THE REGULATION

OF

DIVISION OF HIGHER PLANT CELLS

by

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This thesis is entirely my own work and at no time
has been submitted for another degree.

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I certify that this statement is correct.

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ABSTRACT

Acer pseudoplatanus L (English sycamore) cell suspension cultures provide an excellent system for controlled investigations into the regulation of cytokinesis. They exhibit 60-70% division synchrony on subculture, and produce predictable growth patterns in batch culture.

Previous studies in these laboratories with partially synchronized cultures led to the hypothesis that a critical "trigger" concentration of endogenous IAA (approximately 500-750 ng 10^8 cells) is vital prior to each mitosis. In this present study a more effective induction technique has been employed. The cells were found to rapidly become nitrate and phosphate limited in batch culture. The addition of these nutrients after each cell division resulted in several highly synchronous divisions (80-90%). In these cultures the IAA levels remained at a relatively stable level (50 ng 10^8 cells). Thus, the original hypothesis has been modified and it is now proposed that a critical minimum endogenous concentration of IAA is required to maintain cytokinesis. This IAA level may be optimal for an IAA binding protein.

The nutrient feeding system was adapted for automation with the aid of a computer interface. This automated system ensures that the synchronization technique is more reliable and less time consuming.

The endogenous cytokinins were studied during the first cell division. The individual levels were found to fluctuate with total cytokinin-like activity reaching a maxima coincident with mitosis and/or cytokinesis. Z was the major cytokinin detected. This implicated the cytokinins as possible G2 --> M triggers.

The intracellular pH levels were estimated by ^{31}P NMR spectroscopy. The cytoplasmic pH remained stable (approximately 7.5) during synchronous divisions and decreased as asynchrony developed. This may be a reflection of the nutrient status of the cells and/or the level optimal for the distribution of IAA and/or other growth regulators.

The cell cycle appears to be controlled by the various growth regulators and their receptors. It is apparent that the concentrations, availability and distribution of these substances is vital for the complex regulated cycle to ensue.

ACKNOWLEDGEMENTS

ABBREVIATIONS

AA	Acetic Anhydride
ABA	Abscisic Acid
ATP	Adenosine-5'-triphosphate
BAP	Benzyladenine
Bq	Becquerel
4-Cl-IAA	4-chloro-indole-3-acetic Acid
CV	Cell Viability
2,4-D	2,4-Dichlorophenoxyacetic Acid
d	Dilution Factor
DHZ	Dihydrozeatin
DHZOG	Dihydrozeatin-O-glucoside
DHZR	Dihydrozeatin riboside
DM	Dry Mass
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DVM	Digital Voltmeter
ECD	Electron Capture Detector
EDTA	Ethylenediaminetetra-acetate
GO	Possible state for arrested cells
G1	"Gap 1" in cell cycle
G2	"Gap 2" in cell cycle
GA_n	Gibberellin $A1 - A_n$
GC-MS	Combined Gas Chromatography-Mass Spectrometry
GLC	Gas-Liquid Chromatography
HPLC	High Performance Liquid Chromatography
IAA	Indole-3-acetic Acid
IAN	Indole-3-acetonitrile
IAOx	Indole-3-acetaldoxine

IPA	Isopentenyl adenosine
IPP	Isopentenyl pyrophosphate
IPYA	Indole-3-pyruvic Acid
IR	Infra Red
K_1	Rate of Synthesis
K_2	Rate of Consumption
M	Mitosis
MCV	Mean Cell Volume
MI	Mitotic Index
2-MIP	2-methylindolo-2,3:4'-pyr-6-one
mRNA	Messenger Ribonucleic Acid
N	Cell Number
NPA	N-1-naphthylphthalamic Acid
NAA	Naphthalene Acetic Acid
NMR	Nuclear Magnetic Resonance
ODS	Octadecylsilyl
PAA	Phenylacetic Acid
PCV	Packed Cell Volume
Pi	Inorganic Phosphate
PVP	Polyvinylpyrrolidone
r	Regression Coefficient
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
rpm	Revolutions per minute
S	DNA synthesis
SE	Standard Error
SIM	Selective Ion Monitoring
TCA	Trichloroacetic Acid
TEA	Tetraethyl ammonium chloride

TEAB	Trimethyl ammonium bicarbonate
TFA	Trifluoroacetic Acid
TNH_2	Tryptamine
TOL	Tryptophol
tRNA	Transfer Ribonucleic Acid
UV	Ultra Violet
VDU	Visual Display Unit
Z	Zeatin
Z eqvs	Zeatin equivalents
Z7G	Zeatin-7-glucoside
Z9G	Zeatin-9-glucoside
ZOG	Zeatin-O-glucoside
ZR	Zeatin riboside
ZROG	Zeatin riboside-O-glucoside

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1.0   INTRODUCTION

1.1   Endogenous_Plant_Growth_Substances

The genotype of a plant ultimately determines the way in which it grows and develops but environmental factors interact with these genotypic controls to "tune" many aspects of growth and development (Hill, 1980).

Growth regulating substances, sometimes termed "phytohormones" play a fundamental role in the control of growth and development. The term "phytohormones" can be misleading as it implies that plant "hormones" can be regarded in exactly the same manner as the classically defined animal hormones which are secreted by glands or specialized organs and have very specific effects at points remote from the site of synthesis (Hill, 1980). Conversely, plant growth substances are produced by unspecialized cells, (Sheldrake, 1973), frequently have multiple effects on plants depending upon other factors present and appear not always to have a separation between the site of synthesis and the site of action (Trewavas, 1981).

In fact, it is difficult to define plant growth substances in such a way that would incorporate each of them in every combination and circumstance. Went and Thimann (1937) attempted to define a phytohormone. They suggested that it is

"a substance which being produced in
one part of an organism, is transferred
to another part and there influences a
specific physiological process".

Generally, a growth substance is an organic substance
produced endogenously by a plant, which at low
concentrations can enhance, inhibit or otherwise affect
growth. The resultant effect is usually exhibited at a
site divorced from the site of synthesis (Hill, 1980).

Plant physiologists have split the plant growth
substances, for convenience, into five main groups.
These are, the auxins, the cytokinins, the gibberellins,
abscisic acid (ABA) and the inhibitors, and ethylene. It
is essential to remember that no one group of such
substances can properly be studied in complete isolation
from the other groups as there are complex interactions
between them within the plant. At least some
understanding of all of the major plant growth substance
groups is therefore a necessity if the regulation of
plant growth and development is properly to be
understood.

1.1.1 Auxins

Early research into phototropism, (the bending of plant
organs in response to unilateral illumination), led to
the eventual discovery of the group of plant growth
substances now known as the auxins. Darwin and Darwin
(1881) reported some fundamentally important experiments
with phototropically responding seedlings of the

ornamental canary grass,(<u>Phalaris canariensis</u>), the coleoptiles of such plants proved to be very suitable for such experiments. By covering the apex of coleoptiles and exposing them to unilateral light it was shown that the site of light perception was in the apex but that the curvature response occurred somewhat below the apex. This work inevitably led to the hypothesis that the plant detects the external stimulus of light in one region but transmits a signal which produces a physical response in another region.

The concept that this transported signal actually had a chemical basis was proposed by Boysen-Jensen (1911) after he demonstrated that the signal was still viable after the detecting site had been physically removed and then replaced. This hypothesis was supported by the work of Paal (1919) who excised the tips of the coleoptiles of <u>Coix lacryma</u> and then replaced them on one side of the coleoptile stumps. The coleoptiles curved away from the side covered by the tip, without a unilateral light stimulus. Paal proposed the hypothesis that the tip produced a growth-accelerating substance which passed across the moist contact between the tip and the stump, and down that side of the coleoptile. Thus, the treated side grew faster than the other and therefore the coleoptile curved.

Later, Went (1926) isolated the chemical signal by allowing it to diffuse from excised oat (*Avena sativa*) coleoptile tips into agar blocks. These agar blocks containing the chemical, if placed on one side of the decapitated coleoptiles in the dark, produced curvature of the stumps comparable with Paal's earlier experiments (Went, 1928). Agar blocks which had not been in contact with excised coleoptile tips produced no curvature. These experiments led to the development of a simple biological assay, (bioassay), for auxin-like activity. Went plotted a graph of the number of coleoptile tips placed on the agar against the degree of curvature of the coleoptile stumps, he thus produced a dose-response curve (Went, 1928). Thus, quantitative estimates of the growth substance could be made by allowing extracted plant samples to diffuse into agar blocks and observing the degree of curvature produced by the sample-treated blocks on the coleoptile stumps. Using this technique, rich sources of the growth substance were found which facilitated the eventual identification of the substance (Fogg, 1975).

Plant physiologists called the growth substance revealed by these early experiments, "auxin", which was derived from the Greek word "auxein", meaning "to grow". Researchers then attempted to isolate and chemically identify this growth promoting substance. However, initial attempts were hampered by the fact that it is present in minute quantities in most plant tissues,

(Audus, 1953). Kögl and Haagen-Smit (1931) isolated three compounds with growth promoting activity from human urine, maize oil and malted barley. These were named Auxin A, Auxin B and heteroauxin (Kögl et al., 1934). However, further work did not reproduce the isolation of Auxins A or B from these sources and it became clear that the major auxin-like activity present was actually heteroauxin (Wildman and Bonner, 1948; Bennet-Clark et al., 1952). Heteroauxin was identified by Kögl and Kostermans (1935) as indole-3-acetic acid (IAA) which had been known of for a number of years (Ellinger, 1905). Later, Vliegenthart and Vliegenthart (1966) unequivocally identified Auxins A and B by mass-spectrometry as cholic acid and thiosemicarbizide, respectively, neither of which are active in auxin bioassays.

Indole-3-acetic acid appears to be the principal auxin in a great variety of plants. It has a relatively simple structure:
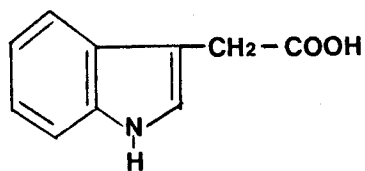
Indole-3-acetic acid
(IAA)

IAA has been found to have various roles in plant growth
and differentiation, the classical ones being in the
control of both phototropism and geotropism
(Dolk, 1936). Pincus and Thimann (1948) defined an
auxin as:

> "An organic substance which promotes growth
> (ie. irreversible increase in volume) along
> the longitudinal axis, when applied in low
> concentrations to shoots of plants freed
> as far as practicable from their own inherent
> growth promoting substances. Auxins may, and
> generally do, have other properties, but
> this one is critical."

It is now known that IAA is present in most plants and
is thought to be the principal auxin in the higher
plants (Thimann, 1969). Elliott and Greenwood, (1974)
have unequivocally identified IAA in the roots of
Zea mays with the aid of combined gas chromatography/
mass spectrometry (GC/MS). Other indole auxins have also
been found in higher plants; 4-chloro-indole-3-acetic
acid (4-Cl-IAA) has been identified in Pisum sativum
seeds both as the free acid (Marumo et al., 1978) and in
the methyl ester form, (Gander and Nitsch, 1967). Not
all auxins are indoles, Wightman and Rauthan (1974) have
demonstrated the presence of the non-indolic auxin
phenylacetic acid (PAA) in a range of shoot tissues.

There has been much research into the biosynthesis of
IAA in higher plants, which has revealed four pathways
which are listed overleaf under the names of their main
intermediates:

(i)   Indole-3-pyruvic acid (IPYA)

(ii)   Tryptamine (TNH$_2$)

(iii)   Indole-3-acetaldoxine (IAO$_x$)

(iv)   Tryptophol (TOL)

In all four pathways the primary precursor for biosynthesis is tryptophan, an amino acid, (Schneider and Wightman, 1978).

Initially it was believed that auxin was produced in the tip of the shoot but later evidence suggests that hormones are synthesized in nearly all parts of the plant (Sheldrake, 1973). This generalized production of growth substances deviates from the classical "hormone" approach; however, localized synthesis and polar transport is known to take place in some instances (Pilet and Elliott, 1981). Specific sites that have been noted for IAA biosynthesis in higher plants include young leaves (Wheeler, 1968), immature ovaries and developing seeds (Nitsch, 1965).

The polarity of auxin transport has attracted considerable research, which has been facilitated by the application of radio-labelled IAA to isolated organs and whole plant tissues. IAA transport in maize roots is preferentially acropetal (Pilet, 1964; Shaw and Wilkins, 1974) and occurs predominantly in the stele (Greenwood et al., 1973). Shoot applied IAA has been found to move into the root system (Davies and Mitchell, 1972). In

maize the application of labelled IAA to the cut mesocotyl surface or to the caryopsis resulted in the appearance of radioactivity in the root (Batra et al., 1975). Pernet and Pilet (1976) demonstrated that although IAA applied to maize root tips enters the root cap its basipetal transport is very limited.

It is believed that the biological activity of IAA may be controlled by oxidation to various metabolites (Hinman and Lang, 1965; Ricard et al., 1972) or by conversion to various conjugates such as the aspartate form (Andreae and Good, 1955), inositol derivatives (Piskornik and Bandurski, 1972) or the glucose ester, (Schneider and Wightman, 1974).

