CHAPTER I

INTRODUCTION

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(1) ALKALOIDS

The alkaloids are organic, nitrogenous bases which occur chiefly in plants, but to a lesser extent also in microorganisms and in animals. This definition, of course, has many exceptions and reservations e.g. colchicine and ricinine which are not basic; ephedrine, muscarine and mescaline which are not nitrogen containing heterocyclic compounds; and a group of alkaloids isolated from animals and microorganisms, e.g. batrachotoxin A (from columbian arrow poison frog Phyllobates aurotaneia, castormine (from Canadian beaver), muscopyridine (from musk deer), pyocyanine (from Pseudomonas aeruginosa), and gliotoxin (from fungus Trichoderma viridae). An extensive study in animals and microorganisms may invalidate the traditional exclusive relationship between alkaloids and higher plants (Hughes and Genest, 1973).

The marked physiological activity of many alkaloids provided the stimulus for early investigations leading to the isolation and characterization of such well known representatives as strychnine, morphine, quinine, nicotine and cocaine. Frequently the physiological activity of an alkaloid manifests itself in an extreme toxicity; on the other hand, many alkaloids have therapeutically useful pharmacological properties at

sublethal dosage and have become established as valuable drugs in general medical practice.

During the early 1950s the field of alkaloid biosynthesis moved from the realms of fruitful and often very farsighted speculation into the phase of experimental investigation. Biologically oriented researchers started studying the physiological aspects of alkaloid formation in many plants for practical reasons, e.g., agronomists to grow crop plants of low alkaloid content and pharmacognosists to grow medicinal plants of high alkaloid content.

The compounds from which most alkaloids are derived include :- (a) the amino acids (Anthranilic acid, Lysine, Nicotinic acid, Ornithine, Phenylalanine, Tryptophan and Tyrosine); (b) the organic acids (Acetic and Mevalonic acid); and (c) sources of one carbon fragments such as Methionine and Formic acid. Alkaloids can be classified according to the type of heterocyclic rings into pyrrolidine, piperidine, pyridine, quinoline, isoquinoline, tropane, pyrrolizidine, quinolizidine and indole.

(2) Alkaloid synthesis in plants

Generally plants synthesize alkaloids in young tissues, but not necessarily in the actively growing cells. Ricinine, as an example, is synthesized in germinating seedlings of Ricinus communis beginning at day 5, the alkaloid being mainly found in

the cotyledons and in etiolated seedlings (Skursky and Waller, 1969). The synthesis terminates as soon as the endosperm is consumed, whereas in illuminated plants it continues (Schiedt, et al., 1962). On the other hand, there are clear cases where alkaloid synthesis occurs in older tissues. In Lupines, the synthesis of alkaloids does not start until 2 weeks after germination and it continues until the plants start flowering (Wiewiorowski and Podowinska, 1966). The synthesis of alkaloids has also been well documented (Evans and Patridge, 1957; Evans and Major, 1968; Evans, et al., 1972) in roots. The onset of flowering, in many cases, slows down or stops alkaloid synthesis. Generally the site of highest concentration of alkaloids may not be the site of synthesis.

(3) Role of alkaloids in plants

Regarding the role of alkaloids in plants, very little work has been carried out. Fairbairn and his school (1966, 1967, 1968) investigating the rapid turn over of alkaloids in Conicum maculatum, Papaver somniferum and Atropha belladona suggested that alkaloids are active participants and not the end products in plant metabolism. For example, morphine, after being fed to the poppy plant was converted into new alkaloidal compounds. These compounds are then translocated into the seeds, and there is some evidence that they may play an essential role in the production of viable poppy seeds. Fraenkel (1959) suggested that some alkaloids may serve to protect plants against insects.

