CHAPTER IV

DISCUSSION

D I S C U S S I O N

Plant tissue culture is one of the most dynamic and promising tool for research in experimental biology for the study of various basic problems in plant physiology, plant biochemistry, etc. Plant cell and tissue cultures have enabled us to increase our knowledge tremendously in many areas. Recent advances in this field have made possible the investigations of growth and differentiation at the levels of tissues and cells. The present research endeavour concentrated on : initiation and establishment of callus cultures of _Nicotiana tabacum_ L. var. Anand-2 and _Gossypium hirsutum_ L., cv. Sankar-5; the examination of growth responses and accumulation of total and reducing sugars; accumulation of extractable starch; organogenetic responses as influenced by growth substances; the physiological changes during growth of callus; the study of certain enzymes during shoot and root differentiation both in light and dark; and lastly the study of these enzymes in cotton callus tissues which remain unorganised even when cultured on shoot and root differentiating medium of tobacco. An attempt was made to find out what is actually contributing for organogenetic development and what is not, by comparing the changes in enzyme activities in a differentiating tobacco callus and a non-differentiating cotton callus tissue.

A. Initiation and establishment of callus cultures of
    tobacco and cotton.

Callus was induced from the floral buds of N. tabacum L.
var. Anand-2 as described in Chapter III, Section A, Expt. 1.
The tobacco callus tissue was healthy and fast growing and
was maintained in 30 day cultural cycles. Chapter III,
Section A, Expt. 2, describes the induction and establishment
of callus cultures derived from the anthers of cotton.
Katterman et al. (1977) described the successful initiation
and transfer of viable callus of cotton G. barbadense L.
using Cleland's reagent, dithiothreitol, a strong antioxidant
(Cleland, 1964). They used high levels of auxin in the medium
described by Beasley and Ting (1973). On the contrary, Anita
Rani and Bhojwani (1976) reported that the stem and hypocotyl
segments of both Gossypium hirsutum and Gossypium arboreum
did not callus on White's medium alone; while on Murashige and
Skoog's medium supplemented with 1 mg/l kinetin, 1 mg/l NAA
and 40 mg/l adenine, 80% of the explants revealed prolifera-
tion. In the present study, callus was initiated from the
anthers of cotton without using any antioxidants in MS medium
supplemented with 2 mg/l each of IAA, NAA and kinetin. The
callus was initially brown but in course of subcultures, it
turned green and maintained a high rate of growth. Calli of
different plant species may vary in their texture, friability
and coloration. Stem callus of Citrus grandis gave callus
strains that were greenish-white, compact, nodular, and slow
growing, or that were highly friable and fast growing (Mitra
and Chaturvedi, 1972).

Cultural conditions for <u>Gossypium</u> sps. have been defined also by Smith and Price (1978). Using high light intensity (9000 Lux) at 30°C, they incorporated high doses of hormones, especially cytokinin (e.g. 10 mg/l of 2 ip) for prolonged cultures of some cotton species.

B - I. <u>Hormonal influence on growth and accumulation of total and reducing sugars in callus tissues of Gossypium hirsutum.</u>

In the present study, four auxins : IAA, IBA, NAA and 2,4-D and two cytokinins namely kinetin and BAP, and one gibberellin (GA_3) were tested for their ability to support callus growth and accumulation of total and reducing sugars.

First the above hormones were tested singly and then auxin-cytokinin, auxin-gibberellic acid and auxin-cytokinin-gibberellic acid interactions were studied. The results are given in Chapter III, Section B-I, Expts. 3-6.

A glance at tables 2 to 5 revealed that anther callus of cotton could grow in the absence of exogenous hormonal supply. Growth on fresh and dry weight basis increased 2.9 folds and 3.3 folds respectively during the course of culture for 4 weeks. Increasing concentrations of native auxin IAA stimulated growth, but at 5 mg/l level it was inhibited. The other three synthetic auxins IBA, NAA and 2,4-D inhibited growth of cotton callus with increase in hormonal concentration from 0.2 mg/l to 5.0 mg/l.

Moyed and Tuli (1968) have demonstrated that low levels of 3-methyleneoxindole - the degradation product of IAA - stimulated growth, whereas its higher concentrations antagonized it. Low 3-methyleneoxindole level can be achieved by either low IAA oxidase activity or by high 3-methylene-oxindole reductase activity. So at higher concentrations of IAA (5 mg/l), it may be that the rate of IAA destruction was high, resulting in low endogenous IAA and high 3-methyleneoxindole levels, which did not favour growth. On the other hand, the reverse situation should hold good for stimulation of growth at low levels of IAA.

In view of the known regulation of cell growth by IAA and the inverse correlation of IAA oxidase content with growth (Galston and Dalberg, 1954), it appears likely that the enzymatic destruction of IAA is important in regulating the amount of growth substance in the plant. Also, monophenols stimulate IAA oxidase activity resulting in low growth values; whereas diphenols inhibit IAA oxidase activity thus stimulating growth (Thimann et al., 1962; Furuya and Galston, 1961; Lee and Skoog, 1965 a, 1965 b; Van overbeek, 1966; Tomaszewski and Thimann, 1966).

Maximum cotton callus growth was obtained with the use of IBA in the medium. This cannot be explained because IBA oxidising enzyme has yet not been worked out fully. The literature at present is also scanty on how it affects the

primary and secondary metabolism of the cells in culture.
Bapat and Narayanaswamy (1977) have reported, on the
contrary, that IBA did not influence significantly the
growth of _Achras_ _sapota_ endosperm cultures.

Growth of cotton callus on NAA medium was almost
similar to that of IAA. Increasing concentrations of NAA
inhibited growth like that of IBA. NAA is reported to
stimulate the invertase activity which will result in better
utilization of carbohydrate source i.e., sucrose (Glasziou
et al., 1966). It is also known to enhance RNA content by
as much as 50% (Leaver, 1966), which would result in
increased synthesis of structural and functional proteins.
NAA is found to accumulate rapidly in plant tissues and is
then metabolized slowly to a series of unidentified deriva-
tives (Glasziou et al., 1968). The optimum level of NAA in
the system can thus be maintained resulting in rapid growth
of callus.

Compared to other three auxins, 2,4-D showed lesser
growth response. This could be explained by the following
arguments. Firstly, the degradation of 2,4-D in the plant
cells is known to be slow. It was shown to metabolize ten
times slower than IAA (Bendana et al., 1965), due to possible
lack of 2,4-D oxidizing enzyme system. Secondly, the rate of
2,4-D transport is very slow (McCready, 1963). Thirdly,
2,4-D is known to interfere with glucose metabolism (Bourke

et al., 1962) and to increase its catabolism via pentose phosphate pathway (Humphreys and Dugger, 1957).

In many cases, however, 2,4-D is found more effective than either IAA or NAA or IBA in callus induction (Yeoman, 1973) and in the maintenance of active growth and cell division (Tulecke et al., 1965; Gamborg et al., 1968; Rawal, 1979). Lamport (1964) and Simpkins et al. (1970) demonstrated the effectiveness of 2,4-D at low concentrations in promoting high growth rates and cell separation in sycamore suspension cultures. Growth as measured by increase in fresh and dry weight and total cell number was more rapid in NAA medium than that in 2,4-D medium in callus cultures of Cucumis melo (Fadia and Mehta, 1973); but in the case of mesocarp and endosperm cultures of Achras sapota (Bapat and Narayanaswamy, 1977) and also in callus cultures of Arachis hypogaea (Shailaja Rama Rao, 1978) growth was more on 2,4-D than that on IAA or NAA containing medium.

The indispensibility of kinetin for growth of callus tissues has been demonstrated by Murashige and Skoog (1962) and Steward et al. (1969). Kinetin induced growth is acredited to enhanced RNA and protein synthesis (Skoog et al., 1967; Letham and Williams, 1969; Kende and Gardner, 1976). Kinetin has, further, been reported to have additive effect to that of auxin for growth promotion of spruce and sycamore callus cultures (Steinhart et al., 1961; Digby and

Wareing, 1966). Investigations on a range media (Schenk
and Hildebrandt, 1972) have shown that tissues of dicoty-
ledonous plants are generally more dependent on an external
cytokinin supply than are tissues of monocotyledonous plants.
In the present study kinetin stimulated growth of cotton
callus (Table 3). Increasing concentrations of kinetin also
increased both fresh and dry weight of the tissues. 6-
benzylaminopurine (BAP) at 0.04 to 1.0 mg/l level increased
dry weight of the tissues but at higher level (2.0 mg/l) it
suppressed growth.

Murashige (1964, 1965) working with tobacco callus, and
Nickell and Tulecke (1959) working with various monocots and
dicots, found GA_3 to inhibit growth. These results were
confirmed in our laboratory earlier when tobacco tissues were
cultured on GA_3 medium (Rawal, 1979). Gibberellin is known to
influence oxidative or peroxidative enzyme activity resulting
in a decrease in IAA degradation. Also it was proposed that
gibberellin induced the formation of a compound which
inhibits the oxidative degradation of IAA. The evidence for
this possibility is, however, extremely tenuous; for (a) such
an effect must occur within 24 hours to account for the
increases in auxin levels noted (Kuraishi and Muir, 1964; Nitsch
and Nitsch, 1959, 1963; and Radiovova and Runkova, 1963); and
because (b) the effect must be a pronounced one since increase
in auxin level of upto 200 times have been recorded (Nitsch

and Nitsch, 1963). Watanabe and ·Stutz (1960) observed an increase in an inhibitor concentration in some tissues under some light conditions following GA_3 treatment. In the present study, callus cultures of cotton showed higher growth values at 25. mg/l level, while at 100 mg/l GA_3 level growth was much reduced. This inhibition of growth by GA_3 at 100 mg/l level is in agreement with the findings of Galston (1959) who reported an increased inhibitor content in pea seedlings due to GA_3. However, no change was found in the oxidative activity in dwarf pea stems over a 100-fold concentration range.

Of all the auxin-cytokinin combinations tested, 2 mg/l of IAA and 1 mg/l of BAP with 2% sucrose supported maximum growth (Table 4). Of all the auxin-gibberellin interactions tried maximum growth was supported by NAA + GA_3 (Table 5). Though on fresh and dry weight basis, 0.2 mg/l NAA, 1.0 mg/l BAP and 25.0 mg/l GA_3 in combination supported maximum growth of all the auxin-cytokinin-gibberellin interactions tried, during the subsequent sub-cultures the growth was found to decline very significantly due to some inexplicable reasons.

Both auxins and cytokinins readily form conjugates in plants. IAA and 2, 4-D can be converted to relatively inactive glucosides or glucosyl esters (Feung, 1975), a phenomenon often occuring in monocot tissue, or to the active amino acid conjugates (Feung et al., 1976) which are

more common in dicot tissue. Similarly cytokinins can form
a variety of glucosides, some active and some relatively
inactive. More recently amino acid conjugates of cytokinins
have also been detected (Wang, 1978). Since such conjugates
are formed from many exogenous compounds neither conjugation
mechanism can be considered unique to the two plant growth
regulators. It is more likely that the pathways represent
inactivation or storage mechanisms. The ease of hydrolysis
of some of these conjugates has led to the assumption that
the free acid or base is responsible for any biological
activity observed. Thus conjugation may be a way of preserving
the biological activity of the plant growth regulators
(Everett et al., 1978). An understanding of the molecular
action of auxin and cytokinin is further complicated when
note is taken of the interactions in tissue cultures which
can occur between these two hormones. The effect of cyto-
kinin on both auxin conjugation (Lau and Yang, 1973) and
degradation (Lee, 1974) represent direct evidence for one
facet of this interaction. However, there is far more
circumstantial evidence which indicates that hormones can
drastically alter each others metabolism or may indeed have
some common sites of action.

