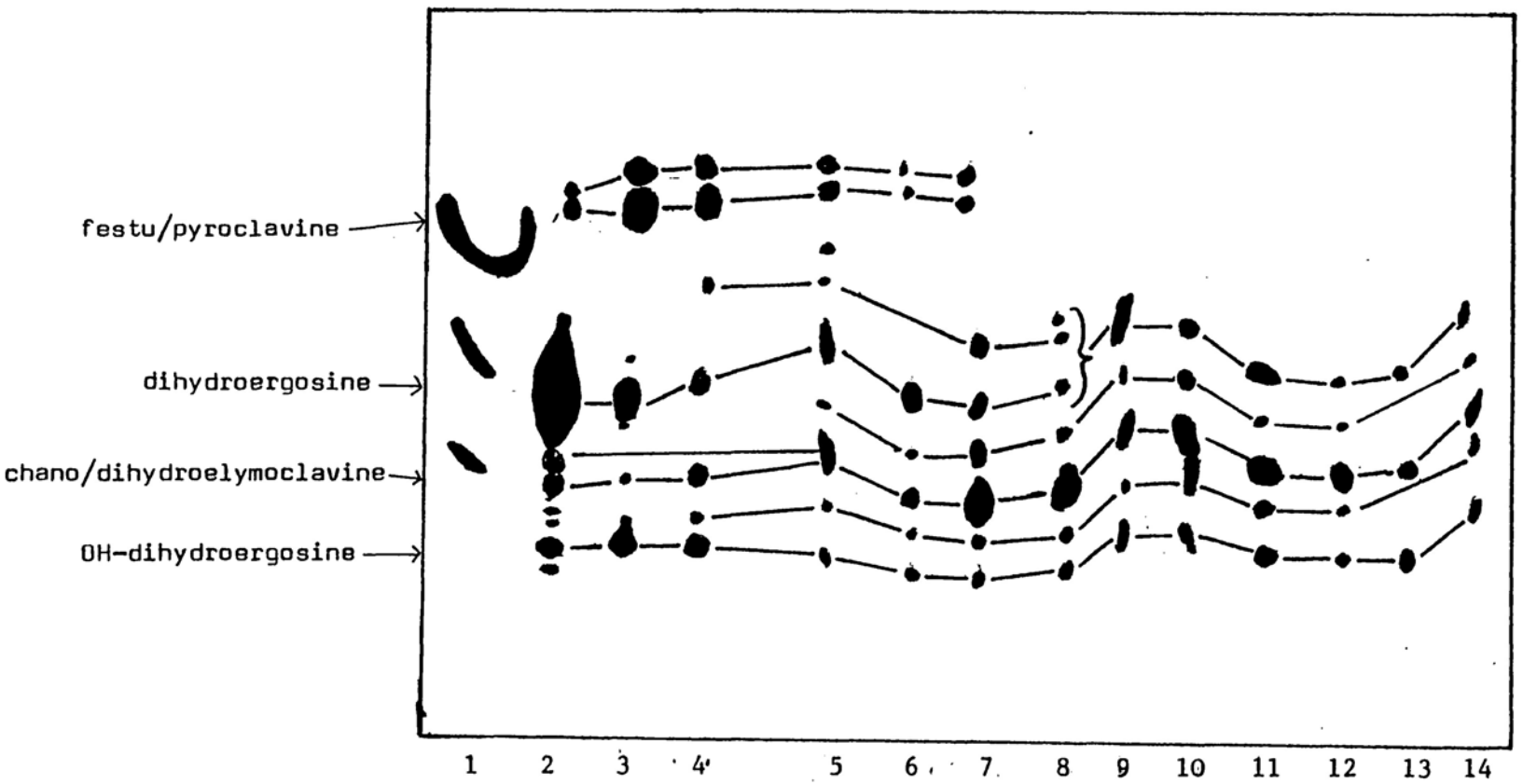


Fig 36 TLC of fractions collected from HPLC



Fig 37 TLC of S. sorghi Alkaloids using $CHCl_3$: MeOH : NH_4OH (95:7:5)

different R_f regions of alkaloid. Since the minor alkaloids which were the desired objective to isolate have R_f values less than dihydroergosine, (DHE), solvent systems which would cause migration of DHE to a very high R_f region whilst clearly separating the slower R_f alkaloids, were sought. Adsorbents and solvent systems tested were as follows:

(a) Alumina ($Al_2 O_3$)

Three solvent systems were tested Xylene/Mek/ MeOH/NH_4OH 10:9:5:0·5; DCE/MeOH/NH_4OH 9:1·5:0·5; and $CHCl_3$/MeOH/NH_4OH 95:7:5), of which none effected good separation of alkaloids.

(b) Kieselguhr

Alkaloid was detected only at the solvent front in all solvent systems tested ($CHCl_3$/MeOH/NH_4OH 95:7:5; Xylene/Mek/MeOH/NH_4OH 10:9:5:0·5; and DCE/MeOH/NH_4OH 9:1·5:0·5).

(c) Silica

The solvent systems $CHCl_3$/MeOH/NH_4OH (9:1:0·01) and CH_2Cl_2/MeOH/NH_4OH (9:1:0·01) effected the same separation as $CHCl_3$/MeOH/Ammonia (95:7:5). $CHCl_3$/EtOH/NH_4OH (9:1:0·01) caused good separation of alkaloids especially in the high R_f region where festuclavine and pyroclavine were separated from each other and also pulled further away from DHE than occurs in original system. The effect of other solvents tested in order of increasing polarity are shown in Fig 38.

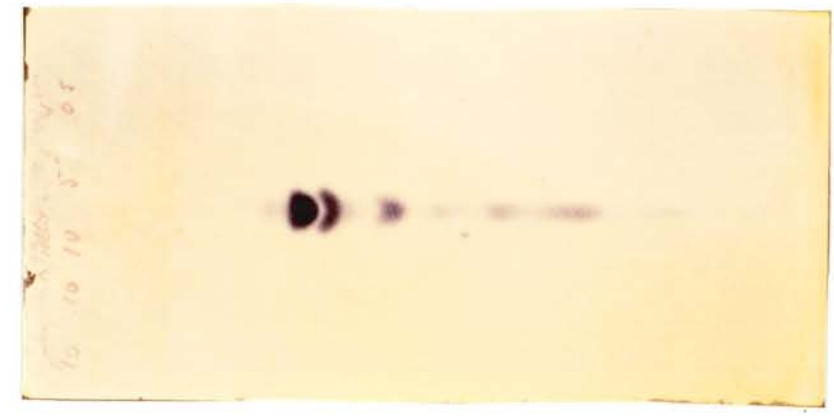
(iii) Isolation of S. sorghi Alkaloids by Preparative TLC

Isolation of individual, in particular minor, alkaloids from a S. sorghi alkaloid mixture (combined fractions 6,7 and 8 from HPLC, Fig 36), was attempted using preparative TLC. The minor alkaloids with R_f values less than OH-dihydroergosine were separated, both from the other alkaloids and from each other by the solvent system xylene/Mek/MeOH/NH_4OH (10:10:5:0·5). Four regions of minor alkaloid were observed but insufficient material was obtained from each for further analysis.

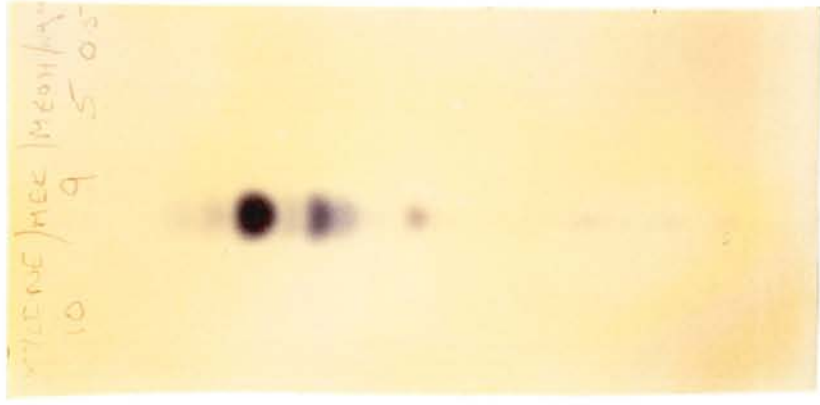
The other alkaloids were recovered and rechromatographed in the solvent system $CHCl_3$/MeOH/NH_4OH (95:7:5), giving six distinct regions of alkaloid. When analytical chromatograms were made from the alkaloid region, each was found to contain several alkaloids (Fig 39). Further attempts to resolve the components of fractions 2-7 (Fig 39) still did not yield pure alkaloids (Fig 39). Some of

Fig 38 Effect of Various Solvent Systems on Separation of S. sorghi Alkaloids by TLC.