For a long time it was believed that alkaloids were the end products of metabolism which were not metabolised any further. But later research has shown that this concept is not generally true. There are many well documented examples of partial or complete breakdown of alkaloids. Willuhn (1966) has reported that the steroid alkaloids in the fruits of Solanum dulcamara are metabolised almost completely as the fruit ripen. Tracer experiments have also demonstrated the metabolism of various alkaloids in their producing organisms; Elymoclavine in Ipomoea rubro-caerulea shows rapid degradation in the leaves, leaves being the primary site of alkaloid synthesis, the leaves have the lowest alkaloid content of all the tissues (Gröeger et al., 1963; Mockaites, et al., 1973)

(4) Ergot alkaloids

Ergot alkaloids are basically indole alkaloids with an Indole nucleus. But ergolines are unique among them in that the "C" ring closure is on the 4th position of indole nucleus. For a long time, the only source of ergot alkaloids was the sclerotium of the fungus Claviceps purpurea (Fries) which grows on rye and which is commonly known as ergot. The ascomycetes of the genus Claviceps grown either parasitically or saprophytically were the commonest sources of alkaloids until 1960. The presence of alkaloids in fungi other than Claviceps species was established only after 1961 when fumigoclavine-A, fumigoclavine-B and festuclavine were isolated from Aspergillus fumigatus by

Spilsbury and Wilkinson (1961). Taber and Vining (1958) had reported that Penicillium roquefortii produced small amounts of clavine alkaloids, and this has been confirmed by Agurell (1964) after isolating cotaclavine from Penicillium chermesinum.

The most surprising source of ergoline alkaloid was discovered by Hofmann and Tscherter (1960) when the seeds of Rivea corymbosa (L.) Hallier f. and Ipomoea tricolor Car. were found to contain lysergic acid amide, isolysergic acid amide and chanoclavine. Der Marderosian (1967) observed that ergot alkaloid containing genera were largely restricted to Ipomoea, Rivea and Argyreia and the common alkaloids were simple lysergic acid derivatives and clavines.

(4) (a) Pharmaceutical importance

Ergot is a drug prepared from the dark purple spur shaped bodies found in the seed head of diseased rye plant. Barger (1931) and Stoll (1952) explored in detail the use of ergot in medicine. Very large doses of ergot alkaloids bring about acute poisoning, the symptoms of which are essentially due to central stimulation. The general symptom, a burning sensation in the limbs, is commonly given a religious significance and hence called as St. Anthony's fire or St. Martial fire to describe the affection.

The ergot alkaloids have a rich and varied history as therapeutic agents and several alkaloids are currently used in

the treatment of miqraine and in the control of Postpartum hemorrhage (Floss et al., 1973). Barger (1931) had reported the lactation inhibitory effect of these alkaloids. Schelesnyak (1954) reported the ability of ergotoxine to inhibit deciduoma formation. Floss et al. (1973) and Meites and Clemens (1972) confirmed ergoline inhibition of lactation, the development of certain hormone-dependent mammory tumors and nidation via an inhibition of Prolactin secretion from the anterior pituatory. Even though the role of Prolactin in human breast cancer is not clear, Salih et al. (1972) have observed that a significant percentage of human breast cancers show Prolactin dependence. Further, Cassady and Floss (1977) have summarised some medicinal uses of ergot alkaloids (Table 1).

Table 1. Medicinal uses of ergot alkaloids

U S E	D R U G
1) Treatment of postpartum hemorrhage.	Ergonovine, Methyl ergonovine
2) Migraine headache	Ergotamine, Dihydro ergotamine
3) Serotonin antagonism	Methyl sergide
4) Treatment of peripheral and cerebral circulation problems	Hydergin, (dihydro ergotoxine) Nicer goline
5) Psychotherapy	LSD-25

(4) (b) Chemistry

All ergoline alkaloids except chanoclavine (Hofmann, et al., 1957) contain the tetracyclic ring structure which has been given a trivial name as Ergoline (Jacobs and Gould, Jr. 1937). Among the several known indole alkaloids, ergolines are unique in that the "C" ring closure is on the 4th position of indole nucleus (Fig. 1).