Experiments described in Section B-I clearly indicated
that the accumulation of total and reducing sugars in cotton
callus cultures was influenced by exogenous hormonal supply.
Maximum accumulation of total sugars was supported by

0.2 mg/l IAA in the medium; whereas maximum accumulation
of reducing sugars was noticed on 2.0 mg/l IAA (Table 2)
(Section B-I. Expt. 3). However, a correlation between
accumulation of sugars and callus growth could not be
drawn. 2.0 mg/l IBA supported maximum accumulation of total
sugars, while 5.0 mg/l IBA gave rise to maximum accumula-
tion of reducing sugars, where the growth was minimum. Of
all the auxins tested, IBA supported maximum growth as well
as maximum sugar accumulation (Table 2). 0.2 mg/l NAA
supported maximum growth and also maximum accumulation of
reducing sugars. On the other hand, 2.0 mg/l NAA supported
minimal growth, but total sugar accumulation was maximum.
Like NAA, 2,4-D also showed maximum growth on 0.2 mg/l,
where accumulation of reducing sugars was high. 2,4-D at
1.0 mg/l level, exhibited maximum accumulation of total
sugars.

Of the two cytokinins tested KN supported maximum
growth, while BAP at 2.0 mg/l level supported maximum
accumulation of total and reducing sugars. GA_3 at lower
concentrations i.e., at 5.0 mg/l supported maximum accumu-
lation of total sugars, but reducing sugars were higher at
50.0 mg/l. A correlation between growth and sugar accumula-
tion was not noticed in any of the above treatments (Section
B-I. Expt. 4. Table 3).

Among auxin-cytokinin interactions, 2,4-D (0.2 mg/l)

+ BAP (1.0 mg/l) supported maximum accumulation of total and reducing sugars but growth was minimum on this medium. Though growth was moderate, 2.0 mg/l IAA + 25.0 mg/l GA_3 registered maximum accumulation of total and reducing sugars. Out of the auxin-cytokinin-gibberellin combinations, maximum accumulation of total sugars was noticed on 2,4-D + BAP + GA_3 medium where growth was minimum. On the other hand, maximum accumulation of reducing sugars was seen on NAA + KN + GA_3 medium. Effect of phytohormones on the accumulation of sugars is not worked out extensively in cultured plant cells. However, total and reducing sugar accumulation much depends on enzymatic activities. Amylase and invertase are known to produce reducing sugars and these enzymes are much influenced by phytohormones (Bourke et al., 1962; Humphreys and Dugger, 1957; Wedding et al., 1967; Palmer, 1968; Glasziou et al., 1966; Jacobsen, 1970; Varner and Chandra, 1964). Also the possibility of direct and indirect effects, such as influence of auxin levels on sucrose metabolism cannot be ignored (Wiggans, 1954).

B - II. <u>Influence of carbohydrates on growth and accumulation of total and reducing sugars in callus cultures of G. hirsutum.</u>

Experiments conducted with the callus cultures of cotton to examine the influence of different sugars on growth, morphogenetic response if any, and total and reducing sugar

accumulation, are described in Chapter III, Section B-II, and Expts. 7-9. Though a wide range of different sugars was used in the medium as a source of carbon, no morphogenetic response was observed in callus cultures of cotton. Different concentrations of mono, di, tri and polysaccharides and also sugar alcohols were used in the basal medium to study their influence on growth. The different carbohydrates tested were glucose, fructose, galactose, arabinose, mannose, sorbose, sucrose, maltose, lactose, raffinose, starch, myo-inositol, mannitol and sorbitol.

Out of the monosaccharides tested, only glucose and fructose supported good growth. Among di, tri and poly-saccharides, sucrose, maltose and starch supported growth but none of the sugar alcohols used supported callus growth. Increasing concentrations of these carbohydrates glucose, fructose, sucrose, maltose and starch also increased the growth as measured by fresh and dry weight and also the accumulation of total and reducing sugars.

Carbon source is the invariable constituent of culture media used for growing plant tissues. The carbohydrate nutrition of plant callus and organ cultures has an important bearing in understanding their metabolism. The effects of certain carbon sources on the growth of tissue cultures have been reviewed by Gautheret (1959), White (1951), Goldsworthy (1964), Street (1966, 1969) and Maretzki et al. (1974). The

usefulness of sugar as carbon source may vary with its
quality and quantity for the cultivation of tissues from
a variety of plant species. Gautheret (1941) determined
optimum sucrose concentration for the growth of carrot
root callus cultures. Later (Gautheret, 1945) equimolar
concentrations of other sugars were used and their nutritive
value was compared with that of sucrose. Most of the plant
tissues grow successfully on either sucrose, glucose or
fructose but they may vary widely in their ability to use
other carbon sources. Maltose has been reported to support
growth of plant tissue by several workers (Simpkins et al.,
1970; Anstis and Northcote, 1973). Different species of the
same genus (Mathes et al., 1973), different cultivars of
same species (Chong and Taper, 1972), different organs of
the same plant (Mathes et al., 1973) and even different
clones derived from the same parent callus cultures (Arya
et al., 1962) have been found to differ in their response
to carbohydrate when grown in vitro.

Marigold tissue recorded increased weight on mannose,
maltose and cellobiose; periwinkle tissue on galactose,
maltose, lactose, cellobiose and raffinose; and tobacco on
maltose and cellobiose (Hildebrandt and Riker, 1948). The
divergence among species in their preference for different
carbohydrates was demonstrated in a direct comparison of
tissue isolated from five species (Hildebrandt and Riker,
1949), and the comparative effectiveness of hexoses,

pentoses, disaccharides and polysaccharides for callus
growth of different species, including those isolated from
tumor tissue, has been more generally reviewed (Gautheret,
1959). Galactose has been also shown to promote growth of
many cultured species (Mathes et al., 1973; Meryl Smith and
Stone, 1973; Thorpe and Laishley, 1974; Nickell and Maretzki,
1970), but mannose utilization is usually poor (Mathes et al.,
1973; Meryl Smith and Stone, 1973; Nickell and Maretzki, 1970).
In the present study galactose, arabinose, mannose, sorbose,
lactose and raffinose failed to support growth of cotton
callus. On the other hand, lactose and raffinose have
supported growth and proved a suitable energy source for
some species as reported by Mathes et al. (1973), Thorpe
and Laishley (1974) and Nickell and Maretzki (1970).

Natural polysaccharides are generally not utilized by
plant tissues although Hildebrandt and Riker (1949, 1953)
have reported utilization of pectin, dextrin and soluble
starch by tumor culture of Catharanthus roseus and Chrysan-
themum frutescence. Soluble starch was found to be effective
as a carbon source for virus tumor tissue of Rumex acetosa
(Nickell and Burkholder, 1950; Brakke and Nickell, 1955),
corn endosperm tissue (Straus and La Rue, 1954), callus
cultures of Rubus (Karstens and Cats, 1960) and Juniperus
(Constabel, 1960, 1961, 1963). In the present study, it was
found that cotton callus has the capacity to utilize
exogenously supplied starch to a considerable extent. There

is evidence that utilization of starch and maltose by tissues in culture is dependent upon the secretion of the appropriate hydrolytic enzymes into the culture medium (Street, 1969; Jaspars and Veldstra, 1965; Maretzki et al., 1971; Brakke and Nickell, 1951).

Sugar alcohol myo-inositol has been shown to be essential for spruce tumor tissue (Risser and White, 1964), Fraxinus callus cultures (Wolter and Skoog, 1966) and Convolvulus arvensis cells (Earle and Torrey, 1965). Hendre et al. (1975) reported that growth of maize, wheat, rice and sorghum was not significantly affected when its concentration was varied from 0 to 200 mg/l. However, cotton callus tissues failed to grow on inositol, mannitol and sorbitol at the concentrations tried. Chong and Taper (1972) have reported that callus tissues of apple could be successfully grown on sorbitol as the sole carbon source. The growth rate on sorbitol was equivalent to, and in some cases greater than, that on sucrose. Mannitol, however, is usually either not available for growth (Homes, 1967; Goris, 1971), or sustains only poor growth (Hildebrandt and Riker, 1949).

C - I. <u>Physiological studies with Amylase, Invertase,</u>
<u>MDH, G-6-PDH and FDPA as well as sugars and starch</u>
<u>during growth of callus tissues of tobacco and cotton</u>

Metabolic changes associated with wounding or ageing
of plant organs have been investigated by many authors.
However, very few details are available with higher plants
which picturise metabolic events, the extent and timing of
enzyme synthesis and their activities during growth of
callus in culture.

The results obtained while following the carbohydrate
metabolism in tobacco and cotton tissues during growth in
culture are described in Chapter III, Section C-I, Expts.
10-11. The callus tissues of tobacco exhibited only lag and
exponential phases of growth, <u>the stationary was absent</u> in
the culture regime provided. Throughout lag and exponential
phases of growth, specific amylase activity declined.
Contrary to this, invertase activity per unit protein basis
increased during lag and early exponential phases of growth.
However, the enzyme activity declined during late exponen-
tial phase of growth. The specific MDH activity was highest
during the lag phase. Later on it declined. Likewise
activities of G-6-PDH and FDPA per unit protein basis shot
up during lag phase, but declined during the exponential
growth phase.

As in the case of tobacco, in cotton also both lag and exponential phases of growth were observed, the stationary phase being absent. All the enzymes studied : amylase invertase, MDH, G-6-PDH and FDPA showed higher activities during the lag period. The activities either remained steady or declined during exponential growth phase.

In both the tissues, total and reducing sugars accumulated fast during lag period but declined thereof very fast. Total starch accumulated in tobacco during lag period, but in cotton it declined during the same period. During exponential and post-exponential phases of growth starch content slightly declined in tobacco but it increased in cotton.

Few investigations have reported activity levels of enzymes during the culture cycle of cell and tissue cultures from higher plants. Such studies have showed that the activities of enzymes during the lag, exponential and stationary phases varied. During the growth cycle of Paul's Scarlet Rose, sucrose was hydrolysed and by the end of the exponential phase only reducing sugars were present in the medium (Nash and Boll, 1975). The reducing sugars were found to be glucose and fructose in equal amounts. A similar inversion of sucrose has been observed during the growth of tomato roots (Weston and Street, 1968), tobacco callus (Thorpe and Meier, 1973) and Acer suspension

cultures (Simpkins et al., 1970). The inversion of sucrose
has been attributed to surface-located hydrolytic enzymes
(Weston and Street, 1968) and more specifically to a cell-
wall bound invertase (Thorpe and Meier, 1973). Wall bound
invertase generally occurs in higher plants. Its activity
can fluctuate considerably and seems to depend on the stage
of development of the tissues in which it is found (Lyne
and ap Rees, 1971; Jaynes and Nelson, 1971; Shannon and
Dougherty, 1972). Moreover, Klis and Hak (1972) have shown
that the activity of acid invertase in cell walls of
Convolvulus callus increased markedly upon the transfer of
tissue fragments to fresh nutrient medium.

Clearly the higher activities of amylase and invertase
in the present study account for the large amounts of
reducing sugars during the lag phase of growth. The high
levels of reducing sugar in sucrose medium during the initial
growth phase in rose cells similarly suggest high invertase
activity (Nash and Boll, 1975).

The MDH activity both in tobacco and cotton callus
cultures markedly increased in the first few days when there
was hardly any growth at all. Studies with Nicotiana (De
Jong, 1967) and Paul's Scarlet Rose (Nash and Davies, 1975)
cell suspension cultures have shown a rapid rise in total
MDH activity till the mid-exponential phase of growth
followed by a very rapid decline during the remainder of

the exponential period. In the present study, however, the situation varied with the tissue; for in tobacco callus the MDH activity was high during the lag phase, while with cotton callus it was high during the lag as well as exponential period and then declined during the post-exponential phase. Givan and Collin (1967) have demonstrated high respiratory rate during the lag phase of growth in Acer pseudoplatanus cell suspensions. The respiratory rate declined with the progress of growth as also did cellular nitrogen content. Thus the energy required for exponential growth is produced prior to its onset both in case of tobacco and sycamore.

