CHAPTER VI

GENERAL DISCUSSION

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The main objectives of the present investigation, as stated in the Introduction (Chapter I), were to establish the callus and suspension cultures of Datura metel L., Cassia fishtula L. and a hybrid variety ('Sankar 4') of cotton, Gossypium hirsutum L., on a completely defined medium. Besides examination of growth, studies were also designed to investigate the physiological and biochemical changes associated with growth and polyphenol production in the above tissues subjected to different cultural parameters. Correlation, if any, with certain allied enzymes was also attempted. To what extent the evidences obtained in the present investigation have realised the aim now remains to be assessed.

It was clear from the results obtained in Experiment 3-1 that Datura anther callus which was originally initiated and maintained on white's complex (coconut milk containing) medium grew fairly vigorously when transferred onto a completely defined medium (Table 3, Chapter II). The growth of the cell suspensions obtained later by transferring the callus pieces to agitated liquid medium was much more rapid as compared to that of the cultures on solid medium. The maximum growth of cells

in suspension was achieved in 15 days and the growth when plotted against regular intervals of time showed a clear sigmoid curve. After prolonged lag phase, growth was most pronounced between 10 and 15 days during which there was maximum increase in fresh and dry weights of the cells.

The completely defined (modified MS) medium (Table 3, Chapter II) was also found to support maximum growth of the callus tissues of Cassia and cotton originally initiated and maintained on complex medium (Experiment 3-3). When a comparison of the growth dynamics of both the tissues was made, it was observed that the lag phase in cotton tissues extended upto day 5, followed by a rapid growth phase which extended upto day 15. On the other hand, in Cassia callus cultures the growth showed marked raise from the beginning and it registered steady increase during the course of culture for 20 days. Final growth recorded after incubation for 20 days was higher in cotton tissues as compared to that in Cassia (Experiment 3-4).

The pattern of growth and the final growth values (Final wt./Initial wt.) observed in the above three

tissues compared quite favourably with those of most other plant tissues cultured on chemically defined media (Street, 1966).

Studies on carbohydrate requirements of Datura cells showed that sucrose was the most suitable as it supported the highest growth of tissue measured in terms of increase in fresh and dry weights (Experiment 4-1). Glucose and fructose supplied individually and together in equimolar concentration supported about equal amount of growth; while maltose and starch were found to be poor energy sources. Of the sucrose concentrations tested, 2% was found to be optimal for growth (Experiment 4-2).

Since Gautheret (1945) studied experimentally the carbohydrate requirement and critically examined the ability of various sugars to support growth of carrot root callus, similar studies involving number of normal and tumor tissues were made by many workers (Nickell and Burkholder, 1950; Street, 1957; Tiwari, 1968). Dextrose, laevulose and sucrose at concentrations from 0.5 to 4.0 per cent were reported excellent sources of carbon for tissues of normal and crown gall of marigold, paris-daisy, periwinkle, sunflower and tobacco hybrid (Hilderbrandt and Riker, 1949, 1953). Further, out of sixteen carbon

sources tested for growth of spruce cells, 0.5 per cent sucrose was reported best by Risser and White (1954). Fructose was proved beneficial in some instances (Hilderbrandt and Riker, 1953; Weinstein et al., 1959) but poor in the case of other tissues (Pollard, Shantz and Steward, 1961; Tulecke et al., 1962).

The studies referred above indicate that though, by and large, sucrose was preferentially utilized, callus tissues derived from different plant species differ in their ability to utilize carbohydrates. Such differences have been reported to occur even in closely related callus cultures as illustrated by the difference in response to added sugars shown by single - cell clones derived from a single - parent culture (Arya et al., 1962).

Examination of growth responses to different levels of 2,4-D at a fixed concentration of kinetin and also to different concentrations of kinetin at a fixed level of 2,4-D showed that the combination of 2.0 mg/l 2,4-D with 0.4 mg/l kinetin supported the highest growth of Datura cultures (Experiment 4-3, 4-4). The suggestion that the effect of kinetin was additive to that of auxin for stimulating the growth of the tissues was made by Steinhart (1961) and Digby and Wareing (1966) working with spruce and sycamore cultures respectively.