The biogenetic precursors of the ergoline ring system of ergot alkaloids have been recognised for quite some time (Weygand and Floss, 1963). One part of the molecule is derived from L-tryptophan, which is incorporated with all the carbon and hydrogen atoms and the nitrogen atom of the side chain except the carboxyl group (Fig. 2). Another part represents an isoprene unit derived from Mevalonic acid and a methyl group (Ramstad, 1968; Floss, 1971) transferred from Methionine to the nitrogen atom derived from the amino group of Tryptophan (Baxter, et al., 1964). Isopentenyl pyrophosphate (Van Tamelen, 1953) and its isomer dimethylallyl pyrophosphate (Harley-Mason, 1954) have been recognised as "active isoprene" units which originate from Mevalonic acid (Plieninger, 1958). Birch et al. (1960) and Baxter et al. (1961, 1962) have shown by degradation studies that C-2 of mevalonate becomes C-17 of the tetracyclic ergolines. It has been quite clear that γ, γ-dimethylallyl pyrophosphate reacts with the 4-position of the indole moiety of tryptophan to give rise to 4-(γ, γ-dimethylallyl)- tryptophan (Plieninger et al.,

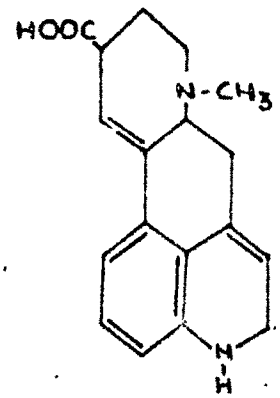
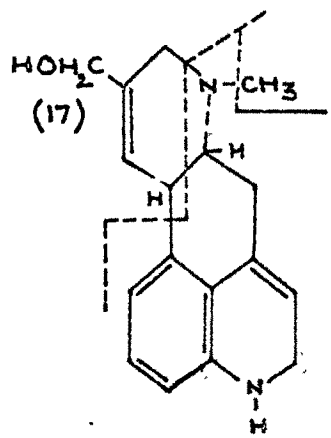
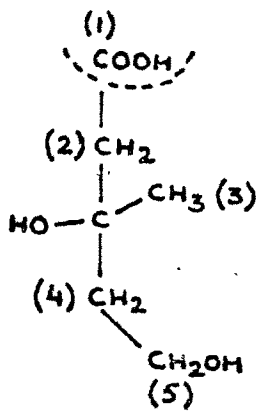


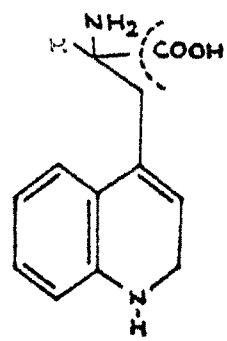
FIG. 1: LYSERGIC ACID



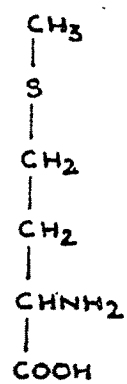
ELYMOCLAVINE



MEVALONIC ACID



TRYPTOPHAN



METHIONINE

FIG. 2: BIOSYNTHETIC ORIGIN OF THE ERGOLINE RING SYSTEM

1964). Plieninger's group (1967) have presented additional evidence for the intact incorporation of this compound into ergot alkaloids. Soon after Robbers and Floss (1968) as well as Agurell and Lindgren (1968) have demonstrated its formation by the ergot fungus under conditions which inhibit alkaloid synthesis.

(4) (c) Biogenesis of ergot alkaloids

Extensive studies on clavine alkaloid interconversions in ergot fungus have been done by Agurell and Ramstad (1962) and accordingly the main pathway of biosynthesis involves hydroxylation of agroclavine to elymoclavine which in turn is converted into lysergic acid derivatives (Mothes et al., 1962) (Fig. 3). The carboxyl group of lysergic acid forms a peptide linkage with an amino group of variety of amino acids or peptide residues to yield the therapeutically useful ergot alkaloids.

The alkaloids produced in ergot fungus are derived from Tryptophan, Mevalonic acid and the methyl group of Methionine (Weygand and Floss, 1963). Arcamone et al. (1962) have shown that various Tryptophan analogs - some of which are effective in other microorganisms as feed back inhibitors of tryptophan synthesis - increase alkaloid formation. Much work has been carried out on tryptophan synthetase, the enzyme which catalyses the reaction -

$$\text{Indole} + \text{Serine} \longrightarrow \text{Tryptophan} + H_2O$$

The tryptophan synthetase activity has been correlated with alkaloid production in fungi.