Since Went's early Avena sativa bioassay for auxins a large number of other such bioassays have been developed. These include the split pea curvature assay (Went, 1934), the Avena sativa straight growth test, (Bentley, 1950), and root growth inhibition tests (Moewas, 1949). However, research into auxin activity is complicated by the fact that auxin precursors can cause responses in bioassays because of conversion to IAA. A good example of this is indol-3yl-acetonitrile (IAN) which was extracted from cabbage (Jones et al., 1952); this compound was later found only to be active in auxin bioassays when the nitrilase enzyme converted it to IAA (Bentley and Housley, 1952; Bentley and Bickle, 1952). Thus, although physiological assays are informative they

need the back-up of molecular chemistry. However, chemical identification can also be difficult because of the minute quantities of auxin present in plants and the large losses incurred during the extraction procedures. Nevertheless, various sensitive physico-chemical techniques have been employed.

IAA has been found to have various roles in plant growth and differentiation, the classical ones being in the control of both phototropism and geotropism (Dolk, 1936). Another major role of IAA in the higher plants is the stimulation of cell expansion. It is believed that this effect is achieved by the promotion of cell wall loosening, (Heyn, 1931). Cell enlargement can actually take two forms; firstly, a flaccid cell can regain its turgor, this being a reversible process, and secondly, a turgid cell can enlarge, as in stem elongation, this being an irreversible process. IAA induced cell expansion probably involves more than a simple physical stretching of the cell wall, this is assumed because the cell expansion is highly temperature sensitive and inhibited by KCN treatment (Ray and Ruesink, 1962). Thus, an energy requiring process may occur which is promoted by IAA and produces or stimulates a cell wall loosening factor. Various models have been developed in an attempt to explain this cell expansion. Cleland (1968) believes there may be a biochemical modification which results in a loosened cell wall, and thus irreversible expansion. Other

workers, (Burstrom et al., 1967, Masuda, 1968) suspect that the process is brought about by an initial biochemical cell wall loosening followed by an elastic, reversible expansion. This loosened cell wall under turgor pressure is modified biochemically to produce a rigid wall and thus irreversible expansion.

Another auxin effect is the rapid extrusion of protons from plant cells into the free space and wall solution (Cleland, 1973, 1976). A pH reduction is known to affect the mechanical properties of cell walls in vitro, (Rayle et al., 1970), and also rates of cell elongation in vivo, (Bonner, 1934; Rayle and Cleland, 1970). Such evidence led to the hypothesis of "acid-growth", (Rayle and Cleland, 1970; Hager et al., 1971; Ganot and Rheinold, 1970). This theory proposes that IAA affects cell wall loosening by the stimulation of a proton pump which actively transports protons into the cell wall solution and free space. There may then be an enzymic or non-enzymic hydrolysis of some of the cross-links within the cell wall matrix. A model was proposed (Hager et al., 1971) in which a membrane-bound ATPase is linked to an ion transport pump. Possibly there is an electrogenic mechanism of proton extrusion associated either actively or passively with cation uptake (Cleland, 1976; Cleland and Lomax, 1977). However, there is evidence in the literature which tends to conflict with the acid-growth hypothesis. For example, IAA is capable of increasing the rate of elongation

after acid-stimulated elongation has subsided, (Vanderhoef and Stahl, 1975), and this elongation tends to be transient whereas auxin stimulated growth may continue for as long as 24 hours (Rayle and Cleland, 1970). It is therefore apparent that much further research is needed in the field of acid-growth, particularly into the mechanism by which auxin causes proton extrusion. Acid growth may be only a partial explanation for the role of auxin in cell expansion.

IAA treatment is also known to cause increases in protein and ribonucleic acid (RNA) synthesis, (Fan and Machlachlan, 1967; Datko and Machlachlan, 1968). Continual protein synthesis appears to be required for auxin induced cell expansion, the proteins involved are relatively unstable and are constantly re-synthesized, (Cleland, 1970). IAA stimulation of RNA synthesis is mainly directed towards ribosomal RNA, (Davies et al., 1968; Trewavas, 1968).

IAA is not only a promoter of growth, it can also have an inhibitory effect. Auxins from a plant's apex have been shown to inhibit the growth of lateral buds, (Thimann and Skoog, 1933). Pilet (1961), noted that for very young maize roots a low concentration of IAA promoted growth, whereas higher concentrations of IAA were growth inhibitory. Growth of older roots was inhibited by all IAA concentrations tested. Pilet concluded that the auxin content of such roots increased

with age and eventually reached a "supra-optimal" level such that the application of auxin caused an inhibition of growth.

IAA's role in the cell cycle is not fully understood, mainly because of the complexity of the processes and the interactions with the other plant growth regulators. However, for many years IAA has been implicated as a cell division factor; Avery and co-workers (1937) noted that the movement of IAA correlated with the rate of initiation of cambial division; Das and co-workers (1956) demonstrated that IAA when present with kinetin induced mitosis and subsequently cytokinesis in excised tobacco pith tissue. In the absence of IAA no mitoses occurred, and in the absence of kinetin some mitoses occurred, but only a few of these cells went on to divide. Other workers have also noted the necessity of IAA for the initiation of DNA synthesis and mitosis in tobacco pith tissue (Patau et al., 1957; Naylor et al., 1954). Cooke and Meyer (1981) demonstrated that auxin was necessary from the beginning of the culture period in order to induce the mitotic cycle in tobacco mesophyll protoplasts; they also noted that cytokinin was only essential later in the culture period for the development of the mitotic apparatus (Meyer and Cooke, 1979). Generally, it is believed that auxins stimulate DNA polymerase activity and DNA synthesis, both prerequisites for mitosis, (Yeoman and Mitchell, 1970).

Digby and Wareing (1966) experimented on the effect of IAA on the growth of Acer pseudoplatanus cell suspension cultures. They found that cell division and cell expansion were enhanced by an IAA concentration of 2.5 mg dm^{-3}; however, at 10 mg dm^{-3} the cell's division was inhibited, but cell expansion was still promoted. The results of similar experiments by Street and co-workers (1968) emphasize the problems encountered in this field of research. They found that the highest level of cell division was effected at 10 mg dm^{-3} of IAA and that no enhancement of cell expansion occurred until concentrations of > 10 mg dm^{-3} were used. Clearly the roles of the plant growth regulators are not clear-cut and it must be remembered that there is a vast number of other factors which are involved in plant cell cycle regulation.

Very close correlations have been observed between IAA levels and mitotic indices, the most interesting results are from work involving synchronized cultures, (Section 4.0). In synchronously dividing Chlorella cells the levels of endogenous free IAA per cell were found to double in the period just preceding cytokinesis, (Grotbeck and Vance, 1972). Nishinari and Yamaki (1976) noted that the levels of IAA per cell in synchronized tobacco cell cultures fluctuated in parallel with the number of cells in mitosis.

Elliott et al., (1987) working on the same line of Acer pseudoplatanus cells used in this present study, also provided evidence of an IAA regulatory effect on mitosis. The levels of endogenous IAA through four consecutive cold-induced synchronous divisions showed that increases in the amount of IAA per cell prior to each cell division paralleled similar changes in the mitotic indices. IAA levels were also observed to increase during the period of cell expansion before cell division. The action of IAA on cell expansion in this line of Acer pseudoplatanus cells has also been reported by Moloney et al., (1983). However, Robinson (1982) found that during the first synchronous division there was no cell expansion but the levels of endogenous IAA did increase. Elliott et al. (1987), also working with the same strain of cells in continuous culture, indicated a correlation between endogenous IAA levels and specific growth rates. Further data on these cells has been provided by Firby (1985) who reported two peaks of intracellular IAA levels when she investigated the first synchronous division after inoculation. The first peak appeared to precede DNA synthesis and it was thus postulated as a trigger or possible initiator for DNA replication in the cell cycle. The second peak either coincided with or immediately followed mitosis, and therefore could be related to cytokinesis rather than mitosis.

Elliott et al. (1987) also found high levels of IAA in used media; however, when radioactively labelled IAA was supplied no apparent movement of the label into the cells took place (Robinson, 1982). This may suggest that such IAA does not play a role in the regulation of growth processes within the cell, although it must be remembered that a cell suspension culture cannot be regarded as being representative of a whole plant. Within a whole plant, IAA is transported away from sites of synthesis, (Elliott, 1977); in a closed suspension culture there is likely to be an accumulation of IAA that would in normal circumstances have been transported elsewhere. The roles of both IAA and 2,4-D in the culture medium and cells are by no means fully understood. Moloney et al. (1983) suggested that 2,4-D may trigger the cell's metabolism during the lag phase and the cell then becomes able to produce sufficient auxin in the form of IAA. It is quite probable that if IAA does act as a trigger for cell division, it does so in conjunction with other plant growth regulators, notably the cytokinins. In this present study the roles of both IAA and the cytokinins in the regulation of the Acer pseudoplatanus cell cycle are investigated further.

1.1.2 Cytokinins

Research into the group of plant growth regulating substances that we now know as the "cytokinins", was initiated early this century when Haberlandt (1913)

produced evidence of a substance in plants which could stimulate cell division, (cytokinesis). He noted that phloem diffusates induced cell division in potato parenchyma. Later, in 1921, he investigated the cell division promoting effects of wounding and found that rinsing the wounded area suppressed cell division, but cell division could be restored by applying crushed tissue to the affected area. Van Overbeek and co-workers (1941), reported the presence of a growth promoting substance in coconut milk, this factor was later found to be zeatin riboside, (Letham, 1968).

The advent of plant tissue culture techniques enabled more extensive research into the cytokinins. Jablonski and Skoog (1954) employed these techniques with Nicotiana tabacum and discovered that in the presence of an auxin supply the cells grew somewhat slowly and primarily by cell expansion. However, when fragments of vascular tissue were added to such cultures, cytokinesis resulted. The calluses subsequently produced could be sub-cultured but the medium had to contain a factor such as coconut milk or yeast extract to sustain cell division.

Later, Miller and co-workers (1955, 1956) isolated and identified kinetin (6-furfurylamino purine) from autoclaved herring sperm DNA. In fact, kinetin was produced during autoclaving by the dehydration and migration of a deoxyribose moiety from the 9 position of

adenine to the N6 position, (Skoog and Leonard, 1968). Kinetin, like most of the cytokinins is a substituted purine; this has led researchers to look for a connection between the cytokinins and nucleic acids with purine bases in the cell division response.

The cytokinins are not all structurally related, the definition of a cytokinin is therefore essentially a physiological one. It is now universally accepted that a cytokinin is a compound that promotes cell division in cultured callus tissue grown on a defined medium with all the necessary organic and inorganic nutrients and growth factors including an exogenous supply of auxin. This definition includes non-naturally occurring cytokinins, for example, kinetin.

Although the first synthetic cytokinin, kinetin, was isolated in 1956, (Miller et al.), it was not until 1963 that Letham first identified an endogenous cytokinin, zeatin (Z). With the discovery of so many other cytokinins it soon became clear that most naturally-occurring cytokinins are N6-substituted purines (Horgan, 1978; Matsubara, 1980). However, there are a variety of other substances which exhibit some cytokinin-like activity in bioassays, sometimes interacting with other factors. A good example is diphenyl urea which may produce its effects by being built into a cytokinin molecule of the substituted

adenine type. Diphenyl urea is actually a naturally
occurring cytokinin and was isolated from coconut milk
(Shantz and Steward, 1955).

Compounds such as zeatin and its derivatives, which
possess an isoprenoid side chain attached to the N6
position of the purine nucleus are the most widely
occurring natural cytokinins (Letham and Palni, 1983).
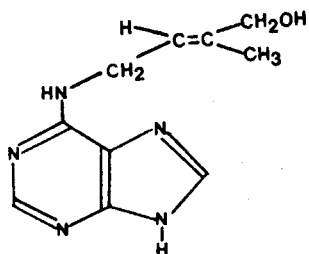
Zeatin (Z)

Saturation of the side chain results in the formation of
dihydrozeatin (DHZ) and related compounds which are more
resistant to cleavage by "cytokinin oxidase" type
enzymes, (Whitty and Hall, 1974).
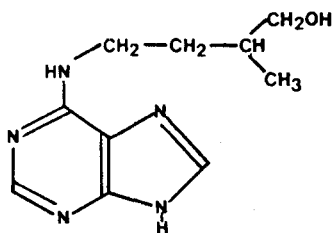
Dihydrozeatin (DHZ)

Glucosylation of the hydroxyl group of the side chain of
zeatin and dihydrozeatin results in the formation of
cytokinin O-glucosides. Other substitutions of the
purine nucleus at the 3, 7 and 9 positions produce
cytokinin ribosides, ribotides and N-glucosides.
Different combinations of glucose, ribose and
phosphoribose substitutions together with the occurrence

of methylthio derivatives and alanine conjugates lead to the presence of a large number of naturally occurring cytokinins.