Examination of the effect of presence or absence of light at two auxin levels in Experiment 4-10 revealed that high level of auxin ($5x10^{-5}M$) supressed growth both in dark and in light, the inhibition being more pronounced in the latter. Even at optimal auxin level, light of high intensity had adversely affected growth. The inhibitory effect of high light intensity on growth, however, was overcome by the presence of GA_3 in the medium as observed in Experiment 4-11. Similar antagonism between GA_3 and light effects was described by Lockhart (1956). Light inhibited the growth of etiolated peas and gibberellin application promoted growth to about the same extent.

Studies on the nitrogen requirement in completely defined medium showed that the standard culture medium containing both ammonium nitrate and potassium nitrate as nitrogen sources supported maximum growth of Datura cell suspensions (28 fold increase in fresh weight). Of the individual nitrates tested in single dose, ammonium nitrate enhanced growth considerably more; doubling the level of ammonium nitrate, however, had no significant effect on growth.

Casein hydrolisate had been shown to be capable of meeting the nitrogen requirement of some cultured

tissues such as sunflower crown gall tissue (Riker and Gutsche, 1948) maize-endosperm tissue (Straus and La Rue, 1954; Tamaoki and Ullstrup, 1958), mature embryos of Hordeum (Harris, 1956) and Cucumis callus cultures (Fadia, 1971). In the present studies, however, the substitution with casein hydrolisate as sole organic source of nitrogen did not prove as effective as the balanced supply of ammonium and potassium nitrates (Experiment 4-5).

It was clear from the experiments described in Chapter IV that polyphenols could be detected in measurable quantities in Datura cell culture. The highest accumulation of polyphenol content was registered (during 5 to 10 days) before the cells embarked upon the phase of most rapid growth (10 to 15 days). It is now well established that rapid growth as measured by increase in fresh and dry weights of the tissue is preceded by active cell proliferations (Torrey et al., 1962; Mehta et al., 1967) which in the case of Datura cells corresponds with the period of maximum polyphenol synthesis. This, however, differs from the observations made by Davies (1972) in Paul's Scarlet rose cell cultures. Examining the pattern of accumulation of polyphenolic compounds in relation to

growth of the tissues he found that polyphenol accumulation was essentially restricted to late and post exponential growth phases.

The superiority of sucrose, from amongst the different sugars tested, for the maximum production of polyphenolics was established from Experiment 4-1 and 4-2. With the increase in the level of sucrose in the medium there was enhanced polyphenol accumulation in the tissues (Experiment 4-2), clearly indicating the availability of sucrose as a limiting factor in polyphenol production. Working with rose cells, Davies (1972) has also demonstrated a close correlation between the cessation of polyphenol synthesis and exhaustion of the medium with respect to available carbohydrate. Comparison of the fold wise increase in growth and polyphenol content on day 15 clearly showed that increase in sucrose level influenced polyphenol production more markedly than it did growth.

It is reasonable to support that the universally occurring aromatic amino acids are made from the carbohydrates, following essentially the same pathway in higher plants as in microorganisms in which it was worked out in great detail by Davis (1958). Further, there is good evidence that phosphoenolpyruvate (PEP)

and D-Erythrose 4-phosphate (E-4-P) required for the synthesis of shikimic acid are formed in higher plants (Neish, 1960). The well established Emoden-Meyerhof-Parnas (EMP) pathway produces PEP, while E-4-P required for the synthesis of shikimic acid is formed in pentose phosphate pathway as shown in the chart in Chapter I. Shikimic acid is of wide spread occurrence and some enzymes of its pathway have already been found in higher plant cell cultures (Gamborg, 1966; Zucker, 1972). Furthermore, experiments using tracer techniques have shown that shikimic acid is readily converted to a variety of aromatic amino acids in number of plant species. In light of above it is reasonable to realise the role of sucrose in the enhancement of the synthesis of aromatic amino acids which are now established as precursors for the formation of phenolic compounds.

Plant growth substances are known to influence the production of secondary metabolites (Furuya, 1967; Staba, 1969). In the present studies, effects of 2,4-D supplied at different concentrations on polyphenol production in Datura cell suspensions were examined in Experiment 4-3. The results clearly showed that the effect of raising the auxin level had delayed the initiation of polyphenol

synthesis. In absence of 2,4-D, however, the polyphenols accumulated even during the initial 5 days of cultures. Rapid polyphenol accumulation from 5 to 10 days was followed by high growth rate during the subsequent period of 10 to 15 days at all auxin levels. 2,4-D level optimal for growth (10^{-5}M) also promoted maximum polyphenol production; while like growth the polyphenol accumulation was adversely affected at sub- and supra-optimal concentrations, suggesting that to a limited extent the polyphenol synthesis can be controlled by auxin concentration in the medium.