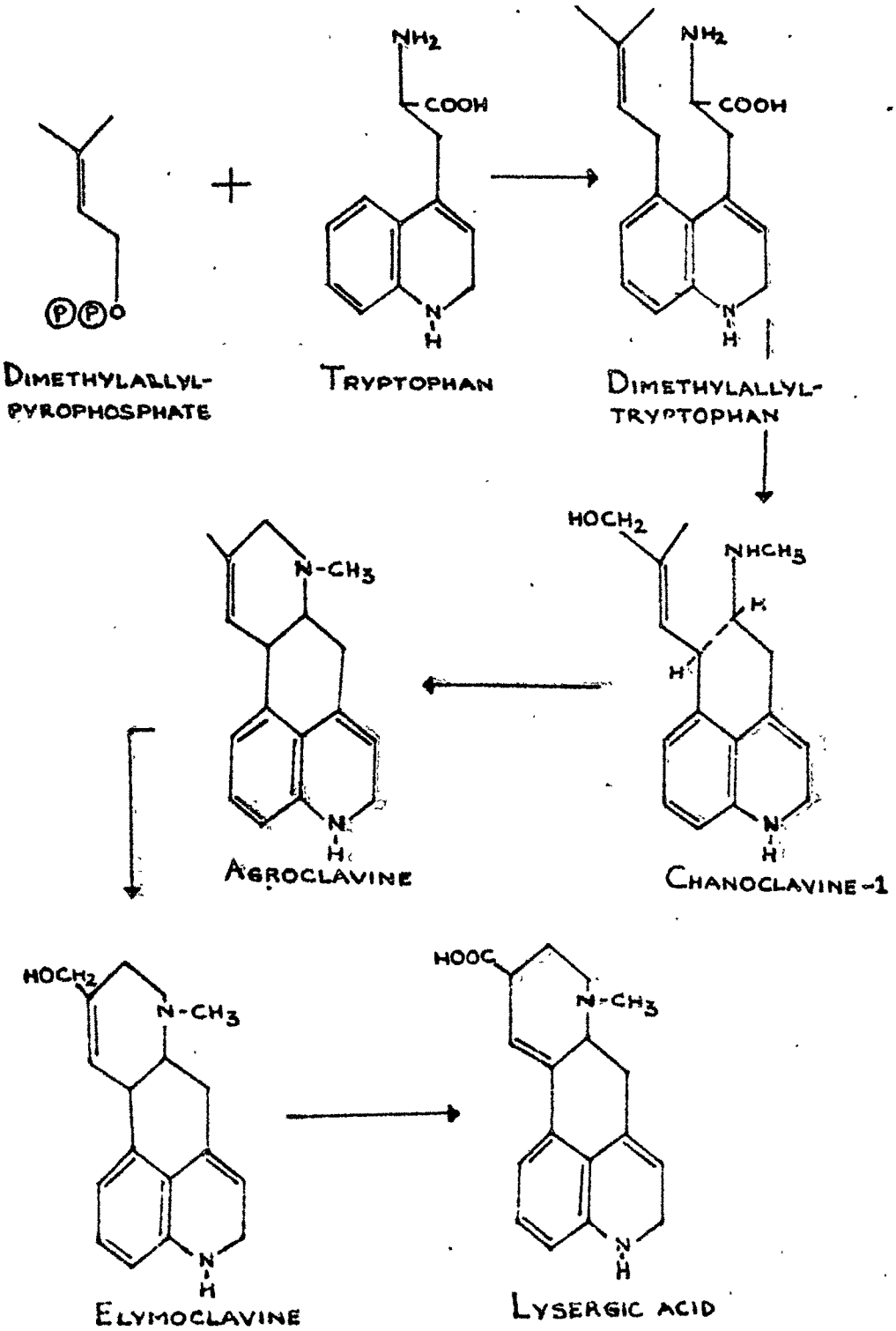


FIG.3: BIOSYNTHESIS OF LYSERGIC ACID

It has been shown for quite some time (Tyler, 1961) that the addition of Tryptophan to cultures of the ergot fungus in many cases increased the alkaloid yield and this had been attributed to its role as an alkaloid precursor. Efforts have also been made to determine the time course of the appearance and disappearance of the tryptophan biosynthetic enzymes in Claviceps during the culture period. Several groups have reported an increase in tryptophan synthetase activity at the beginning of idiophase in alkaloid producing strains (Robbers et al., 1972 b; Schmauder and Gröeger, 1973). In two strains the activity of the enzyme reaches a maximum at the time of most rapid alkaloid formation and then it decreases dramatically. It was also observed that a similar stimulation of alkaloid synthesis could be obtained by adding various tryptophan analogues to the culture.

The effect of Tryptophan was also demonstrated in cultures where alkaloid synthesis was suppressed by increased levels of inorganic phosphate (de Waart and Taber, 1960). Biffi et al. (1959); Prokofieva-Belgovskaya and Popova (1959) and Bernlohr and Novelli (1960) have observed that the production of secondary metabolic products, such as antibiotics by fungi, eubacteria and actinomycetes was commonly associated with slow-growth and with low concentration of orthophosphate in the medium. Similar observations were also made by Taber and Vining (1958, 1959) in relation to production of ergot alkaloids by Claviceps purpurea.

The phosphate inhibition of alkaloid synthesis was overcome

