CHAPTER IV

GENERAL DISCUSSION

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This research endeavour had four directions of special emphasis : first, initiation and establishment of callus and suspension cultures of Crotalaria juncea L. on a completely defined medium; second, examination of growth responses and the production of phenolic compounds in the above tissue subjected to different cultural parameters; third, investigation of the development of certain allied enzymes in relation to the production of phenolic materials; and four, isolation, purification and characterization of L-phenylalanine ammonia-lyase, a key enzyme in the pathway leading to the synthesis of phenylpropanoid compounds. Besides these, experiments were also designed to examine the changing patterns of growth, phenolic compounds and the development of few related enzymes during the germination of Crotalaria seeds.

The results obtained in the germination experiments are presented in Chapter III, Section A. Rapid increase in fresh and dry weights from the beginning indicated prompt germination of the seeds after overnight soaking in water. No lag phase was observed and 0-72 hours constituted rapid growth period. The maximum production of phenolic compounds

was registered during the rapid growth phase. Thus, in Crotalaria seedling, polyphenol synthesis was essentially restricted to the growing and dividing cells.

The developmental pattern of peroxidase activity, in individual parts and in the seedling as a whole, showed close correlation with the production of phenolic compounds; pointing to an important role of peroxidase in the regulation of phenolic biosynthesis. Further, their respective maxima occurred in different parts of the seedling (polyphenols in cotyledon and peroxidase in hypocotyl) and the level of polyphenols and peroxidase on unit basis also showed variation among the different parts of the seedling. This indicated that though peroxidase might have regulatory role in phenolic biosynthesis, it has as well other physiological roles in growth and development.

Maximum foldwise increase in IAA oxidase activity after the culture period of 5 days was recorded in hypocotyl (10.63 fold) and minimum in the root (1.63 fold). This is in contrast to the accumulation of phenolic compounds in which maximum increase was registered in the root (2.0 fold) and minimum in the hypocotyl (1.43 fold). This seemed to suggest regulation of IAA oxidase activity by the endogenous

level of phenolic materials. Though most of the polyphenolic compounds are inhibitory to this oxidase activity, few are known to enhance its activity (Gortner and Kent, 1958). Many-fold increase of IAA oxidase in hypocotyl may possibly be due to more production of phenolic compounds which might have promotory effect. However, this needs further confirmation by chromatographic separation and quantitative estimation of individual phenolic compounds in each of the parts of the seedling. The physiological role in growth and development of this oxidase activity is evident from its variable levels and unequal increase in different parts of the seedling. The mechanism of its action is discussed later.

The importance of phenylalanine ammonia-lyase (PAL) in the synthesis of phenolic compounds is obvious from the correlationship obtained between them in individual parts of the seedling. The observed level of PAL in hypocotyl was more than in the cotyledon, whereas the level of polyphenols was more in cotyledon than in hypocotyl. It seemed that PAL level varied not with the concentration of the phenolic compounds but with the quality of phenolic materials. Or, this discripancy may be due to variability of phenol reagent to react with various phenols (Goldstein and Swain, 1963). Compared to phenylalanine deamination, tyrosine deamination

terminated a day earlier, suggesting domination of phenylalanine pool over tyrosine pool in the synthesis of phenolic compounds in Crotalaria. Further, though TAL terminated a day earlier than PAL, the level of TAL was higher than PAL throughout the germination period in root and in the later period in hypocotyl. Obviously, tyrosine pool is also preferentially utilized in Crotalaria for polyphenol biosynthesis.

In Chapter III, Section B, 1, are described the experimental details for the induction of callus from Crotalaria seedling and its different parts. Callus was formed from practically all parts of the seedling and on all the media tested. However, the initiation was best on the medium containing 2.0 mg/l 2,4-D, 2.0 mg/l IAA and 2.0 mg/l kinetin. Initiation of callus was observed first from the root, gradually shifted to the cotyledon via hypocotyl. Callus, thus formed, was fast growing and within 30 days no part of the seedling was visible in the culture flask.

For the culture of a number of callus tissues, auxin is the only essential supplement which needs to be added to the basal medium supplying inorganic ions and sugars. Among the natural and synthetic auxins, indoleacetic acid (IAA), naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic

acid (2,4-D) are most widely used for the culture of various normal tissues (reviewed by Gautheret, 1955). The above three auxins were tested for their ability to support growth and the results are summarized in Section B, 2. Best growth was recorded on the medium with 2,4-D as auxin supplement.

Superiority of 2,4-D over other auxins for the growth of Crotalaria cells can be explained by at least two points. First, the degradation of 2,4-D in the cell might be less than the other two auxins, possibly, owing to the absence or low activity of the 2,4-D oxidizing enzyme system. This point is further strengthened by the observation that 2,4-D metabolites nearly ten times slower than the IAA (Bendana et al., 1965). Second, different rates of transport of different auxins in the tissue might be responsible. The rate of 2,4-D transport is reported to be very slow (McCready, 1963). The ultimate step of growth by cell enlargement is a coincident enlargement of the protoplast by water uptake and a yielding of the cell wall. Experimental evidence indicates that the stimulatory action of auxin softens the cell wall by increasing its plasticity (Tagawa and Bonner, 1957; Cleland, 1958) and thereby ensuing a swelling of the cell by simple osmotic water uptake. 2,4-D, because of its

slow rate of transport and metabolism, possibly maintains this action for most of the culture period.

That kinetin has an additive effect on tissue growth to that of auxin was demonstrated by Steinhart (1961) in spruce and by Digby and Wareing (1966) in sycamore cultures. Incorporation of kinetin in the medium has been found to be essential for the growth of many callus cultures (Murashige and Skoog, 1962; Steward, 1969). But the results obtained in the present study clearly indicated that for the growth of Crotalaria seedling callus kinetin was not essential and it had no additive effect to that of auxin for stimulating the growth of the tissues. Clearly, Crotalaria cells synthesized sufficient cytokinins and have no exogenous requirement. Gamborg et al. (1968) made similar observation. They showed that soybean cultures do not require kinetin for growth and may produce adequate quantities of cytokinins to satisfy their own needs and even the lower concentration of exogenous kinetin depressed growth. Higher kinetin level in the medium may adversely influence the quantity and physical properties of the extracellular hemicellulose appearing in the culture, the synthesis of protein and the balance established in the cells between protein and

soluble nitrogen (Street, et al., 1969). Overall result of this influence will be the reduction of the growth. Incorporation of kinetin into the nutrient medium for Crotalaria resulted into supraoptimal level of cytokinins and hence the reduction in growth.

Crotalaria callus grown on a medium containing 2.0 mg/l 2,4-D and subcultured at monthly intervals on similar auxin enriched media continued growth indefinitely with undiminished vigour. Increasing the auxin content of the medium led to the reduction in the growth of the tissue. Though little is known of the action of plant growth regulators on the changes in chemical content of plant tissues, experiments with plants have shown that RNA and protein synthesis are involved in the action of auxin (Noodén and Thimann, 1963; Key and Shannon, 1964; Vajranabhaiah and Mehta, 1976). However, Skoog and Robinson (1950) demonstrated that auxin has a catalytic function in carbohydrate metabolism which is related to its effect on growth. Wiggans (1954) found that supraoptimal auxin level led to a decrease in the reducing sugar concentration. The same may be the case with RNA and protein synthesis. Working with Cucumis cell suspensions Vajranabhaiah and Mehta (1976) demonstrated adverse effects of high auxin

content on nucleic acid metabolism and protein synthesis. In Crotalaria also supraoptimal level of 2,4-D might have adversely affected the RNA and protein synthesis and carbohydrate metabolism and thus led to the reduction in growth.

The progress of growth showed typical sigmoid curve for both the callus and the suspension cultures (Section B, 4). However, growth of the callus tissues was found faster than that of the suspension cultures. Leaching out of certain chemicals and different rates of ion uptake might be responsible for this result. Growth of the suspension cultures could have been improved by manipulating the cultural conditions, but no such attempt was made as the callus tissues grew quite satisfactorily.