Some authors have further shown that the growth of plant cells either as callus (Komamine et al., 1969; Thorpe and Laishley, 1973) or in liquid suspensions (Fowler, 1971; Givan and Collin, 1967) is accompanied by not only in total respiratory activity but also in the absolute and relative capacities and activities of the EMP pathway and pentose phosphate pathway (PPP). Work with batch suspension cultures suggested that the enhanced respiratory activity during the lag phase of culture was characterised by an increased PPP activity relative to the EMP pathway. There-after during the exponential growth the respiratory activity per cell declined and so also the absolute capacity and relative activity of the PPP. Marked changes were noted

during growth in the activities of key EMP pathway and PPP enzymes and also in the level and pattern of oxidative phosphorylation (Wilson, 1971).

In the present study peak values of G-6-PDH activity were noticed during early exponential and lag phases of growth respectively in tobacco and cotton tissues. Similar activity for the enzyme FDPA was also observed in both the tissues. These findings support the earlier work of Fowler (1971), and Wilson (1971) who suggested that lag phase is characterised by intensive metabolic activity and that the balance of metabolism is directed towards biosynthesis which required the development of reducing power generated through the PP pathway. Shimizu et al. (1977) working with Acer pseudoplatanus batch cultures also supported similar view.

C - II. Physiological studies with GOT, ME and PEPC and total and reducing sugars during growth of callus tissues of tobacco and cotton in the dark.

Of the many physical factors that influence growth and differentiation, light plays a vital role. Very few physiological studies have been hitherto made with callus cultures grown in the dark. In the present study, the role of dark fixation of CO_2 which utilizes phosphoenolpyruvate derived from carbohydrate as a substrate is examined during growth

in culture of tobacco and cotton tissues.

The control of intracellular pH in a cell may involve the regulation of intracellular organic acid levels, and Davies (1973) has suggested that phosphoenolpyruvate carboxylase activity lowers the intracellular pH by generating organic acids through a process of dark CO_2 fixation. He further suggested that malic enzyme by catalyzing the decarboxylation of malate has an opposite effect. Supporting the work of Davies, Bown and Lampman (1971) demonstrated the presence of these two enzymes in Avena sativa coleoptiles. Later Bown et al. (1974) showed the stimulation of Avena coleoptile growth by CO_2, and a correlation between malate accumulation and IAA-stimulated growth was revealed by Haschke and Luttge (1975). In addition, two recent papers demonstrated IAA stimulation of CO_2 fixation after a lag period of 60 minutes (Haschke and Luttge, 1977; Dymock et al., 1977), indicating that promotion of CO_2 fixation is secondary to IAA stimulation of H^+ extrusion and growth which occur with lag periods between 10 and 20 minutes (Jacobs and Ray, 1976; Cleland, 1976). Further, the PEP carboxylase from maize leaves was inhibited by physiological concentrations of oxaloacetic acid (Lowe and Slack, 1971).

Growth of tobacco and cotton tissues when grown in dark was reduced when compared with that in light. In tobacco

callus tissues, GOT activity per unit protein declined sharply during the lag phase of growth, but reached a peak during late exponential phase. In contrast to this, peak specific activity of GOT was obtained much earlier in cotton tissues (6 days earlier than in tobacco). Dhindsa et al. (1979) have noticed, however, peak GOT activity on day 4 in tobacco callus, the activity remaining more or less unchanged thereafter. Unlike this, in the present studies the GOT activities declined after attaining peak values in both cotton and tobacco callus.

Both malic enzyme and PEP carboxylase activities could not be detected on day 0 in tobacco and cotton tissues. Peak specific activity of ME was observed on day 3 i.e., during lag phase of growth in tobacco as well as in cotton. PEPC exhibited the peak value on day 3 in cotton callus, while in tobacco the peak was attained on day 9. All the three enzyme activities were higher in cotton callus when compared with tobacco.

CO_2 has been shown to be essential for the initiation of growth in sycamore cell cultures (Gathercole et al., 1976). During non-autotrophic carbon fixation, malate is known to act as an osmoregulatory solute in turgor-driven cell growth (Dhindsa et al., 1975); and it can, through its decarboxylation, also provide NADPH for reductive biosynthesis (Ting and Dugger, 1965; Fowler, 1974). Dhindsa et al. (1979)

have shown an increase in malate content with the growth
of tobacco callus. If malate inhibits PEP carboxylase
activity, the later activity should decrease with an
increase in growth. This is confirmed in the present
study with cotton and tobacco callus in both of which the
PEP carboxylase activity declined during rapid callus
growth (Expts. 12, 13). The malate inhibition of labelled
bicarbonate incorporation into a labelled oxaloacetic acid
derivative (Hill and Bown, 1978) demonstrated that malate
inhibition was not due to a mass action effect in the assay
which couples PEP carboxylase and MDH in malate production.
The correlation of malate accumulation with IAA-stimulated
growth in coleoptile tissue (Haschke and Luttge, 1975)
suggested that the compensating mechanism may be malate
production through CO_2 fixation. This suggestion supported
the earlier observation that the maximum growth response
to IAA required the presence of CO_2 (Bown et al., 1974). The
continuous build-up of malate in tobacco callus (Dhindsa
et al., 1979) seemed to indicate that malate maybe acting
primarily as an osmotic solute to regulate cell expansion
between successive cell divisions. Although no evidence is
presented here, this interpretation is in keeping with the
previously reported role of malate in cotton fibre growth
in vitro (Dhindsa et al., 1975) and also in stomatal opening
(Allaway, 1973).

D. Hormonal effect on growth and development of Amylase, Invertase, MDH, G-6-PDH and FDPA and total and reducing sugars in callus cultures of cotton.

It is a tacit assumption that the regulation of plant growth and differentiation has its origin partly in changes in the pattern of enzyme activities. Even if this is admitted to be true, it does not greatly advance our understanding since the questions which are then raised reveal the extensive ignorance that prevails. For example, is the whole pattern of enzymes changed or just a related group, or only one? If many enzymes change, are all the changes of equal significance, or is only one enzymic event crucial to the physiological response? Is there a specific temporal order in the enzyme changes, or does the interlinking of metabolic pathway account for apparent sequences of events? There are many reports in the literature that growth substances either increase or decrease the enzymic activities. To mention a few, auxins have stimulated the activities of ATPase in oat coleoptile (Hager et al., 1971), RNA polymerase in soyabean hypocotyl (O'Brien et al., 1968), DNA polymerase in soyabean hypocotyl (Leffler et al., 1971) etc. Cytokinins are also shown either to inhibit many enzyme activities such as protease and RNase in several leaves (Kende, 1971; Srivastava, 1968) or promote enzymes like RNA polymerase, DNA polymerase in several calluses (Kende, 1971;Vajranabhaiah

and Mehta, 1977). Gibberellins are known to stimulate the activities of α-amylase (Yomo and Varner, 1971), RNA polymerase in pea stem (Johri and Varner, 1968), invertase in Artichoke tuber discs (Leaver, 1966), etc..

Every growth substance can apparently under appropriate conditions regulate the synthesis of RNA and probably also the synthesis of DNA, and in turn cell division. Also every growth substance can regulate the activity of one or more hydrolytic enzymes. Of these, an interesting group are the cell wall polysaccharides, since these have to pass through the plasmalemma in order to reach their substrates.

In the present study on cotton callus lower concentration of IAA (2.0 mg/l) stimulated both growth and the activities of hydrolytic enzymes like amylase and invertase as well as those of the EMP and PP pathway enzymes e.g. MDH, G-6-PDH and FDPA when compared with the higher concentration (5.0 mg/l).

Lower concentration of NAA (0.2 mg/l) promoted invertase and G-6-PDH activity, while higher concentration (2.0 mg/l) enhanced the activities of amylase, MDH and FDPA.

2,4-D at the two concentrations (0.2 and 5.0 mg/l) tested did not show any marked effect on amylase activity in cotton callus. Higher concentration, on the other hand, (5.0 mg/l) promoted invertase activity. MDH showed higher

activities at 5.0 mg/l 2,4-D level till day 15 and there-
after higher MDH activity was noticed at 0.2 mg/l level.
In contrast, the FDPA was higher at 0.2 mg/l 2,4-D level
till day 15 and thereafter it was more at 5.0 mg/l level.
G-6-PDH was, however, promoted at low 2,4-D level (0.2 mg/l).

Of all the auxins tested, NAA at 2.0 mg/l level was
more effective in promoting the activity of amylase; while
2,4-D at both the concentrations tested was highly inhibitory.
The differential effect of NAA and 2,4-D as far as amylase
activity is concerned has seldom been reported in literature.

At 0.04 mg/l kinetin amylase was considerably inhibited;
whereas higher concentration (2.0 mg/l) had stimulatory
effect. The higher rates of nucleic acid and protein synthesis
reported to occur in washed slices of plant storage organs
are probably associated with the increased levels of inver-
tase and IAA oxidase (Edelman and Hall, 1965); though, on
the other hand, there are several enzymes notably starch
phosphorylase and amylase which fail to show higher activi-
ties in washed tissue (Palmer, 1968). Similarly, in the present
studies with cotton callus amylase always failed to show
higher activities during early stages of growth unlike other
enzymes. Only towards the termination of culture growth,
amylase registered higher activity. This is probably due to
higher degradation of endogenous starch which is needed for
the growth of the tissues. This view is substantiated further

when the endogenous level of starch was determined as
mentioned earlier in the text.

Though GA did not support good growth of cotton callus,
the activity of amylase was found quite high. Specific
activity of amylase was higher on 100 mg/l GA containing
medium than on 25 mg/l GA containing medium. It is well
known that GA induces the de novo synthesis of amylase in
the endosperm of barley (Paleg, 1960 a; 1960 b; Yomo, 1960;
Chrispeels and Varner, 1966; Jacobsen, 1970; Jacobsen and
Knox, 1970). This enzyme induction depends on RNA and protein
synthesis, as determined by the use of inhibitors (Varner,
1964; Varner and Chandra, 1964; Chrispeels and Varner, 1967).
Higgins et al. (1976) have found that (in the hormone-
treated tissue) the level of translatable mRNA for α -amylase
increased parallel with the increased rate of enzyme synthesis.
However, it was not so with in vitro grown tissues of cotton,
since the amylase activity was high towards the termination
of culture period, but not during the initial lag phase.

Among the plant growth regulators, GA_3 has been known
to increase the activity of amylase. In the present study
NAA was also found to stimulate the activity of amylase in
cotton callus cultures at 2.0 mg/l level.

In Daucus carota (Ricardo et al., 1972) and in
Convolvulus arvensis callus (Klis and Hak, 1972) only acid

cell wall invertases have been found. This acid cell wall invertase was, at least in part, tightly bound to the cell wall. In Acer pseudoplatanus cultures, acid as well as neutral invertases exist (Copping and Street, 1972). Although the ratio of bound to soluble enzymes shifted, depending on the phase of the culture growth cycle, both these enzymes were partly bound to cell wall fractions.

In the present study acidic, neutral and alkaline invertases, both wall bound and cytoplasmic, have been detected in callus cultures of Gossypium hirsutum (unpublished data); but only neutral cytoplasmic invertase is reported here, since it has been postulated that neutral invertase is predominantly in the cytoplasm. This has led to the hypothesis that the neutral invertase is responsible for sucrose hydrolysis in the cytoplasm in cells having less sugar demand and in which sucrose is being stored (Hatch and Glasziou, 1963; Bacon et al., 1965; Hawker, 1969; Ricardo and ap Rees, 1970).

Of the three auxins tested, IAA at 2.0 mg/l promoted invertase activity, while 2,4-D at 0.2 mg/l level inhibited it. Higher concentration of kinetin (2.0 mg/l) promoted neutral invertase activity in cotton callus cultures when compared to that in 0.04 mg/l kinetin containing medium. Of all the hormones tested, 2.0 mg/l kinetin gave high values. While GA at 25 mg/l inhibited the invertase activity,

100 mg/l GA promoted it considerably. The enhanced enzyme activities in all above treatments have been noticed during the initial lag or the exponential growth phase in cotton.

