CHAPTER I

I N T R O D U C T I O N

INTRODUCTION

Recent advances in plant tissue culture raise the possibility of using aseptically cultured higher plant cells for the production of metabolites by standard microbiological methods. Clearly these advances also open up possibilities for studying both metabolic pathways and the genic control in plant cells, and the intrinsic and extrensic factors which control the growth and differentiation of the tissue cells of higher plants (Street, 1963).

The advances which would follow from the successful establishment of cultures of isolated cells of higher plants (free cell cultures) were first formulated by Haberlandt in 1902. From that date and for a number of years, Haberlandt and his students made many unsuccessful attempts to determine the special cultural requirements of cells isolated from higher plant organs. The first successful plant tissue cultures were, however, not such free cell cultures but cultures of isolated seedling roots (White, 1934) and, very quickly following this, of the callus tissue which arise from cambial tissue in response to appropriate chemical stimuli (Gautheret, 1939; White, 1939).

Callus tissues derived from many plants can now be grown on the surface of agar culture media which 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 (Mehta, 1965).

Several groups of workers have successfully dispersed and grown such callus tissues in agitated liquid medium. Steward and Shantz (1955) established by 'chance', the suspension of free cells and cell aggregates by transferring callus pieces to agitated liquid medium. They further demonstrated that such suspension cultures could be serially propogated 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).

There are many useful things which can be done with such suspension cultures as they exist to-day. One such important use which is receiving much attention of late, is in studying the problems of growth, development and associated physiological and biochemical changes (Staba, 1969). Unlike intact plants, where there are

highly complex problems of interrelationships and interferences due to gradients of many sorts, in cell cultures the requirement of growth as well as physiological and biochemical changes associated with growth could be examined more precisely under controlled conditions. It was with this idea that the present studies on growth and its underlying physiological processes and biochemical changes were initiated.

The importance of cell suspensions for biochemical studies is clearly evident from the past success achieved in understanding intermediary metabolism using bacterial cell suspensions and in studies of photosynthesis using algae cell suspensions. Similarly, the isolated plant cells or cell suspensions produced in culture have been used in a variety of problems, including the study of cell division (Muir et al., 1954; Torrey, 1957; Steward et al., 1958), susceptibility of tissues to virus infection (Hilderbrandt, 1958; Bergman, 1959), cellular differentiation (Steward et al., 1958), the production of cell metabolites (Tulecke and Nickell, 1960), relationship between growth, respiration and protein synthesis (Givan and Collin, 1967) and relationship between cell division, cell expansion and cell separation (Henshaw et al., 1966). Still many problems

remain untouched. The production of many plant cell suspensions has been achieved mainly with complex media containing coconut milk, yeast extract or others which limit the usefulness of the technique where analysis of chemical changes is desired. Considerable progress has been made by several workers (Torrey and Reinert, 1961; Henshaw et al., 1966; Stuart and Street, 1969; Raj Bhandari et al., 1969) in evolving simple, completely defined, synthetic media for studying growth, cell differentiation and associated physiological changes. Taking advantage of these reports, a completely defined medium has been evolved, in the present studies, by avoiding coconut milk for studying growth, morphogenesis, the production of secondary plant products and closely related enzymes, in Datura, Cassia and cotton tissues.

The carbohydrate requirement of callus cultures was first studied by Gautheret (1945) who examined the ability of various sugars to support the growth of carrot cultures. Similar studies, involving tissues from a number of dicotyledons, were subsequently made by Hilderbrandt and Riker (1949, 1953), Arya et al. (1962), Simpkins et al. (1970), Rao and Mehta (1968) and Fadia and Mehta (1973). These studies revealed that

most tissues grow best when supplied with sucrose, dextrose or laevulose as carbohydrate source.