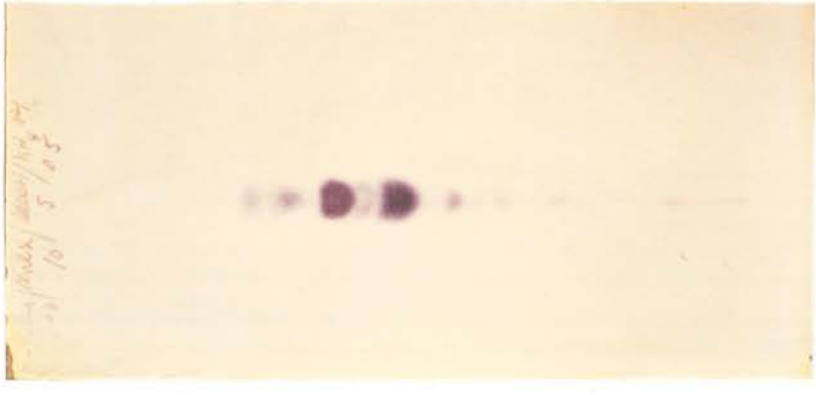
1. Acetone/Mek/MeOH/NH_4OH (10:10:5:0•5)
2. Xylene/Mek/MeOH/NH_4OH (10:10:5:0•5)
3. Xylene/Mek/MeOH/NH_4OH (10:9:5:0•5)
4. Xylene/Mek/MeOH/$CHCl_3$/NH_4OH (10:10:10:5:0•5)
5. Xylene/Mek/MeOH/NH_4OH (10:9:8:0•1)
6. Xylene/Mek/MeOH/NH_4OH (10:7:3:0•05)
7. Xylene/Mek/MeOH/NH_4OH (10:9:7:0•05)
8. DCE/MeOH/NH_4OH (9:1•5:0•01)



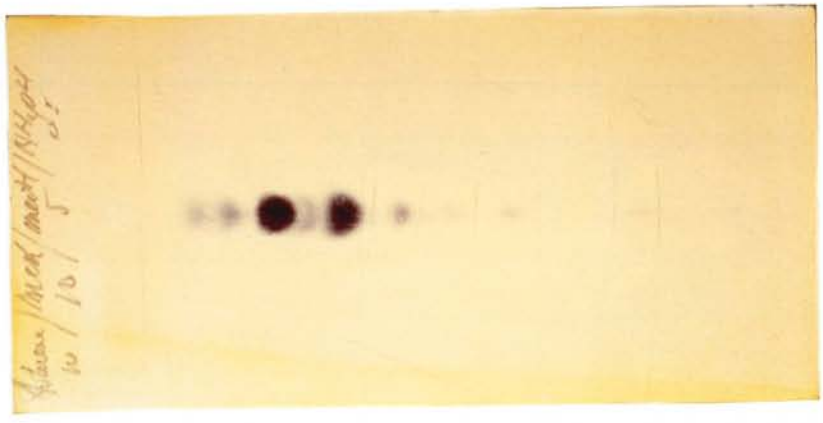
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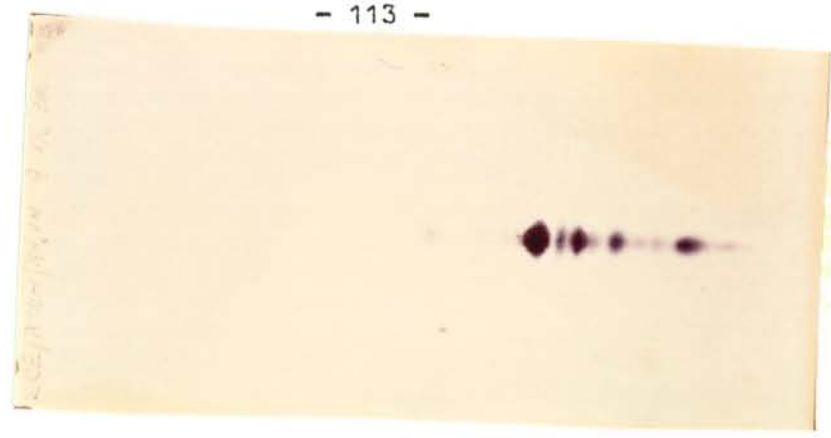
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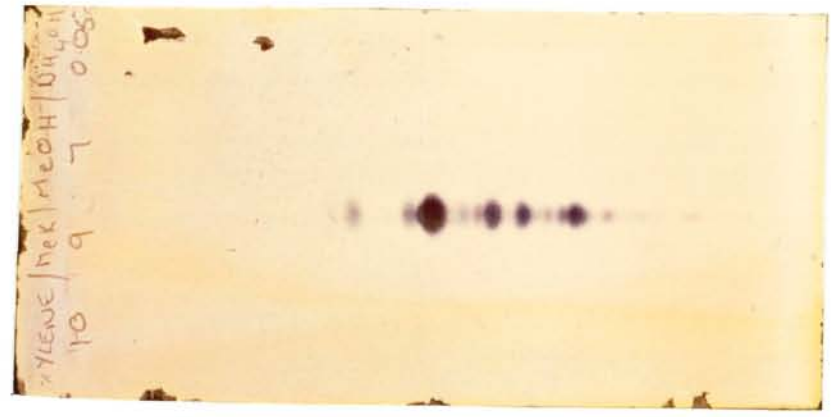
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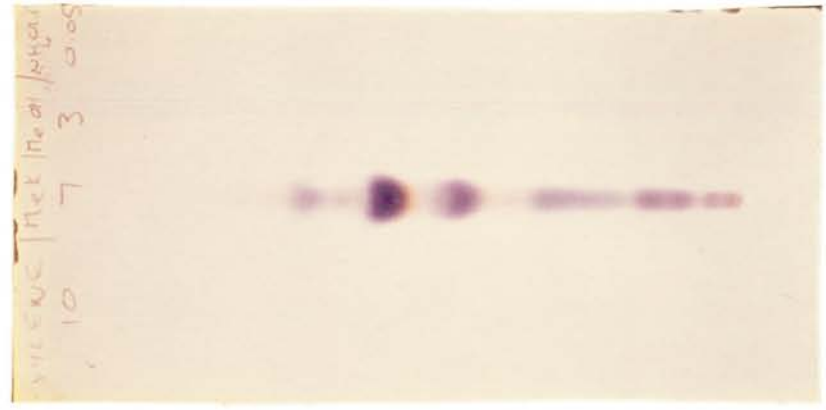
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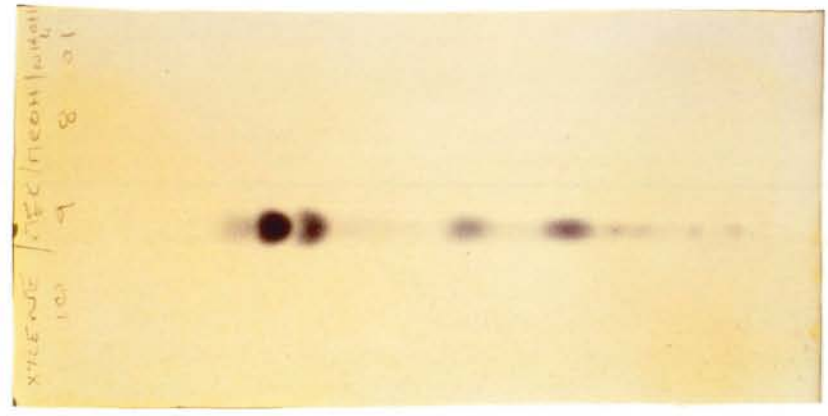
8


XYLENE | hex | MeOH | NH4OH
10 9 7 0.05

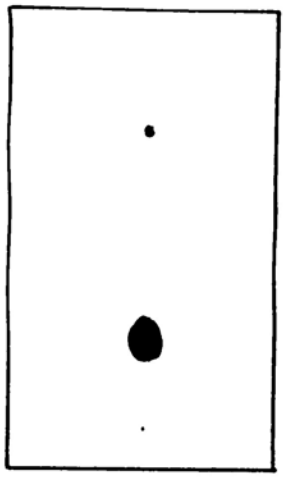
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XYLENE | Hex | MeOH | NH4OH
10 7 3 0.05