The side chains of the N6 substituted purines dictate how a compound will behave in a cytokinin bioassay. The activity tends to increase with chain length up to an optimum of five carbons among N6 alkyl derivatives. (Skoog et al., 1967). Another important factor is the unsaturation of the side chain, its presence produces a dramatic increase in activity over the saturated analogue. A polar group can also exert an effect. The hydroxyl group positioned in 4-hydroxy isopentenyl adenine (zeatin) makes this compound much more active than the 2- or 3- hydroxy analogues (Leonard et al., 1969). Skoog and co-workers (1967) demonstrated that the 8-azapurine cytokinins are considerably less active than their purine analogues. Another non-adenine compound exhibiting cytokinin activity is 6-benzyl thio purine (Kulaeva et al., 1967) which is more active than N6 benzyl adenine.

Generally, the naturally occurring cytokinins are adenine bases, ribosides and ribotides, with additional substitution also occurring, such as O-glycosylation or methylthiolation (Burrows et al., 1970). Because there is a large number of such compounds it is essential to

determine the precise molecular structure of the cytokinins in plant extract fractions which demonstrate activity in bioassays.

After the discovery of the synthetic cytokinin, kinetin, various workers reported cytokinin-like activity in a variety of plant sources, but the actual isolation and identification of these substances was impeded by technical problems. Difficulties were encountered in extracting the small quantities of cytokinins which are present even in large amounts of plant material, and the methods that were developed were somewhat time-consuming. This probably explains why so few data are available about the relationships between endogenous cytokinin levels and physiological responses. The development of gas chromatography/mass spectrometry (GC/MS) and high performance liquid chromatography (HPLC) for the purification, separation and identification of the cytokinins has provided more concrete evidence for their growth substance role, based upon correlative physiological evidence (Wareing, 1976). However, cytokinin activity in a plant may represent a multiplicity of known and unknown compounds, this makes the use of bioassays a crucial part of the study of cytokinins.

Bioassays can be employed exclusively to estimate the total cytokinin-like activity extracted from a tissue and can also be used in conjunction with Sephadex LH-20

chromatography or HPLC to attempt to estimate the levels of the individual cytokinins present. A dose-response calibration curve is prepared for the bioassay, often using kinetin, benzyl adenine or zeatin as standards. The cytokinin activity present in the extract is then read off this calibration curve and quantified in terms of ng equivalents of the standard used. It must be remembered however, that such bioassay procedures are limited in their accuracy because not all cytokinins produce identical dose-response calibration curves.

Various cytokinin bioassays are routinely used by researchers. The first cytokinin bioassay developed was the tobacco callus assay (Miller et al., 1956). The assay depends upon the large increase in the fresh mass of the callus when the cells undergo division. The amount of cytokinin present determines the cell division and thus the fresh mass of the callus. This bioassay is relatively sensitive but has the disadvantage that supraoptimal levels may result in a depression of maximal growth, leading to underestimates of cytokinin levels. The soybean callus system (Miller, 1963) has the advantage over the tobacco callus system as concentrations as high as 1 mg dm^{-3} are still suboptimal. There are various bioassays based on this principal of utilisation of cell division inducement, the advantages of such bioassays are high sensitivity, sterility and specificity (Letham, 1978) but they do have the disadvantage of a long incubation time before a

result is actually obtained. This problem led to the development of more rapid, though probably less sensitive, bioassay systems.

The ability of cytokinins to promote expansion of excised leaf or cotyledon tissue was utilized by Letham (1968) in the excised radish cotyledon bioassay. The inhibition of leaf senescence, first reported by Richmond and Lang (1957) was utilized by various workers using tobacco (Letham, 1967), oat (Vagar and Bruinsma, 1973) and bean leaves (Seth and Wareing, 1965). The leaf senescence bioassays tend to be relatively insensitive to low cytokinin concentrations, (Letham, 1967). The Amaranthus bioassay (Bigot, 1968) uses the property of cytokinins to promote betacyanin pigment synthesis in the dark. The bioassay used in the present study of cytokinins depends upon the stimulation of chlorophyll production in etiolated cucumber cotyledons (Fletcher and McCullagh, 1971; Fletcher et al., 1973). Fletcher and McCullagh (1971) reported this bioassay to be more sensitive to low cytokinin concentrations than the callus bioassays, however, this has been disputed by Hosford (1984). Nevertheless this is a convenient and sensitive assay which has recently been revised to an even more sensitive form (Fletcher et al., 1982).

The synthesis of cytokinins does not necessarily take place in all plant tissues. Misleading conclusions can be made when attempting to establish the sites of

synthesis of such substances because high levels of cytokinins in particular organs may be due to "sinks" or "pools" rather than sites of synthesis. The ability of cytokinins to promote cell division has directed researchers to zones of high meristematic activity as possible sites of synthesis. Goldacre (1959) suggested that cytokinin production may be a normal accompaniment of cell division and thus proposed the root meristem as a possible site of synthesis. Since then the vascular cambium, buds and developing seeds have also been implicated.

Mothes (1960) produced evidence that roots could be sites of synthesis when he demonstrated that the senescence of tobacco leaves was delayed by either kinetin application or the formation of roots on the petioles. Roots are now widely accepted as major sites of synthesis and it is believed that the cytokinins move in the xylem to the shoot where they are involved in the control of development (Letham and Palni, 1983). Cytokinins have also been detected in cultured roots (Koda and Okazawa, 1978; van Staden and Smith, 1978) and in cultured root tissue of pea (Short and Torrey, 1972a), soybean (Sargent and King, 1974) and carrot (Mizuno and Komamine, 1978). Root apices have also been shown to contain high levels of cytokinins (Short and Torrey, 1972b; Feldman, 1975). Kriesel (1976) and Wang,

Thompson and Horgan (1977) demonstrated that increased cytokinin levels in explants were associated with root development.

The cambium often appears to contain high levels of cytokinin-like materials. This site was considered as a possible region of synthesis when a rise in activity was observed from the xylem sap of canes of grape during cold storage, even though no visible roots were present (Skene, 1972). Similar observations were made with artificially chilled poplar twigs (Hewett and Wareing, 1973). However, root initials are known to be present in one year old poplar stems and it is therefore possible that the root primordia are the actual source. Another explanation for high levels of cytokinins in the xylem sap is that the cambium may be a site for inactively stored cytokinins. It is believed that such storage compounds could be glucosides.

It is now generally believed that the cytokinins in buds comes from the roots and stems of the plant. Girdling experiments performed on willow cuttings in autumn indicate that the cytokinin glucosides increase in the buds and the phloem below the girdle (van Staden and Brown, 1978). During bud swelling these glucosides decreased while a compound which co-chromatographs with zeatin riboside increased. This may indicate that the buds have the ability to convert storage cytokinins to

active forms. The glucosides accumulated below the girdle and therefore it is likely that they were transported from the roots.

Seeds and fruits contain high levels of diverse cytokinins. Burrows and Carr (1970) demonstrated that cytokinin levels within pea seeds reached their highest levels when most endosperm was present, and decreased with age. This implies that if seeds are a site of synthesis they are only such in their early developmental stages.

Cytokinins are known to be present in both the xylem sap (Skene, 1975) and the phloem exudate (Hall and Baker, 1972; Vonk, 1978, 1979) of many plants. It would appear that if the major site of synthesis is in the roots, then the cytokinins are distributed to the growing points via the phloem and xylem. Translocation in the phloem is now considered to be bidirectional, and van Staden (1976a,b) has suggested that in woody plants there may be recycling of the cytokinins in autumn.

Other tissues have also been implicated in cytokinin synthesis, such as cultured lateral shoots (Wang and Wareing, 1979). In general, cytokinin synthesis has been associated with meristematic tissues (Feldman, 1975).

There is considerable evidence in the literature of cytokinins affecting the growth of a variety of intact plants and also plants in cell and tissue culture. In fact, Miura and Miller (1969) suggested that all plants have the ability to synthesize cytokinins provided that the mechanism is induced. It is widely accepted by plant physiologists that cytokinins are involved in cytokinesis (Miller, 1961; Fosket, Volk and Goldsmith, 1977); that they retard senescence, possibly by affecting protein synthesis, (Richmond and Lang, 1957); and that they bring about the mobilisation of nutrients within plant tissues (Mothes and Engelbrecht, 1961). Cytokinins also appear to be involved in apical dominance (Wickson and Thimann, 1958). An early property of cytokinins to be discovered was their ability to control some aspects of morphogenesis such as bud formation (Skoog and Tsui, 1948; Skoog and Miller, 1957). The role of the cytokinins and auxins in morphogenesis has been found to depend upon their relative levels. Skoog and Miller (1957) demonstrated that high levels of auxin with low levels of kinetin favoured root formation, whilst low levels of auxin and high levels of kinetin favoured bud formation.

The discovery of the delay of senescence (Richmond and Lang, 1957) by cytokinin application lead to information regarding nutrient mobilisation. Mothes (1961) showed that localised applications of cytokinins resulted in a movement of nutrients. Later, Muller and Leopold (1966)

demonstrated a movement to and an accumulation at the site of application. They proposed that this may be the basis of cytokinin controlled bud growth and the regulation of apical dominance.

There have been various investigations into the role of the cytokinins in plant growth and differentiation. Sitton et al., (1967) analysed the cytokinin content of the root exudate of sunflower during vegetative and flowering periods. A steady increase in cytokinin content was observed until the formation of the first flower bud, then the level decreased to a minimum at flowering. The decrease appears too early to be the primary regulator of senescence, Leopold and Kreidman (1975) suggested that the control process involves a change from root to shoot synthesized cytokinins. The majority of research performed in this area has not included the identification of the cytokinins concerned. However, Horgan et al., (1973) have identified the major cytokinins in Populus robusta and changes in cytokinin activity have been followed during chilling and bud burst (Hewett and Wareing, 1973). A rise in cytokinin activity was noted prior to or at bud burst, a similar trend was observed in sycamore sap (Purse et al., 1976). It would appear that the majority of the processes which involve cytokinins also involve a complex interaction with the other plant growth substances.

The cytokinins have for a long time been associated with the nucleic acids and so researchers were not surprised to detect cytokinin activity in tRNA preparations (Zachau et al., 1966; Hall et al., 1967). Both DNA and RNA have been regarded as cytokinin sources for the cell. The biosynthetic pathway resulting in tRNA cytokinins is known in micro-organisms and animal cells (Kline et al., 1969). The crucial step in the pathway is the transfer of the Δ^2-isopentenyl side chain from Δ^2-isopentenyl pyrophosphate (IPP) to the adenosine of the tRNA to give the cytokinin N^6-(Δ^2-isopentenyl) adenosine (IPA). It has been suggested that the same route is used in plants, and it has been demonstrated that IPA is synthesized in tobacco pith tissue by the attachment of an Δ^2-isopentenyl-group, originally derived from mevalonic acid, to preformed tRNA, (Chen and Hall, 1969). An enzyme system has been isolated from maize which can transfer the isoprene unit from IPP to the adenosine of tRNA in vitro (Holtz and Klambt, 1978). However, intact cytokinins have also been shown to be incorporated into tobacco tRNA (Walker et al., 1974) implying that the IPP transfer pathway is not a unique system for cytokinin incorporation with tRNA.

It is believed that IPA has no cytokinin activity when it is an integral part of the tRNA molecule, (Chen and Hall, 1969). The biologically active compounds are produced when the tRNA components are released as nucleosides or nucleotides during tRNA catabolism.

Pathways exist in higher plant tissues by which IPA can be converted to the free cytokinins such as the zeatin derivatives, (Hall, 1973).

In bacteria it has been proposed that tRNA turnover could account for all of the detectable cytokinin content (Hahn et al., 1976). However, such turnover is unlikely to account for the total cytokinin requirements of the higher plants. Short and Torrey (1972b) demonstrated that there was approximately 27 times more cytokinin in a free form than bound in the tRNA in pea root tips. Wareing et al., (1977) pointed out that if the cytokinins present in certain species of tRNA are released during RNA degradation it is difficult to understand why leaves require a supply of cytokinin from elsewhere in the plant, unless the amounts of cytokinin released by tRNA degradation are indeed insufficient. Further evidence for the existence of tRNA independent cytokinin biosynthetic pathways is the fact that plant tissue known to contain cytokinin in its tRNA may be dependent on added cytokinin when grown in culture (Burrows, 1976).

Whatever their biosynthetic pathways the cytokinins are universally recognised as promoters of cell division. However, they have not yet been assigned a specific role in the regulation of plant cell division. They are thought to act via an effect on protein synthesis in the G_2 of the cell cycle or in the transition from G_2 to

mitosis (Everett et al., 1978; Fosket, 1977), possibly by inducing the synthesis of specific proteins required for the functioning of the mitotic apparatus (Fosket and Short, 1973; Fosket and Tepfer, 1978). Fosket and Short, (1973), showed that soybean tissue cultures arrested by cytokinin deprivation contained twice the extractable DNA of those grown in the presence of zeatin. They proposed the hypothesis that the cells had arrested prior to mitosis after completing a round of DNA replication. Further work has implicated cytokinin-induced activation of specific, stored, cytoplasmic mRNAs which bring about the synthesis of proteins (Tepfer and Fosket, 1978). A change in the proteins synthesized following treatment with cytokinins has been reported by Fosket (1977) and Jouanneau (1970) working with tobacco; this occurs prior to mitosis. In nuclei prepared from coconut milk kinetin treatment resulted in the stimulation of RNA synthesis (Datta and Sen, 1965) and protein synthesis (Roychoudhury et al., 1965). Kulaeva and Romanko (1967) demonstrated that 6-benzyl-adenine stimulated protein synthesis in vitro in isolated tobacco chloroplasts. Cytokinins have also been found to affect a number of enzymes involved in secondary metabolism. An important example is the stimulation of nitrate reductase activity by cytokinins (Roth-Bejerano and Lips, 1970).