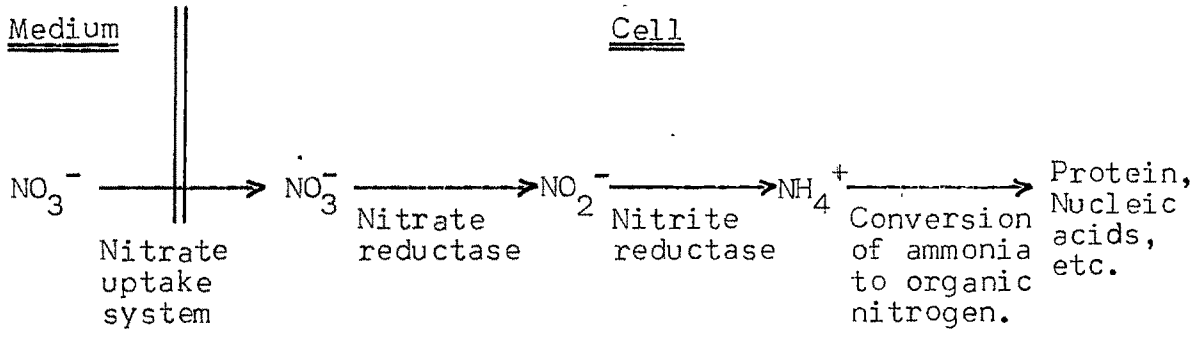
Tissues vary considerably in their ability to utilize different carbohydrates. Sucrose is usually the carbohydrate of choice in standardized cell culture media (Murashige and Skoog, 1969; Gamborg et al., 1968; Rao and Mehta, 1968; Stuart and Street, 1969; Nickell and Maretzki, 1969; Schenk and Hilderbrandt, 1972). Superiority of sucrose over other carbohydrates was also established for the growth of Crotalaria callus cultures (Chapter III, Section C, 1).

An apparent preference for sucrose over its constituent monosaccharides sometimes can be traced to sucrose hydrolysis, a problem that arises during autoclaving of media (Ball, 1953; Koblitz et al., 1965). Further, experimental evidence suggests that although sucrose hydrolysis can occur, because of either autoclaving or presence of surface-bound enzymes, this is not generally a prerequisite for sucrose uptake. In contrast to this, extracellular hydrolysis is essential for uptake of other di-, oligo- or poly- saccharides and form the limiting factor for the utilization of these carbohydrates. However, a fuller understanding of this problem demands elucidation of the mechanism of sucrose uptake by the cells.

Maximum production of polyphenols in the tissues grown on sucrose as carbohydrate source can also be explained by its rapid utilization for protein synthesis as well as protein turnover. Some of the intermediates of this system, L-phenylalanine and L-tyrosine, are the precursors of phenolic compounds. In light of the above, superiority of sucrose for the production of phenolic materials over other carbohydrates becomes evident.

Nitrogen was shown to be growth-limiting in a number of cultured tissues (Henshaw et al., 1966; Filner, 1966; Nash

and Davies, 1972; Young, 1973). In cultured Crotalaria tissues higher level of nitrogen was inhibitory to growth (Chapter III, Section C, 2A), optimal level being 840 mg/l nitrogen. Supraoptimal nitrogen level might possibly disturb the Krebs cycle as was also observed in liver mitochondria where higher concentration of ammonia affected the conversion of isocitrate to α-ketoglutarate (Katunuma et al., 1966). The major source of nitrogen in MS medium for Crotalaria tissues is in the form of nitrate. It is assimilated in the cells as shown below :



The supraoptimal level of nitrogen increases ammonium ions in the cells which in turn might disturb Krebs cycle and thus also protein and nucleic acid synthesis. The overall result would be the reduction in growth.

Most products of phenylpropanoid pathway, such as lignin, flavonoids, cinnamic acid, benzoic acid etc., are

devoid of nitrogen. However, it is suggested that the biosynthesis of these compounds is in some way related to the nitrate metabolism of the cells (Hahlbrook, 1974). Increase in nitrogen content of the medium has decreased the relative amount of phenolic compounds in Crotalaria callus cultures. Thus, increasing the nitrogen level from zero to 1260 mg/l has reduced accumulation of phenolic materials from 1.221% to 0.767%. However, total phenolics increased with increase in nitrogen level upto 840 mg/l (optimal for growth). This is possibly because enhanced nitrogen level of the medium upto 840 mg/l promoted growth but decrease in polyphenol content per cell with increase in nitrogen is not as pronounced as the increase in growth. The point became more clear when polyphenol content of the tissues grown on White's medium was examined. White's medium, which contains very little nitrogen (52 mg/l), supported good production of phenolic compounds both per culture and on unit basis. A similar relation was observed in Crotalaria seedling where considerable reduction in the production of phenolic compounds was recorded during the period of maximum increase in cellular nitrogen (Shah et al., 1976). Further, Westcott and Henshaw (1974) have presented evidences to indicate antagonism between tannin synthesis and nitrogen metabolism. Increase in nitrogen could, possibly, cause a premature

limitation on the availability of carbon for polyphenol synthesis by diverting more of the available sugar into primary metabolites. An interaction between nitrogen source and carbohydrate supply influencing pigment formation has been reported for cultured red cabbage embryos (Szweykowska, 1959).

The Crotalaria callus cultures grew best on a balanced supply of ammonium and potassium nitrates. When tested singly, potassium nitrate was found better than ammonium nitrate. Less of growth on ammonium salt may be attributed to difficulties in absorption or to inhibition of metabolic reactions directly or indirectly associated with nitrogen assimilation. The ambient pH could be decisive for absorption of various nitrogen sources (Street, 1966): pH-growth responses curves indicated that nitrate nitrogen when pH is acidic (4.7 - 4.9 pH approx.), ammonium nitrogen when pH is neutral (7.0 - 7.2 pH approx.) and nitrite at pH 5.0 - 6.0 supported maximum growth. When nitrate and ammonium is added together in the medium, growth of the tissue causes a less pronounced alkaline drift. The later point explains why Crotalaria cells grew best on a balance supply of ammonium and potassium nitrate as nitrogen source (Chapter III, Section C, 2C).

For many tissues, natural products rich in amino acids such as casein hydrolysate and yeast extracts have been shown to be capable of meeting the nitrogen requirements. Casein hydrolysate and yeast extracts have been found suitable as the sole sources of nitrogen for Crotalaria callus tissues (Chapter III, Section C, 2D). This indicated that the absorbed amino acids are actively deaminated in the tissue cells, or that their deamination readily yielded ammonium to the sites involved in the synthesis of essential metabolites. Yeast extract is reported also to contain traces of auxin (Dormer and Street, 1949) which might be one of the factors responsible for its effect on growth.

Unlike growth-responses, ammonium nitrate supported more polyphenol production than potassium nitrate. Less growth was recorded in the tissues when the ratio of ammonium nitrogen and nitrate nitrogen was 1 : 1 (i. e. when ammonium nitrate was the sole nitrogen source) in the medium. Obviously in such cases the production of primary amino acids (essential for growth) will be less which in turn affect the trans-aminases and other enzymatic reactions leading to the production of essential metabolites. Due to this, deamination of aromatic amino acids, such as phenylalanine and tyrosine, are favoured which resulted into more production of secondary

metabolites. Enhanced synthesis of polyphenols on yeast extract and casein hydrolysate may also be due to the presence of aromatic amino acids which are direct precursors of the phenolic biosynthesis.