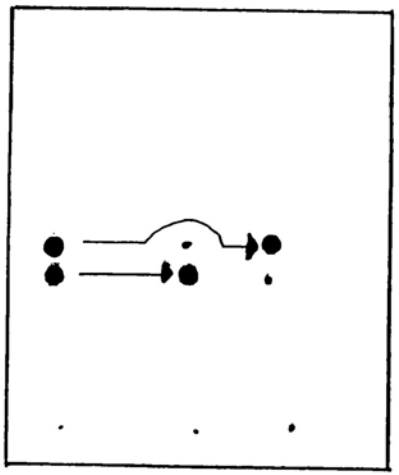
6


XYLENE | HEX | MeOH | NH4OH
10 9 8 0.1

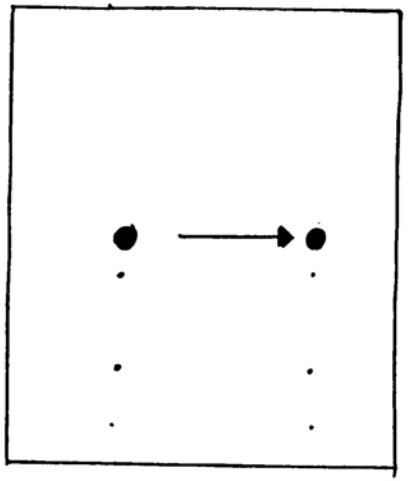
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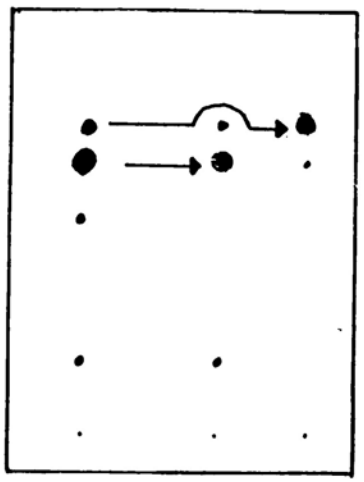
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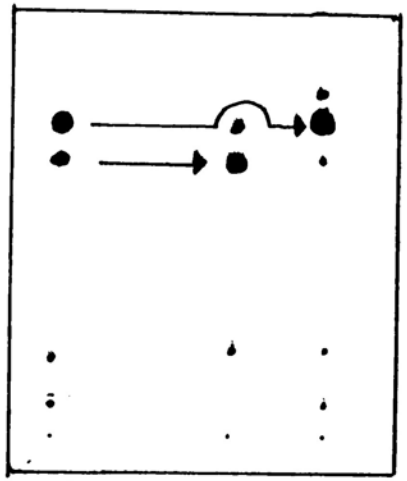
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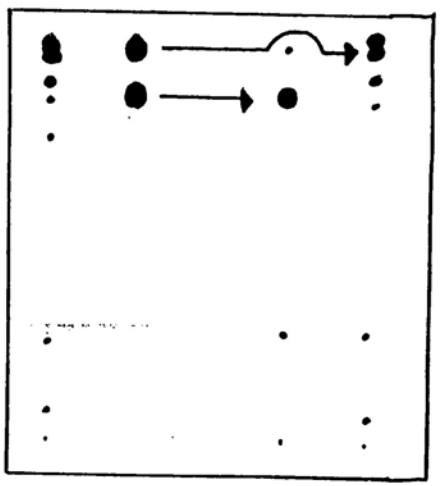
FRACTION 3



FRACTION 4



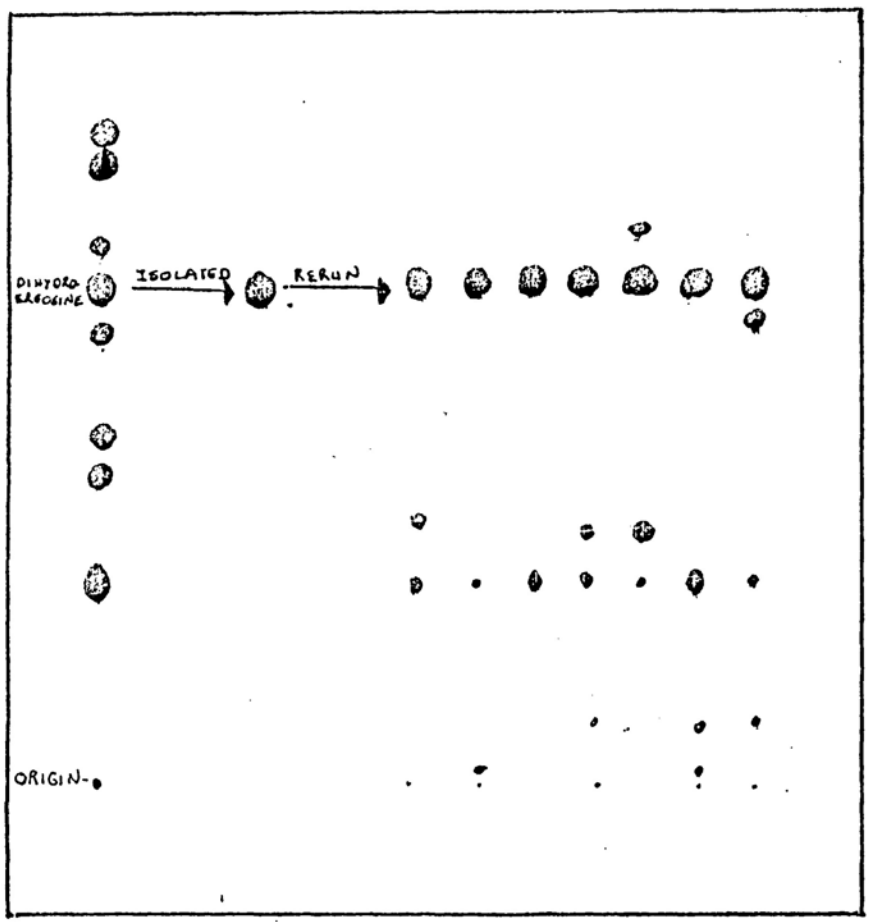
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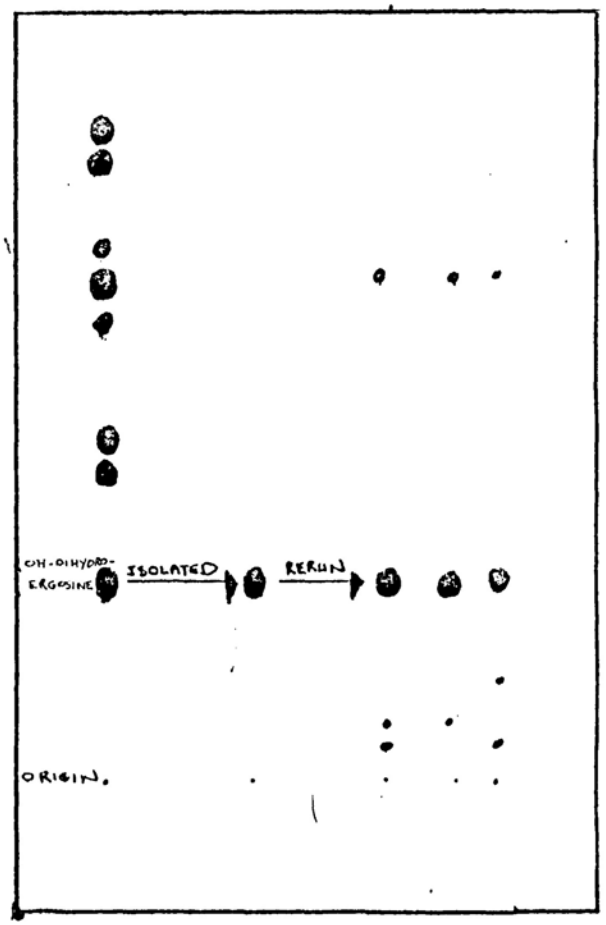
FRACTION 6

Fig 39 Isolation of DHE from S. sorghi alkaloid mixture.

the alkaloids in these mixtures had R_f values quite different from the main alkaloid, implying that they cannot have co-chromatographed at an earlier preparative stage. From these results it must be concluded that some structural changes are occurring. Further possible evidence for molecular rearrangement or decomposition of alkaloids is seen in the principal product of S. sorghi; chromatographic purification of dihydroergosine generally also yields traces of an alkaloid having an R_f value similar to OH-Dihydroergosine and vice-versa. Dihydroergosine ostensibly purified by preparative chromatography also sometimes contains an alkaloid which does not correspond to any seen in the original crude alkaloid extract (Figs 40, 41). A minor alkaloid extract appearing in crude extracts co-chromatographs with the unnatural product OH-dihydroergosine and it is possible that the former is structurally interconvertible with dihydroergosine. Structural changes in alkaloids could be mediated by silica which is a very efficient catalyst for oxidation. Other adsorbents were tested (expt 3.3(ii)) but did not satisfactorily separate crude alkaloid mixtures. If silica caused oxidation of alkaloid the proportion of the low R_f material to dihydroergosine should increase with the time of exposure to silica. In this connection, sample b (Fig 40) was applied to a silica plate and left for 5, 10, 15 and 20 minutes before developing, but no change in the proportion of alkaloids occurred (Fig 42). The alkaloids could have been light and/or oxygen sensitive; however, chromatography of alkaloids under argon and/or in the dark did not appear to offer any advantage. For any one alkaloid, the purest sample available was explored by electron impact mass spectrometry. Apart from confirming the identity of dihydroergosine and OH-dihydroergosine, mass spectra of the other alkaloids which had R_f values less than dihydroergosine were consistent with dihydroelymoclavine or chanoclavine-like compounds ($M^+ - 256$). An alkaloid having an R_f value greater than dihydroergosine, chromatographically therefore near to Festuclavine/Pyroclavine showed an important ion (m/e 240) identical to their molecular ion.



(a) Dihydroergosine



(b) OH - dihydroergosine

Fig 40 Isolation of DHE and OH-DHE from S. sorghi Alkaloid mixture.