The literature does not show a clear-cut relationship
between the cytokinins and cell division; this area is
reviewed in more detail in Section 3.0. It is likely
that the cell division inducing effect is caused by
complex interactions of the plant growth substances. For
example, there is a close association between cytokinins
and auxins; a number of plant tissues will only undergo
mitosis if cytokinins, or auxins, or both, are present.
Nevertheless, some plant physiologists argue that
cytokinins are a specific trigger for cytokinesis,
(Torrey, 1961).

Mackenzie and Street (1972) investigated cytokinins in
Acer pseudoplatanus cell suspension cultures. They
extracted two cytokinins which they called Cytokinin 1
and Cytokinin 2. Their Cytokinin 1 corresponded to a
cytokinin found naturally in water melon and cucumber
seeds and their Cytokinin 2 co-chromatographed with
zeatin riboside. Mackenzie and Street noted a rapid
rise in endogenous cytokinins preceeding cell division,
and later the levels declined in the linear phase.
Short and Torrey (1972a) working on pea root callus
observed a similar rise in cytokinin content early in
the culture period, and a peak when cell division
occurred. Mackenzie, Konar and Street (1972) attempted
to induce cell division in Acer pseudoplatanus cells by
externally supplying cytokinins to cultures containing
such low inoculum densities that they would not
normally grow, or if they did, they would exhibit an

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excessively long lag phase. They noted that the addition of cytokinins reduced this lag phase and that zeatin appeared to produce this effect at lower concentrations than kinetin. The longer the cells were in the stationary phase, the less likely they were to divide. This was probably due to their declining endogenous cytokinin activity and also a decline in their ability to take up added cytokinins. They proposed the hypothesis that during the lag phase the cells develop a critical level of cytokinin activity by an excess of endogenous synthesis over loss and internal degradation. Once cell division had been initiated the subsequent growth was apparently unaffected by low cytokinin levels. At high concentrations cytokinins can inhibit growth. However, in a growing Acer pseudoplatanus cell suspension culture they found that the final cell density was independent of the supply of external cytokinins. This implies that once cell division has been initiated by a critical cytokinin level, the external levels do not affect the growth rate or final cell yield. This observation , however, contrasts with the work of Helgeson et al. (1969) on tobacco callus; they noted that the growth rate is controlled by the external cytokinin supply.

The work carried out by Robinson (1982) in these laboratories, does not totally agree with that of Mackenzie and Street (1972). She also investigated Acer pseudoplatanus cell suspension cultures and noted

that the cytokinin-like activity increased but in this case remained high during the linear phase of growth before progressively declining into stationary phase. This is consistent with the proposal that cytokinins are involved in cell division. Cytokinin-like activity was found in the medium, though the amount remained at a fairly constant level throughout the cycle. Mackenzie and Street (1972) also analysed the used medium and found it to contain Cytokinin 1, low levels of Cytokinin 2 and a cytokinin that co-chromatographed with zeatin. In cold induced synchronous divisions, Robinson (1982), observed an increase in cytokinin activity prior to each division and a decline to a low level during periods in the cold. The mitotic index of these cells increased in parallel with the cytokinin-like activity; this reinforces the view that cytokinins are required for mitosis in Acer pseudoplatanus cells.

Firby (1985), also investigated the cytokinins of cultured Acer pseudoplatanus cells in this laboratory. She reported that two peaks of cytokinin activity were present after inoculation, one at approximately 20 hours and the second just preceeding cytokinesis. Both of these peaks were characterized by rapid increases and decreases in cytokinin activity. The 20 hour peak was believed to be associated with protein synthesis. A very similar cytokinin activity pattern has been reported by Elliott et al. (1986) in Beta vulgaris suspension cultures.

The site at which cytokinins are active is not known,
although it is believed that they act on plant
metabolism as mediators, promotors or inhibitors at a
level close to, though not necessarily at the genome,
(Burrows, 1975). It is thought that the different
forms of cytokinins exhibit different roles in the whole
plant. Generally, nucleotide derivatives may be
associated with cytokinin uptake and transport across
membranes (Laloue et al., 1977) and may also be
important translocated forms of cytokinin (Skene, 1975;
Letham, 1978). The formation of 7- and 9-glucosides,
alanine conjugates and side chain cleavage to adenine
and its derivatives may be mechanisms for lowering the
physiological level of cytokinin activity (Letham and
Palni, 1983). The O-glucosides are thought to be
storage forms of cytokinin; they are relatively stable
and are known to accumulate in mature leaves
(Wareing et al., 1977; Palmer et al., 1981a). The free
bases per se may be the active cytokinin form (Letham
and Palni, 1983). The regulation of the interchange
between "storage" and "bound" forms of cytokinin to the
free base may be controlled by enzymatic activity.

Whitty and Hall (1974) identified a "cytokinin oxidase"
type enzyme in the kernels of Zea mays, which cleaves
the sidechains of the isopentenyl type, but does not
attack saturated side chains.

The cytokinins are a very complex group of plant growth regulators and despite much research, the basic questions of cytokinin function and mode of action are largely unanswered. In many instances cytokinin action involves numerous responses which cannot easily be categorized as primary or secondary, or easily quantified. The aim of this present study is to assess the quantitative changes of the various cytokinins present in Acer pseudoplatanus cell suspension cultures throughout their growth cycle and to attempt to correlate any such levels with batch culture growth cycle and cell cycle regulation.

1.1.3 Gibberellins

The early research into the gibberellins was as a result of observations in plant pathology. The fungus Gibberella fujikuroi, causes a disease in rice plants known as "Bakanae", meaning "foolish seedling" (Hill, 1980). The principal symptom of affected plants is hyperelongation of the stems and leaves resulting in very spindly and fragile plants which usually fall over, hence the name, "foolish seedling". In 1926 Kurosawa discovered that the fungus-free filtrate from a culture of the fungus produced this same symptom when applied to healthy rice seedlings. Thus, it appeared that the fungus produced a substance that caused stem and leaf elongation. Yabuta (1935) isolated a crystalline solid from the fungus which induced stem hyperelongation. Later, Cross et al., (1959) using ultra-violet (UV),

infra red (IR) and nuclear magnetic resonance (NMR) spectroscopy, was able to assign a structure to this substance, known as gibberellic acid.
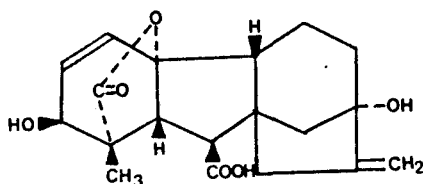
Gibberellic Acid (GA.3)

The other gibberellins have structures closely related to GA.3; they tend to be weak acids, as are auxins. The known gibberellins are referred to by numbers which are approximately in chronological order of their discovery. Besides the "true" gibberellins based on the gibberellane structure, there are a few other compounds with different structures which exhibit GA-like activity in some bioassays, such as kaurenoic acid (Katsumi et al., 1964). However, such compounds may be converted to gibberellins by the plant.

Probably the most dramatic effect of the gibberellins can be seen on application to dwarf varieties; the plant often attains a "normal" height, (Brian and Hemming, 1955). They also induce premature bolting of rosette-type plants (Lang, 1956a), stimulation of flowering, (Lang, 1956b), breaking of bud dormancy (Brian et al., 1955), promotion of seed germination (Lona, 1956) and stem elongation (Phinney, 1961). They

are also known to stimulate de novo synthesis of some enzymes, such as α-amylase, protease and ribonuclease (Chrispeels and Varner, 1967).

Gibberellins are believed to have a role in the onset of bolting and flowering after induction by light and high temperature (Phinney and West, 1960). Flowering in gymnosperms is also linked with gibberellic acid levels (Ross and Pharis, 1976); this may be due to the ratio of non-polar to polar gibberellins (Pharis et al., 1976). There has also been a suggested role for gibberellic acid in the geotropic responses of roots and shoots (Railton and Philips, 1973).

Research has suggested various sites for GA synthesis in the higher plants, but there seems to be considerable indirect evidence to implicate the root system as a major biosynthetic site, (Nitsch, 1957; Butcher, 1963; Carr et al., 1964; Phillips and Jones, 1964; Reid and Carr, 1967). There is also evidence of GA inter-conversion, with some GAs being more biologically active than others, (Spray et al., 1984; Ingram et al., 1984).

The mechanism by which gibberellins stimulate growth is not fully understood. Some workers have suggested that they may act similarly to auxins, having a role in cell expansion (Lockhart, 1960). However, Cleland et al., (1968) found no effect of gibberellic acid on hypocotyl

wall extensibility. There is evidence of gibberellin-like compounds in cell and callus cultures; Lance et al., (1976) detected gibberellic acid in Nicotiana tabacum calluses. Hall (1979) studied endogenous gibberellins in the line of Acer pseudoplatanus cells used in the present study. A link was found between the greening of cultures and the gibberellin content of the cells. A possible role for gibberellins as factors regulating the G1/S transition in Acer pseudoplatanus cells was proposed and GA.3 has been found to stimulate cell division at low inoculum densities (Hall, 1979).

A large number of known gibberellins exist and therefore there are problems both in identification and with the use of bioassays for quantitative research. The gibberellin bioassays have been reviewed by Bailiss and Hill (1971). Undoubtedly, many of the gibberellin precursors are also active in some routine gibberellin bioassays. The gibberellins present a major problem to plant physiologists because of their numbers, their interconversions and their complex interactions not only amongst themselves but also with the other plant growth regulators (Ingram et al., 1984).

1.1.4 Abscisic Acid and the Inhibitors

For a long period of time plant physiologists suspected that if growth promoters existed, it was likely that inhibitors did too. The first evidence came from extracts which were purified by thin layer chromatography, the bioassays of which showed inhibitory zones. Bennet-Clarke and co-workers (1952) noted the so-called inhibitor - β complex, and later Eagles and Wareing (1963) found it to be present in birch leaves.

The activity of this inhibitor increased under short day conditions, Eagles and Wareing (1964) believed it had a role in controlling bud dormancy, hence they suggested the name "dormin".

The inhibitor was also extracted from sycamore leaves (Robinson and Wareing, 1964) and from the pods of yellow lupins (Rothwell and Wain, 1964), where it was believed to be involved in the abscission of immature fruitlets. Ohkuma et al., (1963) isolated an abscission enhancing substance from cotton fruits and named it abscisin II. Cornforth and co-workers (1965) later identified both dormin and abscisin II as 3-methyl-5 (1-hydroxy-4 oxo-2,6,6 trimethyl-2-cyclo-hexene-1-yl) cis-trans 2,4pentadienoic acid; this chemical is now known by the generally accepted name, abscisic acid (ABA), (Addicott et al., 1968).
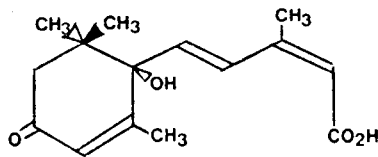
(+)-(s) abscisic acid (c,t-ABA)

Abscisic acid is an optically active compound; the (+)s enantiomer is the naturally occurring form. The 2-trans-geometrical isomer is also naturally occurring but is rarer and also biologically less active. Abscisic acid and 2-trans-abscisic acid interconvert in solution when illuminated to a 1:1 equilibrium mixture. This is an important factor to be considered in ABA estimations. However, the two compounds are chromatographically distinct, (Lenton et al., 1968; Rivier et al., 1977).

The biosynthesis of ABA is not fully understood, although mevalonic acid has been implicated as a precursor, (Milborrow, 1975a,b).

ABA is now known to be involved in the control of several plant growth processes; notably, bud dormancy, abscission, transpiration rate and root geotropism, (Addicott, 1970). It has been identified as an

endogenous substance of roots of several species (Walton et al., 1976), and has been found in the root cap of <u>Zea mays</u> (Rivier et al., 1977). ABA has also been shown to be an inhibitor of root elongation (Pilet, 1970), Pilet and Elliott (1981) suggested that maize root elongation and georeaction are regulated by the interaction of acropetally moving IAA, with growth inhibitors, (probably including ABA), transported in a basipetal direction.

Bud dormancy cannot be easily correlated with ABA levels; in potato tubers it is thought that there is an interaction of growth regulators. The ABA-like compounds act as inhibitors whilst gibberellins stimulate the buds to develop (Rappaport and Wolf, 1969). The application of ABA to seeds has been found to inhibit germination (Rudricki et al., 1968) suggesting a possible role for ABA in seed dormancy.

The role of ABA in abscission is well reported. ABA has been found to promote leaf abscission in excised leaf explants of apple (Pieniazek, 1971), <u>Coleus</u> (Dorffling and Bottger, 1968), and bean (Jackson and Osborne, 1972). Fruit abscission is also regulated by ABA (Rothwell and Wain, 1964) and it has been shown to be the factor causing the abscission of immature lupin fruitlets (Cornforth et al., 1966).