The observed ability of increased auxin level to repress the synthesis of phenolic compounds in Datura cells is similar in many respects to the reported effects of naphthalene-acetic acid (NAA) on anthocyanin synthesis in cultured Haplopappus cells (Constable et al., 1971). Earlier Blakely and Steward (1961) had found by microscopic observation of Haplopappus cells that anthocyanin accumulation depended on the auxin concentration of the medium containing coconut water as an essential ingradient. Other reports on the effect of hormonal levels on phenylpropanoid synthesis included the observations of Berlin and Barz (1971) that the substitution of either NAA or IAA for 2,4-D led to a marked increase in coumesterol accumulation in

suspension cultures of Phaseolus aureus. The inhibitory effects of high auxin concentrations on the production of polyphenols as observed in the present investigations and also by Davies (1972) is apparently not limited to phenylpropanoid derivatives; since other secondary metabolites e.g. nicotine accumulation in tobacco callus cultures has been reported to be inhibited by high concentrations of IAA (Tabata et al., 1971).

Contrary to 2,4-D effects kinetin concentration has no pronounced delaying influence in the initiation of polyphenol production. The maximum polyphenol accumulation was attained at the same kinetin concentration ($2x10^{-6}$M) which supported highest tissue growth. Supra-optimal kinetin levels, however, supressed growth of the tissue without any adverse effect on polyphenol production. Similarly, in absence of kinetin there was less adverse effect on polyphenol production than on growth; which seemed to suggest that kinetin had less direct role in the regulation of polyphenol production in Datura cells. However, the picture obtained in the case of Cassia callus cultures was different (Experiment 4-12, Table 18) when IAA was used instead of 2,4-D to enhance morphogenetic responses.

Examination of interaction between light and auxin on polyphenol production in Datura cells in Experiment 4-10 clearly indicated that light stimulated the polyphenol synthesis; the stimulatory effect of light being more pronounced at high auxin ($5x10^{-5}$M 2,4-D) level than at its low (10^{-5}M) dose. Furtner, the inhibitory effect of high auxin ($5x10^{-5}$M) level on the production of polyphenolics was completely reversed by light.

The gibberellin effects on polyphenol production, on the other hand, was quite different from that of auxin (Experiment 4-11). In low concentrations (10^{-6}M), GA_3 enhanced polyphenol production in presence and absence of light; while higher levels of gibberellin markedly reduced polyphenol production both in light and dark. This clearly indicated that the GA_3 effect varied with the concentration and was independent of light. However, a general unfavourable effect of GA_3 treatment on the production of alkaloids, a group of phenolic compounds, in various plant organs was reported by some workers (Ambrose and Sciuchetti, 1962; James and Sciuchetti, 1964; Masoud et al., 1968).

When a relationship between inoculum size and the volume of the medium on one hand and growth and polyphenol

production on the other was studied, it was observed that in a fixed volume of medium, maximum growth and polyphenol production were recorded at low inoculum size (100 mg). With the increase in inoculum size, there was gradual decline in growth value and also in polyphenol accumulation. Further, there was observed marked lag in growth as well as initial delay in the polyphenol development with the decrease in inoculum size. Both of these can be attributed to the 'conditioning' effect of the medium by the different inoculum sizes tested. Moreover, the close relationship between volume of the medium and inoculum size with polyphenol production and total growth attained clearly implied that both growth and polyphenol production were limited by the supply of some essential nutrients in the medium. This conclusion was further substantiated by the observation that both growth and polyphenol accumulation continued to increase in low inoculum size beyond the period at which they started declining in higher inoculum size (Fig. 21 and 22). The latter could be explained by the different rates at which the essential nutrients are exhausted from the medium. The difference in time sequence of maximum polyphenol accumulation (5 to 15 days) and rapid growth of the tissue (15 to 20 days), however, seemed to suggest that the limiting nutritional factors for plyphenol production and growth were different. Rao (1971) working

in this laboratory observed that the growth of Datura tissue was limited by nitrate supply and not by the supply of inorganic ions, sucrose or the release of endogenous inhibitors. In their studies with Parthenocissus triouspidata suspension cultures, Henshaw et al. (1966) had demonstrated that the final yield of cells per culture was determined by volume of the medium rather than by the initial cell population. The same authors have further shown that the decline in cell division in Parthenocissus cells was also brought about by the depletion of the nitrates from the medium.