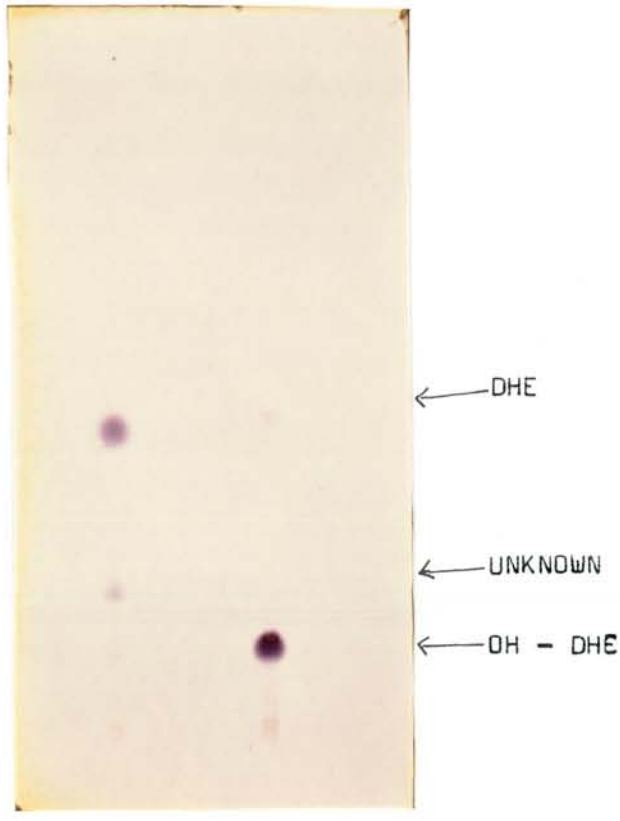
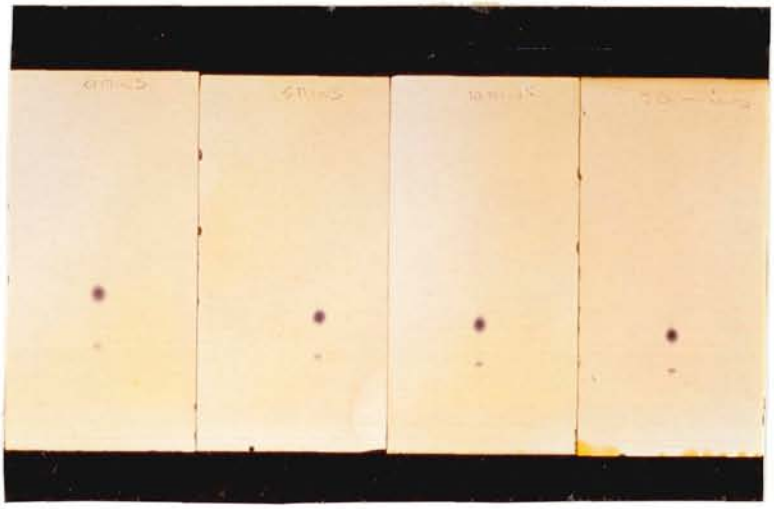


Fig 41 Presence of Minor Uncharacterised Alkaloid in dihydroergosine extract.



0 mins 5 mins 10 mins 20 mins

Fig 42 Effect on dihydroergosine of exposure to silica for various lengths of time.

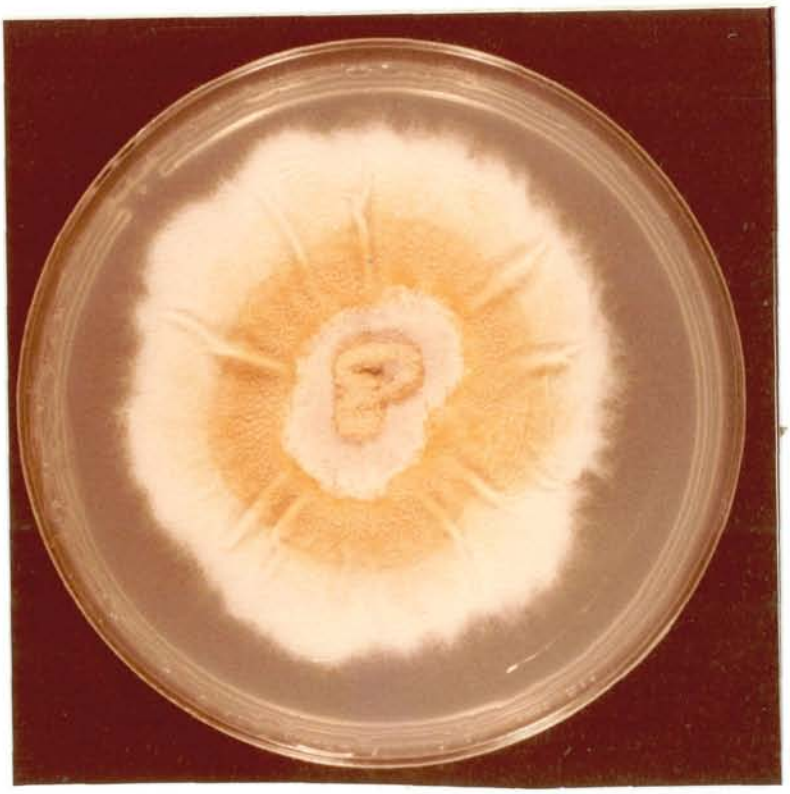
4. CLAVICEPS PURPUREA

4.1 Exploration of the Effect of Asparagine and Aspartic Acid on Growth of C. purpurea

Claviceps purpurea (29/4) was grown on asparagine, and aspartic acid amino nitrogen agar to test respectively for sphacelial and sclerotial growth (Mantle & Nisbet, 1976). On asparagine agar (Fig 43) yellow colouration was observed, due to profuse sporulation. A ring of slightly sclerotial growth appeared around the centre of the culture. In contrast mycelia on aspartic acid (Fig 43) exhibited a very compact pigmented sclerotial-like form of growth. The effect of varying the concentration of aspartic acid relative to asparagine in the medium was explored in cultures grown for 21 days (Table 18). Growth was predominantly sphacelial with a few sclerotial patches when the batched concentration of the total nitrogen source consisted of less than 80% aspartic acid nitrogen. At 80%, regions of sphacelial and sclerotial tissue were approximately equal. Homogen s sclerotial growth was only seen on aspartic acid nitrogen alone and this was accompanied by a reduction in sporulation by approximately 10^2 fold. Alkaloid was only detected in mycelia in which growth was predominantly sclerotial. It is clear that aspartic acid has to constitute a substantial proportion of the nitrogen source in order that the fungus is constrained to produce sclerotial tissue.

Three strains of C. purpurea (29/4, 12_2, AG14) differing with respect to pathogenicity, morphology and alkaloid production were grown on membranes applied to media in which asparagine or aspartic acid were the nitrogen sources. Membranes and mycelia were transferred to fresh medium every four days for 20 days when they were harvested and analysed for dry weight, spore concentration and alkaloid (Fig 44).

Strain 12_2 did not produce alkaloid but sporulated profusely on both media. The other two strains produced similar alkaloid yield on asparagine but the aspartic acid nitrogen source markedly increased alkaloid production in strain 29/4 while decreasing it in strain AG14. The latter effect may be consequent on aspartic acid supporting rather less biomass production than asparagine in this strain. The compact cushion-like, non-sporulating morphology consisting of sclerotial-like



(a) Asparagine



(b) Aspartic Acid

Fig 43 Growth of C. purpurea on asparagine amino N medium and on aspartic acid amino N medium.

% ASPN	DRY WT (g)	SPORE COUNT	ALKALOID
0 (ASN ONLY)	1•251	$9•6 \times 10^9$	0
10	1•425	$10•24 \times 10^9$	0
20	0•977	$11•2 \times 10^9$	0
30	1•18	$9•12 \times 10^9$	0
40	1•124	$12•48 \times 10^9$	0
50	1•268	$10•72 \times 10^9$	0
60	0•972	$1•059 \times 10^9$	0
70	1•01	$18•88 \times 10^9$	0
80	0•99	$7•36 \times 10^9$	0
90	1•3	$6•88 \times 10^9$	0•416
100 (ASP ONLY)	0•973	$9•6 \times 10^7$	1•124

TABLE 18 : Effect of varying the relative concentrations of Aspartic - N to Asparagine N on dry wt., spore concentration and alkaloid production of C. purpurea 29/4