The change in the transpiration rate due to water stress is connected with ABA levels. Wright (1969) found an increase in inhibitor-β in detached wheat leaves after they had begun to wilt. ABA is known to accumulate in the leaves of water stressed mesophytic plants (Wright, 1978; Walton et al., 1977). Applications of ABA to intact leaves or isolated epidermal strips initiates stomatal closure, (Horton, 1971), and thus transpiration is dramatically reduced. It is believed that the closure of the stomata may be due to starch accumulation in the guard cells which increases the cells' water potential; ABA may inhibit α-amylase synthesis thus resulting in such a build-up of starch (Mansfield and Jones, 1971). The precise stimulus responsible for the elevation of ABA levels in water stressed tissues is not known. It is, however, generally accepted that water stress does result in elevated ABA levels, which in turn results in an increase in the water potential of the guard cells. As well as an accumulation of starch in the guard cells an efflux of K^+ ions has also been reported, (Mansfield and Jones, 1971), in ABA treated guard cells. The ABA stomatal closure response is very rapid and is thus unlikely to be primarily controlled by the inhibition of α-amylase synthesis; it is probable that the rapid response is achieved initially by the ion efflux, with the inhibition of α-amylase synthesis reinforcing the response.

The role of ABA in root geotropism is complicated by its interactions with IAA. The classical Cholodny-Went theory of tropisms (Cholodny, 1926; Went, 1926) explained geotropism in roots in terms of IAA alone, as ABA had not been identified at the time. They suggested IAA was transported from the root apex asymmetrically and in a basipetal direction but Pilet (1964) found acropetal transport of IAA in roots. Later, Juniper et al., 1966, found the root cap to be the site of geoperception and an inhibitor was found to be produced in the root cap (Wilkins and Wain, 1974). It is possible that the georesponse is caused by an interaction of oppositely transported ABA and IAA. This work highlights the difficulties encountered when working in the field of growth regulators as they rarely have effects not involving some interaction with other plant growth substances.

Many researchers have investigated the effect of added ABA on tissue cultures. It has been found to inhibit cell division in tobacco (Li et al., 1970) and rose cultures (Gamborg and La Rue, 1971) by 50% at a concentration of 10^{-5} mol dm^{-3}, whilst enhancing soybean callus cell division in the presence of kinetin (Sankhla and Sankhla, 1968). A 50% increase in cell division has been reported in cultured slices of Helianthus tuberosus (Minocha, 1979; Watson and Halperin, 1980) when 5 mg dm^{-3} ABA was supplied. Hosford (1984) found no inhibitory action of ABA on cell division in sugar

beet cells at concentrations up to 10^{-4} mol dm^{-3}. Likewise, Hall (1979), working with the same strain of _Acer pseudoplatanus_ cells used in this present study, found no inhibitory affect on cell suspension cultures at concentrations of 10^{-5} mol dm^{-3}.

The role of ABA in plant growth regulation is undoubtedly a complex one. Research into this area has been further complicated by the rapid fluctuations in endogenous whole plant ABA levels depending upon the plant's water status.

1.1.5 Ethylene

Ethylene is a difficult growth regulator to investigate because of its gaseous state at normal temperature. As it is the simplest olefin possessing only one functional group it is difficult to believe that its mechanism of action can be very specific.

$$H_2C=CH_2$$
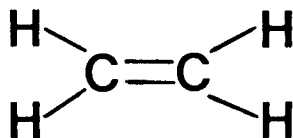
Ethylene

Smoke and coal gas were known for a long time to affect the tropic response of both roots and shoots, but it was not until 1901 that Neljibow treated pea seedlings with ethylene and found that it caused growth to be stunted, thickening of the stem and loss of the geotropic

response. The induction of flowering in pineapples exposed to smoke was also found to be due to ethylene, (Rodriguez, 1932).

Elmer, (1932, 1936), noted that a volatile substance originating from ripe apples inhibited the normal sprout development of germinating potatoes. The apical growth was dramatically inhibited, resulting in the development of small stem tubers rather than normal sprouts. It was observed that immature apples did not have this effect.

Kidd and West (1932) reported that a variety of fruits produce a vapour that has the affect of stimulating ripening. Smith and Gane (1933) and Kidd and West (1934) underlined the fact that such fruits were required to be ripe if they were to produce such effects.

Denny and Miller (1935), Denny, (1935, 1936) and Gane (1935) observed an epinastic response of leaves caused by a substance evolved by a variety of plants' leaves, fruits, flowers and roots. Various workers, including Denny and Miller (1935) postulated that this substance, that was produced in a volatile form by plants, was ethylene. In 1934, Gane reported that ethylene had been identified amongst the gaseous products evolving from ripe apples.

Early researchers were hindered by the difficulties involved in assaying a gas. Thus, the introduction of gas chromatography (GC) for ethylene assays has enabled the production of ethylene to be estimated very accurately (Burg and Thimann 1960). The many roles of ethylene in plant growth and development are therefore beginning to be revealed.

Ethylene has an inhibitory effect on plant growth. Burg and Burg (1968) demonstrated that it reduces the elongation of pea stems. Goeschl et al., (1967), observed that ethylene produces lateral swelling of shoots; it was suggested by Radin and Loomis (1969) that this swelling was associated with the radial enlargement of roots such as radish. A loss of geotropism in roots and shoots has also been suggested to involve ethylene (Chadwick and Burg, 1967; Zobel, 1973).

Principally, auxins and ethylene have opposing effects. The auxins induce stem elongation, whereas ethylene causes inhibition of stem elongation (Burg et al., 1971). Hall (1952), suggested that the process of abscission was brought about by a combined effect of IAA, an abscission retardant, and ethylene, an abscission inducer.

Ethylene production is often stimulated by auxins, and the relationship between ethylene production and the levels of applied or endogenous auxin is usually very

close. Burg and Burg (1968) demonstrated that as the applied IAA concentration increased there was a point reached where the IAA was actually inhibitory to stem growth in pea; this level was the level needed to stimulate ethylene production. Ethylene has also been found to reduce IAA levels in pea seedlings (Burg et al., 1971), this may be due to changes in auxin transport, (Burg and Burg, 1967). It is difficult to test hypotheses concerning the relationship between IAA and ethylene production as several other compounds are known to induce ethylene production, for example, abscorbic acid and cycloheximide.

The roles of ethylene are numerous and varied. Another classic role worthy of note is that of the triggering of the climacteric rise in respiration and also the subsequent change in colour, texture and sweetness of fruit (Burg and Burg, 1965).

The biosynthesis of ethylene is believed to involve the precursor methionine (Liebermann and Mapson, 1964). Further work has provided evidence of the uptake and conversion of ^{14}C-methionine to ethylene in apple tissue (Liebermann et al., 1966).

Ethylene is probably the least studied of the plant growth regulators in plant tissue cultures. This is almost certainly because of the problems involved in sampling and assaying for a gas whilst maintaining

sterility. However, there has been some work in this area with Acer pseudoplatanus cell suspension cultures. Stuart and Street (1969) noted that low inoculum density cultures grew better in the presence of certain conditioning factors originating from high inoculum density cultures. One such conditioning factor was known to be volatile and this led to the hypothesis that a critical low level of ethylene was required for optimal growth. Mackenzie (1970), supplied ethylene to low inoculum density cultures but found there to be no apparent stimulatory effect; higher levels of ethylene were observed to inhibit growth. This inhibitory effect was utilized by Constabel and co-workers (1974), to induce synchrony in soybean cultures.

Conversely, ethylene appears to stimulate growth in Nicotiana tabacum callus, (Huxter et al., 1978). A correlation was noted between growth rate and ethylene production and treatment with ethylene biosynthesis inhibitors caused an inhibition of growth. This growth inhibition was partially counteracted by supplying the cultures with ethylene. These apparently conflicting results again emphasise the problems inherent in the interpretation of plant growth regulator research.

1.2 Criticisms_of_the_Classical_Theories_Relating_to
Endogenous_Plant_Growth_Regulators

The classical Cholodny-Went (Cholodny, 1927; Went, 1928)
approach to growth regulator action relies upon two
basic precepts. Firstly, "without growth substance, no
growth", and secondly, "when there is little auxin,
there is little growth, when more auxin, more growth".
Since this original early theory there have been several
critical appraisals of work based on this principle and
some researchers have even reported inverse
relationships between the level of auxin present and the
amount of measurable growth. Van Overbeek (1932) found
that dark grown Lepidium seedlings elongated
considerably but contained negligible levels of
endogenous diffusible auxins; however, light grown
seedlings were shorter and yet contained much higher
levels of auxins. Later, Laibach and Meyer (1935) and
Fiedler (1936) even claimed to have demonstrated growth
to occur in the absence of detectable auxins. The
advent of such data obviously led researchers to
question the simplistic concept of the original
Cholodny-Went theory. A detailed criticism was later
published by Mer (1969).

Mer had previously performed his own experiments on
etiolated oat plants and concluded that there was no
correlation between extractable auxin and either rate,
or increment of growth, (Dattaray and Mer, 1964). He
postulated that the measurable auxin was either auxin

that was surplus to requirements if growth had ceased, or else that it was to be used for future growth. The earlier work of Went (1928) was then subjected to re-analysis by Mer (1969). Went had concluded that there was a linear relationship between the degree of curvature of coleoptiles and auxin concentration. However, on re-analysis it was revealed that the relationship is actually nearer to a logarithmic form, with the degree of curvature being proportional to the logarithm of the IAA concentration.

Much of the early work with the plant growth regulators involved application of such substances to plants. More recently, plant physiologists have been strongly criticized for this experimental technique because it is now believed that the effect of added plant growth regulators may be quite different to that of the endogenous substance. In fact, Milborrow and Garmston (1973) demonstrated, using radioactively labelled compounds, that ABA synthesized _in situ_ is compartmentalized and that applied ABA did not enter these compartments.

This obviously complicates the issue for plant physiologists attempting to correlate both external and internal levels of plant growth regulators with growth responses. Even the simple technique of measuring auxin content by allowing diffusion into agar blocks is complicated by factors such as the ease of diffusion

from different compartments, the auxin transport system and whether there is continued auxin 'production, (Went and Thimann, 1937). The more modern assay techniques frequently involve extraction by solvents. In these cases also, the growth substance is usually extracted from the plant material regardless of whether or not it is compartmentalized; thus an over-estimate of the amount of the substance actually being utilized by the plant at that time may be obtained. Conclusions drawn from experiments involving such extraction procedures rely upon the assumption that there is only one pool of the growth substance and that the amount in that pool is proportional to the growth response. In many instances this may not be the case. It is now widely believed that the rate of metabolism of the plant growth regulators is more important than the actual pool size in correlations with growth responses, and that there may be a turnover of plant growth substances, (Heslop-Harrison, 1964).

The concept of a rate of turnover can be explained by the model equation:

$$A \xrightarrow{K_1} B \xrightarrow{K_2} X$$
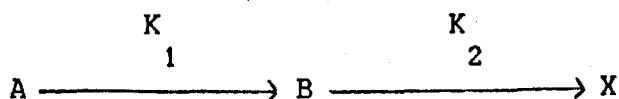
where Reiner (1953) defined the rate of turnover of B as the number of molecules of B newly formed, or the number of molecules of B present which are converted to X per unit time, whichever is the smaller. K_1 and K_2 are rate

constants.   Mer (1969) and Dennis (1977) assumed that
IAA is consumed and metabolized by the plant during the
complex process of growth.   Using the above "turnover"
equation, Mer attempted to justify why some researchers
could not find any apparent correlation between IAA and
growth.   He   considered   four   different   possible
situations.

Firstly, if the rates of synthesis and consumption (K_1
and K_2) are both low, the IAA level will be high or low
depending on whether it was high or low when the rate
constants equilibriated.   Similarly, in the second
possible situation, if the rate constants are both high,
the IAA levels may be high or low.   These two model
situations could explain the different growth rates that
have   been observed with the same apparent auxin
contents, the growth is controlled by the rate of
consumption and not by the actual auxin level.   Thirdly,
the rate of synthesis may be high but the rate of
consumption low, ($K_1 > K_2$). The extractable levels will
be high but the pool is not being utilized, thus low
growth levels may be observed.   Finally, the rate of
synthesis may be low but the rate of consumption high,
($K_1 < K_2$), resulting in a decrease in the pool level; in
this case the rate of growth would be regulated by the
rate of auxin synthesis.   When these four possible
idealised situations are considered one can see how
unrealistic it may be to assume that the extractable
pool size alone regulates growth.

Although this theory does not completely lack logic there appears to be little biological evidence to support it. There is some controversial evidence from Zeevart (1971) working with gibberellins in spinach. He administered a GA synthesis inhibitor to long and short day plants and tentatively concluded that in the long day state the rates of synthesis and consumption of GA increased without altering the pool size. However, the methanol extraction technique employed by Zeevart has been criticized by Browning and Saunders (1977) and Hall (1979). Apparently, under certain conditions part of the gibberellin pool is not extractable with methanol, and a detergent treatment is necessary.