In view of the results obtained in Experiment 4-5 nitrate cannot possibly be a main limiting factor for polyphenol synthesis; for enhanced nitrate level had no stimulatory effect on polyphenol production. This indicated that the rate of polyphenol synthesis and the accumulation of total phenolics were independent of the nitrogen status of the medium, as has also been suggested by Davies (1972).

The role of amino acids in the synthesis of secondary plant products is well known (Chan and Staba, 1965; Staba and Jindra, 1968). L-phenylalanine and L-tyrosine are

well recognised intermediate aromatic amino acids in the biosynthesis of phenylpropanoid compounds (Davis, 1958; Sprinson, 1960). When these amino acids were incorporated into the medium, individually, L-phenylalanine at $5x10^{-4}$M concentration considerably enhanced the total polyphenol production over control (Experiment 4-6); L-tyrosine, on the other hand, did not prove to be as efficient a precursor for polyphenol production in Datura metel L. suspension cultures (Experiment 4-7). As shown in the chart in Chapter I, Introduction, not all species can convert the tyrosine pool intermediates to phenolic cinnamic acids. This ability has been demonstrated only in some members of the family Gramineae (Neish, 1964). On the other hand, it is believed that in most of the species the intermediates of phenylalanine pool can be readily converted to clinnamic acid derivatives whose further conversation leads to the formation of phenolic compounds. This would explain the stimulatory effect of phenylalanine and the ineffectivenss of tyrosine on polyphenol production on Datura cell cultures.

To examine the relationship, if any , between morphological differentiation and polyphenol production Cassia tissues were subjected to IAA/Kinetin interactions.

as described in Experiment 4-12. The results obtained showed that in low auxin medium (0.05 and 0.1 mg/l IAA alone) where there was initiation of roots from the callus, polyphenol accumulation was significantly low; clearly suggesting an inverse correlation between morphogenesis and polyphenol production.

Though there are very few reports in literature concerning correlation between morphological differentiation and total polyphenol production, there are many attempts elucidating the relationship between differentiation and production of specific alkaloids (Chan and Staba, 1965; Staba and Jindra, 1970; Konoshima et al., 1970). West and Mika (1957) suggested that the presence of atropine in the root callus of belladona was related to conditions of culture incompatible with active proliferation and conducive to differentiation. Working with the callus and the suspensions of the same species, Atropa belladona L. Raj Bhandary et al. (1969) showed that conditions leading to synthesis of the principle alkaloids of belladona are only achieved in association with the organisation of root structure. Studies on nicotine production in tobacco cultures revealed that

there was an intimate relationship between nicotine production and differentiation phenomenon. Tabata et al. (1971) clearly demonstrated that the nicotine production in callus cultures of "Bright yellow" tobacco can be regulated by growth regulators and also showed that the nicctine production was intimately associated with bud formation. In tissue cultures of Scopolia parviflora also a close relationship was demonstrated between organogenesis and alkaloid production (Tabata et al., 1972).

The observations made in the present studies with Cassia callus cultures are however, comparable with studies made by Kaul and Staba (1968) and Stohs et al. (1969) which revealed that undifferentiated Dioscorea deltoidea tissue cultures produced more diosgenin, whereas root-differentiated tissue cultures produced very small amounts.

Since substances like carbohydrates, auxins and cytokinins are involved practically in every aspect of metabolism of higher plants, it is logical to believe that the diverse and apparently unrelated biological

effects are explainable mainly in terms of their role in nucleic acid metabolism. Further these substances are known to affect several enzyme systems which play an important role in the physiological and biochemical processes, including regulation of plant growth and biosynthesis of phenolic compounds. Peroxidase and indoleacetic acid oxidase (IAA oxidase) enzymes are known to play a major role in the regulation of plant growth (Audus, 1972). Further, the enzyme peroxidase can be implicated in several reactions related to phenolic biosynthesis (Mason et al., 1957). Attempts were next made, therefore, to study the effects of sucrose, 2,4-D and kinetin on peroxidase and IAA oxidase enzymes which as mentioned above are closely associated with growth and biosynthesis of phenolic compounds in Datura cell cultures.