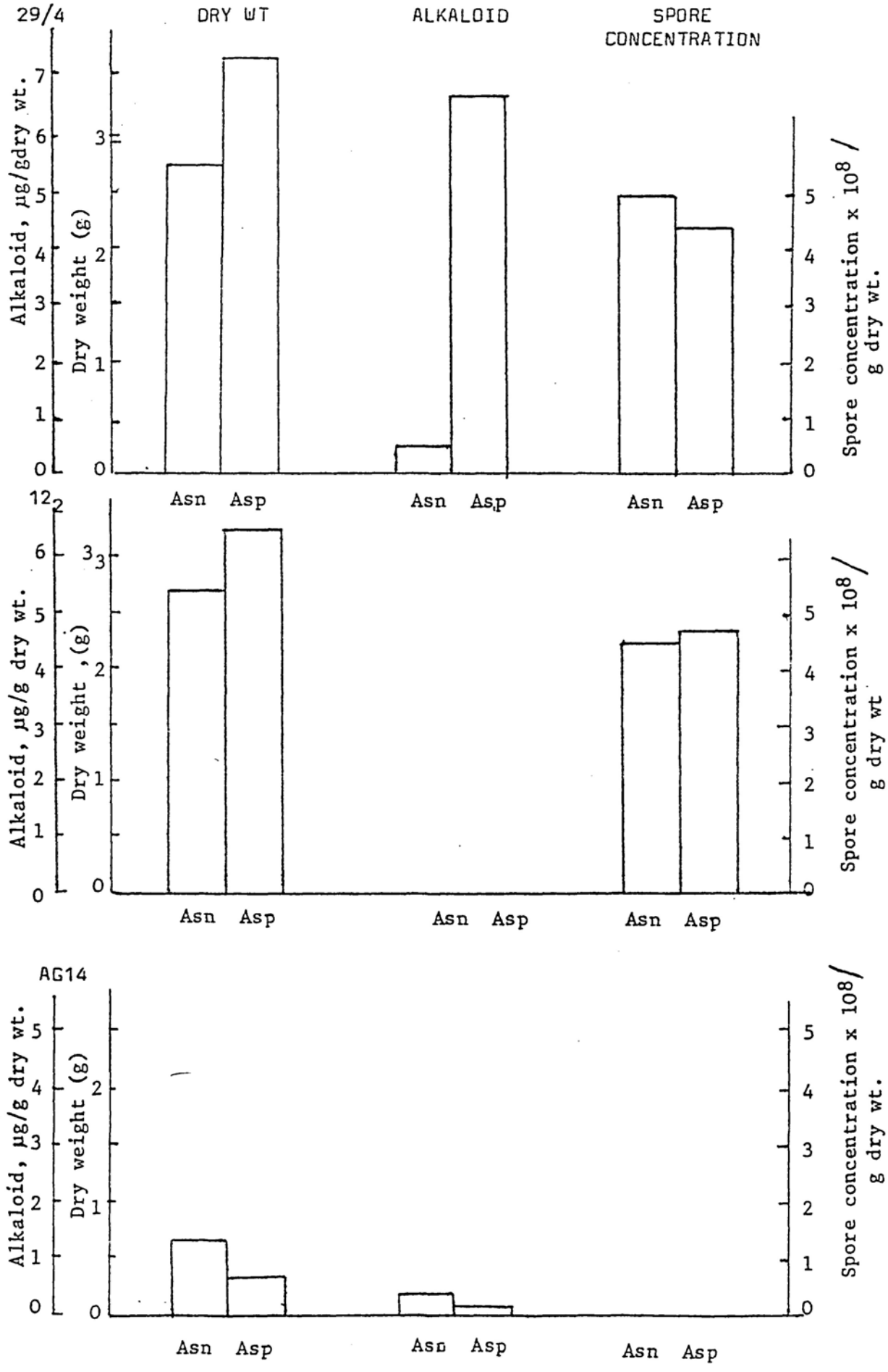


Fig 44 Dry Weight, Alkaloid and Spore Concentrations of 29/4, 12/2 and AG14 grown on asparagine, and aspartic acid amino N media.

cells of AG14 was similar on both nitrogen sources. By contrast in strain 29/4 there was a clear distinction between sphacelial growth on asparagine and the heavier purple-pigmented sclerotial-like growth on aspartic acid. The alkaloid yield of strain 29/4 when frequently transferred to new aspartic acid medium in this experiment was much greater than that obtained without transfer (expt. 4.1). This is consistent with previous findings (Mantle & Nisbet, 1976). Whenever alkaloid was produced the characteristic qualitative composition was expressed irrespective of the medium nitrogen source. The principal alkaloids were peptide-type but strain AG14 produced a minor, non-UV fluorescent alkaloid which did not correspond to any of the clavine alkaloids with R_f values of a similar order (chanoclavine, elymoclavine, lysergol, penniclavine and setoclavine). Further characterisation studies were precluded by the small amount of material.

4.2 Exploration of the Apparent Failure of a C. Purpurea Strain to Produce Alkaloid in Parasitic Culture

The most active alkaloid-producing region, the next 2mm segment after excision of the proximal 1mm (Nisbet, 1975), in the sclerotia of the ergotoxine-producing C. purpurea strain 29/4 and the corresponding region in the apparently non-alkaloid producing strain 12_2, was thinly sliced, washed three times with and finally resuspended in 0·02M phosphate buffer at pH7. To these suspensions 1μ Ci of ^{14}C-tryptophan plus a trace of cold tryptophan was added and the mixture incubated at 24°C for 24 hours on a rotary shaker (100 r.p.m.). The sclerotial slices were filtered, washed with distilled water, freeze-dried and powdered in a tissue grinder. Amphoteric and basic alkaloids were extracted sequentially, chromatographed and autoradiographed.

A region of radioactivity at the same R_f value was detected in the amphoteric fraction of both strains 29/4 and 12_2. Each radioactive region co-chromatographed with unlabelled dimethylallyltryptophan. No radioactivity was detected in the ether extracts. This is not surprising for strain 12_2 since it did not produce basic alkaloids, but it was an unexpected result for strain 29/4 from the tissues of which ergotoxine was extracted. Comparison of amphoteric alkaloid extracts of strain 12_2 with or without previous incubation with ^{14}C-tryptophan showed a considerable increase in the amount of DMAT present in the former; in the extract of the latter only a trace of DMAT was

observed. It should be noted that the sclerotial tissue of strain 12_2 used for incubation with ^{14}C-tryptophan was from the region in which ergot alkaloid biosynthesis is, in alkaloid-producing strains, most active. By contrast, the material used as the 'control', although ideal in being from the same sclerotia, was all older tissue which would be expected to contain the maximum content of any components of the ergot alkaloid biosynthetic pathway. Thus one might have expected more DMAT in the controls and the reverse result obtained may be more marked than appears. It is clear that the apparently non-alkaloid-producing strain, 12_2, is capable of synthesising DMAT and that the addition of tryptophan stimulates this first pathway specific step in ergot alkaloid biosynthesis. The block in alkaloid biosynthesis in strain 12_2 would appear therefore to be at the next step in the pathway.

In strain 29/4, the apparent absence of metabolism of ^{14}C-tryptophan to other alkaloids beyond DMAT in the pathway could be due to using an unsuitable phosphate buffer rather than to any inability of the fungus to effect these transformations.

4.3 Effect of Hydroxy-L-Proline on Alkaloid Production by a Strain of C. purpurea, 117/2/SF

Following the success of redirecting alkaloid biosynthesis in S. sorghi by addition of OH-pro, and considering also the success of Beacco et al (1978) in redirecting C. purpurea alkaloid biosynthesis, an ergotamine-producing strain of C. purpurea (117/2/SF) was tested for its ability to produce hydroxyergotamine when fed with OH-pro. Shake-flask cultures were fed at various stages during alkaloid production with OH-pro so as to give a final medium concentration of 1 mg/ml (Table 19). Neither growth nor alkaloid production appeared to be inhibited by OH-pro, and at the end of the fermentation no OH-pro could be detected in the filtrate nor in an amino acid extract of the mycelium. There was no evidence of any novel alkaloid. This experiment was repeated and the same results obtained.

^{14}C-OH-pro (2 μCi) was fed to a shake-culture of strain 117/2/SF at 3 days when alkaloid production had just begun, for confirmation that OH-ergotamine could not be synthesised. Two days later, when the alkaloid titre of the filtrate was 275 μg/ml the mycelium, in

TIME OF ADDITION (DAYS AFTER INOCULATION)	ALKALOID TITRE OF CULTURE FILTRATE AT TIME OF OH-Pro ADDITION	FINAL ALKALOID TITRE OF CULTURE FILTRATE (12 DAYS)
4	120 µg/ml	460 µg/ml
6	320	530
8	390	475
10	420	460

TABLE 19 : Alkaloid Yield of C. purpurea, 117/2/SF fed with OH-Pro at stages during the fermentation.

which most of the alkaloid is retained, was harvested and extracted for alkaloid. Autoradiography of the alkaloid autoradiogram showed no signs of radioactivity. Thus strain 117/2/SF appears to be able to assimilate OH-pro from the medium, but not to incorporate it into alkaloid.

Hydroxyproline was also unable to replace proline in verruculogen biosynthesis by Penicillium simplicissimum and by Aspergillus fumigatus.

4.4 Investigation of an Uncharacterised Amino Acid Substance (AAS)

Previous investigations of the free amino acid composition of Claviceps purpurea ergots during parasitic development (Corbett et al, 1974) showed the presence of an amino acid substance (AAS), the retention time of which in the autoanalysis did not correspond to any common amino acid. This substance constituted the principal free amino acid in sclerotia and was detected in developing ergots coincident with the formation of alkaloid. It was not detectable in the sclerotia of strains which did not synthesise alkaloid. Techniques for isolation and purification of this amino acid have been explored in recent years, but difficulties in obtaining a pure sample compounded intrinsic problems in structural studies. For example, the free acid was insufficiently volatile for an electron impact mass spectrum to be obtained and thus mass spectrometric analysis had been confined to methyl and acetyl derivatives. Considerable data had been generated but no firm conclusion concerning the structure of the free acid could be made (P.G. Mantle and E.S. Waight, private communication). The free acid also had not been amenable to NMR spectroscopy. The best method of qualitative analysis of AAS in fungal extracts involved high-voltage electrophoresis and the amino acid had a characteristic behaviour in analytical ion-exchange chromatography (Chromaspec).

A pale yellow, freeze-dried sample (50 mg) of AAS which had been prepared from commercial ergot, and separated from other amino acids by preparative high-voltage electrophoresis (H.V.E.) was available (P.G. Mantle) for structural studies, summarised in Fig 45. Approximately 1 mg was hydrolysed for 24 hrs (6N HCl/100^{o}C) to test whether the sample was contaminated with any peptide. H.V.E. showed no hydrolytic products, and it was concluded that the sample was peptide-free. This also confirmed the stability of AAS to this treatment.