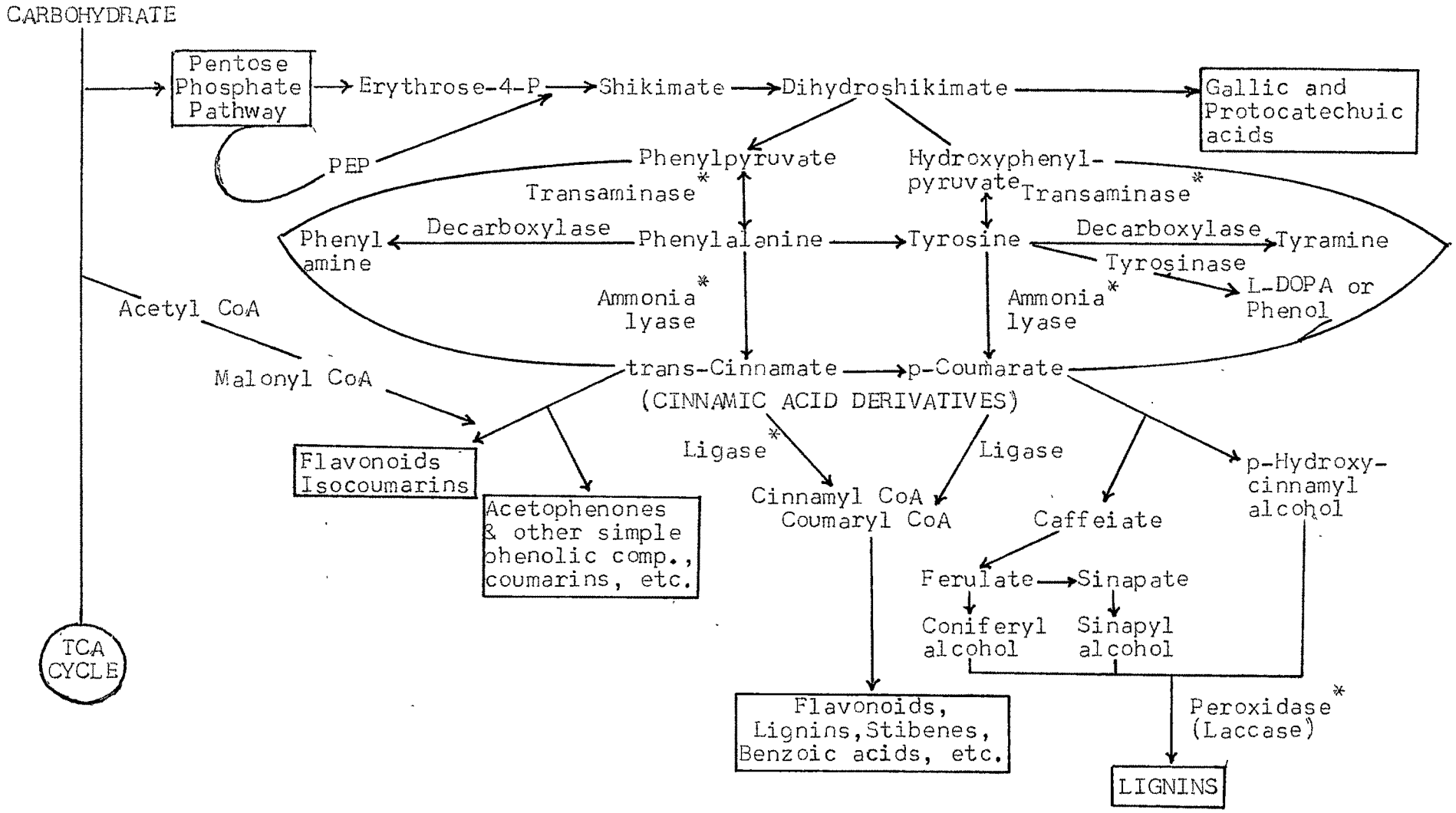
Urea as a sole nitrogen source failed to support growth. This may possibly be due to the lack of proper hydrolysis or the presence of biuret which is toxic to growth (Burris, 1959). The latter point is more evident from the inhibition of growth even when urea is added to the standard nitrate containing medium (Chapter III, Section C, 2E). The cyanic acid formed from urea during the culture period could also be responsible for the observed inhibitory effect of urea. Absolutely no growth on urea as a sole nitrogen source suggested that it inhibited the production of primary metabolites, presumably, including phenylalanine and tyrosine - the immediate precursors of polyphenol synthesis. Thus, less accumulation of phenolic compound was registered when urea is incorporated into the medium. However, the exact mechanism of urea action in polyphenol biosynthesis remains uncertain.

Working with tobacco cell cultures, Filner (1965) observed that the cells grew well on nitrate, or a complete mixture of amino acids, or both nitrate and a complete amino acid

mixture, but the cells could not grow on nitrate plus an incomplete mixture. Crotalaria cells too failed to grow satisfactorily when L-phenylalanine or L-tyrosine or both were incorporated into the medium containing nitrate, the inhibition of growth was more marked with L-phenylalanine as an additive (Chapter III, Section C, 3). Among the hypotheses which were considered to account for the amino acid effects was the idea that they inhibit growth by inhibiting the biosynthesis of other amino acids. Since cells growing on MS medium must utilize the nitrogen from nitrate to synthesize amino acids, the pathway of nitrate assimilation would seem a likely place for one amino acid to prevent the synthesis of others. L-Phenylalanine seems to be more inhibitory than L-tyrosine as more reduction in growth resulted with L-phenylalanine.

Both the amino acids, L-phenylalanine and L-tyrosine, are direct precursors of polyphenol synthesis. In light of this, increase production of phenolic materials on unit basis is obvious when these are incorporated into the medium. Although phenylalanine is more readily deaminated than tyrosine in Crotalaria cells grown on standard MS medium, addition of tyrosine has enhanced the production of phenolic compounds

substantially. Less activity of TAL in normal course was attributed to the presence of enzyme tyrosinase which limit the availability of tyrosine for TAL. It may, therefore, be possible that addition of tyrosine inhibits or limits the activity of tyrosinase (either feed-back inhibition or substrate saturation) diverting more of tyrosine for TAL to support maximum polyphenol synthesis. On the other hand, phenylalanine may be distributed among its degradative pathways (Chart I) limiting its availability for PAL and hence little increase in phenolic compounds. Further, tyrosine is nearly four times less inhibitory to growth than phenylalanine (Section C, 3). The effect of these amino acids on growth is due to their inhibition of nitrate assimilation pathway, particularly the glutamate generating system (Filner, 1965). The synthesis of phenylalanine and tyrosine is dependent on the transaminasion reactions involving glutamate. This means the endogenous synthesis of phenylalanine is less than tyrosine. The overall result is that tyrosine supported maximum whereas phenylalanine enhanced little production of phenolic compounds. An antagonism was also observed both for the growth of the tissue and for the production of phenolic compounds when these two amino acids were added together to the medium.



IMPORTANT INTERCONVERSIONS AND PROBABLE MAJOR BIOSYNTHETIC PATHWAYS FOR PHENYLPROPANOID COMPOUNDS

(Enzymes marked with * are examined in the present investigation)

CHART I.

A number of metabolic processes have been discussed (Kefeli and Kadyrov, 1971) in relation to the mechanism of action of phenolic growth inhibitors, including effects on ion transport, protein synthesis, hormone deactivation, and energy metabolism. For any given plant tissue, one or more of the above processes might be associated with the inhibition. Six phenolic acids tested in the present investigation also inhibited growth of Crotalaria callus cultures (Chapter III, Section C, 4). The degree of inhibition varied with the phenolic acid and the concentration tested. Cinnamic acid, p-coumaric acid and chlorogenic acid in presence of 2,4-D were strong inhibitors even at low concentration, while hydroxybenzoic acid, ferulic acid and caffeic acid in presence of 2,4-D were effective as inhibitors only at high concentrations. In absence of 2,4-D, only ferulic and cinnamic acids were found effective inhibitors at low concentration, while others were strong inhibitors only at higher doses. On the contrary, low levels of hydroxybenzoic and chlorogenic acid stimulated growth over the control. The interesting results were obtained with caffeic acid in absence of 2,4-D. Increasing concentration of this phenolic acid promoted growth. Caffeic acid has been implicated in IAA metabolism (Zenk and Muller, 1963; Leopold and Plummer,

1961) and has been shown that it did not alter metabolic processes of isolated mitochondria (Demos, et al., 1975). Further, it is generally accepted that all the compounds which alter respiration or coupling in the isolated mitochondria also inhibit growth. Assuming that caffeic acid in absence of 2,4-D did not alter metabolic processes of isolated mitochondria in Crotalaria also, its nill effect on growth as an inhibitor could be realized.

In presence of 2,4-D, all the six phenolic acids inhibited the synthesis of phenolic compounds (Chapter III, Section C, 4). This inhibitory effect of phenolic acids may be attributed to their reported inhibitory effect on ion uptake (Glass, 1974). The active absorption of sugars, glucosides and inorganic ions were found to be inhibited by phenolic acids. The ultimate result of this would be less production of essential metabolites some of which are intermediates in the phenylpropanoid pathway. The stimulation of phenolic compounds by caffeic acid in absence of 2,4-D can similarly be traced to its effect on growth discussed above. Hydroxybenzoic acid and ferulic acid also promoted polyphenol synthesis on percentage basis at higher concentration in absence of 2,4-D. This effect seems to be only due to the more inhibition of growth than the phenolic compounds, resulting into higher accumulation of phenolic materials per cell.