It was observed in Experiment 4-2 that for the maximum accumulation of polyphenolic compounds, there should be an adequate supply of carbohydrate, particularly sucrose. When the kinetics of the development of peroxidase and IAA oxidase enzymes were studied both in presence and in absence of sucrose, it was observed that the increase in concentration of sucrose also enhanced the synthesis of peroxidase as well

as IAA oxidase enzymes (Experiment 5-1). The maximum increase in peroxidase activity was observed from day 5 to day 15 and that of IAA oxidase from day 0 to day 10. The progressive changes in peroxidase activity at all sucrose levels showed a close relationship with the progressive accumulation of total phenolics in the tissues (Experiment 4-2, Fig. 10); this correlation suggested that the peroxidase might play an important role in phenolic biosynthesis. Peroxidase is also considered by several authors to be involved in the biosynthesis of lignin, a highly complex aromatic secondary plant product (Siegel, 1956; Freudenberg et al., 1958; Freudenberg, 1959; Higuchi and Ito, 1958). Other lignin-like polymers were also reported to have been formed from ferulic acid and H_2O_2 by peroxidase activity present in tissue sections (Stafford, 1960a and 1960b). Mazelis (1962) showed that peroxidase in presence of Mn^{++} and pyridoxal phosphate catalised the oxidative decarboxylation of amino acids leading to the formation of several phenolic compounds.

The early inactivation (i.e. terminated on day 10) of IAA oxidase in Datura cells, under different sucrose levels, might be due to the inhibitory effect of some of the accumulated phenolic acids on IAA oxidase system;

since naturally occurring phenolic acids like chlorogenic and caffic acids form competitive inhibitors of IAA oxidase system (Rabin and Klein, 1957; Nitsch and Nitsch, 1962; Zenk and Muller, 1963; Pilet, 1964; Tomaszeswski and Timann, 1966) thereby regulating growth.

The results obtained on the effects of 2,4-D on peroxidase, IAA oxidase, polyphenol accumulation and growth revealed that 2 mg/l 2,4-D was optimal for all the above four parameters. There was greater accumulation of total phenolics in tissues with high peroxidase activity in high auxin ($2x10^{-5}$M and $5x10^{-5}$M) containing medium; whereas growth and IAA oxidase activities were high in low auxin (10^{-6}M) medium. Detailed analysis of auxin response to polyphenol accumulation and growth on one hand, with enzyme activities on the other, indicated a close correlation between peroxidase pattern and polyphenol accumulation and between growth and IAA oxidase pattern. Wolter and Gordon (1972) have similarly observed that a close correlation existed between peroxidase activity and the process of lignin synthesis in Aspen tissue cultures under the influence of 2,4-D. ~~That~~ The presence of chemical cofactors can greatly modify the activity of IAA oxidase has opened up possibilities of growth control through the regulation of IAA oxidase in the tissues. The demonstration by Goldacre et al. (1953), that the stimulation of IAA oxidase

activity by synthetic auxin 2,4-D was due to a small trace of 2,4-dichlorophenol as an impurity led to the realisation that a wide range of monophenols would enhance the reaction. In contrast to such monophenols, there is a considerable number of polyphenolic substances which block IAA oxidase action and thus produce synergism with native IAA (Nitsch and Nitsch, 1962; Tomaszewski, 1964). Since monophenolic and polyphenolic compounds are easily and naturally interconverted in plant tissues, it is easy to see that such interconvertions have been proposed as a further mechanism for adjusting IAA levels via the control of IAA oxidase activity.

The present results further showed the initial delaying effects of 2,4-D on peroxidase formation. This inhibition which lasted for nearly 70 to 100 hrs was not observed in tissues grown on 2,4-D free medium. Lavee and Galston (1968) also reported similar effects of auxin (IAA) on peroxidase formation *in vitro* cultures of *Palargonium* pith. Thus these results clearly showed that contrary to certain reports in literature (Boll, 1965; Zenk, 1966), peroxidase is not necessarily a constitutive enzyme, unaffected by auxin. It is rather highly subject to external control, and one such controlling factor is

the level of auxin in the medium. The results obtained in the present investigations further strengthen the view proposed by earlier workers (Galston and Dalberg, 1954; Lavee and Galston, 1968) that the regulation of peroxidase activity by auxin could be important in controlling auxin degradation, growth and the production of secondary plant products like lignin synthesis.