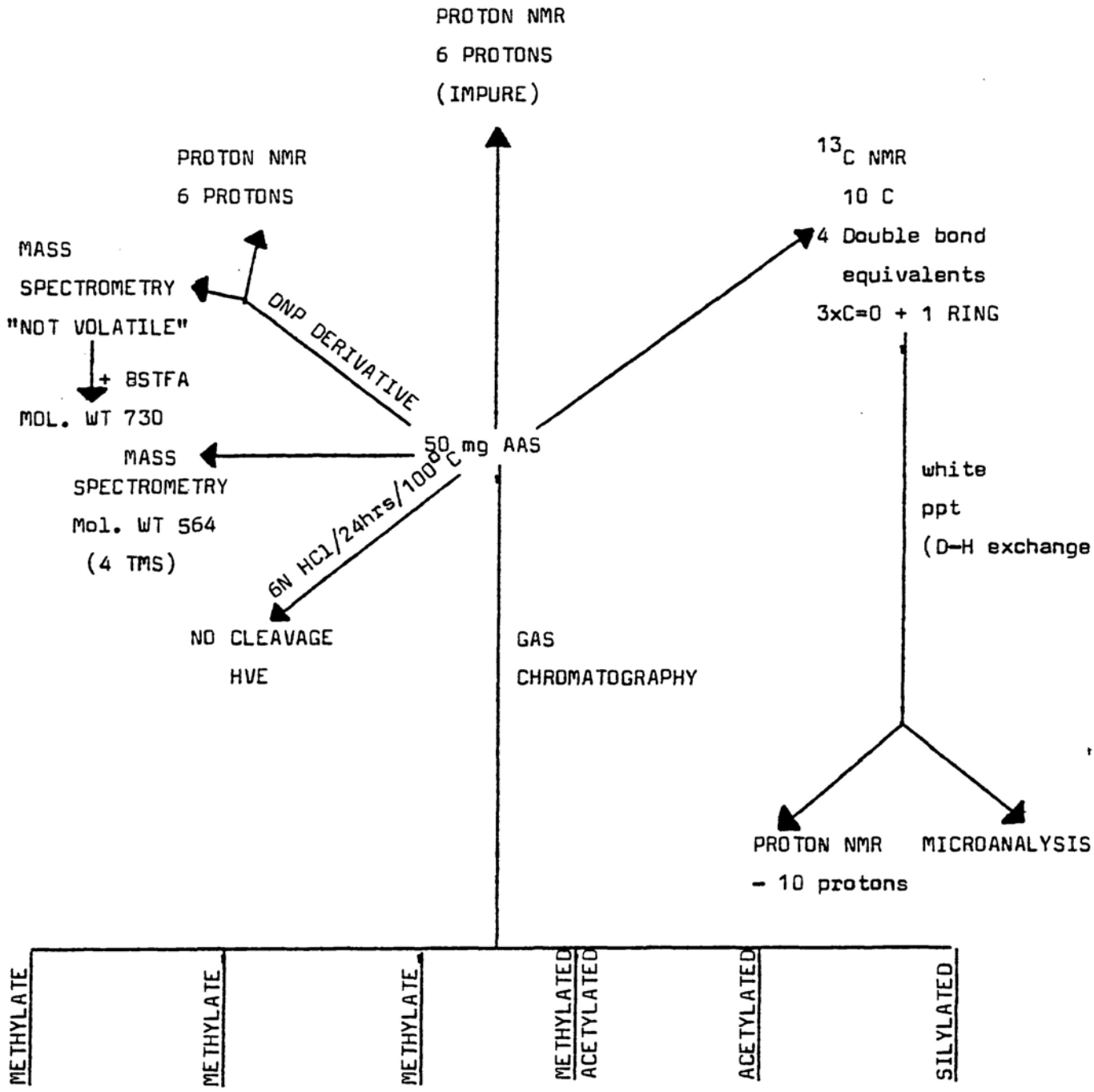


Fig 45 Structural Studies of AAS

Several derivatives of AAS were prepared for gas-liquid chromatography, but none yielded useful information.

Proton Nuclear Magnetic Resonance (NMR) spectroscopy of AAS revealed 6 protons, but also showed evidence of contaminating base material. A DNP (dinitrophenol) derivative of AAS was prepared, and separated from other contaminating material by preparative TLC. Proton NMR spectroscopy of the DNP derivative again showed 6 protons. Electron impact mass spectrometry was unsuccessful due to the non-volatile nature of this derivative, consequently á trimethyl silyl (TMS) DNP derivative was made using Bis (trimethyl silyl) trifluoracetamide (BSTFA). The free amino acid was also silylated using BSTFA. From electron impact mass spectrometry of these derivatives (Table 20), it was calculated that AAS had a molecular weight of 276 corresponding to a molecular formula of $C_{10}H_{16}N_2O_7$.

^{13}C NMR spectroscopy of AAS showed ten carbons (Table 21), and it was calculated that there are four double bond equivalents, which probably correspond to 3 C=O bonds and one ring structure. Other information obtained from the spectrum was that one CH (ppm 58) is attached to a CH_2. The CH_2 groupings appear to be in aspartic/glutamic acid type environments, and it is likely that a $^{OH}\!\diagdown\!CH\!\diagup\!^{CO_2H}$ grouping occurs. The tube plus sample of AAS in D_2O used in ^{13}C NMR spectroscopy, was left to stand in the laboratory for two days and surprisingly a white precipitate formed. This precipitate was separated from the supernatant and treated with distilled water to reverse any deuterium exchange. Proton NMR spectroscopy showed that the white compound was a purer form of AAS (AAS_2). Ten protons were now seen, and the base contaminant of the original crude AAS had now disappeared. Micro-analysis of AAS_2 (Table 22) did not agree with the expected molecular formula of $C_{10}H_{16}N_2O_7$, but a considerable amount of ash remained after combustion. Since this ash cannot be due to phosphorous, sulphur, or halogens (mass spectrometric evidence), and was unlikely to be inorganic salt, it is assumed to be mainly the ash often found (B. Johnson) after CHN analysis of amino acids. If the percentage of CHN found is recalculated allowing for 11·52% ash a molecular formula of $C_{10}H_{16\cdot8}N_2O_7$ is obtained. As there is an even number of nitrogens there must be an even number of hydrogens, therefore the microanalytical result was consistent with the molecular formula $C_{10}H_{16}N_2O_7$ deduced from mass spectrometry. Insufficient material was available for Infra-red Spectroscopy.

TRIMETHYL SILYLATED DNP DERIVATIVE OF AAS.

m/z	MEASURED MASS	CALCULATED MASS	COMPOSITION
511	511.1667	511.1679	$C_{20}H_{31}N_4O_8Si_2$
613	613.2114	613.2180	$C_{24}H_{41}N_4O_9Si_3$
730 (M^+)	-	-	$C_{28}H_{51}N_4O_{11}Si_4$ (DEDUCED)

TRIMETHYL SILYLATED AAS

345	345.1651	345.1669	$C_{14}H_{29}N_2O_4Si_2$
447	447.2123	447.2167	$C_{18}H_{39}N_2O_5Si_3$
459	459.1791	459.1804	$C_{18}H_{35}N_2O_6Si_3$
549	549.2280	549.2302	$C_{21}H_{45}N_2O_7Si_4$
564 (M^+)	-	-	$C_{22}H_{48}N_2O_7Si_4$

TABLE 20 : Electron Impact Mass Spectrometry of AAS Derivatives

PPM	BOND TYPE
28•5	CH_2
31•5	CH_2
32•5	CH_2
51	CH
58	CH
71	CH
75•5	$-\overset{|}{\underset{|}{C}}-$
173•5	C = O
174	C = O
175	C = O

NMR in D_2O

TABLE 21 : ^{13}C Chemical Shifts of AAS

	C	H	N
% THEORY	43•48	5•84	10•14
% FOUND	38•86	5•46	8•95
DEVIATION	- 4•62	- 0•38	- 1•19

% ASH = 11•52

ASH FREE	C	H	N
% FOUND	43•92	6•17	10•11
DEVIATION	+ 0•44	+ 0•33	- 0•03

TABLE 22 : CHN Microanalysis of AAS

5. DISCUSSION

The first *in vitro* production of alkaloids by *Sphacelia sorghi* was reported by Mantle (1973). As was found in early fermentation studies on *Claviceps fusiformis* (Buck *et al*, 1968), a glucan accumulated during the growth in surface culture of *S. sorghi*, therefore predictably and actually precluding a shake-culture process. In submerged fermentation increased viscosity of the broth due to glucan production impairs oxygen transfer and therefore adversely affects growth and the production of secondary metabolites involving oxidatives steps. Mixed culture of *S. sorghi* with a glucanase-producing, alkaloid non-producing strain of *C. purpurea* provided an effective system for the production of *S. sorghi* alkaloids and is the first reported instance of advantageous mixed culture of ergot fungi. *C. purpurea* glucanase autolysed the glucan produced by *S. sorghi* restoring the medium to one of low viscosity and consequently improving oxygen transfer and aerobic growth. Whilst the possibilities of mixed culture were being explored, a glucanase-producing isolate of *S. sorghi* arose spontaneously, so allowing for the first time successful submerged fermentation of *S. sorghi* for the production of alkaloids in pure culture. The amount of alkaloid produced by *S. sorghi* in mixed culture with *C. purpurea* was less than that achieved by the variant of *S. sorghi* above. The *C. purpurea* strain, 12_2, used was found to have a block in its alkaloid biosynthesis subsequent to dimethylallyltryptophan (DMAT). If, instead of alkaloid biosynthesis being blocked by a genetical error, an inhibitor to the step DMAT $\longrightarrow$ Chanoclavine I was being produced, this inhibitor could possibly leak into the culture medium and similarly inhibit *S. sorghi* alkaloid biosynthesis.