Though growth of the tissue was reduced at higher sucrose concentration (4%), increasing the sucrose level of the medium led to increased production of phenolic compounds (Chapter III, Section D, 1). 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 has been worked out in great detail (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) as shown in the chart I. Shikimic acid is of wide spread occurrence and some enzymes of its pathway have been found in higher plant cell cultures (Gamborg, 1966; Zucker, 1972). The shikimic acid pathway is now well established as providing the precursors for the synthesis of the aromatic amino acids in both microorganisms and higher plants. Further, in plants, secondary metabolites such as phenolic compounds, alkaloids, flavonoids, etc. also arise from this pathway (Yoshida, 1969). In light of this the role of sucrose in increased production of phenolic compounds by stimulating the production of aromatic amino acids is evident.

Increase in the sucrose content of the medium also

enhanced the activity of peroxidase, IAA oxidase, PAL as well as TAL enzymes. The function of sucrose in the development of these enzymes is, however, not well understood. Metabolism of exogenous sucrose could either provide a source of ATP for the synthesis of these enzymes or induce the said enzymes by increasing their respective substrate concentrations. The latter point can only be considered valid if the substrate concentration in the tissue is lower than its saturation level. The stimulation of these enzymes by increasing sucrose content have been reported in a number of cases : peroxidase in Cassia callus cultures (Shah et al., 1975) and Datura cell suspensions (Mehta et al., 1974), IAA oxidase in Datura cell suspension (Subbaiah, 1974), PAL in strawberry leaf disks (Creasy, 1968 a; 1968 b) and in Paul's scarlet rose cell suspensions (Davies, 1972b).

The concentration of phenolic substances in Crotalaria callus cultures was influenced by exogenous hormones. The polyphenols accumulation was less in the cells grown on higher concentration of auxin (2,4-D or NAA) (Chapter III, Section D, 2 and 3) indicating that auxin repressed the polyphenol production. However, increasing the auxin

(either 2,4-D or NAA) content of the medium from 0.2 mg/l to 2.0 mg/l stimulated the production of phenolic compounds. This is probably due to adsorption and gradual degradation of auxin by the cells which is tolerable only upto 2.0 mg/l concentration. Increasing the auxin concentration above 2.0 mg/l supressed the phenolic production.

The observed ability of higher 2,4-D concentrations to repress synthesis of phenolic materials in Crotalaria cultures is similar in many respects to the reported effect of NAA on anthocyanin synthesis in cultured Haplopappus cells (Constabel et al., 1971) and the effect of 2,4-D on polyphenol synthesis in Cassia callus cultures (Shah et al., 1976b) and Paul's scarlet rose cell suspensions (Davies, 1972). The effect of raising the 2,4-D level above the optimal was one of delaying the initiation of polyphenol synthesis. Thus, increasing the 2,4-D content to 10.0 mg/l (5 folds) delayed the initiation by over 5 days. Since accumulation apparently terminated at approximately the same stage at all the 2,4-D levels, the net result was that total polyphenol content was reduced at the high 2,4-D dose. Furthermore, since the rate of growth was less affected than the phenolic compounds by these different 2,4-D levels, it is clear that increasing 2,4-D content must also reduce the rate of polyphenols

synthesis per unit basis. For 0.2, 2.0 and 10.0 mg/l 2,4-D treatments, the estimated maximum rates of phenolic synthesis (µg/100 mg dry wt.) were 900, 1100 and 875, respectively.

On the other hand, increasing concentration of NAA showed no delaying influence on the formation of phenolic compounds. The biosynthesis and accumulation of phenylpropanoid compounds in plants is controlled by specific genes (Feenstra, 1960; Harborne, 1965). The Crotalaria cells presumably possess these genes since phenolic substances readily accumulated in the cells. NAA or 2,4-D might repress the manifestation of the genes, when the concentration exceeds 2.0 mg/l. However, the possibility of indirect effects, such as higher auxin level reducing the sugar concentration (Wiggans, 1954) which in turn decreases the production of phenolic compounds, can not be ruled out.

The results obtained on the effects of 2,4-D or NAA on peroxidase, IAA oxidase, PAL and TAL revealed that 2.0 mg/l auxin (either 2,4-D or NAA) was optimal for all the four parameters.

The present results further showed the initial

suppression of peroxidase formation by 2,4-D which lasted for over more than 5 days. No such effect was noticed in case of NAA treatments. Thus, the response of peroxidase development to 2,4-D and NAA was very much similar to that of polyphenol production where also no initial delaying effect of NAA was observed in contrast to 2,4-D. Several reports showed the regulation of peroxidase by auxin (Galston and Dalberg, 1954; Lavce and Galston, 1968; Shah et al., 1976b) and suggested that this could be important in in controlling auxin degradation, growth and the production of secondary plant products like lignin. The present investigation further strengthened this view.

Both the auxins had no delaying influence on the development of IAA oxidase activity. Auxin had a dual effect, depending upon its concentration, 2.0 mg/l being optimal. Similar effect was demonstrated in tobacco callus cultures by Lee (1971a) who observed the changing patterns of IAA oxidase isoenzymes as influence by auxins (IAA, 2,4-D, 2, 4, 5-T and 2, 4, 6-T). The demonstration by Goldacre et al. (1953) that the stimulation of IAA oxidase activity by synthetic auxin (2,4-D) was due to a trace of 2,4-dichlorophenol (DCP) as an impurity led to the realization

that a wide range of monophenols would enhance the reaction. In contrast to such monophenols, there is a considerable number of polyphenolic compounds which block IAA oxidase action and thus produce synergism with native IAA (Nitsch and Nitsch, 1962; Tomaszewski, 1964). Since monophenolic and polyphenolic substances are easily and naturally interconverted in plant tissues, it is easy to see that such interconversions have been proposed as a further mechanism for regulating IAA levels via the control of IAA oxidase activity.

Effects of hormones on PAL activity have been studied by several workers in different plant tissues (Reid and Marsh, 1969; Ruberye and Foske, 1969; Davies, 1972b; Subbaiah, 1974). Davies (1972b), while examining the effects of 2,4-D on PAL activity in Paul's scarlet rose cell suspensions observed the regulatory role of auxin on the rate of enzyme reaction. Similar effects of 2,4-D and NAA were also recorded on the development of PAL activity in Crotalaria callus cultures. The response of TAL activity to 2,4-D and NAA is similar to that of PAL.

Experiments with plants and plant tissues (Biswas and Sen, 1959; Noodén and Thimann, 1963; Key and Shannon, 1964;

Vajranabhaiah and Mehta, 1975) have shown that RNA and protein synthesis are involved in the action of auxin. Continuous RNA synthesis is required to sustain the increase in PAL levels has been demonstrated by Davies (1972b). In light of this and our own results obtained using cycloheximide, which are discussed later, the influence of auxin recorded in the present investigation on peroxidase, IAA oxidase, PAL and TAL synthesis is obvious.

Nickell (1958) showed that promotion, no effect or repression in growth may be obtained with different plant tissue cultures in response to GA_3 treatments. In Crotalaria callus cultures 10.0 mg/l GA_3 in presence of 2.0 mg/l 2,4-D was recorded optimal for growth. However, no significant difference was registered with this treatment over the control (2.0 mg/l 2,4-D alone). On the other hand, sub- and supra-optimal levels were inhibitory as compared to the control. The stimulatory or inhibitory effects of GA_3 on growth might be due to its effects on both cell division and cell extension (Street, 1966; Lalchandani, 1970).

Further, GA_3 could mediate growth through its effect on auxin metabolism, but the mechanism of action has not yet been settled. A sparing action of GA_3 on auxin is

suggested by the work of Pilet (1957), Stutz and Watanable (1957), Kuraishi and Muir (1963) and others. One possibility is that GA_3 might increase the rate of auxin production and the other is that GA_3 might decrease the rate of IAA destruction by lowering the level of IAA oxidase and (or) by increasing the level of IAA oxidase inhibitors. A "three-factor" interaction of growth regulation was proposed by Brian and Hemming (1958); the third factor has something to do with auxin metabolism. The sparing action of GA_3 on auxin appeared to depend on the prior interaction of GA_3 with the third factor. The results obtained with Crotalaria callus cultures has indicated no sparing action of GA_3 on auxin as evident from the increase in IAA oxidase activity after GA_3 treatments.