In _Acer pseudoplatanus_ cells, high activity of both acid and neutral invertases correspond to periods of active growth and cell division (Copping and Street, 1972) and there is a similar trend in _Saccharum_ sps. (Maretzki et al., 1974). Their results were confirmed in the present study of cotton callus tissues where high activity of soluble neutral invertase corresponded to periods of active callus growth.

The relationship, or lack thereof, between cell wall invertase and cell growth has been discussed well by Edelman and Hanson (1971 b), Copping and Street (1972), Klis et al. (1974) and Thorpe and Meier (1973). Glasziou and Waldron (1964) have reported that in sugar cane stalks low concentrations of IAA increased the acid - invertase level, while higher concentrations suppressed the activity. Present studies support this view with soluble neutral invertase. However, Edelman and Hall (1965) showed that invertase was inhibited by IAA.

Glasziou et al. (1966) presented evidence that the auxin NAA, stimulated the development of invertase in sugar cane disks. The RNA content increased by about 50 per cent

within 24 hours of washing carrot disks and thus preceded
the increase in invertase (Leaver, 1966). In the agreement
of the results of Glasziou et al. (1966), present studies
with cotton also showed development of neutral soluble
invertase activity at low concentration of .NAA (0.2 mg/l),
but higher concentration (2.0 mg/l) proved inhibitory. The
later response is similar to that reported for the invertase
bound to the cell walls of carrot root and artichoke stem
tubers (Edelman and Hall, 1964). Low level of 2,4-D
(0.2 mg/l) inhibited invertase development to a considerable
extent and this inhibition was not that much when the level
of 2,4-D was higher (5.0 mg/l). Palmer (1968) had observed
that 2,4-D was active in preventing the induction of high
levels of phosphate metabolism and also invertase in plant
tuber tissues (Palmer, 1968).

More detailed studies have yet to be made of the
enzymic activity of soluble neutral invertase and its
hormonal regulation in vitro.

Cell growth depends on carbohydrate utilization for
two primary functions : formation of major cell components,
and as an energy source. The assimilation of ^{14}C-sucrose
into a rapidly turned over soluble pool, and its extensive
utilization for protein synthesis as well as protein turn-
over, was demonstrated by Steward and Bidwell (1966) in
carrot cells. The balance between utilization for biosynthesis

or respiration shifts, depending on the stage of cell
development. Increased rates of respiration in vivo was
observed by many authors (Laties, 1963; ap Rees and Beevers,
1960; Click and Hackett, 1963) when tubers of many plants
were sliced in thin disks and washed in water. Also
dramatic increased rates of respiration in vitro was
observed in tobacco (Bellamy and Bielesky, 1966), Acer
pseudoplatanus (Givan and Collin, 1967), Rosa sp. (Nash
and Davies, 1972), etc. In each case oxygen consumption
increased twofold or more until the 4 to 6th day of culture.
This was followed by a period of lessening oxygen demand
over the subsequent 8 to 14 days. Respiration rate changes
in Saccharum sp. cultures (Maretzki et al., 1974) and the
activity of MDH in the present study of cotton callus are
similar to those mentioned above. These similarities are
likely to be more apparent than real, since growth rates
depend not only on origin of the culture but also on
culturing conditions. Nevertheless, the high mitochondrial
activity that develops a few days after cells are trans-
ferred to fresh medium - a feature that all cell cultures
have in common is significant. Also, peak respiration rate
is correlated with active protein and nucleic acid
synthesis preceding the phase of rapid cell division
(Givan and Collin, 1967). Clearly the plant growth
substances seemed to play a regulatory role in the activity
of MDH in callus cultures of cotton in the present study.

In Acer pseudoplatanus lowest activities throughout
the growth cycle were found in phosphofructokinase for the
EMP pathway, and in glucose-6-phosphate dehydrogenase and
transketolase for the PP pathway (Fowler, 1971). These
enzymes were, therefore, considered rate-limiting. According
to Maretzki et al. (1974) G-6-PDH is not a rate-limiting
enzyme for the PP pathway in Saccharum sp. In the present
study with cotton callus also G-6-PDH for PP pathway and
FDPA for EMP pathway do not seem to be rate-limiting enzymes.
On the other hand, there is similarity in the changes most
of these enzymes undergo in their activity during the growth
cycle, and these enzymes have activity peaks that coincide
with those for oxygen consumption also (Komamine and Shimizu,
1975). Working with Acer pseudoplatanus cell suspension
cultures Shimizu et al. (1977) have reported that respiratory
activity per cell was highest during intensive metabolic
activity. During this lag phase they found high levels of
ATP, NAD and NADPH and also demonstrated the peak in energy
charge of the culture. Once cell division has commenced
there is a gradual change in the pattern of metabolism.
Although the rate of oxygen uptake began to decline, the
level of ATP was maintained, but the level of NADPH and the
energy charge declined. This indicated that the balance of
metabolism had altered and that the demand for biosynthetic
reducing equivalents had decreased.

Present study was carried out with callus cultures
of cotton and such a system is limited in its potential
for regulatory studies because of the transient nature of
the growth phases and the inflexibility of growth control.
Such restrictions may be overcome by the use of continuous
(chemostat) cultures since these systems allow the mainte-
nance of steady states of cellular activity in a particular
growth phase at a specific growth rate (Fowler, 1977).
Still, the higher activities of G-6-PDH, and FDPA in cotton
callus cultures during the early lag phase or end of the
lag phase are in agreement with the results of earlier
workers.

As reported by Shimizu et al. (1977) the occurrence of
a high level of NADPH after 4 days of culture is concomitant
with an increased catalytic capacity of the PPP and an
increase in activity of the PPP relative to the EMP pathway
(Fowler, 1971). These observations of Shimizu et al. (1977)
and the present studies with cotton callus support the view
that the PPP is most active in early lag phase of growth
and that its function is concerned with the production of
NADPH for biosynthesis. Whether the increase in PPP activity
relative to the EMP pathway as observed by Shimizu et al.
(1977) in Acer cultures during the first few days of culture
is brought about or not at the expense of EMPP metabolic
flux due to partial inhibition of phosphofructokinase by
increasing ATP levels needs further study.

It is a general observation that cells with a high growth rate have a high activity of both the PP pathway and glycolysis, whereas in cells with a low growth rate the oxidation of carbohydrate is predominantly through glycolysis (Fowler, 1978). Working with chemostat cultures Fowler (1978) has found that at low and median growth rates PPP enzymes had low activities relative to equivalent glycolytic enzymes. However, at high growth rates this situation was reversed with G-6-PDH, a key pentose phosphate pathway control enzyme, having a significantly higher activity than the equivalent regulatory enzyme from glycolysis, phosphofructokinase. The increase in activities of hexokinase and G-6-PDH activity compared with pyruvate kinase may indicate that a major drainage of carbon precursors is occurring from the PP pathway, e.g. as pentose sugars for nucleic acid synthesis, so that the level of carbon flow through pyruvate kinase into the tricarboxylic acid cycle is substantially less than that entering the pathways at the hexose level.

Though few, hormonal control of glycolysis has been demonstrated in cell cultures. Relatively few studies are made on the hormonal regulation of MDH activity in vitro. present studies with callus tissues of Gossypium hirsutum revealed that among auxins, IAA at both the levels tried (2.0 and 5.0 mg/l) had a marked stimulatory influence on

the MDH activity. Whereas NAA at 0.2 mg/l level had
suppressed the enzyme activity. Kinetin at 0.04 mg/l level
had a promotary effect in comparison with 2.0 mg/l level.
GA_3 at 25 mg/l level increased MDH activity in the lag
phase, but later on the activity was higher on 100 mg/l GA_3.

The activities of G-6-PDH and aldolase were enhanced
in absence of exogenous auxin in Saccharum sp. cell cultures
(Maretzki et al., 1974). Among the three auxins tested in
the present study, 2,4-D was found to promote the activity
of G-6-PDH. Contrary to the reports of Maretzki et al.
(1974) who worked with sugarcane cell cultures, present
studies indicated that in the presence of exogenously
supplied auxins, G-6-PDH activity was increased. Kinetin at
the two concentrations (0.04 and 2.0 mg/l) tested was not
as effective as 2,4-D in cotton callus cultures. Also GA_3
had no significant control over the activity of G-6-PDH at
100 mg/l level, but at 25 mg/l level it had a promotary
effect. In Acer pseudoplatanus cells the substitution of
high kinetin (2.5 mg/l) for low kinetin (0.25 mg/l) concen-
tration, lowered the specific activity of G-6-PDH (Bergmann,
1963). Despite a lower respiration rate, total glucose
consumption remained unaffected. Therefore, Bergmann
concluded that a non-oxidative pathway for glucose predomi-
nated when kinetin concentration was high. On the other hand,
present studies with cotton revealed that high kinetin

(2.0 mg/l) favoured G-6-PDH activity when compared to low
kinetin (0.04 mg/l).

Cytokinins have also been reported to reduce activity
of the EMP pathway in _Nicotiana_ _tabacum_ (Bergmann, 1963)
and of the PP pathway in _Nicotiana_ _glauca_ cultures (Scott
_et_ _al_., 1964). Out of IAA, NAA and 2,4-D tested with cotton
callus cultures, NAA at 2.0 mg/l level stimulated considerably
the activity of FDPA when compared to the other auxins. This
stimulation of FDPA by NAA in the callus cultures of cotton
is hitherto a new report. Kinetin at low level (0.04 mg/l)
enhanced the activity during lag phase, but thereafter the
activity was higher at higher level (2.0 mg/l). Maretzki
_et_ _al_. (1974) have reported that FDPA activity increased in
the absence of exogenous auxin. Present studies also support
this view to some extent. When compared to 25 mg/l of GA_3,
FDPA activity was higher on 100 mg/l GA_3 containing medium.
In other words, higher GA concentration had promoted aldolase
activity.

Since cotton is a fast growing tissue, both EMP and PP
pathway enzymes have a high activity as was also observed by
Fowler (1978) in sycamore cell suspensions.

E. Effect of carbohydrates on growth and development of
Amylase, Invertase, MDH, G-6-PDH and FDPA and total and
reducing sugars in callus cultures of cotton.

As already described, when plant cells and tissues are
cultured aseptically, they cannot meet their energy require-
ments autotrophically since the normal functions of chloro-
plasts are frequently absent or blocked. Therefore, in plant
cell, tissue and organ cultures, it is necessary to substitute
suitable carbon sources. Plants translocate a restricted
number of carbon compounds, viz. sucrose, oligosaccharides
related to sucrose, and sugar alcohols. Of these, sucrose is
pre-eminent (Ziegler, 1975). It seems reasonable then, that
sucrose or any other carbohydrate source from which glucose
or fructose can become readily available, should serve best
the energy needs of cells grown in culture.

Callus cultures of many plants grow mainly either on
sucrose or glucose or fructose. Very few plant cells grow
on maltose and starch. In the present studies it is shown
that callus cultures of Gossypium hirsutum could be grown
successfully and equally well either on glucose or fructose
or a mixture of the two or sucrose. Growth of the tissues
was, however, relatively less on maltose and starch
containing media.

Along with plant growth substances, the quality and

quantity of carbohydrate source that we supply exogenously
played a pronounced role in maintaining the growth and
metabolic events of the cells and tissues (Simpkins et al.,
1970; Copping and Street, 1972; Maretzki et al., 1974).
Though a synchronous growth or differentiation system would
be ideal for proper understanding of the biochemical and
physiological events of growth or differentiation (Komamine
et al., 1978), present studies were carried out with cotton
tissue grown as callus.