by exogenous Tryptophan, which suggested that it was in some way mediated through Tryptophan (Robbers et al., 1972). Mevalonic acid alone was found to be having no effect and Tryptophan + Mevalonic acid had about the same effect as Tryptophan alone. It was not clearly understood whether the phosphate inhibition of alkaloid synthesis can be overcome by tryptophan analogues. Robbers et al. (1972) obtained very little restoration of alkaloid synthesis with 5-methyl tryptophan; but Gröeger and co-workers (1973) were able to induce alkaloid synthesis in phosphate inhibited cultures with Tryptophan as well as 5-methyl tryptophan.

DMAT synthetase, the first pathway - specific enzyme of ergot alkaloid biosynthesis, has been isolated from mycelia of Claviceps species, strain SD 58. This enzyme catalyzes the formation of DMAT from dimethylallyl pyrophosphate and L-tryptophan (Fig. 4). The formation of DMAT in the ergot fungus have already been demonstrated by Robbers and Floss (1968) and Agurell and Lindgren (1968). Later Heinstein et al. (1971) and Lee et al. (1976) have been successful in isolating dimethylallyl pyrophosphate : L-tryptophan dimethylallyl transferase (DMAT synthetase) from mycelia of Claviceps species, strain SD 58.

There are some reports on the effect of Tweens (surface reactants) on growth and alkaloid production in fungi. Tweens are surface reactants available as Tween-40 (palmitic acid), Tween-60 (stearic acid) and Tween-80 (oleic acid). The positive roles of Tweens on ergot alkaloid production has been established (Mizrahi and Miller, 1969; Řehaček and Basappa, 1971).