An alternative interpretation, as was suggested by Lee (1971 b), is that oxidation of IAA by the GA_3-promoted IAA oxidase might lead to the formation of intermediates and products more active than IAA in modifying growth. This is supported by Nitsch and Nitsch (1959) and Kuraishi and Muir (1964) who reported an increase in diffusible auxins other than IAA by GA_3 and by Meudt (1967) and Tuli and Moyed (1969) who found certain products of enzymatic oxidation of IAA were more active as auxins than IAA.

In the present experiments, since auxin was supplied at its optimal concentration, any change in the level of endogenous auxin would mediate the growth.

The stimulation in the relative amount of phenolic substances was recorded at low GA_3 level, but not significant changes were registered at optimal and supraoptimal (for growth) concentrations. Further, though the low GA_3 level supported maximum polyphenols on unit basis, more production of total polyphenols was recorded at concentration optimal for growth. This is because the difference in growth at low (1.0 mg/l) and optimal (10.0 mg/l) GA_3 levels was more pronounced than the relative amount of phenolic compounds produced. GA_3 effect on the production of phenolic materials in Crotalaria callus cultures is similar in many respects to the observed effect of GA_3 on pigment formation by Nickell and Tulecke (1959). They made an interesting observation that GA_3 affects the colour of all the cultures tested irrespective of their growth responses. They concluded that apparently GA_3 affected one or more enzyme systems involved in pigment formation which is unrelated to the growth effects.

Like other growth substances, GA_3 also showed considerable influence on peroxidase, IAA oxidase, PAL and

TAL enzyme activities. In all the cases 10.0 mg/l ~~mg/l~~ GA_3 concentration was found optimal. It is also interesting to note that at this concentration of GA_3 no significant difference with the control (2.0 mg/l 2,4-D alone) in the activity of these enzymes was recorded, but the sub - and supraoptimal levels of GA_3 had marked effects.

The dual effects of GA_3 on peroxidase and IAA oxidase activities have been reported in a number of cases (McCune, 1961; Galston and McCune, 1961; Birecka and Galston, 1970; Lee, 1971b; Lee, 1972). The results obtained in the present study showed that 10.0 mg/l GA_3 had no significant effect on peroxidase activity, but 1.0 (low) and 50.0 (high) mg/l GA_3 levels inhibited the development of peroxidase activity. However, the stimulation in specific activity of peroxidase was observed both at 10.0 and 50.0 mg/l GA_3 concentrations, suggesting that the effect of GA_3 is possibly mediated through its effect on protein synthesis.

The present results showed an increase in the level of IAA oxidase after GA_3 treatment, stimulation being more pronounced at the lower levels (1.0 and 10.0 mg/l). Similar type of observations were made by Boldue *et al.* (1970),

Ockerse *et al.* (1970) and Lee (1971b). Lee (1971b) working with tobacco callus cultures demonstrated that actinomycin D and cycloheximide inhibited the increase in IAA oxidase isoenzymes, an indication of a requirement for both RNA and protein synthesis. In *Crotalaria* also cycloheximide inhibited the IAA oxidase activity (Chapter III, Section D, 6). GA_3 has been shown also to regulate the synthesis of RNA (Chandra and Varner, 1965; Johri and Varner, 1968) and specific enzyme proteins (Filner and Varner, 1967; Jacobson and Varner, 1967). It is likely, therefore, that GA_3-dependent increase in IAA oxidase was a result of action of GA_3 at the level of RNA synthesis.

Our results showed marked variation in the amounts of PAL and TAL in tissues grown on different GA_3 concentrations. As cycloheximide inhibited both PAL and TAL activities (Section D, 6), it is reasonable to realize the effects of GA_3 on PAL and TAL enzymes through its effects on RNA and protein synthesis as was the case with other two enzymes discussed above. An alternative possibility is that GA_3 acts through its effect on the reported (Walton and Sondheimir, 1968; Zucker, 1968) PAL inactivating protein. All the above effects of GA_3 on nucleic acids and protein

synthesis could also be interpreted in light of its suggested role as a derepressor of genes (Cleland, 1969).

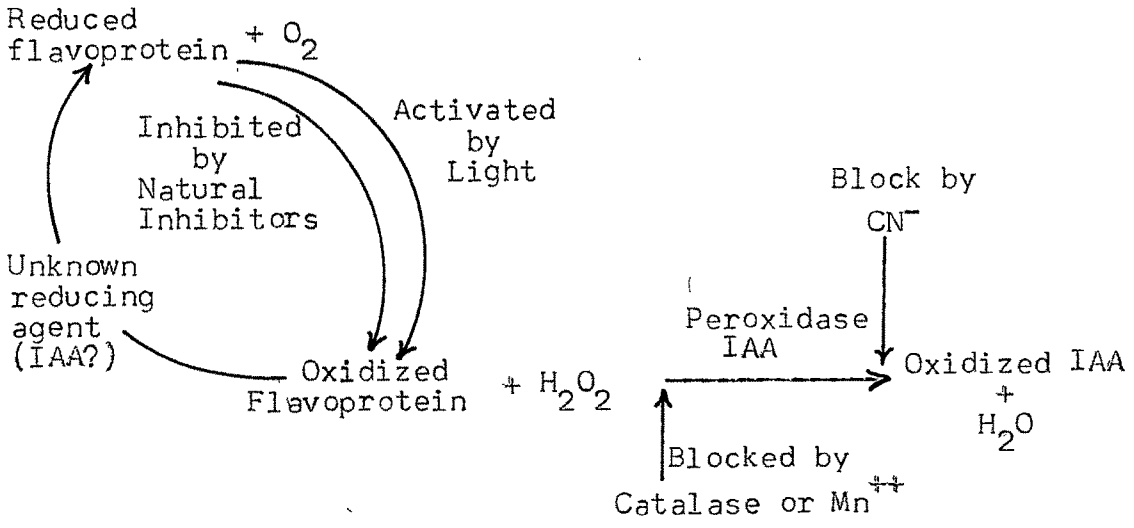
The growth of the cultures exposed to light was low in presence of both the auxins (2,4-D and NAA) at all the concentrations tested (Section D, 2 and 3). Light is not essential for the growth of thecultured tissues as such tissues depend upon an external supply of sugar. Though low light intensities were favourable to continuous growth of many cultures, relatively higher light intensities were inhibitory to growth (Street, 1966). One possibility of inhibitory effect of light on growth might be due to the photooxidation and photodecarboxylation of auxin as demonstrated by Zenk (1968). Light is also known to affect the enzyme systems such as peroxidase (Rao et al., 1969) and IAA oxidase (Galston, 1950; Galston and Baker, 1951; Galston et al., 1953). The results obtained in the present investigation also showed similar type of light action on peroxidase and IAA oxidase. Light is also reported to affect other metabolic events such as protein synthesis (Zucker, 1972) and carbohydrate metabolism (Creasy, 1968 b). In view of this, the role of light could well be realized in the regulation of growth.

Contrary to the above results, GA_3 at 10.0 and 50.0 mg/l stimulated growth in presence of light (Chapter III, Section D, 4). Thus, the inhibitory effect of light on growth was removed by GA_3 at 10.0 and 50.0 mg/l concentrations. Involvement of phytochrome in the light effects has been proposed by Liverman and Bonner (1953) who showed that the red-light effect on growth is reversed by far-red light in the usual phytochrome manner. Lockhart (1961) also interpreted GA_3 effect as relieving the red-light inhibition of growth. However, blue light effects could be mediated through the proposed (Galston, 1960; Wald, 1960) β-carotene or riboflavine pigment systems. But this is not yet well settled.

Haskins (1955) noted no significant difference in peroxidase activity in etiolated and green corn seedlings, but Rao *et al.* (1969) observed an 8-fold difference in the development of peroxidase in light-grown and dark-grown thalli of the liverwort *Riccia plana*; the higher level being reached in darkness. They therefore, concluded that light was inhibitory to the development of peroxidase activity. In contrast to this, the results obtained in the present study showed no inhibition of peroxidase activity by light in presence of various hormones tested except at higher 2,4-D and GA_3 concentrations. The light mediated peroxidase

activityc could possibly be interpreted in terms of its effects on IAA oxidase system, which is discussed below.