Nitrates are most commonly used by tissues grown *in vitro* as source of nitrogen for their growth. Substitution with ammonium salts or nitrates has not been very successful (Riker and Gutsche, 1948; Heller, 1953, 1954). However, a balanced supply of nitrates and ammonium salts has been shown to support better growth of certain callus cultures than the nitrates alone (Steward *et al.*, 1958; Shantz and Steward, 1959; Bhatt, Fadia and Mehta, (in press). Effects of different amino acids as source of nitrogen on growth and metabolism have been discussed in detail by Street (1966). Casein hydrolysate either acid or enzyme hydrolysed has also been used extensively in tissue culture media, but with varying results (Nitsch and Nitsch, 1957; Staba, 1962; Steinhart *et al.*, 1962; Arya, 1965; Vasil and Hilderbrandt, 1966; Tiwari and Arya, 1967).

Besides normal nutritional requirements, growth of plants and their isolated tissues is dependent upon the availability of growth substances (Audus, 1972). They are known to play important role in cell metabolism, predetermining the course of cell division, growth,

differentiation and cell hypertrophy (Butenko, 1968). Of these growth substances, auxins are the most important supplements which need to be added to basic medium for the successful culture of normal callus and suspension cultures. The plants synthesize sufficient auxin to enable them to grow and develop, completing the normal life cycle. But isolated tissues and cells synthesize auxin in suboptimal amounts needing an additional supply of auxin in nutrient medium for their continuous growth in culture.

Over and above auxin, for many callus cultures, and for most suspension cultures, the culture medium must also contain vitamins and other reduced nitrogen compounds. Vitamins particularly thiamin and pyridoxine are reported to be essential and stimulatory for the growth of the plant cultures. Both these vitamins are involved as prosthetic groups or co-enzymes in enzyme systems essential to all living cells (Lohaman and Schuster, 1937; Lichstein, Gunsalus and Umbreit, 1945; Racker, de la Haba and Leder, 1953). Attempts have been made, therefore, to incorporate these vitamins in the synthetic medium for successful growth of callus and suspension cultures.

Many synthetic media which are evolved for the successful culture of plant cells contain cytokinins, another class of growth substances. Kinetin which is the most widely used cytokinin is known to possess growth stimulating properiety (Miller, 1961). Incorporation of kinetin into nutrient medium has led to substantial enhancement in growth of a number of callus and suspension cultures (Murashige and Skoog, 1962; Steward, 1969; Lalchandani, 1970). In addition to its growth promoting properiety, kinetin has also been used in many physiological and biochemical investigations. Letham (1967) has recently reviewed the effects of kinetin on various aspects of growth and metabolism. Kinetin treatment affects the nucleic acid synthesis, particularly that of RNA which in its turn affects the protein synthesis and also other physiological process like morphogenesis and scenescence (Patau et al., 1957; Kuraishi, 1959; Osborne, 1962; Osborne and Susan, 1963; Bhatt and Mehta (in press) . Kinetin is also shown to affect enzyme activities in excised plant parts and cultured plant cells (Lavee and Galston, 1968; Lee, 1972).

Several reviews on the possible adaption of plant cell cultures as an important tool for the production

of useful compounds by industry appeared in early sixtees, among them notably are by Nickell (1962), Staba (1963) and Street (1965). The question whether there were opportunities for the culture of plant tissues on an industrial scale was posed again in 1966 by Tulecke, who answered it hopefully by stating thus: 'if tissue culture as a technique continues the same rate of advance as in recent years, it is reasonable to predict that some commercial use will be made in the next decade'.