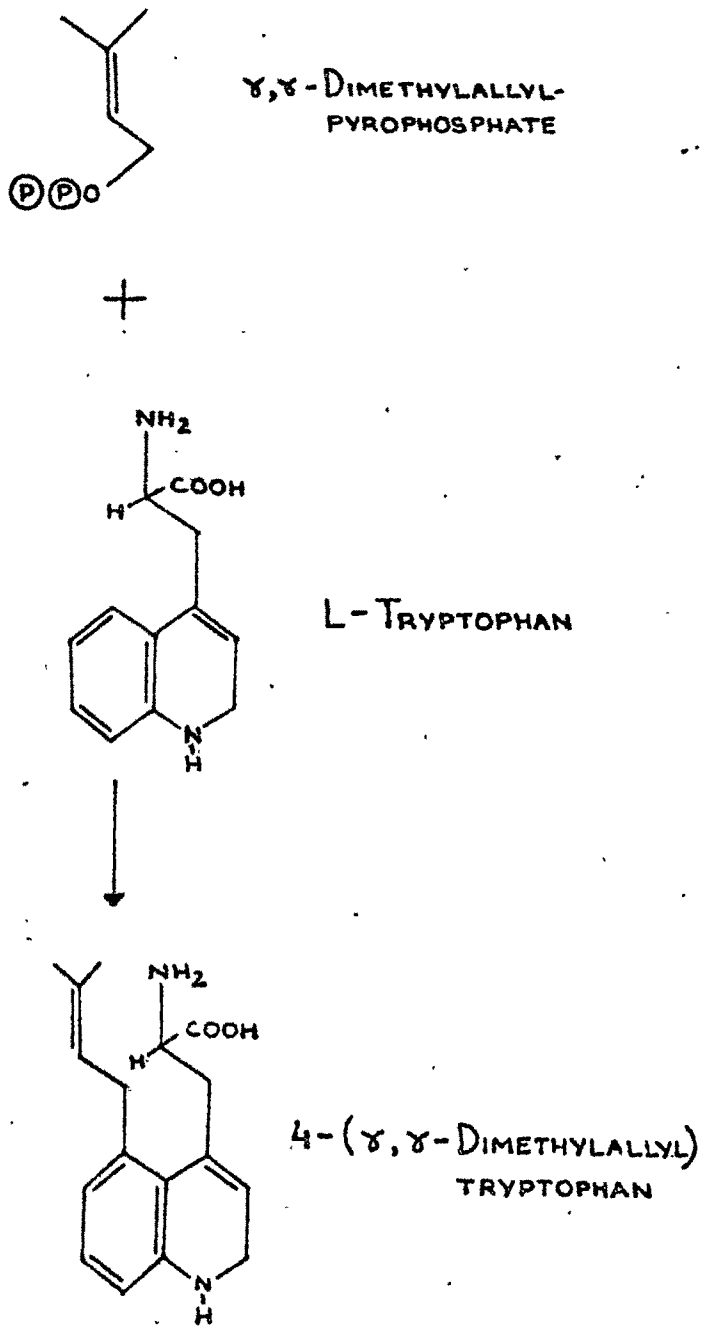


FIG. 1: FORMATION OF DMAT

The above observations with ergot alkaloid production in Claviceps formed the background for present studies with a higher plant. The effects of various above mentioned parameters were examined with tissues isolated from the intact plant.

(5) Plant Tissue Culture as a tool to study alkaloid production

In fungi and other microorganisms the structure of the intact organism is so simple that the entire organism can be cultured in media under defined conditions. Studies of physiological processes like, for instance, the nutritional/ hormonal requirement for growth and differentiation - morphological as well as biochemical - in higher plants were not feasible until the advent of plant tissue and cell cultures. The differentiation can be morphological involving initiation of organ primordia. It could also be biochemical; for the opening up of the particular biosynthetic pathways too is clearly the outcome of a particular kind of cell differentiation (Street et al.,1965). Tissue cultures as an experimental tool is all the more handy with higher plants because, unlike in fungi and microorganisms, in higher plants the synthesis of secondary metabolites generally takes place in one part of the plant, whereas it is stored in another part of the plant. Study of such processes is rather difficult when a cell is developing within a framework of an organised plant body in which there is reciprocity between different parts and gradients of all sorts. A frequently employed technique, therefore, is to remove from the plant,

a group of cells constituting either an entire organ or a particular type of tissue. These cells can then be transferred to a nutrient medium where their growth and nutrition; metabolism and morphogenetic patterns can be examined under a wide variety of deliberately varied conditions. These techniques are collectively referred to as "Tissue Culture".