Disappearance or appearance of carbohydrates from the
medium during cell growth has been determined. In Rosa sp.
the total carbohydrate in the medium (initially 2% sucrose)
disappeared by day 12 in culture with a low density inoculum.
When a high density inoculum was used carbohydrate disappeared
by day 8 in culture (Nash and Davies, 1972). Disappearance of
the exogenous carbohydrates was similarly rapid in Ipomoea
sp. cultures (Rose et al., 1972). In present studies with
cotton there was rapid accumulation of carbohydrate during
initial culture growth, and the carbohydrate content declined
towards the termination of the culture period.

Working on Pogostemon cablin callus, Jones et al.
(1973) found that during the first 48 hours, decrease of
carbohydrate in the medium corresponded to a high rate of
sucrose utilization for cell dry weight production, which
was accompanied by a high MDH activity. About 50% of the

sucrose in the medium used for callus growth was converted to cell dry weight. Like in many plant cell cultures (Carceller et al., 1971), present studies with cotton callus also showed that increasing level of carbohydrate increased the dry weight of the cells. The total carbohydrate content of Ipomoea sp. cells increased continually first 48 hours (Rose et al., 1972) but in Saccharum sp. not until well into the linear growth phase. In Saccharum sp. as well as in callus of tobacco (Thorpe and Meier, 1974 b) endogenous reducing sugars initially increased, then decreased to 50% or less of their maximum value by day 10 in culture. Brown and Short (1969) showed a similar pattern for the UDP-glucose concentration in Acer pseudoplatanus cells. Cotton callus cultures studied here also followed a similar pattern.

Amylase activity was higher on 1% glucose medium than on 2 and 4%. Increasing concentrations of fructose likewise inhibited amylase activity. When a mixture of glucose + fructose was used the enzyme activity was higher at 2% glucose + 2% fructose level. Sucrose at 4% inhibited amylase activity markedly. As in the case of glucose, fructose and sucrose, maltose also at 1% level favoured amylase activity, while at 2 and 4% levels it inhibited the enzyme activity. Soluble starch when added into the medium, stimulated specific activity of amylase at 2% level

but again inhibited it at higher concentration (4% level).

Glucose, fructose and sucrose can reach the cytoplasm intact, while other di- oligo- or polysaccharides are probably hydrolysed extracellularly. Extracellular amylases are essential for utilization of starch by plant cell and tissue cultures since it is a heavy molecular weight polysaccharide. Karstens and De Meester-Manger Cats (1960), Nickell and Brakke (1950), Straus and Campbell (1963), Jaspars and Veldstra (1965), Marvin and Morselli (1971) and Maretzki et al. (1971) have reported the release of these amylases into the surrounding medium. Three to four days after inoculation of the tissue onto starch containing medium, the medium surrounding the tissue had showed white patches, probably an indication of release of amylase into the medium. Amylase activity was detected in the spent medium in the present studies also. Maretzki et al. (1974) have further observed that amylase release by Saccharum sp. cultures was increased by replacement of sucrose with starch. By and large, specific amylase activity was higher on 1% maltose medium than on other mono, di and polysaccharides incorporated media.

Enzyme maltase has been detected in callus cultures of cotton grown on maltose containing medium during the present investigation. Earlier Klis (1971) had reported the presence of α -glucosidase in cell wall fragments of Convolvulus arvensis.

Increasing concentrations of glucose, fructose, glucose + fructose, sucrose, maltose and starch have inhibited soluble neutral invertase in callus tissues of Gossypium hirsutum. All these sugars at 4% level were more inhibitory than at 2 or 1% level. Glucose at 4% level especially has inhibited invertase activity in the present studies which could be attributed as feedback inhibition. The glucose effect on suppression of many catabolic enzymes in micro-organisms has long been known (Epps and Gale, 1942; Monod, 1947). Formation of both inducible and constitutive enzymes (invertase) maybe suppressed by addition of glucose to the culture medium (Magasanik, 1961). In immature storage tissue of sugar cane, control of invertase level appears to be mediated through auxin which may increase or decrease the level depending on concentration, and a feedback control involving the level of sugar present in the metabolic compartment (cell cytoplasm) (Sacher and Glasziou, 1962; Sacher et al., 1963). Sugars such as glucose, fructose and sucrose inhibited or reduced the invertase activity (Glasziou and Waldron, 1964 b). Present studies with cotton callus cultures has thus analogies with the glucose effect in micro-organisms and also confirm the results of Glasziou and his co-workers who worked with sugarcane. Further Glasziou et al. (1966) have reported that glucose increased the rate of destruction of messenger RNA required for the production of invertase. This however, needs further

investigation at molecular level. In the present study
with cotton callus, the inhibition by glucose, fructose and
sucrose of soluble neutral invertase, however, did not
appear until several days after inoculation. Contradicting
the above results, Copping and Street (1972) have reported
the highest invertase levels in the glucose grown Acer cell
cultures. However, for both Acer pseudoplatanus (Copping
and Street, 1972) and Saccharum sp. (Maretzki et al., 1974)
the evidence against invertase control of cell growth is
more compelling : cells grow equally well on glucose or
sucrose; and either sugar elicits similar patterns of
invertase activity during the growth cycle.

As was seen in the earlier experiments (Chapter III,
Section D) the activities of MDH, G-6-PDH and FDPA increased
immediately after transfer of the tissue and thus peak
values could be seen during early lag phase or at the end
of lag phase in most of the instances. These results are
similar with the findings of many earlier workers (Givan and
Collin, 1967; Nash and Davies, 1972; Komamine et al., 1969;
Kikuta et al., 1977). Givan and Collin (1967) working with
sycamore suspension cultures have reported that protein
content and rate of oxygen uptake rise sharply early in the
culture period, during which no significant increase in dry
weight can be observed. Similarly, Komamine et al. (1969)
have shown increased rate of respiration during the first

few days, when growth of carrot root cultures could not
be distinctly measured. Accompanying the rise in respiration,
the C_6/C_1 ratio also increased. As callus developed, the
respiratory rate and C_6/C_1 ratio gradually decreased.
Respiratory and glucose metabolism of cultured carrot root
tissues showed remarkable increases in activity of the
glycolytic - TCA pathway during the first 4-6 days of
culture and the predominance of the PP pathway during
callus development of carrot root explants.

Likewise Kikuta et al. (1971) have shown that callus
of potato tuber starts exponential growth with a 5-day lag
period, at which time marked production of DNA occurs. In
the lag period, respiration is very much resistant to
malonate and the activity of G-6-PDH passed its peak which
suggested that the PP pathway was involved in the initial
lag phase of callus development. Earlier Gibbs and Beevers
(1955) had observed that immature plant tissues respired
glucose to a large extent via the glycolytic pathway, and
as the tissues differentiated and aged, the participation
of the PP pathway was increasingly pronounced. Fowler and ap
Rees (1970) supporting this view showed that carbohydrate
oxidation in the relatively undifferentiated cells of the
root apex was mainly via the glycolytic pathway and that
differentiation involved an increase in the capacity and
activity of the PP pathway.

In cotton, it appeared that both glycolytic and PP
pathways were active simultaneously. Either 1 or 2% level
of carbohydrate favoured the enzymic activities, but higher
levels (4%) reduced the specific activities of MDH, G-6-PDH
and FDPA in general. Sucrose, maltose and starch at 1%
level have highly favoured MDH activity, but 4% sucrose
inhibited MDH activity at least during the lag phase. Another
highlight was the stimulation of FDPA activity by 1% glucose
and 2% starch (Tables 23, 38). However, regulation in an
isolated system lacking a source to sink relationship will
always require a more cautious interpretation. It is
important to note further that the metabolic activities
measured in an asynchronous system would only represent an
average value of that parameter in cells at various stages
in the cell cycle (Komamine et al., 1978).

F.   Organogenesis in callus cultures of Nicotiana
      tabacum L.

Experiment conducted with the callus cultures of
tobacco to invoke morphogenetic responses is described
in Chapter III, Section F. Earlier studies of Rawal (1979)
in our laboratory on callus cultures of tobacco showed that
manipulation of IAA and sucrose resulted either in the
induction of roots or shoots or both. Hence, an attempt was
made here to regulate morphogenesis by manipulating IAA and

sucrose. Rawal had also observed that except IAA, none
of the tested growth hormones such as IBA, NAA and 2,4-D
nor kinetin, MAP and adenine induced organogenesis in
tobacco callus. Low concentrations of IAA (0.175-0.3 mg/l)
favoured shoot differentiation, whereas higher levels
(0.5-2.0 mg/l) favoured root differentiation. Still higher
concentrations of IAA (3.0-4.0 mg/l) brought about complete
suppression of organogenesis. The concentration of sucrose
also in the medium seemed to have marked influence on
organogenetic development. However, the sucrose mediated
effect on organogenesis was apparently restricted to low
concentrations of IAA (0.3 mg/l). Sucrose at 1% in the
medium (IAA- 0.3 mg/l) did not support organogenesis;
however, it induced shoot differentiation at 3% sucrose and
roots at 6% sucrose levels. Manipulation of sucrose concen-
trations between 2-6% levels did not influence organogenesis
in presence of higher levels of IAA (2.0 mg/l) in the
medium; as roots were differentiated invariably. The
increase in sucrose concentration at low (0.3 mg/l) as
well as high (2.0 mg/l) IAA levels in the medium, consi-
derably influenced the time taken for organogenetic
responses. For instance, IAA at a concentration of 0.3 mg/l
with 2% sucrose induced shoots in four weeks. With the
increase of sucrose level to 3% (keeping the concentration
of IAA constant at 0.3 mg/l) shoots were differentiated
within 15 days. Similarly, in presence of 2.0 mg/l IAA,

2% sucrose induced roots in four weeks. However, with the
increase of sucrose level to 3 and 6% in the same medium,
differentiation of roots occurred within 12-15 days. A shift
in morphogenetic pattern was also observed in Citrus callus
by Chaturvedi and Mitra (1975) when Zeatin was added into
the medium in place of BAP.

Mehra (1972), Kshirsagar (1974) and Kshirsagar and
Mehta (1978) have demonstrated the influence of sucrose
levels on the differentiation of gametophyte and sporo-
phyte from the fern callus in vitro. The influence of
sucrose in inducing morphogenetic shift and in reducing
the time course for organogenesis in tobacco callus tissues
seemed to be a very complex process indeed. This might
involve many biochemical pathways and possibly part of
the sucrose might be acting as an osmoticum also as shown
in Chapter III, Section I. Rawal and Mehta (1981, in press)
have recently observed that IAA at 0.3 mg/l in presence of
6% sucrose favoured root differentiation with higher
activity of MDH than the shoot forming system on 3% sucrose
medium. This clearly indicated the higher energy requirements
for rhizogenesis than for caulogenesis. In the present study
also this view is substantiated when sucrose is partially
replaced by mannitol as an osmotic agent (Chapter III,
Section I). Osmotic requirement for root differentiation
is markedly higher than for shoot formation. Also, the
activity of IAA oxidase was low in root forming system than
in the shoot differentiating system. This seemed to suggest
higher endogenous auxin requirement for root differentiation

than for shoot differentiation. Thirdly, high sucrose levels have been already shown to support higher phenolic synthesis (Shah and Mehta, 1978; Kavi Kishore and Mehta, unpublished). Hence, a phenomena of auxin-phenolic synergism or antagonism could be envisaged. Such an auxin-phenol interaction has been observed to promote the rooting of the Rubus, but not in strawberry (James, 1979). Also high rates of shoot growth in Rubus have been demonstrated on phenolic free media (Boxus, 1974; James and Newton, 1977).

Besides being the source of energy, carbohydrate would provide more reducing power (NADH or NADPH) at higher levels, which might facilitate early organogenetic response.