Like peroxidase, light enhanced the activity of IAA oxidase in presence of all the hormones tested. However, the higher level of 2,4-D, NAA and GA_3 reversed the promotory effect of light on IAA oxidase in Crotalaria callus cultures. The IAA oxidase system, as studied in crude extracts from etiolated peas by Galston (1950), Galston and Baker (1951), and Galston et al. (1953), has been shown to consist of a flavoprotein enzyme which produces hydrogen peroxide, and a peroxidase which oxidizes IAA by means of the peroxide as illustrated in the scheme below. These workers have shown



(Interrelation of the flavoprotein and other enzyme constituents of the IAA oxidase system and its activation by light as proposed by Galston et al., 1953).

that light activation of the flavoprotein enzyme occurs by the removal of naturally occurring inhibitors. Dialysis of the crude enzyme preparation greatly increases its activity in the dark, and the purified enzyme shows very little light activation. However, when the dialysate is added back, the inhibition is reimposed, and is reversible upon illumination.

From the work of Fang and Butts (1957) with Kidney bean, pea and corn plants, it appears that light may have a direct effect upon the function of the enzyme, different from that effecting its induction. Upon feeding IAA-1-^{14}C and measuring the subsequent production of respiratory $^{14}CO_2$, they found that the destruction of IAA proceeded much more rapidly in light than in the dark and in corn the IAA catabolism was completely dependent on light.

Auxin is reported to influence the synthesis of phytochrome (Clarkson and Hillman, 1967). GA_3 regulation of phytochrome action is discussed earlier. Assuming that light influences the peroxidase and IAA oxidase through phytochrome, it is easy to realize the reversibility of light effect by higher level of 2,4-D, NAA and GA_3. In support of this, Galston (1969) showed that red light

increased auxin breakdown in pea internode sections and Schopfer and Plachy (1972) demonstrated phytochrome mediated peroxidase activity. Further, evidence that phenolic inhibitors and co-factors for IAA oxidase may be responsible for the action of light has also been found (Engelsma and Meijer, 1965).

The stimulation of PAL and TAL activities in Crotalaria callus cultures by light in presence of different hormones was observed (Sections D, 2, 3 and 4). Since Zucker's report (1965) on the induction of PAL by light in potato tuber tissue the photoinduction of this enzyme has been studied in a great number of other plants (Nitsch and Nitsch, 1966; Durst and Mohr, 1966; Scherf and Zenk, 1967; Engelsma, 1967 a, 1967 b, 1974; Wong et al., 1974).

The requirement for light in PAL and TAL induction is not clear. Durst and Mohr (1966) obtained a doubling of PAL activity in mustard seedling subjected to continuous far-red irradiation, indicating that induction is mediated by the phytochrome. Finding that PAL synthesis was inhibited by CMU and that added carbohydrate would not stimulate induction in darkness, Zucker (1969) concluded that photosynthesis is

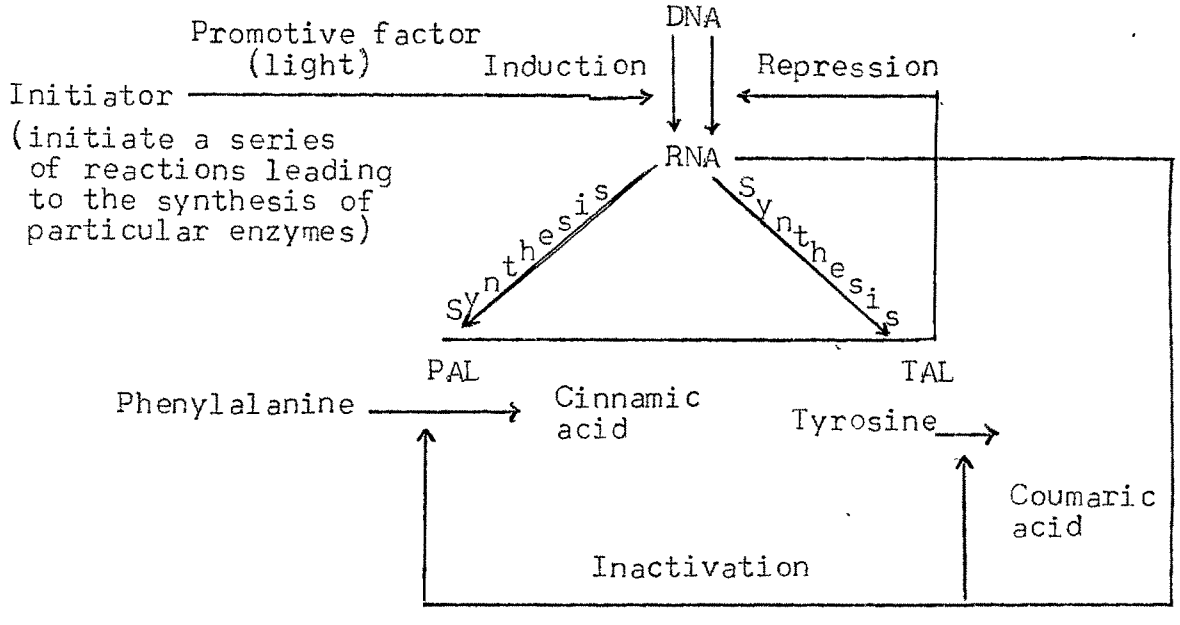
required for the induction of the enzyme. High intensity light can remove the requirement of sucrose in strawberry leaf disks (Creasy, 1968 b), presumably the higher rate of photosynthesis produces enough sucrose for PAL induction. However, high concentrations of sucrose cannot substitute for the requirement of light (Zucker, 1969) in Xanthium which indicated that the function of light is not solely to supply carbohydrate. Light could facilitate the uptake of external carbohydrate (Creasy, 1968 b) which causes accelerated rate of PAL synthesis. Another possible role of light is to provide a photosynthetic product which is required for the synthesis of PAL or to protect PAL against inactivation.

Engelsma (1967 a) showed that the effect on PAL of a saturating dose of blue light is greater than that of saturating red or far red light. He suggested that a distinction has to be made between the effect of red and far red light, which is probably mediated by phytochrome, and that of blue light which must be mediated by another pigment (in addition to phytochrome which absorbs also in the blue region). He proposed (1974) that the effect of blue light, at least as far as it is not mediated by phytochrome, might be due to trans-cis-conversion of (hydroxy) cinnamic acids in those cell compartments where a suitable photoreceptor

is present. The action spectrum (Engelsma, 1967 b) is in agreement with the concept of a flavin type of photoreceptor.

mRNA synthesis requirement for the induction of PAL was suggested by Zucker (1972). He further indicated that requirement of mRNA synthesis and the possible existence of an operon in phenolic biosynthesis are consistent with the gene activation theory of phytochrome action (Mohr, 1966). The phytochrome effects is also believed to be due to its effects on membrane properties (Smith, 1970; Borthwick, 1972).

The specific activity of PAL in dark samples was as high as that in light and in some instances even higher than that in light, suggesting that light also influenced the rate of inactivation of the enzyme. A similar phenomenon has been observed in potato tuber disks (Zucker, 1968) and in gherkin seedlings (Engelsma, 1967a). Thus, in Crotalaria callus cultures, it seems that light influences both the inactivation and synthesis of PAL. The nature of inactivating process is uncertain, but the effect of cycloheximide indicates that it depends on de novo protein synthesis. A tentative scheme proposed by Engelsma (1967a) is modified and illustrated below to explain the action of light on PAL and also on TAL.



Though the difference recorded between the PAL synthesis in presence and absence of light was not pronounced due to comparatively low light intensity used (1000 lux), the results clearly showed that there was good synthesis of PAL in absence of light but in presence of sucrose. This observation suggested that there could be two pools of PAL, the synthesis of one pool may require both sucrose and light and that of another only sucrose. Similar suggestion was made by Wong et al. (1974) for PAL in Strawberry leaf disks.

No literature is available on the photoinduction of TAL. The present study indicated photoinduction of TAL very much similar to that of PAL. This is obvious in view of the fact

that TAL performs an analogous reaction to PAL and that the activity of TAL resides in the PAL enzyme protein itself. The latter is further substantiated by the enzyme purification studies discussed later.