First attempts in growing isolated plant cells in culture were made by Haberlandt (1902) to resolve the problem of cellular differentiation and inter cellular relationships. However, he failed in maintaining them viable in culture. Since then many attempts have been made notably by Kotte (1922 a, b) and Robbins (1922 a, b) to meet nutritional requirements of cultured cells of diverse types derived from various plant species (Bobilioff-Preisser, 1917; Kemmer, 1928). However, the first ever success was achieved not with isolated cells but with the excised seedling: roots of tomato (White, 1934). This was followed by almost simultaneous successes in growing continuously in culture the tissues from cambial cells under suitable conditions (Gautheret, 1939; Nobecourt, 1939; White, 1939). Since then callus tissues derived from different parts of plants belonging to diverse taxa have been grown on the surface of agar culture media. The latter contain a utilizable sugar, inorganic salts and various organic growth factors (Gautheret, 1959). Such cultures can be serially subcultured by subdivision of the tissue masses provided that the tissue

fragment transferred on each occasion is above a critical size (Caplin, 1954; Mehta, 1965). A definite correlation between the tissue growth attained, on one hand, with the ratio of inoculum size and volume of the medium, on the other, has been established (Henshaw et al., 1966).

The interest and emphasis shifted from the culture of organs and tissues to that of free cells when Steward and Shantz (1955) established the suspension of free cells and cell aggregates by transferring callus pieces to agitated liquid medium. They further demonstrated that such suspension cultures also could be serially propagated in liquid medium. Since then the suspension cultures have been initiated from callus tissues derived from different parts of a wide range of plant species (Mehta, 1965).

Unlike intact plants where there are highly complex problems of interrelationships and interference due to gradients of many kinds, in cell cultures the requirements of growth as well as physiological and biochemical changes associated with growth and differentiation (morphological as well as biochemical) can be examined more precisely under controlled conditions. Suspension cultures derived from callus tissues provide an excellent research tool for determining the precise nutritional requirements and metabolic activities of cells after liberating them from the constraints of the organisms as a whole. Of course, what nutrition, hormones etc., they obtained from their adjoining

organs and tissues when they were parts of a whole, has now to be exogenously supplied.

Carbohydrate requirements of callus culture was first studied experimentally by Gautheret (1945) who examined the ability of various sugars to support the growth of normal carrot tissue cultures derived from the cambial cells of the root. Similar studies were carried out by many researchers working with diverse plant tissue cultures. Dextrose, laevulose and sucrose have been reported as excellent sources of carbon for tissues of marigold, Parisdaisy, Periwinkle, sunflower and tobacco (Hildebrandt and Riker, 1949). Fructose was found to be beneficial in some instances (Nickel and Burkholder, 1950; Hildebrandt and Riker, 1953; Weinstein et al., 1959); but poor in the case of other tissues (Pollard et al., 1961; Tulecke et al., 1962). The above studies revealed that sugars not only provide with carbon skeleton for the synthesis of varied compounds, primary as well as secondary, but also constitute the source of energy for all the metabolic activities (Simpkins et al., 1970). However, of all the sugars tested sucrose was found to be most effective for the growth of the majority of tissues grown in culture. White (1934) had also observed that tomato roots grew ten times more in sucrose medium than glucose. Sucrose as carbon and energy source for majority of plant tissue cultures has been well recognised (Street, 1966; Rao, 1971).

Incorporation of inorganic and organic nitrogenous compounds

in the nutrient medium have marked influence on growth of the tissues. Cultured cells and tissues preferentially use nitrates for their growth. It has been established that cultured plant cells grow preferentially on either ammonium nitrate or potassium and calcium nitrates. However, for many tissues the most suitable inorganic nitrogen source is a balanced supply of ammonium salts with calcium or potassium nitrate (Steward et al., 1958; Filner, 1966; Bhatt et al., 1973).

Besides normal nutritional requirements, growth of plants and their isolated tissues are dependent upon the availability of growth substances (Audus, 1963). They are known to play significant roles in cell metabolism, growth and differentiation (Butenko, 1968). Among these growth substances, auxin is an essential supplement which has to be added to any basic medium for the successful culture of a number of normal callus tissues. The entire plants synthesize required amount of auxin to enable them to grow and develop and complete their life cycle. But isolated cells and tissues synthesize auxins in suboptimal levels, needing an additional supply of auxin in nutrient medium for their continuous growth in culture (Reinert and White, 1956; Paris and Duhamet, 1958; Gautheret, 1959).