Although Mer (1969) and Dennis (1977) produced an excellent critical appraisal of the work performed in this field it must be remembered that their criticisms and conclusions are weakened because the experimental data on which they are based were obtained from studies of elongation growth, using relatively simplistic bioassays, on a wide range of cells, tissues and organs. Higher plant cell suspension cultures provide a uniform system in which the correlative influences of other organs and cell-types in the plant are absent and the differential patterns of distribution of nutrients and growth regulators between cells are removed, (Elliott et al., 1977). From the criticisms of plant growth regulator research it is apparent that in order to understand the role of a regulator in plant growth it

is essential to investigate the levels of precursors, growth substances and metabolites as well as the rates of turnover, export and import. Plant cell suspension cultures are an ideal system for such studies.

1.3 Cell Suspension Cultures

All living organisms are, or were originally, represented by a single cell which must have possessed all the essentials for that organism to grow and reproduce. This single cell is thus termed "totipotent".

As early as 1838 Schleiden proposed the theory that all living plant cells could live as independent units divorced from the whole plant and were capable of eventually producing a complete new organism. In 1902, Haberlandt became aware of the importance of totipotency and he realized that cell cultures could provide information about the inter-relationships and complementary influences to which cells within the multicellular whole organism are exposed, (Krikorian and Berquam, 1969). Unfortunately, Haberlandt was unable to maintain dividing cultures and thus it was not until the 1930's that permanent plant tissue cultures were established. Gautheret, (1937, 1939), used information gleaned from the studies on growth substances, (Went and Thimann, 1937), and plant nutrition in organ cultures (White, 1937), and then successfully cultured calluses of *Salix capraea* and *Daucus carota* on a

nutrient medium containing mineral salts, glucose, cysteine, thiamine and IAA. These cultures had the potential for indefinite subculture.

It was later discovered that <u>Nicotiana tabacum</u> and <u>Tagetes erecta</u> calluses shaken in liquid media of the correct constitution produced a suspension of small aggregates of plant cells, (Muir, 1953). Muir and co-workers (1954) were able to sub-culture these suspension cultures and found them to be highly uniform and thus ideal for investigating the growth and metabolism of the higher plants, and for determining the specific factors which regulate cell division.

Various cell suspension lines were later initiated, one being <u>Acer pseudoplatanus</u> from cells isolated by Lamport (1964). <u>Acer pseudoplatanus</u> cell suspension cultures are regarded by plant physiologists as a good material for growth and cytological studies as there is a high degree of cell separation at the end of the growth cycle and the cell aggregation is low during periods of active cell division, (King and Street, 1973; Street <u>et</u> <u>al</u>., 1965). <u>Acer pseudoplatanus</u> cells have been used in the present study.

1.4 Pattern_of_Growth_in_Batch_Cell_Suspension_Cultures

A batch culture is a closed culture system where a definite volume of cell culture inoculum is grown in a fixed volume of nutrient medium. The culture is agitated in some way to prevent the cells settling and aggregating and to facilitate gas exchange. This agitation may be achieved by shaking the cultures on orbital platform shakers, (Ragesekhar et al., 1971), by slowly rotating the cultures, (Steward et al., 1952), by spinning (Lamport, 1964; Short et al., 1969) or by magnetic stirrers and/or bubbling sterile air through a sparger (Melchers and Engelmann, 1955; Wilson et al., 1971).

In a batch culture the yield of cell material increases for a period and then reaches a maximum. This may be due to a limiting factor such as nutrients or oxygen level, or possibly due to a build-up of a toxic substance. At this stage of growth a sterile sample of the culture must be taken to inoculate into fresh medium, otherwise the culture will eventually die.

The growth patterns in batch cultures are similar when the ratio of inoculant to media is standard and the period between inoculations is standard. Batch cultured cells produce a sigmoidal cell number curve which consists of five distinct growth phases, (Wilson et al., 1971):

(i)   Lag phase (cell numbers are constant).

(ii)   Exponential   phase   (specific   growth   rate
       increases).

(iii) Linear phase (specific growth rate is constant).

(iv)   Growth   decelaration   (specific   growth   rate
       declines).

(v)   Stationary phase (cell numbers are constant).

(Figure 1.1).

During the lag phase the cells absorb water and
nutrients and prepare for division. The volume of
cytoplasm increases as do the numbers of mitochondria,
ribosomes, endoplasmic reticulum and dictyosomes per
cell. In Acer pseudoplatanus cultures there is an
increase in DNA and RNA content per cell (Short et al.,
1969). The DNA level doubles before the onset of the
phase of rapid cytokinesis and then returns to its
original level at the initiation of cytokinesis. This
suggests a high degree of synchrony in the first
division. The RNA increase may be associated with the
synthesis of new enzymic and structural proteins
necessary for cell division.

During the exponential phase the cytoplasm appears dense
with a high density of endoplasmic reticulum and
ribosomes. There are changes in respiratory activity
(Givan and Collin, 1967) and many cells are in mitosis
and cytokinesis. The cells tend to aggregate and
maintain protoplasmic continuity via plasmadesmata
(Yeoman and Street, 1973). There is a decline in RNA
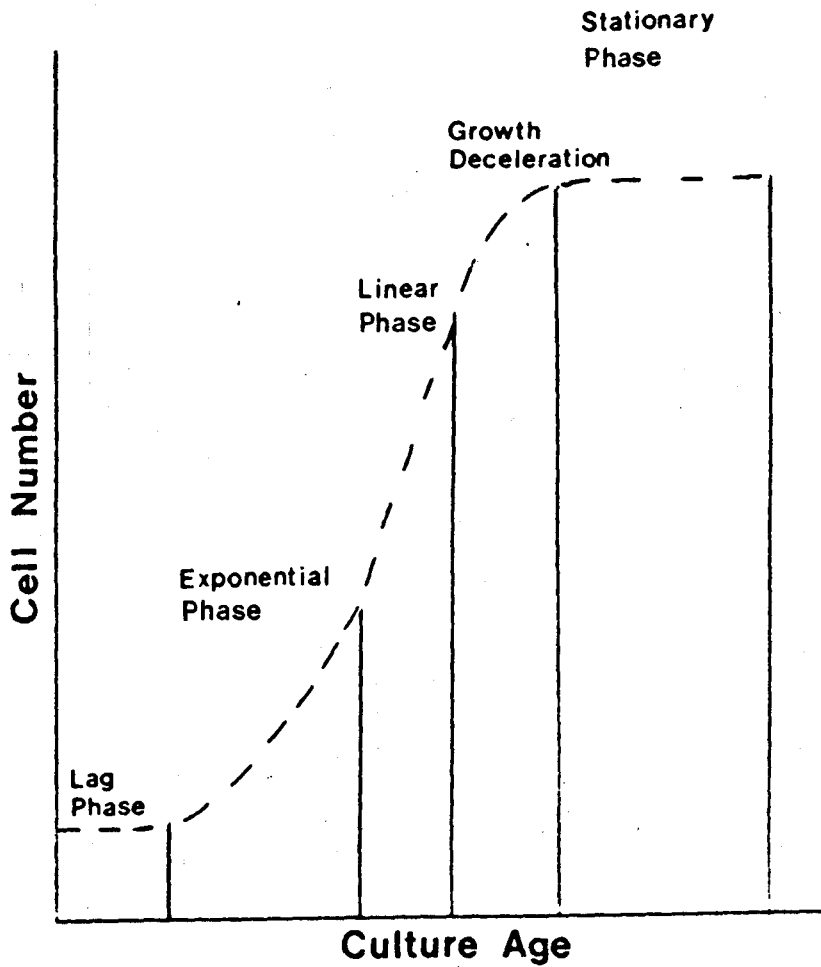
Figure 1.1

Pattern of growth in batch cell suspension cultures.

levels probably due to some catabolism and also a decrease per cell due to cytokinesis, (Short et al., 1969).

The cell aggregates begin to break up as cell division decelerates and the culture begins to enter the stationary phase. During this stage the cells increase in size and become highly vacuolated with a thin layer of cytoplasm which contains only a few organelles. Starch is deposited in the cells and then they eventually senesce and die.

Acer pseudoplatanus cells in batch culture show an initial lag phase which gives rise to a limited phase during which increase in cell number is maintained at a high rate. Following this period the cells divide at a slower rate and increases in cell dry mass and packed cell volume (PCV), merely reflect cell expansion (Henshaw et al., 1966). Analysis of DNA profiles through the growth cycle have shown Acer pseudoplatanus cells accumulate in G1 during stationary phase (Bayliss and Gould, 1974). The other periods of the cell cycle, S, G2 and M are of relatively constant duration, whereas the G1 period is highly variable (Gould et al., 1974).

The growth of cells in culture is greatly affected by the supply of nutrients, aeration and pH. The cells utilize the carbohydrate supplied; Simpkins et al., (1970) suggested that the yield of cells may be limited

by carbohydrate supply. Watson and Fowler (1980) concluded that sucrose was the 'limiting factor. However, nitrate supply can also be a limiting factor. Lewis (1983) and Simpkins et al., (1970) found low nitrate levels produced little cell division in Acer pseudoplatanus cell cultures and similar results were found with low phosphate levels (Wilson, 1976).

The growth pattern of cells in batch culture is very dependent on the initial inoculum density. A low inoculum density may result in a prolonged lag phase or possibly no growth at all. Growth may become unbalanced in cultures resulting in the loss of the coupling between biosynthesis and the cell cycle (Henshaw et al., 1966). If balanced growth is to be achieved, the cells must be transferred into fresh medium regularly and in a standard ratio. The composition of the culture medium is also an important factor for balanced growth with a reproducible pattern. Cell division in Acer pseudoplatanus cell suspensions has been induced by IAA (Digby and Wareing, 1966) and 2,4-D (Street et al., 1968).

Unfortunately, cell suspension cultures tend not to be homogenous and as with any closed system the nutrients inevitably become depleted. As the culture ages the cells begin to aggregate and within these aggregates gradients of nutrients develop (King et al., 1973). Cell aggregation can be prevented by the addition of low

concentrations of cell wall degrading enzymes (Street, 1973a). However, this adds another variable to the system, thus making postulated relationships between plant substances and growth less meaningful. The same separation effect can be induced by the addition of auxins, 2,4-D and kinetin (Torrey et al., 1962; Simpkins et al., 1970).

Batch cell cultures are obviously not perfect systems for studying plant cells, however, they do have the advantages of rapid growth, sterility and relatively easy control of environmental factors. The batch culture can be strongly criticized in that any biosynthetic products of the cells cannot be transported away as they may be in the whole plant. Thus, the cells are probably bathed in abnormally high levels of plant growth substances and metabolic by-products, some of which may be inhibitory or even toxic to the cells at such concentrations. In building up as a pool in the medium these products may produce effects on the cells which would not normally occur in the whole plant, and the cells may eventually adapt to these conditions and become significantly different from the cells of the whole plant. Because of these problems only tentative conclusions can be drawn from any correlations between endogenous and exogenous levels of plant growth substances and physiological changes in cells in batch suspension cultures.

These problems can to some extent be overcome in a "continuous" culture system. In this case a constant flow of sterile nutrient medium is pumped into the cell culture and an equal volume of cell culture, (used medium plus cells), is removed from the culture at the same rate. The growth rate of such a culture adjusts so that new cells are produced frequently enough to replace those being removed. A steady state culture is therefore produced with constant cell density, growth rate and metabolic rate. There are two types of continuous culture - the turbidostat and the chemostat. The turbidostat maintains a constant density of cells by monitoring the turbidity of the cell culture, which is closely related to cell density, and adjusting the dilution rate accordingly (Bryson, 1952). The chemostat maintains a constant growth rate and cell density by a fixed rate of input of a growth-limiting nutrient, for example, nitrate. Research has progressed well in the continuous culture of Acer pseudoplatanus cells and steady states of cell number, dry mass and protein have been achieved, (Wilson, King and Street, 1971). With such a steady state system correlative plant growth substance research data are more interpretable. Lewis (1983), has shown there to be a correlation between endogenous IAA levels and the specific growth rate of Acer pseudoplatanus cells in continuous culture.

Another inherent problem in dealing with cells in batch suspension cultures is that although the first division may be quite synchronous, (that is, the majority of the cells divide within a matter of hours), the degree of synchrony decreases dramatically as the cells become older. Thus, the cells are at different stages in their cell cycles, (Yeoman, 1974), and therefore any apparent correlations between cytological changes and levels of plant growth regulators become meaningless. The ideal system would be a culture that exhibited a high degree of synchrony with each successive division. Thus, any apparent trends, for example, the production of a particular growth substance at a specific point in each cell cycle, would be detectable.

Naturally occurring cell division synchrony is not common amongst the higher plants, this may explain why little is known about the higher plant cell cycle. Most of the synchronously dividing populations that research has concentrated on have been induced by some form of manipulation. However, there are some cases where prolonged synchronous cell division in plant cell suspension cultures has been achieved in a defined medium (Street et al., 1971; Wilson et al., 1971). There are two synchronization techniques routinely employed, these are selection synchrony and induction synchrony (Mitchison, 1971).