Large-scale fermentation of *S. sorghi* yielded alkaloid titres similar to those previously achieved in surface culture (Mantle, 1973). Phosphate was rapidly assimilated from the medium, as occurs in other high alkaloid yielding *Claviceps* fermentations (Arcamone *et al*, 1961, Amici *et al*, 1967, Banks *et al*, 1974). Glucan which accumulated during the initial stages of the fermentation as was found in *C. fusiformis* (Dickerson *et al*, 1970), was subsequently autolysed. Glucan autolysis and alkaloid production appeared to be closely linked. The maximum glucan concentration was over 40% greater in the second 60l fermentation, and the lower alkaloid production, as compared with that of the first

fermentation, might be a reflection of this. Alkaloid production, and possibly therefore glucanase production was associated with swollen lipid-filled mycelium. A similar situation was found in C. fusiformis where the production of β-glucanase, β-glucosidase and of maximum alkaloid yield was associated with the formation of lipid-containing sclerotial-like cells (Banks et al, 1974). Alkaloid production by C. paspali and C. purpurea is also associated with sclerotial-like cells full of lipid. (Arcamone et al, 1961, Mantle et al, 1968).

Degeneration during laboratory culture is a common problem with Claviceps spp. (Kobel, 1969, Mantle 1969, Kobel 1978) and in the present studies this was particularly evident also in S. sorghi. Glucanase producers often changed to non-glucanase producers and high alkaloid-yielding strains tended to change to a form giving only a low yield of alkaloid. Thus, due to the unpredictability of glucanase production many experiments were performed in surface culture.

Colony morphology of S. sorghi was of two distinct types, one white and fluffy, the other cream-coloured and compact. The appearance of surface culture mats was similar to that of the colony used as inoculum. The white fluffy mats produced both macro- and microspores and little or no alkaloid, whereas the cream-coloured compact mats produced mainly microspores and high alkaloid titres.

Axenic culture of S. sorghi is similar therefore to that of C. purpurea and C. paspali (but not to C. fusiformis) in that growth and alkaloid production are distinct phases. Accumulation and subsequent autolysis of glucan during the fermentation resembles the pattern exhibited by C. fusiformis. Loss of macrospore production in high alkaloid yielding S. sorghi isolates may indicate a step towards a non-sporulating form, as is a feature of C. purpurea and C. paspali when alkaloid is being produced in culture. An inverse relationship between conidiation and alkaloid production has also been described for the Claviceps strain, 129, which from its description resembles C. fusiformis rather than C. purpurea (Vorisek, 1974). S. sorghi seems therefore to occupy an intermediate position between C. purpurea / C. paspali and C. fusiformis with respect to alkaloid production in axenic culture.

S. sorghi was able to assimilate the proline analogue, hydroxyproline (OH-Pro), and subsequently incorporate it into the

peptide sidechain of dihydroergosine thereby giving rise to a new alkaloid, 9' OH-dihydroergosine. Many proline analogues were tested (Table 14), but only OH-Pro was incorporated into dihydroergosine. The D-enantiomer of OH-Pro however, appeared neither to be assimilated by the mycelium nor incorporated into dihydroergosine. The optimum time or amount of OH-Pro addition for incorporation into dihydroergosine was not critical, although alkaloid production was significantly reduced at concentrations of OH-Pro > 0·5 mg/ml when added at 5 days. At this stage much of the biomass had yet to be produced. Similar redirection of alkaloid biosynthesis by strains of C. purpurea on addition of appropriate amino acid analogues was reported by Beacco et al (1978) (Table 6). However when OH-Pro was administered to the ergotamine producing cultures of C. purpurea, 117/2/SF, no OH-ergotamine was synthesised. Hydroxyproline did not inhibit growth of C. purpurea in submerged culture, nor was it detectable in the culture filtrate at the end of the fermentation. It appears therefore that C. purpurea is able to assimilate OH-Pro, and consequently might channel it unchanged or, perhaps, after hydrolysis to proline, into the intracellular amino acid pool. Since, when fed with ^{14}C-OH-Pro, C. purpurea produced no radioactive ergotamine, it is unlikely that OH-Pro is hydrolysed to proline. A question arises therefore, concerning the fate of assimilated OH-Pro in C. purpurea. It is intriguing that the multienzyme complex of S. sorghi seems to be unique in accepting OH-Pro as the primer for cyclic tripeptide sidechain biosynthesis. This suggests that an enzyme involved in the biosynthesis of this ergot peptide alkaloid moiety has a broader specificity in S. sorghi than in C. purpurea. Tryptophan analogues cannot be incorporated into the ergoline nucleus. This is probably due to dimethylallyltryptophan transferase being particularly specific for its substrate. Therefore in peptide ergot alkaloid biosynthesis it would appear that there are two separate enzyme systems, one involved in ergoline biosynthesis, in which the enzymes are very specific for the substrate and one synthesising the peptide sidechain which is much less substrate specific.

It may be asked why some Claviceps spp seem to be endowed with an "extra" enzyme system synthesising complex cyclic tripeptide lysergic acid derivatives, while others only form simple lysergic acid amides. Is it possible that in the latter groups the genes coding for the requisite multi-enzyme complex are present but are

always repressed? Could determination of the alkaloids produced by the different Claviceps spp have been in response to the spectrum of nutrients supplied by the particular host plants which they normally parasitise? If not what, if any, ecological advantage do peptide alkaloid producers gain from this complicated pathway? However, although the above questions may be reasonable, it is possible that the process of secondary metabolism, which relieves an embarrassment of excess metabolites that would otherwise accumulate and affect growth, is more important than the actual secondary metabolites (Bu'lock, 1974).

Hydroxyproline was not incorporated into verruculogen, or its hydroxy analogue by Penicillium simplicissimum nor by Aspergillus fumigatus. This indolic tremorgenic mycotoxin is biosynthesised in part from proline (Day, 1980) and represents one of the few other types of fungal secondary metabolites containing this amino acid. Nevertheless the biosynthetic enzyme(s) concerned is insufficiently flexible to accept the foreign hydroxy analogue.

Thus the biosynthetic acceptance of OH-Pro by S. sorghi shown in the present studies has provided a rare if not unique, instance of a 4-OH-Pro derived microbial metabolite. Different isomeric forms appear to be involved where OH-Pro is a secondary metabolite components in other microorganisms e.g. the Streptomycete antibiotic telomycin contains 3 OH-Pro and the toxic peptides of Amanita phalloides contain the allo-4-OH derivative of proline.

In agar culture, concentrations of OH-Pro which failed to diminish alkaloid biosynthesis by established surface cultures of S. sorghi, suppressed growth when incorporated into agar prior to petri dish culture. A concentration of 1 mg/ml reduced growth to 20% of normal, but it was notable that even 10 mg/ml did not completely inhibit growth. It seems that once S. sorghi has entered an active growth phase, as had occurred by day 5 in surface culture experiments, the fungus can tolerate the presence of OH-Pro at an otherwise toxic level, and continue apparently normal growth. It appears that Robbins and McVeigh (1946) have published the only report concerning the effect of OH-Pro on the growth of fungi. Small amounts of OH-Pro stimulated the growth of Trichophyton mentographytes; however growth was completely inhibited at a concentration of 50 µg/ml. Growth stimulation by OH-Pro was not

observed in S. sorghi, but this effect was seen with 3,4 dehydro-DL-proline (Fig 28). Both of these analogues were growth inhibitory at higher concentrations, the latter being more active in this respect. Many common fungi are not adversely affected by OH-Pro; Robbins and McVeigh (1946) list nineteen species of Aspergillus, Neurospora, Penicillium, Phycomyces and Agaricus which were apparently tolerant.

Interpretation of the results on the composition of S. sorghi alkaloids and the role of each in the biosynthetic pathway is difficult. Little is known about the uncharacterised minor clavine alkaloids, but the minor peptide alkaloids appear to be similar to dihydroergosine in that they all incorporated ^{14}C-leucine and ^{14}C-proline. Quigley (1973) also found proline and leucine in a ratio 1:1, as occurs in dihydroergosine, after acid hydrolysis of minor peptide alkaloids. Cyclic tripeptide lysergic acid derivatives readily isomerise at C-8 of the ergoline ring and isomers (isolysergic acid derivatives) usually have different (higher) R_f values in TLC (McLaughlin et al, 1964). Since most of these minor peptide alkaloids have much lower R_f values than dihydroergosine rather more complex structural rearrangement may be involved. It is concluded therefore that the minor peptide alkaloids present in the alkaloid mixture are artefacts of the cultivation conditions and/or the work-up process and that dihydroergosine is the only true peptide alkaloid, constituting the end product of the alkaloid biosynthetic pathway in S. sorghi. Extraction of naturally occurring (Nigerian Sorghum) ergots of S. sorghi seems to support this conclusion in that dihydroergosine is the main alkaloid and only four others, festuclavine, pyroclavine, chanoclavine and dihydroelymoclavine have been observed (Mantle, 1968).