Kinetin effects on growth is discussed earlier. The growth responses to different kinetin concentrations - increasing levels were more inhibitory - is further evidence in favour of the suggestion that the endogenous level of cytokinins in Crotalaria callus cultures was sufficient to support maximum growth and that addition of kinetin (even as low as 0.04 mg/l) to the medium resulted into the supraoptimal concentration for growth.

Kinetin suppressed the polyphenol production in Crotalaria, the increasing level being more inhibitory (Section D, 5). This is in contrast to our results with Cassia callus cultures (Shah et al., 1976b) and Datura cell suspensions (Subbaiah, 1974) where increased production of phenolic compounds was recorded with kinetin treatment. It is interesting to note in this regard that while kinetin was essential for the continuous growth of Cassia and Datura cultures, it was inhibitory to the growth of Crotalaria cultures. Moreover, in all the three cultures, it was

observed that polyphenol synthesis was initiated after the cells embarked upon the phase of most rapid growth. Since kinetin is known to influence cell division, it is reasonable to believe that its effects on polyphenol production might be mediated through its effect on cell division in a particular hormonal milleau. Similar interpretation for kinetin effect on tracheal differentiation has been advanced (Fosket and Torrey, 1969; Fadia and Mehta, 1973).

Kinetin has also been shown to affect enzyme activities in cultured plant cells (Lavee and Galston, 1968; Lee, 1972; Subbaiah, 1974; Shah et al., 1976 b). Like polyphenols and growth, kinetin was also found inhibitory to enzyme development in Crotalaria callus cultures. Though the effects of kinetin on the development of enzyme activities is not well understood, it is possible that kinetin might influence them through its effects on nucleic acids, particularly tRNA. Srivastava (1966) and Fox (1966) observed that cytokinin can exert their biological effects through their incorporation into tRNA; thus influencing amino acid metabolism and protein synthesis. The requirement for both RNA and protein synthesis for the development of peroxidase, IAA oxidase, PAL and TAL was indicated by the inhibition of these enzymes by cycloheximide (Chapter III, Section D, 6). Similar observation

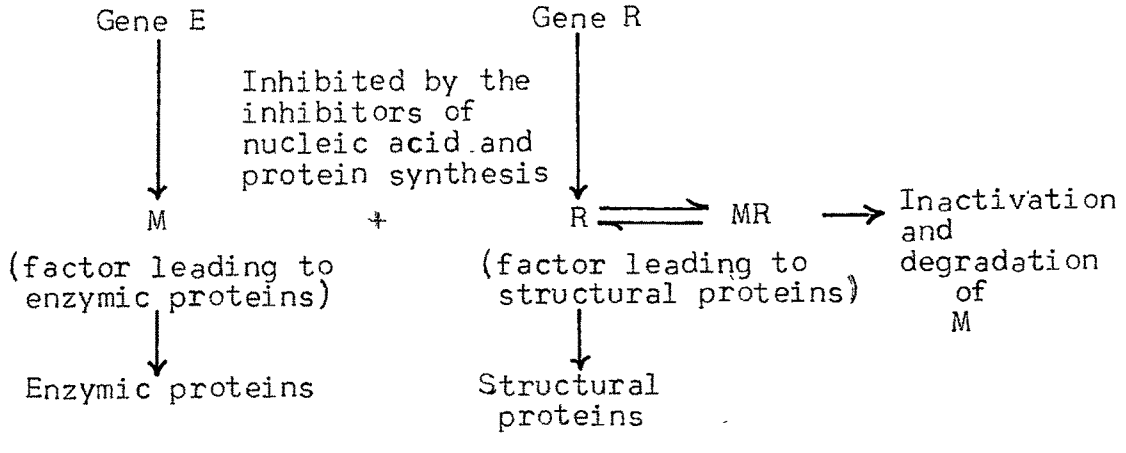
was made for IAA oxidase (Lee, 1971c, peroxidase (Lee, 1972) and for PAL (Zucker, 1972).

Though kinetin has suppressed the IAA oxidase activity over the control and though there was no significant difference recorded in this oxidase activity at various concentrations tested, higher kinetin level supported more activity. This kinetin effect can be attributed to be mediated through its effect on polyphenol production. Phenolic compounds are well-known cofactors (monophenols) and inhibitors (diphenols) of this oxidase activity (Stafford, 1974) and higher kinetin level supported less production of phenolic compounds. Thus, it might be possible that less production of phenolic compounds has favoured the development of oxidase activity but it could not totally overcome the inhibitory effects of kinetin. However, this indicated that kinetin also affected the quality of phenolic compounds produced and it needs further confirmation by examining the qualitative and quantitative changes in phenolic compounds at various kinetin concentrations.

At different concentrations of cycloheximide tested, drastic reduction in growth was registered (Chapter III, Section D, 6). Cycloheximide is a well-known inhibitor of protein synthesis effective at the translation level. Further, according to Ellis and Mac Donald (1970) cycloheximide stimulates the respiration and decreases the inorganic ion

uptake in plant tissue, suggesting an uncoupling action. In light of the above, the inhibition recorded in growth with cycloheximide treatment is obvious.

Like growth, all the four enzymes examined (i.e. peroxidase, IAA oxidase, PAL and TAL) were also inhibited by cycloheximide. This suggested that RNA and protein synthesis are necessary for the action and modulation of these enzymes. This is in agreement with the observations made by Lee (1971c, 1972), Davies (1972b), Klein-eude et al. (1974), Creasy (1974) and others on peroxidase, IAA oxidase and PAL enzymes. However, considerable induction in the specific activity of these enzymes was observed, indicating that inhibitory effects of cycloheximide was more marked on structural proteins rather than enzymic proteins. On this same assumption, the induction recorded in the specific activity of these enzymes could possibly be explained by the model illustrated below:



(Enzyme induction in plant cells (modified after Tomkins et al., 1969))

From the above model it is clear that if cycloheximide inhibition is more pronounced on the structural proteins, the degradation and inactivation of enzymic protein would be less. The overall result would be induction in specific activity of certain enzymes even though the total synthesis of enzyme is less than the control.

It is clear from the results obtained with Crotalaria callus cultures subjected to different cultural conditions that the ability to accumulate phenolic compounds was, in general, restricted to growing and dividing cells. However, these two phenomena can not be related as certain treatments enhanced polyphenol synthesis but inhibited growth. Further, it is also true that cell division and growth can take place in the absence of polyphenol synthesis. Now the question is whether non-dividing cells can synthesis such materials. Answer to this question is uncertain but the present data indicated that though cell division and growth favoured the synthesis of phenolic materials, it is generally not a prerequisite. This is evident from the experiments with tyrosine and/or phenylalanine into the nutrient medium resulted into enhanced production of polyphenols but reduction in growth.

The correlation obtained between polyphenol production

and the development of peroxidase activity under different cultural conditions is quite impressive. This suggested an important role of peroxidase in the biosynthesis of phenolic compounds. Our results with Datura (Subbaiah, 1974) and Cassia (Shah et al., 1976b) also suggested similar correlation. Peroxidases are capable of catalyzing the oxidation of a number of polyphenols. While the physiological function of this reaction is unknown, it could function in controlling the level ofp phenolic compounds in the cell as pointed out by Stafford (1974).

Growth responses and the development of IAA oxidase activity at different concentrations of sucrose, 2,4-D, NAA, GA_3 and kinetin clearly indicated that the enzyme IAA oxidase may be one of the factors regulating growth. Manipulation of this oxidase activity by variation in nutrient medium and thus modulation of growth is further evident from the present data. De novo synthesis of phenolic compounds are known to regulate this oxidase activity (Gortner and Kent, 1958). The results obtained have also suggested similar type of regulation.

A comparison of the estimated rates of polyphenol synthesis with PAL and TAL activities showed that there was

some discrepancy between the times at which their respective maxima occur. Similar observation was made by Davies (1972b) between the rates of polyphenol synthesis and PAL activity in Paul's scarlet rose cell suspensions. He further pointed that a correlation between enzyme activity and the rate of production of the end-product is not necessary to establish the indespensibility of that enzyme to the pathway, provided that the observed level of the enzyme is always adequate to achieve the observed output of the material. The present data raised some doubt about the ability of the observed PAL and TAL levels to sustain the observed rate of synthesis. However, the point is difficult to clearify, as also indicated by Davies (1972b), since it is based on the assumptions that the PAL and TAL activities reflect the in vivo situation, and that the molecular reactivities of all polyphenols synthesized in the cells are similar to that of the standard, chlorogenic acid. In view of the large differences reported for various phenols in their reactivity to the Folin-phenol reagent (Goldstein and Swain, 1963) it seems unlikely that such calculations can be made with confidence. With regard to the relationship between polyphenol synthesis and PAL and TAL activities in Crotalaria callus cultures, as far as timing and amplitude of the responses of both the parameters are concerned, the correlation appeared to be impressive under a variety of

conditions, including variation in initial sucrose, 2,4-D, NAA, GA_3 and kinetin concentrations.