Many callus cultures and most suspension cultures also require other complex growth factors such as coconut milk and yeast extract (Street, 1966). Coconut milk, yeast extract, etc. are found to act synergistically with auxins and enhance growth

considerably (Steward and Shantz, 1955; Torrey and Shigemura, 1957; Mehta, 1966; Rao and Mehta, 1968). These complex supplements, however, limit the usefulness of tissue culture technique when analysis of chemical changes is required. Hence many synthetic media are evolved to replace the above complex supplements. Cytokinin, an another class of growth substances of which kinetin is the most widely used, effectively substitute the complex growth substances of incompletely known composition in stimulating growth (Miller, 1961; Butcher and Street, 1964).

All these aspects formed the background for the present study. The main objective behind the present work is to find out if any correlation can be made between the mechanism of ergot production in a fungus and in higher plants. Vast work has been carried out in Claviceps species and other related microorganisms for ergot alkaloids. But very little work has been carried out in higher plants. Only the presence of these alkaloids are observed in a few plants; e.g. Hofman (1961) observed the presence of these alkaloids in a few plants belonging to the family Convolvulaceae. A few ergoline alkaloids detected in Ipomoea purpurea, Ipomoea violacea and Argyreia nervosa include isoergine, penniclavine, ergometrinine and Ergine. However, beyond the screening of some plants for the presence of ergoline alkaloids, no further work is carried out in higher plants.

Keeping in view the above lacunae, a few plants like Ipomoea purpurea, Ipomoea quamoclit, Argyreia speciosa and

Evolvulus alsinoides were screened for the presence of these alkaloids. Of these, Evolvulus alsinoides showed the presence of ergot alkaloids and hence this plant was selected for further studies. Earlier Varadan et al. (1958) have reported the presence of alkaloids in Evolvulus alsinoides. In soxhlet extraction they detected a new alkaloid and named it as Evolvine. This observation, however, remains to be confirmed as our present studies clearly showed the presence of two specific ergot alkaloids and also different indole derivatives.

Besides, it is medicinally important. It is used in dysentry, bronchitis, epilepsy, leucoderma and for apetite in indigenous Indian medicine. The plant is bitter, pungent and anthelmentic. It is used as a febrifuge with cumin and milk. It is also used as a tonic and vermifuge. Leaves are made into cigarettes and smoked in chronic bronchitis and asthma. It is also used in teething of infants. It brightens the intellect, improves complexion and apetite. It is believed that this plant has the power of strengthening the brain and memory (Chopra, et al., 1956; Kirtikar and Basu, 1975).

In vivo studies with Evolvulus alsinoides indicated the presence of these alkaloids in very small quantities. Callus was, therefore, initiated from the young leaves so as to explore the feasibility of increasing the production of alkaloids by varying the nutritional status and hormonal supplements to the medium and also by feeding the cultures with known precursors.

Possible role of enzyme tryptophan synthetase in the regulation of alkaloid production was investigated. The results obtained in this pursuit are incorporated in the present thesis under the following broad headings :-

(A) "In vivo" studies on alkaloid production in Evolvulus alsinoides L.

(B) Initiation and establishment of callus from Evolvulus alsinoides leaves.

(C) Influence of auxins and cytokinins on callus growth and alkaloid production.

(D) Influence of inoculum : Volume ratio on growth and alkaloid production.

(E) Effect of various sugars and different levels of sucrose on growth and alkaloid production and also on tryptophan synthetase activity.

(F) Effect of total nitrogen concentration on growth and alkaloid production.

(G) Effect of different levels of microelement and macro-element salts on growth and alkaloid production.

(H) Effect of Tweens on growth and alkaloid production.

(I) Effect of precursors on growth, alkaloid production and tryptophan synthetase activity.

(J) Studies on amino acids during the course of culture.

(K) Phosphate inhibition of alkaloids, and its control by Tryptophan and its analogs.

(L) Influence of cofactors of DMAT synthetase on growth and alkaloid production.

(M) Changes in growth and alkaloid production with passages in culture.

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