The callus and suspension cultures derived from plants which synthesize particular secondary plant products such as alkaloids, glycosides and saponins have, in a number of instances, been shown to synthesize these characteristic compounds and even to release them into their culture medium (Dawson, 1942; Solt, 1954; West and Mika, 1957; French and Gibson, 1957; Raj Bhandari et al., 1969; ~~Staba and~~ Kaul and Staba, 1969; Tabata et al., 1971). In many cases it was also shown that the qualitative and quantitative performance of the tissue in forming these secondary plant products may be greatly influenced by cultural conditions (Siegelman, 1964; Staba, 1969; Mehta and Staba, 1970;

Tabata et al., 1971; Davis, 1972). Thus if a higher plant synthesizes some particular chemical of interest to the pharmaceutical industry then it would seem likely that a cell culture will exhibit, under appropriate cultural conditions, the same synthetic capacity. Certain class of plant constituents e.g. resins, tannins, volatile oils may, however, be produced in certain highly localized cells of the plant body. The opening up of particular biosynthetic pathway is then clearly the outcome of a particular cell differentiation. If this is so then to obtain such a constituent from cultured cells we may have to learn both how to grow the cells in culture and how, subsequently to convert them into differentiated cells with the appropriate biochemistry. Of course, if we get this far by combinations of cultural treatments and selected mutants we may be able to improve upon nature and achieve a higher rate of biosynthesis than ever occurs per cell per unit of time in the plant body (Street, et al., 1965).

In the present study a survey for total polyphenols was made in tissue cultures of Datura and Cassia. Nutritional and hormonal effect on polyphenol production in cultured tissues was further examined under different cultural conditions.

In the field of phenylpropanoid biosynthesis most attention has been concentrated on the formation of anthocyanins in a range of pigmented tissues (Blakeley and Steward, 1961; Reinhard, 1967; Constable et al., 1971). Light appears to be of major importance for pigment formation in such tissues, but the extent and timing of the accumulation can also be profoundly influenced by the composition of the growth medium. In Haplopappus gracilis anthocyanin accumulation was inhibited or delayed at elevated auxin concentrations (Constable et al., 1971). Anthocyanins in cultured red cabbage embryos were influenced by the nitrogen/carbohydrate ratio (Szweykowska, 1959).

Polyphenol production in non pigmented tissue cultures has received rather less attention. Flavone glycoside synthesis in suspension cultures of Petroselium hortense has been reported to be light dependent (Halbrock and Wellman, 1970), and the synthesis of a range of polyphenolic material in tea callus cultures was increased on illumination (Forrest, 1969). Tannin synthesis in Juniper callus cultures was reported to be enhanced at high sugar concentrations (Constable, 1968). Deoxyisoflavone synthesis in soyabeen callus tissue was found to be stimulated by 2,4-D and NAA (Miller, 1969). The nicotine

production in tobacco callus cultures was reported to be enhanced by kinetin treatment associated with bud formation (Tabata et al., 1971).

Callus and cell suspension cultures derived from certain species of Atropa, Hyoscyamus and Datura (Solanaceae) have been examined for ability to synthesize tropane alkaloids contained in the original plants (West and Mika, 1957; Chan and Staba, 1965; Staba and Jindra, 1968; Raj Bhandary et al., 1969; Tabata et al., 1969; Konoshima et al., 1970). All these studies indicated that "dedifferentiated" tissues or cells propogated in synthetic culture media produce little or no alkaloids. It was reported that nicotine production in callus cultures of "Bright yellow" tobacco can be regulated by growth regulators and that it is initimately associated with bud formation, a differentiation phenomenon, induced in the cultures (Tabata et al., 1971). Further examination of the tissue cultures of Scopolia parviflora has indicated an intimate relationship between organogenesis and alkaloid formation and furthermore suggested a possible utilization of morphogenetic ability of cultured tissues for efficient production of useful alkaloids (Tabata, et al., 1972). These reports prompted us to examine the relationship between morphogenesis

and production of polyphenols in Cassia callus cultures under different hormonal interactions.

Both auxins and cytokinins are shown to affect enzyme activities in excised plant parts and cultured plant tissues (Morel and Dematriades, 1955; Turian, 1956; Lavee and Galston, 1968; Vajranabhaiah, 1969; Lee, 1972). Morel and Dematriades (1955) have reported that IAA and 2,4-D enhanced the activity of peroxidase and polyphenol oxidase in cultured Jerusalem artichoke tissue.