Like auxin, kinetin also showed considerable influence on peroxidase and IAA oxidase activities (Experiment 5-5) and on total polyphenol production (Experiment 4-4). Kinetin concentration ($2x10^{-6}M$) which was optimal for growth also supported maximum polyphenol production accompanied by high peroxidase and IAA oxidase activities. High kinetin concentrations ($10^{-5}M$ and $2x10^{-5}M$) were, however, found to be more inhibitory on peroxidase and IAA oxidase activities than on polyphenol production. Further, as compared to 2,4-D effects, high concentrations of kinetin did not show much deleterious effects on polyphenol production. Though there was considerable delay in the initiation of peroxidase activity, no lag was observed in IAA oxidase initiation. The close similarity between the patterns of peroxidase activity in tissues at optimal ($2x10^{-6}M$) and sub-optimal

(10^{-6}M) kinetin concentrations with polyphenol accumulation on one hand and the considerable difference in growth supported by these kinetin levels on the other, suggested a close relationship of peroxidase activity with polyphenol production (Fig. 14) rather than with growth (Fig. 15).

Though the effects of kinetin on polyphenol synthesis are not well understood, it is possible that kinetin might influence the polyphenol synthesis through its effects on nucleic acids, particularly on RNA. Srivastava (1966) and Fox (1966) observed that cytokinins can exert their biological effects through their incorporation into tRNA thus influencing amino acid metabolism and protein synthesis. Kinetin has also been shown to enhance the synthesis of enzymes (e.g. tyramine methylpherase in barley roots) and to supress the activities of other enzymes (e.g. ribonuclease and deoxyribonuclease) (Street and Cockburn, 1972). It is further known that lignin and hemicellulose synthesis are initiated while the cultures are actively growing by cell division (Fosket and Torrey, 1969; Carceller et al., 1971). Stockdale and Topper (1966) had proposed that the hormonal milieu in which cell division occurs is the critical factor in the

formation of a specific type of differentiated cell. Since kinetin is known to stimulate cell division, it is reasonable to believe that it might be influencing polyphenol production by enhancing cell division in a particular hormonal environment which stimulates the derivative cells for increased polyphenol production.

The discovery of the enzyme phenylalanine ammonia-lyase (PAL) in barley by Kaukol and Conn (1961) has opened the door for a better understanding on the synthesis of phenolic compounds. It is widely held that phenylalanine is an obligatory intermediate in the biosynthetic pathway leading to the formation of phenylpropoanoid compounds and PAL catalyses the deamination of phenylalanine to trans-cinnamic acid. In turn, the carbon skeleton of trans-cinnamic acid serves as a substrate for the formation of other secondary plant products. These observations were supported by a considerable body of evidence reviewed by Grisebech (1968) and Zucker (1972). Also, as mentioned in Introduction of Chapter V, it is known that PAL responds to several external stimuli such as carbohydrate, hormones, light and others.

Studies on the effect of sucrose levels on the development of PAL activity and the accumulation of

polyphenolics in Cassia callus cultures (Experiment 5-8) revealed that at high sucrose concentrations the development of PAL activity and the accumulation of total polyphenols were more. Creasy (1966) also reported the increase in PAL activity in straw-berry leaf disks with the increase in concentration of sucrose. The requirement of higher levels of external carbohydrate for maximum development of PAL activity was not well understood. However, as suggested by Zucker (1969), it is reasonable to believe that since the enzyme PAL is formed in cytoplasm, the metabolism of exogenously supplied carbohydrate could provide a convenient source of ATP for the synthesis of cytoplasmic enzymes including PAL.

When PAL activity and polyphenol production were examined at different concentrations of 2,4-D (Experiment 5-9), it was found that at low auxin concentration (0.2 mg/l 2,4-D) the initial raise in the enzyme activity was slow followed by a rapid increase. The peak of the activity was observed during 3 to 9 days period; while the highest polyphenol production was recorded from 6 to 15 days. Both PAL activity and total polyphenol production were found maximum in tissues grown on low auxin (0.2 mg/l) medium.