Selection synchrony involves the separation of cells at a specific stage in the cell cycle from the whole population. For example, certain micro-organisms, (Schizosaccharomyces pombe), possess a linear relationship between their size and the stage of the cell in the cycle, so it is possible to select cells at a similar developmental stage (Mitchison and Vincent, 1965). However, selection synchrony is often difficult to achieve under sterile conditions and the population obtained is usually small. Selection methods have not been used with higher plant cells.

Induction synchrony is more commonly used to produce synchronous cultures. This technique involves blocking the cell cycle at a specific point, either by the addition of a chemical or by an alteration of the physical environment (Prescott, 1976). The cells tend to accumulate at a similar stage in the cell cycle, and when the blockage is stopped the release from the affect results in a limited number of synchronous divisions. Various plant cell suspension cultures have been induced to divide synchronously by a number of induction techniques. Synchrony inducers used to date include inhibitors of DNA synthesis, inhibitors of mitosis and starvation/regrowth procedures. Induced synchrony has been achieved by cold treatments, (Okamura et al., 1973; Robinson, 1982), warm treatments, (Fujitsu et al., 1972), anoxia and aeration cycles (Constabel et al., 1974), regimes of pre-starvation and a light/dark pre-

treatment (Nishinari and Yamaki, 1976), periods of specific starvation followed by the reintroduction of auxin (Yeoman and Aitchison, 1976; Nishi et al., 1977), cytokinin (Jouanneau, 1971; Jouanneau and Tandeau de Marsac, 1973), and phosphate (Komamine et al., 1978). A starvation/regrowth treatment for Acer pseudoplatanus cell suspension cultures was achieved in 1973, (King et al., 1973; King and Street, 1973). This regime produced five consecutive cell divisions and nitrate was noted as being the limiting nutrient, (King et al., 1974; Gould and Street, 1975).

Synchronously dividing cell suspension cultures are a very suitable system for studying cytokinesis in higher plant cells. However, some of the limitations of the batch culture are inevitably still present, for example, the unnatural build up of substances in the bathing media. Nevertheless, because the randomness of cell division is reduced, synchronized cultures may facilitate the detection of biosynthetic correlative trends. The main criticism of induced synchrony is that the inducer may produce an unnatural "shock" response and therefore possibly abnormal biosynthetic responses. Although this is a valid comment it must be remembered that the whole cell suspension culture system is unnatural anyway, and thus only tentative conclusions may be drawn. Despite such limitations it is likely that this technique will be of great importance in the study of plant growth substances in the future.

1.5 The Cell Cycle and its Regulation

The cell cycle is defined in terms of the time period of chromosome replication and segregation (Figure 1.2). It is the interval between the completion of mitosis in a cell and the completion of subsequent mitosis in one or both daughter cells. The cell cycle is classically divided into G1, S, G2 and M. These stages in the cycle are defined by the behaviour of the chromosomes, (Howard and Pelc, 1953) as follows:

G1 = Interval between completion of mitosis and the onset of DNA synthesis.

S = Period of DNA replication.

G2 = Interval between completion of DNA synthesis and mitosis.

M = Mitosis.

The biochemical activity resulting in the initiation of DNA synthesis is not fully understood. It is generally assumed that the later part of this G1 period involves some preparation for DNA synthesis. The theory that the G1 period is for the synthesis of enzymes concerned with DNA synthesis is not valid, as these enzymes have been found to be present throughout the whole G1 period. However, both RNA and protein synthesis are essential for the cell to progress through the G1-stage, (Baserga et al., 1965). The synthesis of a new protein is required shortly before DNA synthesis initiates; this suggests that the G1 --> S transition is induced by the production of a protein initiator just before the transition.
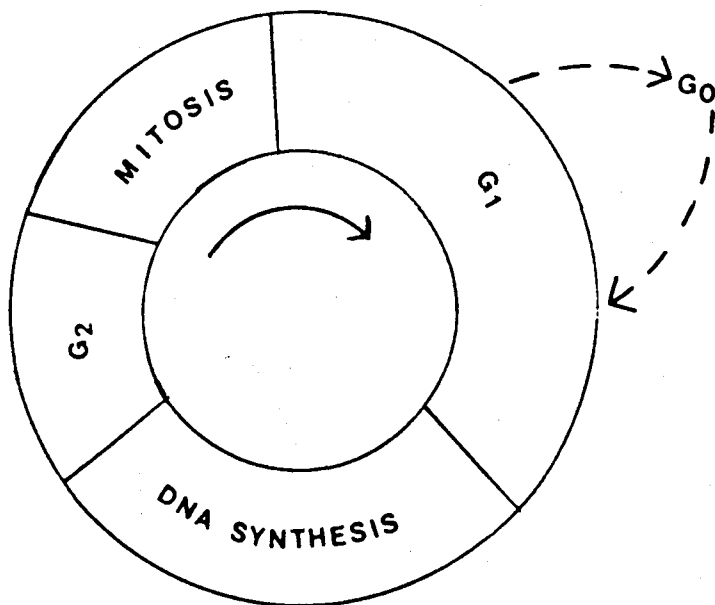
Figure 1.2

The stages of the cell cycle (Prescott, 1968)

The G1-period is notably different from the other stages in the cycle in that its time period is somewhat variable. If cells grown in vitro are exposed to changes in their culture conditions they often have a longer generation time. This is principally due to a lengthening of the G1 period, (Smith and Martin, 1973; Gould et al., 1974). Indeed, some cells and bacteria do not possess a measurable G1 period, but it can be induced by changing the culture conditions (Lark, 1966). This period appears to be the one that cells remain in if their normal pattern has been interrupted. Sometimes this arrested state is termed GO; however, even when no G1-period is detected, the events that induce DNA synthesis still occur just before the cell enters the S phase (Prescott, 1968).

The transition from G1 --> S requires protein synthesis which is also necessary for the maintenance of the S phase; this protein production is controlled at the transcription level as RNA is required for the synthesis. The initiator protein is assumed to be labile as it is necessary for it to be synthesized with each cycle (Prescott, 1968). It is suggested that there is an initiator of DNA synthesis in the cytoplasm of cells in the S period. If an S period cell nucleus is transferred into a G2 cell there is a decline of DNA synthesis, possibly due to the absence in G2 cells of the appropriate initiators (Prescott and Goldstein, 1967).

When S period and G1 period cells are in close proximity the G1 cells are accelerated into the S period, (Dewey et al., 1973); this means there is a high degree of synchrony of replication. Once synthesis has initiated the chromosomes replicate and terminate their replication according to their own programmes. The initiator for replication may be unspecific, pervading the whole cell. The cells in the S period may condition the surrounding medium thus inducing G1 period cells, especially those close by, to enter into S period.

There is also a decrease in alkaline phosphatase during the S period; this inhibits the incorporation of thymidine into DNA and produces changes in cellular membranes. This may be significant in the regulation of cytokinesis, (Baserga, 1968).

The G2 period links the end of chromosome replication with chromosome segregation. In multicellular organisms this period is assumed to be necessary for the condensation of chromosomes and the assembly of the mitotic apparatus. Protein synthesis is essential for successful mitosis, at least into the late G2 period, (Cummins et al., 1966). This is further evidence for the hypothesis that the progression through the cycle depends upon new transcription and synthesis of new protein.

A number of models have been proposed to explain the regulation of the basic cell cycle from the initiation of the G1 period through to the completion of mitosis. The "two principal control points" theory was developed by Van't Hof and Kovacs (1972), in an attempt to explain the results obtained from sucrose starvation experiments on cultured Pisum roots. The carbohydrate starvation of these meristematic cells resulted in the cells arresting in G1 or G2 in a form of stationary phase, (Van't Hof, 1966). The arrested cells were assumed to be metabolically blocked; those in G1 were unable to initiate DNA synthesis and therefore unable to proceed to the S stage of the cell cycle, and those in G2 were unable to enter mitosis. This blockage was overcome if the starvation was ceased. Van't Hof and Kovacs (1972), defined these two points where the cells are arrested in the cell cycle as the "two principal control points", although they admitted that they had not been found to be universal.

Quastler and Sherman (1959), believed that cells made a "decision" shortly after mitosis as to whether they were to divide or differentiate. This period of the cell cycle was later defined as the G0 period; a period of indefinite length for cells not committed to nuclear division. It was proposed that cells left the G0 period randomly and with constant probability per unit time, (Burns and Tannock, 1970). Smith and Martin (1973), furthered this model by defining the S, G2, M and part

of the G1 period as the "B-phase". They suggested that after mitosis the cells enter an "A-phase" in which they do not progress towards cytokinesis. Cells may remain in this "A-phase" for any length of time but always have a constant probability of entering the "B phase". However, it was proposed that all the cells present pass though the A-phase, unlike the earlier mentioned G0-phase, which it was assumed only a proportion of the cells entered. Gould (1977), attempted to explain how an invariant, time independent, transition probability mechanism might be achieved. He suggested that the random "triggering" of cells after division could be effected by the random collision of rare, slowly diffusing molecules.

The classical cell cycle model assumes a stepwise progression through the cycle, each step being dependent on the preceding event. However, cell cycle regulation may be achieved by an independent sequence of events, (Péaud-Lenoel, 1977). Various apparently unconnected pathways may be triggered by an event in the cycle; these pathways may independently proceed on their course and trigger an event before mitosis, which is controlled by the terminal events of the defined number of pathways. Thus, elucidating the numerous factors involved in the regulation of the cell cycle is very complicated.

There has been much research into the estimation of the duration of the periods of the cell cycle (Rembur, 1974). There is some variability amongst the higher plants, but this is frequently within the notoriously variable G1 period. Firby (1985), determined the duration of the phases for the strain of Acer pseudoplatanus used in this present study. The G0 and G1 period was found to be of approximately 45 hours duration, by 58 hours the S period is complete and the cells enter G2. The G2 period lasts approximately 7 hours and mitosis and division eight to ten hours. Thus by 73-75 hours after inoculation mitosis is completed. It was determined that the S, G2, M and D phases constitute a non-expandable time period of approximately 28 hours, with variability only in the G1 phase or the proposed G0 phase.

1.6 Scope of the Present Study

It will be apparent that the role of the plant growth substances in the regulation of the higher plant cell cycle is complex. A major problem encountered by plant physiologists researching in this area is one of finding a suitable plant system with which to work. Cell suspension cultures are relatively simple, virtually homogenous systems, and are divorced from the correlative influences present in the whole plant. They enable accurate growth parameter analysis in the form of cell number, size and biomass. In this present study cell suspension cultures of Acer pseudoplatanus have

been utilized to extend numerous previous studies in these laboratories on the regulation of higher plant cell growth and division.

IAA and the cytokinins have been strongly implicated in the regulation of the cell cycle and cytokinesis. In this study the endogenous cytokinin levels were investigated during the first synchronous division and the subsequent asynchronous growth of a batch culture. The endogenous IAA levels have been determined in previous studies which have revealed substantial fluctuations in levels. Therefore the endogenous IAA levels were redetermined for the first synchronous division under a more frequent sampling regime. Trigonelline was also investigated as a putative G2 arrestor (Tramontano et al., 1982).

Although the first division after transfer of an inoculum into fresh medium is highly synchronous (approximately 70%), in Acer pseudoplatanus cell suspension cultures one must be careful in correlating the endogenous plant growth substance levels with metabolic and physiological processes. This is because any fluctuations in such levels may be attributed to the "shock effect" of the actual transfer of the cells. To some extent this criticism can be overcome by the use of induced synchronous cultures, although it must be remembered that the method of induction may be responsible for any observed trends or fluctuations in

endogenous levels. However, most methods of inducing synchrony do not involve so many traumatic variables as a complete transfer to fresh medium, and therefore such systems have been generally accepted, although any conclusions drawn from them must be tentative. In this study a nitrate/phosphate-feeding induced synchrony regime has been developed in conjunction with a previous worker in these laboratories, (Firby et al., in preparation). This system has been employed to investigate the levels of endogenous IAA during an induced highly synchronous second division. The major problem encountered with this synchrony inducing regime was the crucial timing of the nitrate/phosphate feed if synchrony was to be maintained. Thus, a computer-interfaced automation system has been developed.

The pH of the bathing media has been closely monitored in both asynchronous and synchronous cultures and the vacuolar and cytoplasmic pH levels have also been investigated using ^{31}P Nuclear Magnetic Resonance. The distribution of the observed endogenous levels of IAA is discussed in relation to the vacuolar and cytoplasmic pH levels which may affect IAA compartmentation within the cell.

2.0 GENERAL MATERIALS AND METHODS

The materials and methods used routinely in this study are detailed in this section.

2.1 Plant Material

Cell suspension cultures of Acer pseudoplatanus L were used. The plant material was originally isolated from cambial tissue by Lamport (1964); stock cultures were donated to the department by the late Professor H. E. Street.

2.2 Cell Culture

The Acer pseudoplatanus cells were maintained in batch propagated serial suspension culture in Stuart and Street's (1969) medium, which is based upon Heller's (1953) inorganic salts, lacking both kinetin and urea. Medium was prepared from stock solutions (Table 2.1), which were stored at 4 ^{o}C. All stocks were regularly renewed to avoid contamination by micro-organisms. The final pH of the medium was adjusted to 6.4 using 1 mol dm^{-3} KOH as advised by Stuart and Street, (1969). All medium was autoclaved for 20 minutes at 121 ^{o}C (100 KPa), except for the larger volumes of medium used in the 21 litre batch cultures, which were autoclaved for 45 minutes.