Skoog and Miller (1957) had earlier reported that low concentrations of IAA favoured shoot differentiation in tobacco. Later many workers have demonstrated IAA induction of shoot formation in other systems; for instance, in Pergularia by Prabhudesai and Narayanaswamy (1974), in Anagallis by Bajaj and Mäder (1974), in Cyclamen by Geier (1977), etc. IAA induced root differentiation also has been reported from carrot (Nobecourt, 1939), Rumex (Nickell, 1952), wheat and barley (Gamborg and Éveleigh, 1968), Petunia (Rao et al., 1973), Pergularia (Prabhudesai and Narayanaswamy, 1974), Antirrhinum (Rao et al., 1976);Ipomoea biloba (Bhatt, 1977), Linum (Mehta, 1975), Ipomoea batatas (Kavi Kishore and Mehta, unpublished), etc. The callus may remain undifferentiated though showing prolific growth,

regardless of the hormones and nutrients to which they are exposed. Those showing exuberant growth are least conducive for regeneration as observed in Citrus (Chaturvedi et al., 1974).

Paulet (1965) found that low sucrose in combination with high auxin favoured root differentiation, whereas high sucrose, low auxin and high cytokinin, in combination, favoured shoot formation. Identical situation was demonstrated for tobacco tissue cultures also (Tran Thanh Van, 1977; Gaspar et al., 1977). Maroti and Levi (1977) working with Dianthus caryophyllus have demonstrated improvement in organised shoot differentiation with increasing sucrose concentrations. But present results showed that sucrose was effective in bringing about shifts of organogenetic response from shoot to root differentiation and vice versa only in presence of low concentration of IAA (0.3 mg/l) in the medium.

G - I. Physiological studies with callus cultures of tobacco and cotton cultured on shoot inducing medium in light.

The medium used for shoot differentiation in tobacco callus was MS basal supplemented with 0.3 mg/l IAA and 3% sucrose. Callus tissues of cotton were also cultured on the same medium, but no organogenetic response was observed. Shoot differentiation in tobacco callus occurred between days 13 and 15. Both in cotton and tobacco tissues the parameters examined during 15 day culture period were growth, accumulation of sugars and starch, and the activities

of amylase, invertase, MDH, G-6-PDH and FDPA. The
elucidation of the regulatory mechanism of differentiation
can be more effectively accomplished if information concern-
ing the physiology of the cells which participate in organo-
genesis becomes available. Keeping this in view, on day 15,
non-shoot forming tobacco callus was also analysed for the
above parameters and a comparison was made with that of the
shoot forming portion of the callus. In cotton callus
cultures, lower portion of the callus on day 15 was analysed
and compared with that of the activities of enzymes measured
from upper portion of the callus.

The most dramatic change in shoot-forming tobacco
callus was accumulation of starch prior to organogenesis.
This starch got depleted during shoot induction. On the
other hand, in the non-shoot forming portion of tobacco
callus total starch content was more on day 15. In callus
tissues of cotton, starch accumulated till day 15, unlike
shoot forming part of tobacco callus. Starch accumulation
could be a consequence of either increased synthesis of
it or decreased degradation or a combination of the two.
Starch accumulation has been a conspicuous feature; in
other organogenetic processes both in vivo and in vitro
e.g. the initiation of cotton embryos (Jensen, 1963), the
induction of floral parts in cauliflower (Sadik and Ozbun,
1967), the growth of potato buds following the breakage of

of dormancy (Marinos, 1967) and in developing cotton leaves
in vivo (Chang, 1979). Jensen (1963) had noticed a decline
in stored starch during the development of cotton embryos.
During cotton leaf development in vivo (Chang, 1979) starch
accumulation was characterised by an initial rise to a
maximum at the second to the fourth leaf from the apex.
Then, starch content progressively decreased with leaf age.
From the above literature including the present investigation,
it seems that the accumulation of starch functions in the
initiation of organised structures in plants. The physiolo-
gical significance of starch accumulation in organ initiation,
reflects the high energy requirement of the organogenetic
process. As a source of energy, starch possesses a distinct
advantage. The degradation of starch results in high yields
of glucose-1-phosphate, the subsequent catabolism of which
through glycolysis produces substrate adenosine triphosphate,
without the expenditure of existing high-energy phosphates.

The degradation of starch during organogenesis has been
studied in the present investigation by examining the
activity of amylase during shoot formation in tobacco callus.
Peak amylase activity in tobacco was noticed prior to organo-
genesis, whereas in cotton it was attained on day 21. Several
folds higher activity of amylase was noticed in shoot forming
callus when compared to the non-shoot forming portion of the
callus. Saka and Maeda (1973, 1974) examined the activities

of some hydrolytic enzymes during shoot formation in rice callus. After 10 days in culture, the activity of amylase in shoot-forming regions was higher than in the corresponding callus regions. They also examined α-amylase isozymes and found the formation of a new band during shoot development. Increased activity of starch degrading enzymes during organogenesis in tobacco was also recorded by Thorpe and Meier (1974 a).

Activity of invertase increased prior to organogenesis in tobacco callus studied here. In cotton callus tissues, during the corresponding period activity of invertase either declined or remained steady. As mentioned earlier, the organ initiation process is a high energy requiring one, and during this the degradation products of starch and sucrose must be utilized.Also a continuous supply of free sugars from the medium was required for shoot formation in tobacco as shown by Ross et al. (1973) and Thorpe and Meier (1974 b).

Shoot formation has been shown to be concomitant with high rate of respiration. More pronounced MDH activity prior to shoot formation was noticed in tobacco; whereas, in cotton during this period activity declined considerably. Jensen (1963) had also observed a continued increase in oxygen uptake with the development of the cotton embryo in vivo. A parallel situation was observed in shoot-forming

and non-shoot-forming tissues of tobacco by Thorpe and
Meier (1972). This is a clear indication that the accumu-
lated starch (prior to organogenesis) is serving as a
readily available reserve source of energy for the
organogenetic process.

Support for this idea was obtained when glucose
oxidation and the relative contribution of Embden-Meyerhof-
Parnas and Pentose phosphate pathway (EMP and PPP) therein
was examined. In the days preceding shoot development,
tobacco tissues exhibited higher activities of G-6-PDH and
FDPA; while cotton callus tissues showed significantly lesser
activities during this period. When non-shoot-forming portion
of the tobacco callus was observed, activities of the above
enzymes were several folds lower than that of the shoot
forming tissue. Changes in glucose catabolism during aging
and differentiation of plant tissues have been reported by
several authors (Gibbs and Beevers, 1955; Luštinec and
Pokorná, 1962; Morohashi et al., 1965; Higuchi and Shimada,
1967a, 1967 b; Fowler and ap Rees, 1970). Ashihara et al.
(1974) had found decrease in the activity of the glycolytic
pathway with aging and differentiation in hypocotyls of
Phaseolus mungo seedlings. They had suggested that this
might be due to the decreasing glycolytic enzyme activities
and NADH content. They had further noticed that relative
activity of the PP pathway in glucose catabolism was higher

in the immature part of the hypocotyls than in the inter-
mediary part. This could be attributed to larger amounts
of NADPH and enzymes of the PP pathway. Glycolysis would
lead to the production of ATP by substrate level and
subsequently by oxidative phosphorylation. In case of
cotton tissues, there was a marked increase in the activity
of aldolase when the starch was fed (Chapter III, Results,
Expt. 24). Some, but by no means all, of the reductive
biosynthesis in higher plants use NADPH (ap Rees, 1974).
One role for the pentose phosphate pathway could be the
provision of reducing power for lignin synthesis (Higuchi
and Shimada, 1967 a; 1967 b; Ashihara and Komamine, 1975).
The said pathway provides reducing power (NADPH) for bio-
synthesis of other essential metabolites, like pentose for
nucleic acid synthesis and erythrose for synthesis of
aromatic compounds, via shikimic acid pathway (Davies,
et al., 1964). Wong and ap Rees (1971) had reported that
differentiation of the stele of pea roots was accompanied
by increased activity of the PP pathway relative to that
of glycolytic pathway. Pryke and ap Rees (1976) had noticed
increased activity of the PP pathway relative to that of
glycolysis during extensive lignification in Coleus
explants. This was further confirmed by experiments
investigating the contribution of ^{14}C from specifically
labelled (at C-1 and C-6) glucose to CO_2 released (Thorpe
and Laishley, 1973). More recently Gahan et al. (1979) have

measured the activity of PP pathway during floral induction
in <u>Spinacia</u> <u>oleraceae</u> and concluded that this pathway may be
implicated in providing precursors for the synthetic
processes invoked. This pathway is very important to plant
cells, with as much as 30% of the total glucose passing
through it, although its products may return to the
glycolysis. Thus, any or all of the above mentioned
functions of EMP and PPP might be important during organo-
genesis.

G - II. <u>Physiological studies with callus tissues of</u>
<u>tobacco and cotton cultured on shoot inducing</u>
<u>medium in the dark</u>.

The callus tissues cultured on the same shoot-inducing
medium were incubated in total dark. As stated earlier,
shoots were induced from tobacco tissues within a maximum
of 15 days, whereas cotton callus cultures did not reveal
any organogenetic response. To examine the physiological
changes associated with shoot differentiation of tobacco
callus and the non-shoot-forming callus of cotton the
following parameters were studied : growth, accumulation of
sugars and the activities of GOT, ME and PEPC.

In tobacco callus tissues, activities of GOT, ME and
PEPC increased remarkably between days 9 and 15 i.e., during

the days preceding shoot differentiation. When the non-shoot-forming portion of the tobacco callus grown on the same medium was analysed on day 15, the activities of the enzymes were several folds lower than that of the shoot forming portion. In cotton callus, the activities of GOT, ME and PEPC declined between days 6 and 12 considerably and could not be detected at all on day 15.

It had been shown in earlier experiments (Chapter III, Results, Expt. 26) that during the shoot formation both the glycolytic and the pentose phosphate pathways are enhanced in tobacco callus. This reflected a need for additional reducing power (NADPH) for the high-energy requiring organogenetic process.

The role of dark fixation of CO_2 was examined here which is known to utilize phosphoenolpyruvate derived from carbohydrates as a substrate since the tissues were grown in dark. Malate arising from dark fixation of CO_2 was reported by Dhindsa et al. (1975) to act as an osmoregulatory solute during turgor-driven cell expansion. This malate as reported by Fowler (1974) might also provide reducing power through its decarboxylation by malic enzyme.

Present results showed that the capacities of enzymes involved in malate metabolism increased sharply prior to shoot formation in tobacco. The activities of the enzymes

were higher in shoot forming tissue than in non-shoot-
forming tissue of tobacco and also that in cotton.
Dhindsa et al. (1979) had observed a continuous increase
in malate content with the increase in growth of tobacco
callus, while the increase in malate content in shoot
forming tissue was more dramatic, but transitory. Clearly,
malate decarboxylation through NADP-malic enzyme also was
taking place at a greater rate in shoot forming tissue.
Therefore, the actual amount of malate which accumulated
in tobacco could be less in shoot forming tissue than in
non-shoot forming tissue. This is an indication that there
would be a higher degree of internal CO_2 cycling in shoot
forming than in the non-shoot forming tissue. In support
of this view, shoot forming tobacco callus had been shown
to metabolize (^{14}C) glucose at a faster rate than NSF
tissue, leading to more endogenous CO_2 production (Thorpe
and Laishley, 1973). The depletion of malate during shoot
formation suggested that it might be providing NADPH for
reductive biosynthesis of cellular constituents. This
NADPH would arise from transhydrogenation during which net
hydrogen transfer from NADH to $NADP^+$ takes place as
suggested by Ting and Dugger (1965), and the series of
reactions involved generate the internal cycling of CO_2.
The breakdown of starch via glycolysis would provide PEP
as substrate for dark fixation of CO_2. Similarly, triose

phosphate could enter glycolysis through the PP pathway
and thus be an additional source of PEP. A rapid carbon
flow in shoot forming tissue through malate may be generating
NADPH to meet the additional metabolic requirements of the
shoot-forming process. The malate reactions could be also
playing a role as a pH-stat as suggested by Davies (1973).
More evidence is, however, needed to support this view.