The pattern of p-coumaryl CoA : ligase in Crotalaria callus cultures grown on the control (2.0 mg/l 2,4-D) medium is quite interesting. There was initial delay in the development of its activity. It was virtually absent for the first 10 days of culture period. The activity of this enzyme was picked up only with the maximum increase in the enzymes PAL and TAL. It seemed hence that the development of p-coumary CoA : ligase depended on the activities of PAL and TAL which could provide substrate necessary for the ligase activity. Similar concomitant large increases in the activities of PAL and p-coumarate : CoA ligase was also demonstrated in cell suspension cultures of soybean (Hahlbrock et al., 1971; Hahlbrock and Kuhlen, 1972).

The transamination of phenylalanine and tyrosine by extracts of plant tissues has been demonstrated (Gamborg and Wetter, 1963). Crotalaria tissue extract also exhibited transamination of phenylalanine and tyrosine in the presence of α-ketoglutarate. If shikimic acid pathway (Chart 1) is the exclusive route to phenylpropanoid compounds related to cinnamic acid it would follow that transaminase is an

essential enzyme, since phenylalanine and tyrosine are the sources of cinnamic acids (Brown, 1961; Swain, 1962). Considering the fact that transamination reactions are always reversible, the initial large increase in phenylalanine and tyrosine transaminases could provide evidence that they are generating phenylalanine and tyrosine for further metabolism. However, it could also be possible that they regulate the flow of phenylalanine and tyrosine to the secondary as well as primary metabolites as the reaction is reversible.

Neish (1960) discussed the possible role of phenyllactic acid in the formation of cinnamic acid. A sequence involving transamination of phenylalanine, reduction of the keto acid so formed to phenyllactic acid and dehydration of the later to form cinnamic acid was proposed. An analogous sequence was proposed forthe conversion of tyrosine to p-hydroxy-cinnamic acid. Evidence of an enzymatic nature (Koukol and Conn, 1961; Neish, 1961) has cast doubt on this reaction sequence and instead suggests that cinnamic and p-coumaric acid are produced by direct deamination of the corresponding amino acids. The co-ordination obtained between transanases, ammonia-lyases and p-coumarate : CoA ligase in present investigation also indicated operation of direct deamination

pathway in Crotalaria rather than the one proposed by Neish (1960).

Phenylalanine ammonia-lyase (PAL) has been purified to homogeneity from both the Crotalaria seedlings and the callus derived from it. A purification of 215.6 and 192 folds was achieved from seedling and callus respectively. The purified enzyme catalyses the deamination of both L-phenylalanine and L-tyrosine and the two substrates seem to have a common catalytic site. A similar pH profile with an optimum around 8.8 was recorded for PAL from both the origins.

The carbonyl reagents tested were found to bring about irreversible inactivation of PAL. In spite of several reports on the carbonyl reagents as inhibitors of PAL, the mechanism by which they bring about the inhibition is not well understood. Havir and Hanson,(1968 b) demonstrated that in potato enzyme while the inhibition caused by $NaBH_4$ was irreversible, the inhibition caused by NaCN, semicarbazide and phenylhydrazine could be reversed by removing these reagents. The present data, however, is in agreement with the observation of Kalghatgi and Subha Rao (1975) with Rhizoctonia solani where the inhibitor caused by carbonyl reagents in all the

cases was irreversible.

An investigation into the effects of a range of substrate concentrations on PAL activity from seedling and callus showed that the enzyme did not exhibit exact Michaelis-Menten kinetics. Instead, the Lineweaver-Burk (1934) plots were biphasic and the Hofstee (1959) plots were curvilinear. From these results two Km values were determined, K_m^H was 4.2 mM and K_m^L was 0.8 mM for seedling PAL and K_m^H was 5.0 mM and K_m^L was 0.4 mM for callus PAL. Iredale and Smith (1974) suggested two hypotheses to explain these unusual kinetics : (a) PAL has two substrate binding sites which differ in their affinity for the substrate and interact with each other, or (b) there are two isoenzymes of PAL with differing affinities for phenylalanines. However, evidence obtained from gel filtration and polyacrylamide gel electrophoresis in the present investigation indicated that there is present only one species of PAL with MW of 326,000. Thus, the first hypothesis seems likely to satisfy our kinetic data. Similar kinetic have been observed with PAL from a variety of sources (Havir and Hanson, 1968 a, 1968 b; Attridge et al., 1971; Iredale and Smith, 1974; Kalghatgi and Subba Rao, 1975).

Levitzi and Koshland (1969) suggested the kinetics of

regulatory enzymes exhibiting negative homotropic cooperativity. They also constructed the criteria for the recognition of negative homotropic interactions. These include : (1) the substrate saturation plot looks quantitatively like a Michaelis-Menten curve; (2) the Rs value is greater than 81; (3) the double reciprocal plot is concave downwards; and (4) the Hill coefficient is less than 1. In the case of Crotalaria seedling PAL and callus PAL all of these criteria are satisfied : the substrate saturation curves were quantitatively similar to Michaelis-Menten curve (Fig. E-2); the Rs. values were 86 and 88.48 for seedling and callus enzymes respectively; the double reciprocal plots were concave downwards and the Hill coefficient was 0.2 for the PAL from both the origins. This is, therefore, evident that PAL from Crotalaria exhibited negative homotropic interactions.

The Crotalaria enzyme is made up of two pairs of unidentical subunits (α = 72,000 and β = 90,000) and resembled the wheat (Nari et al., 1972) and Rhizoctonia (Kalghatgi and Subba Rao, 1975) enzyme. The temperature study revealed an activation energy of 7,700 cal. per mol for seedling enzyme and 4,800 cal. per mol for callus enzyme. Further, there were indications that PAL in this plant was a sulphydryl enzyme, similar to the PAL in barley (Koukol and

Conn, 1961), Tobacco (O'Neal and Keller, 1970) and Cucumis sativus (Iredale and Smith, 1974); but in marked contrast to the PAL in potatoes (Havir and Hanson, 1968 c) and Maize (Marsh, Havir and Hanson, 1968 a).

Cynnamic acid and its derivatives were found effective inhibitors of PAL. The inhibitory effect of cinnamic acid on PAL has been reported in various plant tissues (Koukol and Conn, 1961; Havir and Hanson, 1968, 1968 b; O'Neal and Keller, 1970; Iredale and Smith, 1974). This further suggested that PAL is a regulatory enzyme.

From the above discussion, it can be stated that in Crotalaria PAL is a regulatory enzyme exhibiting negative homotropic cooperativity. The regulatory importance of such negative interactions is not yet known (Levitzki and Koshland, 1969), although it is possible that they act to maintain relatively high rates of reactions even at very low substrate concentrations.

Summarizing the discussion, it is clear that Crotalaria callus cultures revealed certain potentialities such as : (1) fairly good growth on completely defined medium with self-sufficient kinetin-synthesizing machinary; (2) higher

accumulation of phenolic compounds than the part from which it was derived, very few of its kind to be reported from the cultured tissues; (3) high degree of regulatory mechanism of polyphenol synthesis, the lowest being 0.15% (on the medium with urea as sole nitrogen source) and the highest being 3.6% (on the medium supplemented with 0.1% tyrosine).Further the enzymatic studies showed that peroxidase was closely associated with polyphenol metabolism, while IAA oxidase was important in the physiology of growth; the later being also regulated by the endogenous levels of phenolic compounds. The relation between PAL, TAL and polyphenol synthesis, as far as the timing and magnitude of the response at various initial concentrations of different cultural parameters, was quite impressive. The coordination in the development of transaminases, ammonia-lyases and p-coumaryl CoA : ligase was also evident. The purification of PAL revealed certain properties which clearly indicates regulatory role of PAL in the synthesis of phenolic compounds.