Chanoclavine I has been isolated from cultures of S. sorghi (Barrow et al, 1974) suggesting that biosynthesis of alkaloids by this fungus follows the usual route up to and including chanoclavine I. However, the present examination of the amphoteric alkaloid fraction revealed no evidence of dimethylallyltryptophan (DMAT), the intermediate between tryptophan + mevalonic acid ⟶ chanoclavine. Barrow et al (1974) could only detect dihydrolysergic acid in their amphoteric alkaloid extracts of S. sorghi cultures, thus leaving chanoclavine as the earliest identified intermediate in alkaloid biosynthesis by this ergot fungus. In the present studies, addition

of ethionine, a methionine antagonist which blocks the N-methylation step in ergoline biosynthesis at a concentration 5 times greater than that effective for C. fusiformis (Szczyrbak, 1972) did not block alkaloid biosynthesis in S. sorghi and subsequently no accumulation of DMAT could be detected. A problem now is whether DMAT actually occurs in S. sorghi alkaloid biosynthesis. If not, what is the biosynthetic route to chanoclavine operated by this ergot fungus? Although cell-free preparations of S. sorghi were capable of metabolising ^{14}C-tryptophan, no radioactivity was detected in DMAT or other alkaloid. However ^{14}C-DMAT did not appear to be metabolised either. Due caution has to be taken in the interpretation of this result as no positive evidence for the efficiency of the cell-free system is available. The result from the ^{14}C DMAT feeding could indicate that S. sorghi is unable to utilise it, thereby suggesting a different route than normal to chanoclavine. Agroclavine, a recognised alkaloid biosynthetic intermediate, inhibits DMAT synthetase in C. fusiformis, where it is an end-product (Floss et al, 1974), but not in C. purpurea where it is not (Gröger and Mair, 1978). This clavine alkaloid is not part of the S. sorghi alkaloid spectrum. However, Quigley (1973) found that when added exogenously to S. sorghi cultures, agroclavine had a very low percentage incorporation and also generally inhibited alkaloid biosynthesis. Consequently agroclavine was rejected as a possible intermediate in the biosynthetic pathway to dihydroergosine. Therefore chanoclavine ⟶ dihydroelymoclavine occurred without the intermediacy of agroclavine. In the present situation neither incorporation of agroclavine nor inhibition of S. sorghi alkaloid biosynthesis occurred.

Three possible interpretations could apply to a failure of agroclavine to inhibit S. sorghi alkaloid biosynthesis:

1. S. sorghi does not produce DMAT synthetase, or the enzyme is insensitive to agroclavine. Quigley (1973) suggested that inhibition of alkaloid biosynthesis by agroclavine occurred at the hypothetical step festuclavine ⟶ dihydroelymoclavine. No enzyme catalysing this reaction has been isolated but if this step had been inhibited in Quigley's experiments, the relevant enzyme in the strain of S. sorghi used in the present studies must have been insensitive to agroclavine. The latter seems most unlikely.

2. Inhibition of DMAT synthetase by agroclavine occurs only in strains of Claviceps where this clavine alkaloid is an end-product. Since in

S. sorghi not only is agroclavine not an end-product, but is not even involved in the biosynthetic pathway, inhibition might not be expected to occur. Feed-back or end-product regulation might be expected to be effected in S. sorghi by dihydrolysergic acid or dihydroergosine.

3. Agroclavine cannot enter the cell, and so cannot reach the site of alkaloid biosynthesis, due to a permeability barrier. Cheng et al (1980) showed that end-product regulation of alkaloid synthesis by elymoclavine in Claviceps fusiformis strain SD58, was considerably more effective in a protoplast preparation where presumably permeability was easier, than in intact mycelium.

The other main biosynthetic steps are shown in Fig 46.

The peptide portion of ergot alkaloids consists of three amino acids, which are specifically ordered, proline always being at the free end of the chain. With the successful incorporation of OH-proline into dihydroergosine, substitution at each of the three amino acid positions has now been achieved in ergot fungi (Table 23). The substitution of proline by OH-pro in S. sorghi reinforces two proposals concerning the biosynthesis of peptide alkaloids;

1. The enzymes involved in the biosynthesis of the peptide sidechain are not strictly substrate specific.

2. The biosynthesis of the peptide portion of these alkaloids is affected by the relative concentrations of amino acids in the internal pool.

Consequently in the future there is a distinct possibility of obtaining new pharmacologically interesting alkaloids by exploring the lack of specificity of some enzymes of ergot fungi.

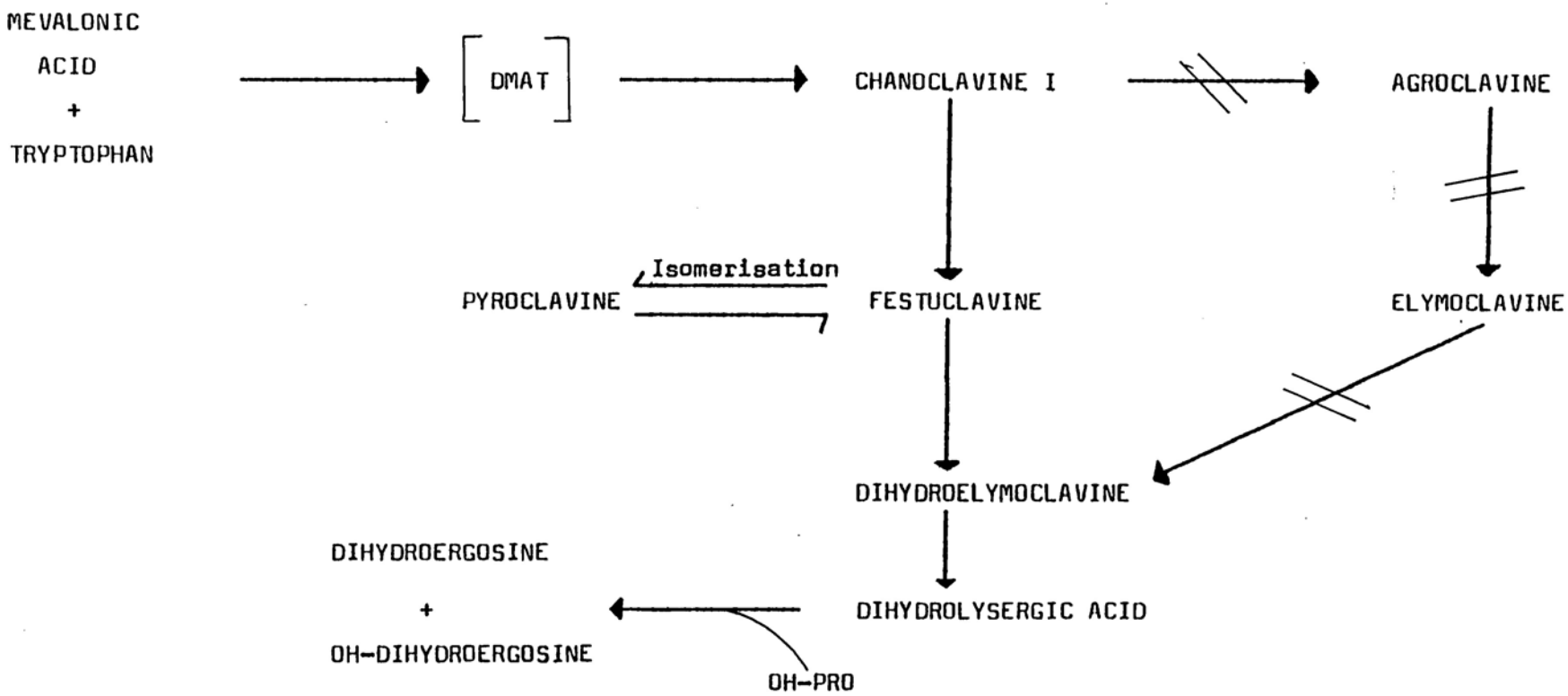


Fig 46 Proposed Biosynthetic Pathway for Dihydroergosine in S. sorghi

Ergot Alkaloid	Amino Acid Composition of Peptide Sidechain	Amino Acid Analogue Incorporated	Reference
Ergotamine	Pro-Phe-Ala	p-chlorophenylalanine p-fluorophenylalanine	Beacco et al (1978)
		∝-amino butyric acid (substituted for alanine ⟶ ergostine)	Floss et al (1980)
Ergocristine	Pro-Phe-Val	p-fluorophenylalanine p-chlorophenylalanine	Beacco et al (1978)
Ergocornine	Pro-Val-Val Pro-Leu -Val	L-norvaline ∝-amino butyric acid L-norleucine 5,5 trifluoroleucine β-OH-leucine	Beacco et al (1978)
Dihydroergosine	Pro-Leu-Ala	L-OH-proline	Present Studies

TABLE 23 : Incorporation of Various Amino Acid Analogues into Ergot Alkaloids.

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