H - I. Physiological studies with callus tissues of
tobacco and cotton cultured on root inducing
medium in light.

The medium used for root differentiation from tobacco
and cotton callus was MS basal supplemented with 2.0 mg/l
IAA and 3% sucrose. The culture vessels were incubated
under continuous light. Roots were differentiated in tobacco
callus between days 13 and 15; but no roots were formed in
callus tissues of cotton.

Starch accumulated prior to rhizogenesis also in tobacco
tissues (Expt. 30). In cotton callus tissues accumulation of
starch was observed till day 6, unlike day 9 in tobacco
tissues (Expt. 31). Histochemical changes underlying shoot
initiation in tobacco callus (Thorpe and Murashige, 1970;
Ross et al., 1973) and rice callus (Maeda and Saka, 1973)
have revealed heavy deposition of starch. But with respect

to root formation, no such reports are hitherto available. Present studies reveal accumulation of starch prior to rhizogenesis in tobacco, which might function in the initiation of organized structures. Accumulation preceded any observable organised development and this accumulation markedly decreased once the organogenetic process started. Perhaps this accumulation reflects the high energy requirements of the organogenetic process as discussed earlier.

Prior to root formation, amylase activity increased in tobacco tissue (Expt. 30). However, the activity dropped between days 12 and 15. This observation coincided with the accumulation of starch. Contrary to this, amylase activity was on continuous increase from day 6 till day 15 in callus cultures of cotton (Expt. 31). When non-root forming portion of tobacco callus was analysed on day 15, the activities of enzymes amylase, invertase and MDH were much less when compared to those in the root forming portions. On the other hand, the activity of enzymes G-6-PDH and FDPA was slightly higher than that in the root forming portion of callus (Table No. 45).

Unlike in shoot forming tissue of tobacco, specific invertase activity was lower than day 0 all throughout the culture period. But in cotton, the specific invertase activity was higher between days 0 and 9 and then it declined. Low activity of invertase in tobacco tissues

maybe attributed to high concentrations of IAA content
in the medium, since this has been shown to inhibit
invertase in cotton callus cultures earlier (Chapter III,
Results, Expt. 14).

The MDH activity/mg protein increased from 0.26 units
on the day preceding root initiation to 2.66 units on the
day of root development in tobacco. In cotton callus also
such an increase in enzyme activity was noticed between
days 12 and 15, though no organogenetic development was
observed.

Specific activity of G-6-PDH in tobacco was on
increase till day 9, and then dropped between days 9 and
15. Same developmental pattern of enzyme was followed in
cotton tissues also.

Prior to rhizogenesis in tobacco, FDPA activity
increased several folds. During this period in cotton,
specific FDPA activity was on decline.

As was noticed earlier in shoot forming tobacco callus,
enzyme activities of amylase, invertase and MDH were higher
in root forming tissues also prior to organogenetic develop-
ment. On the other hand, G-6-PDH and FDPA enzymes showed
higher activities till day 9 but then declined.

From the above observations it was clear that

organogenetic process required more energy which was drawn
from accumulated starch and sugars. This was evident also
when the specific activities of enzymes amylase, invertase,
MDH, G-6-PDH and FDPA were observed. Pronounced enhancement
of the above enzyme activities and the mobilization of
starch from all parts of the tissue was thus indicative
of the intense metabolic activity going on in root forming
regions of the tissue. Stimulation of carbohydrate
oxidation through the EMP glycolytic pathway at the time
of organogenetic development was in keeping with the
suggested role of starch and free sugars. This is also in
agreement with the respiration data. The osmotic role for
the degradative products of starch metabolism and the free
sugars is illustrated under root forming conditions also
elsewhere in the text (Chapter III, Results, Section I,
Expt. 34).

Differentiation is a very complex process, even when
viewed at the level of single cell within the plant body
also. Different types of cells are characterized by
different types of enzymes and structural proteins. This
illustrates, perhaps somewhat simplistically, that
differentiation involves differential gene expression;
different genes are expressed in different types of cell.
Even though specialized cells are characterized by the
presence of specific gene products, many differentiated

cell are totipotent, in that they contain all the genetic
information necessary for growth of a whole plant. The
formation of the cap in the unicellular alga Acetabularia
is regarded as a process of differentiation, which involves
the sequential appearance and disappearance of a number of
enzymes (Harris, 1974; Triplett et al., 1965), the
synthesis of protein (Hammerling, 1963) and the synthesis
of polysaccharides (Zetsche, 1966). It is also pointed that
post-transcriptional events may regulate the expression not
just of one gene, but of all the genes involved in a
developmental sequence. The necessity for protein synthesis
before and during the process of cap formation suggested
that the messenger RNA molecules might be very long lived.
This idea is more likely to be correct than that the
enzymes involved in cap formation maybe stored in inactive
forms in the cytoplasm for long periods. As was shown the
appearance of new polysaccharides and enzymes in the cap
formation of Acetabularia in the present study also during
shoot and root differentiation in tobacco callus, starch
accumulated and enzyme activities increased.

The involvement of sucrose in processes as different
as phloem differentiation and the regulation of plastid
development (Dalton and Street, 1977) illustrates how the
effect of a particular compound may vary according to the
type of cell in which the compound is acting. The effects

of compounds such as sucrose and auxin are clearly
affected by other factors. One such factor is the cellular
environment. Single cells grown in suspension culture do
not differentiate (Street, 1973). Addition of auxin and
sucrose to the growth medium never induces the formation
of xylem or phloem cells in a population of single cells;
whereas, as described in the Introduction (Chapter I),
these substances cause the induction of vascular bundles
in callus tissues. Contact with other cells is therefore,
a prerequisite for differentiation. This is another
indication that cells are sensitive not only to particular
concentrations of growth substances and nutrients, but also
to gradients of concentrations of these substances. A growth
substance can have a wide variety of effects, the actual
effect being dependent on the type of cell. The same is
true of the nutrients, such as sucrose, which affect
patterns of differentiation. The effects of growth substances
and nutrients on gene expression are thus modified by
previous gene expression. Thus the involvement of cellular
interactions and the developmental backgrounds of cells in
the regulation of differentiation illustrates the complexity
of the process of differentiation.

H - II. <u>Physiological studies with callus tissues of tobacco and cotton cultured on root inducing medium in the dark.</u>

The callus cultures were incubated on the same root inducing medium, but under continuous dark. Roots from tobacco callus were differentiated between days 13 and 15; but no roots were formed in callus tissues of cotton.

The appearance of roots from callus tissues of tobacco on day 15 was preceded by a pronounced increase in specific activities of GOT, ME and PEPC. In non-root forming portions of the same callus grown on the same medium, the activities of the above enzymes on day 15 were several folds lower than that in the root forming portions of the callus.

Contrary to these results, the activity of GOT in cotton increased to peak value by day 3. Thereafter the activity decayed till day 15. Malic enzyme and PEPC could not be detected on day 15 in cotton callus cultures, while they increased during the same period in tobacco tissues.

As shown earlier during the formationof shoots from tobacco tissues grown in the dark, activities of the enzymes GOT, ME and PEPC also increased during rhizogenesis; the non-root-forming tissue showing much less activity of all these enzymes.

To meet special metabolic requirements of the root-
forming process, like the earlier discussed shoot-forming
one, a rapid carbon flow through malate maybe generating
NADPH. Increase in the level of NADPH, ATP and energy
charge were also observed in the initial period of culture
when the cell division is maximum (Shimizu et al., 1977).
Continuous build-up of malate in non-shoot-forming tobacco
tissue (Dhindsa et al., 1979) provides evidence for such
a view. Malate in non-organ forming tissues may be acting
primarily as an osmotic agent to regulate cell expansion
between successive cell divisions as shown in cotton fibre
growth in vitro (Dhindsa et al., 1975) and also during
stomatal opening (Allaway, 1973).

As shown earlier (Chapter III, Results, Section H - I,
Expt. 30) accumulation of starch prior to organogenetic
development and its degradation during the process of
differentiation to oligosaccharides could increase the
osmotic potential of the cells resulting in increased water
uptake. The latter would keep proteins fully hydrated and
allow maximum enzyme activity. Thus if, as it appears, both
reducing power and osmoregulation are needed for organo-
genesis, tobacco callus utilized alternative pathways for
fulfilling each of these requirements.

I. <u>Osmotic requirement for shoot and root formation
in callus cultures of tobacco.</u>

Chapter III, Section I, Expt. 34, described the effect
of mannitol as an osmotic agent and possible osmotic
involvement in organogenesis in callus tissues of tobacco.
The reason for selecting mannitol as an osmotic agent being
(i) mannitol does not support tissue growth in tobacco
(Hildebrandt and Riker, 1949); (ii) it has been demonstrated
that mannitol is not metabolised by tobacco (Trip <u>et al</u>.,
1964); (iii) (though) it does enter callus tissues of
tobacco (Klenowska, 1971). Thus potential separation of an
endogenous osmotic requirement from an exogenous medium-
imposed one could be possible.

Mild osmotic stress not only increased callus growth
but also modified the cellular morphology in tobacco
(Klenovska, 1973, 1976) and soybean tissue cultures (Kimball
<u>et al</u>., 1975). The importance of osmotic conditions in the
culture of zygote embryos of <u>Datura</u> (Rietsema <u>et al</u>., 1953),
<u>Capsella</u> (Raghavan and Torrey, 1963) and <u>Hordeum</u> (Granatek
and Cockerline, 1978) have also been documented. Doley and
Leyton (1970), Granatek and Cockerline (1978) have outlined
osmotic-hormonal interactions. Maretzki <u>et al</u>. (1972) have
reported furthermore that small changes in somotic stress
lowered reducing sugar concentrations and a decrease in
invertase activity in sugar cane cell cultures.

The results in Section I and Table 49 suggested that mannitol alone could not give any organogenetic response. Also 1% sucrose with 0.3 mg/l IAA could not evoke any morphogenetic response. Shoots were formed only when 1% sucrose was supplemented with mannitol to give the molar sucrose equivalent to 2%. The dual role of sucrose as an osmoticum and an energy source was indicated by the inability of mannitol to completely substitute for sucrose below the 1% sucrose level. This 1% sucrose level maybe the saturating level for carbon flow through the cell membrane and energy-producing pathways, but an additional 1% sucrose (i.e., total 2% sucrose) was needed to produce the optimum level for turgor generation during shoot formation.

Shoot formation was promoted from 50 to 65% frequency and from solitary to 7 to 9 shoots per callus mass when 2% sucrose was supplemented with mannitol to give the molar sucrose equivalent to 3%. This promotion of shoot formation by mannitol was not a growth response as shown in Table 49. The failure of mannitol to replace the sucrose requirement for growth is consistent with the findings that there are different patterns of malate accumulation by dark fixation of CO_2 during growth and organogenesis (Plumb - Dhindsa et al., 1979). Their results confirm that malate does not accumulate during shoot formation but it does during callus

growth. Studies of Dhindsa et al. (1975) on cotton fiber indicated that levels of potassium and malate in fiber were highest when the rate of fiber growth was maximum and this accumulated malate fulfilled osmotic requirement for growth. This might be true even for tobacco. It is common knowledge that the degradation products of the accumulated starch and free sugars from the medium are important at the site of organ formation.

It was obvious from the present results that osmotic requirements for shoot and root formation are different (Table 49). Number of roots also increased per callus mass with the increase of mannitol content. The success in partially replacing the sucrose requirement for shoot and root formation with mannitol in osmotically equivalent levels supported the view that part of the sucrose is acting as an osmoticum (Thorpe and Murashige, 1970; Barg and Umiel, 1977; Thorpe, 1977).