Effects of hormones on PAL synthesis have also been studied by several workers in different plant tissues (Reid and Marsh, 1969; Ruberye and Foske , 1969). Davies (1972) while examining the effects of 2,4-D on PAL activity and polyphenol synthesis in cell suspensions of Paul's Scarlet rose observed that 2,4-D levels as high as 10^{-3}M had no influence on the rate of enzyme reaction.

In the present studies it was further interesting to note that though the development of PAL activity terminated early (on day 9) during the growth period, the accumulation of polyphenolics continued even after day 9. This suggested that there was some discrepancy between the PAL activity and the synthesis of polyphenolics in the timings at which their respective maxima occur. Further, this also leads to some doubt about the ability of the PAL levels to sustain the observed rate of polyphenol synthesis. As suggested by Engelsma (1968), the accumulated phenolic products may function as natural repressors, since cinnamic acid and subsequent phenolic products (Zucker, 1965; Engelsma, 1968) are known to inhibit PAL synthesis. However, this suggestion needs a detailed analysis of the individual phenolic acids that

are formed during growth cycle along with the development of PAL activity.

The secretion of macromolecules like enzymes (proteins), polysaccharides, amino acids and alkaloids into the culture medium in which the cells of higher plants were grown has been described by a number of investigators as mentioned in the Introduction (Chapter I). In the present studies the release of peroxidase and IAA oxidase enzymes by Datura cells into the liquid medium, under the influence of sucrose, 2,4-D and kinetin was followed during the course of culture. Measurable amounts of both the enzymes were detected even in the controls, and in all the treatments soon after setting up of new cultures. In general, the peroxidase was released in greater quantity than IAA oxidase; the latter being further reduced in presence of 2,4-D and kinetin. The activity of both the enzymes in the tissue as well as in the medium varied markedly with change in sucrose level, 2,4-D and kinetin concentrations in the medium. The patterns of IAA oxidase in the tissue and in the medium were very similar (Experiments 5-1 to 5-6). In case of peroxidase, however, the picture in the medium was different from that in the tissues. During the period 5 to 10 days, when there

was high peroxidase activity in the tissues accompanied by maximum polyphenol accumulation, its release into the medium was significantly reduced. Whether the less detected amount of peroxidase in the medium was due to reabsorption by the tissue or reduced release on account of rapid utilization of the enzyme synthesized in polyphenol production, could not be ascertained in the experiments conducted and this aspect needs to be examined further.

There is hardly any information in literature concerning the influence of cultural parameters on the substances released into the medium. Examination of the cultures for amino acids liberated by cultured sycamore cells was, however, undertaken by Simpkins and Street (1970) on the hypothesis that such release of particular amino acids might reduce the intracellular level to the point of limiting protein synthesis. The same workers further found that the contents of all the amino acids - present in sufficient quantity to permit their accurate measurement - was higher in the high than in the low kinetin medium.

The great value of plant suspension cultures as a potential source of important plant products such as terpenoids, steroids and alkaloids has already been

emphasised earlier. In the light of the foregoing results the culture of plant cells in liquid medium may also prove to be useful to produce plant enzymes in large quantities. The extraction and purification procedures can be greatly simplified if the cells are used rather than their roots, shoots, leaves or sotrage organs, as fibrous materials and storage carbohydrates, lipids and proteins are to a great extent absent from them. The specific activities of the enzymes extracted from cell cultures are reported to be considerably higher than those obtained from the extracts of the differentiated tissues. Zucker (1965), for example, reported that the activity of PAL in potato tuber slices, after an induction period was 13 to 27 nanomoles/hour/gr.fr.wt. The corresponding value, as reported by Gamborg (1966), for potato cell cultures was 410 nanomoles. Similarly, the specific activity of the shikimic dehydrogenase from potato tubers was 37 nanomoles/hour/milligram protein; while the specific activity of the same enzyme from potato cell cultures was 5000 nanomoles. Funken Gamborg has assessed the suitability of cell suspensions derived from several plant species as sources of enzymes involved in the formation of aromatic compounds. Nandi and Ganguli (1961) have also indicated

that most of the enzymes of shikimic acid pathway can be extracted from mung-bean cell cultures. Clearly, in the present studies the peroxidase and IAA oxidase activities were detected in measurable quantities and their activities could be further enhanced by manipulation of the cultural conditions. Possibilities for extraction of the said enzymes, particularly peroxidase from the cells grown in culture and/or from the liquid medium need to be explored in further work along the lines indicated here.