One particular interest in connection with auxin is the auxin destroying enzyme Indoleacetic acid oxidase (IAA oxidase). Thimann (1934) noted that some plant tissues could cause the disappearance of natural auxins, and an enzyme preparation which carried out the oxidative degradation of IAA was separated from peas by Tang and Bonner (1947). Though number of such enzymes have been separated and partly characterised, no unified concept of IAA degrading enzyme has emerged. Tang and Bonner suggested that the pea enzyme was an iron enzyme, probably a peroxidase. Galston et al. (1953) believed it was a peroxidase linked to a flavin. An ability of tyrosinase to destroy IAA has been described by Briggs and Ray (1956).

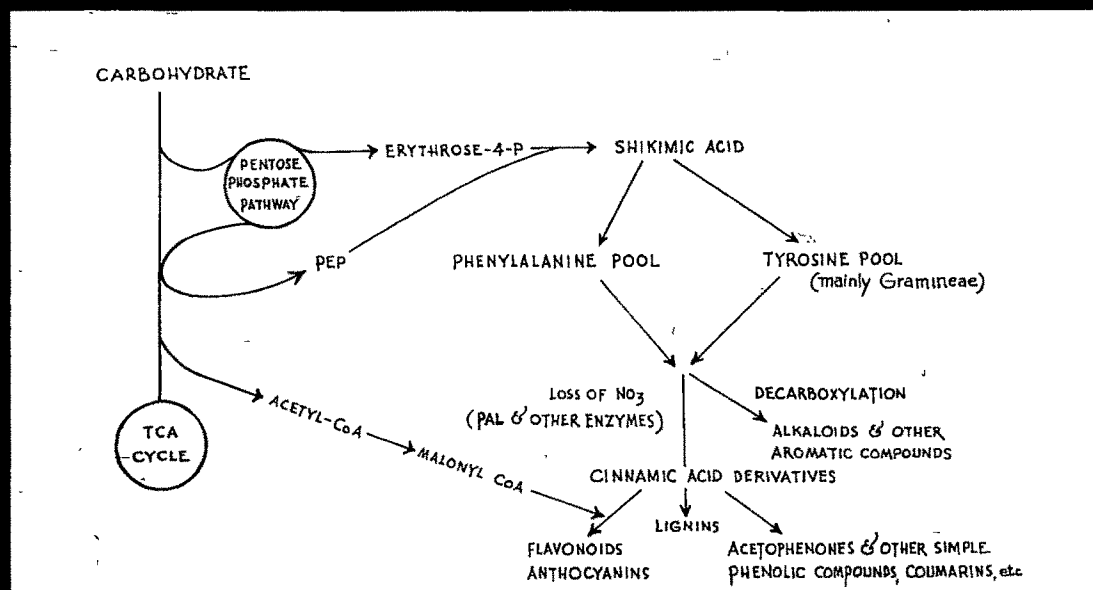
Goldacre et al. (1953), Stutz (1957) and Ray (1958) have demonstrated that peroxidase catalizes the oxidation of plant growth hormone Indoleacetic acid (IAA). Conversely, it is also reported that IAA altered the activity of peroxidase in tissues (Galston and Dalberg, 1954; Lavee and Galston, 1968). Audus and Bakhash (1961) suggested that a mutual interaction between hormone and enzyme may be important in the regulation of plant growth. It is also believed that IAA oxidase activity is a special aspect of peroxidase activity and it is further known that peroxidase is an essential part of IAA oxidase system as it is reported to catalize the oxidation of IAA (Galston et al., 1968). Though IAA oxidase and peroxidase systems have a major role to play in the regulation of growth and differentiation, the exact mechanism has not been well understood. Lee (1972) working with the isoenzyme patterns of peroxidase and IAA oxidase enzyme systems in tobacco tissue cultures, under the influence of different growth regulators, believed that specific peroxidase isoenzymes rather than the level of total peroxidase appear to have a role to play in promotion or inhibition of growth.