These results are also consistent with the hypothesis of Cleland (1967, 1971, 1977) and Zimmerman (1977, 1978) for turgor-driven growth and cell expansion. According to this view, high levels of sucrose and mannitol in the medium would act against the creation of a critical turgor pressure which must be established before cell expansion can occur. This condition of stress would inhibit callus growth, shoot and root formation. This was also evident from the studies

of Dhindsa et al. (1975) during cotton fiber extension by extracellular osmotic agent. Similarly, promotion of shoot and root formation by mannitol, in presence of adequate sucrose as energy source, may reflect the generation of a turgor which results in healthy root and shoot formation.

It was clear from these studies that sucrose was not solely acting as a source of energy and carbon during formation of root and shoot in tobacco callus but also providing appropriate osmoticum. Secondly, osmotic requirements for root and shoot formation were different. The present investigation has, however, no parallel studies in my knowledge, to substantiate this observation.

J. Effect of an inhibitor (Rifamycin) on organogenesis and carbohydrate metabolizing enzymes in callus cultures of tobacco.

Reich et al. (1961) have reported that small amounts of the antibiotic actinomycin D when applied to a tissue culture inhibited RNA synthesis, while synthesis of DNA and protein, at least initially, was not affected. The inhibition maybe on enzyme systems and in several cases may be caused by structural similarities between the normal constituents and the antibiotic.

Rifamycin is a specific RNA polymerase inhibitor in

bacteria, since when applied mouse RNA polymerase was not affected. But evidence exists that in some plants it has activity. Rifamycin is reported to inhibit only the free enzyme before its attachment to DNA, and if this has already occurred and if the nucleotides have been added, the enzyme is protected against the action of these anti-biotics. Earlier work with rifamycin indicates that its inhibition is at a very early stage in the process of transcription; if applied once polymerization of the nucleotides has commenced, it has no effect (Leshem, 1973).

Two concentrations of rifamycin (0.01 and 0.1 mg/l) were added into the standard growth medium of tobacco, and growth was slightly inhibited at 0.1 mg/l rifamycin level. Activities of amylase, invertase and MDH were not changed much, while G-6-PDH and FDPA were many folds higher on 0.01 mg/l rifamycin containing medium than on 0.1 mg/l level. However, growth as well as activities of enzymes tested were several folds higher on the standard growth medium (control) than on rifamycin containing media.

When incorporated into the shoot inducing medium of tobacco rifamycin at lower concentration (0.01 mg/l) delayed the organogenesis from two to four weeks. Also it reduced frequency of response from 70-75% to 50-60%. At higher concentrations of rifamycin (0.1 mg/l), organogenesis was further inhibited with a frequency response of only 5-10%.

Activities of the enzymes tested were higher at 0.01 mg/l rifamycin containing medium than at 0.1 mg/l. When compared with shoot forming medium (control) of tobacco, activities of these enzymes were much suppressed on rifamycin containing media.

When rifamycin (0.01 and 0.1 mg/l) was added into root inducing medium of tobacco, root differentiation was completely inhibited. Secondly, rifamycin at 0.1 mg/l level markedly inhibited activities of the enzymes amylase, invertase, MDH, G-6-PDH and FDPA. In comparison with normal root inducing medium of tobacco (i.e. control), activities of the above mentioned enzymes were inhibited on rifamycin containing media.

The developmental programme of a cell involves, among other things, the appearance and disappearance of specific times of specific proteins. Rifamycin delayed the shoot formation. Is the different concentrations of IAA in the shoot and root inducing media and their influence on RNA polymerase responsible for this differential effect of rifamycin needs to be further studied.

If the effect of rifamycin is on RNA polymerase, then inhibition of protein synthesis should be encountered almost immediately. Although it is widely assumed that much gene expression is regulated at the transcriptional

level, there is very little evidence to support this
assumption. Apparently, the control of the expression of
a number of genes is at the translational and post-trans-
lational levels. However, it is not yet clear whether
translational and post-translational control are more
important than transcriptional control, as has been
suggested by Harris (1974).

The available evidence indicates that messenger RNA
molecules in the cytoplasm of plant cells are long lived.
A rapid decline in the activity of a particular enzyme
must therefore, be achieved by one or more of the following
methods :

(a) Increasing the rate of degradation of the messenger RNA;
detected by changes in enzyme activities, and by changes in
the structure and content of organelles and membrane compo-
nents. Further, different types of cells are characterized
by different types of enzymes and structural proteins. This
illustrates, that differentiation involves differential
gene expression : different genes are expressed in different
types of cell. Inhibitors of protein synthesis and RNA
synthesis when used carefully, can give general information
on the control of gene expression. For instance, actino-
mycin D (inhibitor of RNA synthesis) at concentrations that
completely inhibit seedling growth, does not prevent the
increase in the activities of isocitrate lyase and malate

synthetase in the cotyledons of Citrullus (Hock and Beevers, 1966). Similarly, 6-methyl-purine, at concentrations which abolish RNA synthesis, fails to prevent induction of nitrate reductase in Chlorella (John et al., 1973). In the present study it is seen that rifamycin at higher concentrations (0.1 mg/l) is more effective in inhibiting enzymes such as amylase, invertase, MDH, G-6-PDH and FDPA. It is thus likely that the increases in activities of enzymes of Citrullus and Chlorella are not dependent on the synthesis of messenger RNA, and control is therefore, post-transcriptional. Whereas in Nicotiana tabacum the inhibition of some of the carbohydrate metabolizing enzymes by rifamycin indicate that the activities of these enzymes are dependent on the synthesis of mRNA,

(b) Inactivation of the messenger RNA,

(c) Increasing the rate of degradation of the enzyme. The reduction in nitrate reductase activity under non-inducing conditions is prevented by cycloheximide in Hordeum vulgare (Travis et al., 1969). Similar reductions in the rate of decline of activity in the presence of inhibitors of protein synthesis have been reported for a number of other enzymes. Present physiological studies with rifamycin on callus cultures of tobacco during growth and organogenetic development also indicate that protein degradation as

well as protein synthesis must be taken into account
when considering the control of gene expression.

(d) Inactivation of the enzyme.

The existence of specific inhibitors may cause inactiva-
tion of the enzyme phenylalanine ammonia-lyase as shown
by French and Smith (1975) and deoxyribonuclease in
Chlorella by Schonherr et al. (1970). The possibility of
inhibition of the enzyme activities by rifamycin in tobacco
callus cultures could not be ruled out by inactivation of
the enzyme also as shown above.

On taking cognizance of the entire data obtained
through experimentation in the present study, the following
observations come into focus :

1.   In both tobacco and cotton callus tissues during
        growth, the lag phase of the culture period was
        characterized by an increasing invertase activity,
        respiratory activity (MDH) and also more activity of
        PPP relative to the EMP pathway enzymes.

2.   When the above tissues were grown in the dark,
        there was a decline in growth values. The enzymes
        GOT, ME and PEPC showed higher activities during lag
        phase of growth than in the subsequent growth phases.

3.   Auxins, cytokinins and GA_3 greatly influenced

the enzyme activities of amylase, invertase, MDH, G-6-PDH and FDPA. Of particular interests are the findings that NAA caused pronounced enhancement in the activity of amylase and GA_3 that of FDPA.

4.   Higher concentrations of carbohydrates such as glucose, fructose, glucose + fructose, sucrose, maltose and starch inhibited enzymes such as invertase in cotton tissues. In cotton, both the glycolytic and PP pathways were equally active. While lower concentrations of the above sugars favoured or promoted many enzymes, higher levels (4%) suppressed the activities of MDH, G-6-PDH and FDPA in general.

5.   The most dramatic difference between organ forming and non-organ forming tobacco callus was the accumulation of starch prior to organogenesis. This starch disappeared subsequently and was presumably utilized during the initiation and development of shoot or root primordia. Starch accumulation in cotton tissue was less conspicuous, and it was not mobilised till the termination of culture period.

6.   The utilization of starch involved enhanced rates of degradation of the metabolite during organogenetic process as revealed by high activity of amylase.

7.   The activity of invertase also increased markedly

in the shoot and root forming tissues, than in the non-
organ forming tissues of tobacco and cotton. A conti-
nuous supply of free sugars appeared to be essential
for morphogenesis.

8.   The accumulated starch and free sugars probably
might be serving as a readily available reserve source
of energy for organ generating processes. Evidence in
support of this idea was obtained when the activities
of respiratory enzymes in shoot-forming, root-forming
and non-organ forming tobacco and cotton callus tissues
were compared. Higher activity of MDH was observed
during shoot and root formation in tobacco callus
tissues. MDH activity was by and large substantially
higher during rhizogenesis than during caulogenesis.
These results further indicated that requirements for
energy (ATP) and reducing power (NADH or NADPH) were
higher for root formation than for shoot differentiation.

9.   Examination of glucose oxidation further confirmed
the above findings. Increased activities of enzymes of
both the EMP glycolytic and PP pathways of glucose
oxidation were found in organ forming tobacco callus
than in non-organ forming tobacco and cotton callus
tissues. Stimulation of carbohydrate oxidation through
EMP pathway during organogenesis was in keeping with
the suggested role of starch. This glycolytic pathway

would lead to the production of ATP at substrate
level and subsequently by oxidative phosphorylation.
PP pathway is significant in terms of reducing power
generation for biosynthesis as well as for the production
of highly reactive intermediates for the synthesis of
essential cell constituents like nucleic acids, etc.

10.   Furthermore, the non-autotrophic CO_2 fixation
was markedly higher during organ forming tissues of
tobacco as determined by the activities of malate
metabolism enzymes, viz. PEPC, ME and GOT. Perhaps
malate must be generating reducing power in the organ
forming process.

11.   From the experiments conducted with mannitol as
an osmotic agent in tobacco callus tissues, it is
inferred that sucrose was not acting solely as a
source of energy and carbon during organ formation;
but also providing suitable osmoticum. Secondly,
osmotic requirements for root and shoot formation were
different.

12.   Rifamycin, a specific RNA polymerase inhibitor
delayed shoot formation, while it completely inhibited
rhizogenesis in tobacco callus cultures.

From the present study it becomes quite apparent that organogenesis from unorganised cells is under strict gene control and is accompanied by the synthesis of definite functional proteins. It will be worthwhile to examine the spectral changes in the isoenzyme patterns of various enzymes involved in starch and sucrose metabolism, enzymes of the EMP, PPP and TCA cycle, malate metabolism and many more. Also the changes in the molecular species of RNA cannot be ignored. As Bonner (1965) has pointed, changes in metabolism in which new enzymes originally absent are synthesized or enzymes present show increased synthesis and/or activity must a priori be a cause rather than a result of differentiation. However, this does not indicate how differentiation is regulated at the molecular level.

If we accept the totipotency of all living cells containing a normal complement of chromosomes, then one must ask why is it that certain species are so intractable in culture as in the case of cotton in the present study. This is probably that we have not yet determined the right cultural conditions or the proper additives to the medium. There is something much more fundamental which is involved, and it relates to our complete ignorance of how regulation is controlled at the molecular level. Also, how a single cell among many becomes activated remains speculative. The

role of physiological gradients can explain the formation of loci in which the organogenetic cells may be found. Formation of meristemoids from activated cells is an apparent structural requirement for organogenesis in vitro (Thorpe, 1978). This meristemoid is initially plastic and capable of becoming either a shoot or a root primordia (Bonnet and Torrey, 1965). Perhaps it is this failure of cells to undergo complete dedifferentiation and become mitosis-determined that is at the center of the problem of regulation.

Finally, we have a long way to go before we have mapped the physiological and biochemical events associated with the shift in metabolism which occurs during organ formation. To facilitate this task, we require the development of a system in which the precise location of cells which are synchronously undergoing dedifferentiation and differentiation can be routinely observed and manipulated in situ. Also mention must be made, that these cells should be easily removable and available in large quantities for detailed biochemical studies. Only then we can begin to look at regulation at the molecular level and attempt to answer the fundamental question of what regulates a single cell and sets it apart on the altered pathways of differentiation.

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