The enzyme peroxidase can be implicated in several reactions related to phenolic biosynthesis. Thus horse-

radish peroxidase will catalize the hydroxylation of a number of aromatic compounds in the presence of oxygen and dihydroxyfumaric acid (Mason et al., 1957). Peroxidase is also considered by several authors to be involved in lignin biosynthesis (Freudenberg et al., 1958; Higuchi and Ito, 1958; Freudenberg, 1959). The findings of Mazelis (1962) revealed that peroxidase in the presence of Mn^{++} and pyridoxal phosphate catalyzed the oxidative decarboxylation of amino acids and may well have significance in the biosynthesis of certain phenolic compounds. With this background, IAA oxidase and peroxidase activities were investigated in the present studies under different cultural conditions, and related to polyphenol synthesis.

Another enzyme which plays an important role in the biosynthesis of phenylpropanoid compounds is phenylalanine ammonia-lyase (PAL). There is considerable body of evidence as reviewed by Grisebach (1968), that PAL is responsible for the deamination of phenylalanine, an obligatory intermediate in the biosynthesis of phenylpropanoid compounds in higher plants (Chart I)

Whatever the merits of the case for PAL as the sole channel for phenylpropanoid biosynthesis, it is



PROBABLE MAJOR BIOSYNTHETIC PATHWAYS LEADING TO PHENOLIC COMPOUNDS (After Neish, 1964)

CHART I

clear that many tissues respond in a consistent way to a variety of environmental stimuli by modifying the activity of this enzyme (Davies, 1972). Light induced increases in PAL activity have been reported for a variety of tissues including etiolated seedlings (Durst and Mohr, 1966; Amerhein and Zenk, 1971), potato tuber discs (Zucker, 1965) and cultured parsley cell suspensions (Halbrock and Wellman, 1970). The other factors shown to regulate PAL activity include - ethylene (Hyodo and Yang, 1971), Y-irradiation (Riov et al., 1968), UV light (Hadwiger and Schwochau, 1971b), gibberellic acid (Cheng and Marsh, 1968) and a variety of DNA intercalating agents including actinomycin-D (Hadwiger and Schwochau, 1971a). In the present studies, the effects of auxin and carbohydrate concentrations on PAL enzyme patterns are further examined.

There is ample evidence now that many tissues and cells growing in culture release compounds into culture medium (Street et al., 1965). The release of enzymes into nutrient media by tissue cultures of higher plants is also reported by several workers (Gentile, 1965). Brakke and Nickell (1951) reported the release of α-amylase by tissues of virus-induced tumor from the roots of Rumex

acetosa; Tulecke (1963) described the extracellular degradation of arginine by arginine degrading enzymes in tissue cultures derived from the pollen of Ginkgo biloba L. Maretzki et al. (1971) explained the extracellular hydrolysis of starch, by following the secrets of starch hydrolysing enzymes into medium in sugarcane suspension cultures. Straus and Campbel (1963) reported the presence of peroxidase, IAA oxidase, acid phosphotase and amylase activity in plant tissue culture media. The findings presented in this thesis provide additional data which indicate that the release of enzymes by tissue cultures of higher plants is of common occurrence.

All the above mentioned aspects formed the background for the present studies. The results obtained are incorporated under the following broad heads:-

I. (a) Initiation and establishment of cell suspensions from callus tissues in completely defined medium;

(b) Growth and nutritional studies of the tissues in defined medium.

II. Studies on the changes in polyphenol content in cell suspensions of Datura and callus cultures of Cassia and cotton under varied cultural conditions.

III. Studies on the progressive changes in Peroxidase, Indoleacetic acid oxidase (IAA oxidase) and Phenylalanine ammonia-lyase (PAL) enzymes during growth and their correlation with the synthesis of polyphenolics in *Datura* and *Cassia* tissue cultures.

Examination of the release of Peroxidase and IAA oxidase enzymes into the nutrient medium under different cultural conditions was also undertaken.