Table 2.1

Acer pseudoplatanus medium constituents.

Modified from Stuart and Street, 1969.

	Final Concentration in Culture Medium $(mg\ dm^{-3})$	Stock Concentration $(g\ dm^{-3})$	
KCl	750	37.5	STOCK I
$MgSO_4.7H_2O$	250	12.5	
$NaNO_3$	600	30.0	
$NaH_2PO_4.2H_2O$	133	6.5	
$CaCl_2.2H_2O$	74	3.7	STOCK II
$ZnSO_4.7H_2O$	1.0	1.0	STOCK III
H_3BO_4	1.0	1.0	
$MnSO_4.4H_2O$	0.1	0.1	
$CuSO_4.5H_2O$	0.03	0.03	
KI	0.01	0.01	
$NaFe(EDTA)_2$	1.5	1.5	STOCK IV
Thiamine hydrochloride	1.0	1.0	STOCK V
Calcium pantothenate	2.5	2.5	
Choline chloride	0.5	0.5	
2,4-D	1.0	0.1	STOCK VI
i-inositol	100		Added Dry
Cysteine hydrochloride	10		
Sucrose	20,000		

2.3 Culture Technique

The basic procedures for maintaining sterile cultures have been described elsewhere, (Henshaw et al., 1966; Street, 1973b). Stock cultures ($70cm^3$) were grown in $250cm^3$ Erlenmeyer flasks sealed with a double layer of autoclaved aluminium foil. Autoclaved automatic pipetting units (A. R. Horwell) with wide bore canulae (3mm) were used to transfer fixed volumes of cell culture (usually $10cm^3$), to autoclaved fresh medium (usually $60cm^3$) at 21 day intervals. Where other volumes were used the same ratio of inoculating culture to fresh medium was used. In these cases the transfer was achieved either by decanting over a flame or by joining the old and new culture vessels with silicone rubber tubing and Quickfit joints under absolute alcohol. The cell culture was then allowed to flow into the fresh medium under gravity.

The stock cultures were incubated in the light ($500cd\ sr\ m^{-2}$) at $25^{o}C$ on flat bed reciprocating shakers (L. H. Engineering Co. Ltd.) at 120 cycles per minute as advised by Rajasekhar et al (1971).

All procedures involving aseptic transfer were performed in a sterile room fitted with a twin Laminair flow unit (Pathfinder).

2.4 Sterility

All cultures were checked for sterility by plating out samples onto nutrient agar ($15g$ dm^{-3}). These plates were incubated at 37° C for at least 14 days to ensure there was no contamination.

2.5 Glassware

All glassware used was Pyrex and was routinely cleaned by soaking overnight in Decon 90 (BDH) ($50cm^3$ dm^{-3}) followed by vigorous brushing and several rinses with tap water and finally double-distilled water.

2.6 Chemicals

All chemicals used were analytical grade reagents from BDH (Poole, Dorset), Sigma (Poole, Dorset) and Fisons (Loughborough, Leicestershire).

Organic solvents were re-distilled in all glass apparatus, and stored in dark brown Winchester bottles. Diethyl ether was deperoxidized by redistilling in the presence of iron filings (Vogel, 1962). Butanol was water saturated by shaking with an equal volume of double distilled water and was left overnight to separate out from the remaining aqueous layer.

2.7 Growth Parameters

These methods have been described by Henshaw et al (1966). For cell counts, culture samples of known volume were freed of medium by centrifugation in a Gallenkamp bench centrifuge for 5 minutes at 2,000 g. Then the cells were washed with distilled water before being resuspended in a 1 mol dm^{-3} chromium trioxide solution. The samples were then stored at $4\ ^{o}C$ for no longer than 14 days or counted immediately. To facilitate counting by dispersal of the cell aggregates the samples were incubated in a water bath at $70\ ^{o}C$ for 30 minutes and then agitated using a Whirlimixer for 5 minutes. Samples were diluted with distilled water to a known volume and a few drops of the homogenous sample were placed on a recessed microscope counting slide (1mm deep). Cell numbers were determined by counting 10 fields 5 times under a Vickers microscope with a magnification of X 100. Cell numbers per cm^3 were calculated from the equation:

$$N = \frac{\bar{x}d}{k}$$

Where   N = cell number x 10^{-3} per cm^3.

      $\bar{x}$ = average number of cells per field.

      d = dilution factor.

      k = constant for the microscope and slide used, accounting for the volume of the field.

Packed cell volume (PCV) was determined after centrifugation of a known volume of cell culture at 2,000g for 5 minutes in a graduated centrifuge tube in a Gallenkamp bench centrifuge. The PCV was expressed as the volume of cell pellet per unit volume of culture.

Dry mass was calculated by using a multifiltration unit (Strand Scientific) to filter a known volume of culture under reduced pressure through a pre-weighed glass fibre pad. The cells were washed with double distilled water and then the cells and pad were dried to a constant mass in an oven at $80^{\circ}C$.

2.8 Cytological_procedure_for_cultured_plant_cells
(Bayliss and Gould, 1974)

Samples of cells were washed with 3 x $5cm^3$ double distilled water and fixed with $5cm^3$ of formic acid solution ($500 \, cm^3 \, dm^{-3}$). The cells could be stored in formic acid at $4^{\circ}C$ for 4 to 5 weeks but the nuclei and chromosomes were usually fixed after 24 hours.

The fixative was removed by washing the cells with 3 x $5cm^3$ double distilled water. The cells were resuspended in $5cm^3$ $1 \, mol \, dm^{-3}$ hydrochloric acid preheated to $60^{\circ}C$ and hydrolysed for 12 minutes in a water bath at $60^{\circ}C$. The acid was removed by centrifuging the cell samples in a Gallenkamp bench centrifuge for 5 minutes at 2,000g. The supernatant was discarded and the cells were immediately resuspended in basic fuchsin. The cells were

left for 2 hours for the staining process to be complete. The stain was removed and the cells were washed in $3 \times 5cm^3$ five minute changes of sulphur dioxide water. The water was freshly prepared by mixing $5cm^3$ of $1 \ mol \ dm^{-3}$ hydrochloric acid and $5cm^3$ of $0.45mol \ dm^{-3}$ $K_2S_2O_5$ with $100cm^3$ of double distilled water. The cells were then transferred to aqueous acetic acid $(250cm^3 \ dm^{-3})$.

Squashes of the cell samples were then prepared. The squash was made using the minimum volume of liquid. The cover-slips used were coated with silicone "repelcote", after the squash had been made the cover-slip was carefully removed with a razor blade. The slide was then immersed in a series of solvent concentrations, being left overnight in each of:

       (i)   Absolute alcohol
      (ii)   1:1 (Alcohol:Inhibisol)
    (iii)   1:3 (Alcohol:Inhibisol)
     (iv)   Pure inhibisol

A permanent slide was then prepared using DPX, a neutral mounting medium (BDH).

Microdensitometry was performed on a Vickers' M85 scanning Microdensitometer set to a wavelength of 565nm.

2.9 Cell Viability (Widholm, 1972)

$5cm^3$ cell culture samples were taken, $1cm^3$ of a phenosafranin solution ($1g\ dm^{-3}$) was added to each and the mixtures were agitated for 5 minutes using a Whirlimixer. After standing at room temperature for 5 minutes the samples were centrifuged for 5 minutes at 2,000g in a Gallenkamp bench centrifuge. The supernatant was discarded and the cells were then washed with $5\ x\ 5cm^3$ double distilled water. The cells were then diluted by a known volume of double distilled water and a sample was placed on a microscope slide. The dead cells are stained red by phenosafranin. The total cell number and the number of stained cells were recorded for five fields and the percentage of non-viable cells was estimated.

2.10 Quantitative Analysis of Cytokinins

The extraction technique employed was a modified method (Hosford, 1984) based on the work of Horgan and Kramers (1979). HPLC was used to separate individual cytokinins and the cucumber cotyledon chlorophyll retention bioassay (Fletcher and McCullagh, 1971; Fletcher et al, 1982) was used to estimate total and individual cytokinin activity.

2.10.1 Total Cytokinin Extraction

Samples of cell culture of 400-1000cm^3 were taken for cytokinin analysis. The cells were freed from medium by filtration under reduced pressure using a Buchner funnel and Whatman (No 1)filter paper. The sample was washed with 500cm^3 of ice-cold double distilled water. The fresh mass of the cells was recorded and the sample was frozen at -20°C. When frozen the cells were freeze-dried, re-weighed and stored at -20°C prior to analysis.

To extract the cytokinins the cells were homogenized in a Wareing blender in 200cm^3 of a methanol solution (800cm^{3}dm^{-3}). The sample was stirred in the methanol overnight at 4°C in darkness. The cells were then filtered under reduced pressure using a Buchner funnel and Whatman (No 1) filter paper, and then resuspended in 200cm^3 of pure methanol. The sample was again stirred overnight and then refiltered. The methanolic extracts were combined and concentrated in a round-bottomed Quickfit flask under reduced pressure at 30°C using a Buchi rotary evaporator. The extract was concentrated to approximately 1cm^3.

The extract was taken up in 50cm^3 of acetic acid buffer (pH3) and placed in a screw-topped centrifuge tube. The extract was frozen at -30°C overnight. The sample was thawed in a waterbath at 30°C and was then spun in an

ultracentrifuge at 20,000g for 30 minutes. The supernatant was decanted off and the sediment (containing lipid material) was discarded.

The pH of the extract was adjusted to 8.3 (with glacial acetic acid and 1 mol dm^{-3} KOH) and was partitioned five times against equal volumes of water-saturated butan-1-ol. The aqueous layer was discarded and the combined butanol phases were concentrated to approximately 1cm^3 in a round bottomed Quickfit flask under reduced pressure at 30°C using a rotary evaporator.

The sample was taken up in 5cm^3 of pH3 water (formic acid) and applied to a Sephadex SP-C-25 (H$^+$) ion-exchange column (40cm^3 bed volume) previously equilibriated as follows: Sephadex SP-C-25 (H$^+$) (Pharmacia) (10g) was allowed to swell for 2 days in 2 x 500cm^3 0.5mol dm^{-3} (NH$_3$)$_4$SO$_4$. After filtration it was resuspended in 500cm^3 formic acid solution (70cm^3 dm^{-3}), filtered and resuspended in 500cm^3 of a fresh formic acid solution (70cm^3 dm^{-3}) three times over a period of 8 hours. Finally the Sephadex was filtered, resuspended in 500cm^3 formic acid (10cm^3 dm^{-3}) and stored at 4°C.

The Sephadex was carefully poured into a Quickfit chromatography column until approximately 40cm^3 of column had settled out. The column was eluted with four column volumes of a formic acid solution (70cm^3 dm^{-3})

followed by four column volumes of water adjusted to pH3 with formic acid until the effluent was found to be pH3 (Redgwell, 1980). The sample (pH3) was introduced to the top of the column and the column was eluted with four column volumes of pH3 water. 0.5 mol dm^{-3} NH_4OH was applied to the column, producing a brown band. The sample was collected just before the band was eluted and collection finished after a column volume behind the band had been eluted. This technique purifies the sample by ion-exchange chromatography.

The sample was stored at $4^{\circ}C$ and was then concentrated to approximately 20cm^3 under reduced pressure at $30^{\circ}C$. The pH was adjusted to 5.8 with 0.1 mol dm^{-3} acetic acid and the sample was then passed through a Sep-Pak C18 cartridge (Waters). The Sep-Pak was pre-treated by washing twice with 5cm^3 of methanol followed by 5 x 5cm^3 double distilled water (pH 5.8) using a 5cm^3 gas-tight glass syringe (Hamilton). After introduction to the Sep-Pak the sample was allowed to drip through slowly. The Sep-Pak was again washed three times with 3 x 5cm^3 pH 5.8 distilled water and then with 5cm^3 of a methanol solution ($100\text{cm}^3 \text{dm}^{-3}$). The cytokinin-like compounds were eluted with 5cm^3 of a methanol solution ($500\text{cm}^3 \text{dm}^{-3}$). The eluate was reduced to dryness under reduced pressure at $30^{\circ}C$, resuspended in methanol (2cm^3)

and stored at -20°C in a small stoppered vial prior to further analysis. This technique purifies the sample by a reverse-phase chromatography system.

Total cytokinin activity was estimated by the cucumber cotyledon bioassay (Section 2.10.3).

Individual cytokinins were separated by subjecting the sample to analytical HPLC. The activity of the fractions collected from the HPLC were also quantified using the cucumber cotyledon bioassay.

2.10.2 Separation_of_Individual_Cytokinin_by_HPLC

HPLC is being used increasingly for the isolation of plant growth substances. The method used was based on that reported by Horgan and Kramers (1979).

The sample (in $50mm^3$ of the starting eluant) was injected via a Rheodyne valve and $200mm^3$ injection loop onto an analytical column (5μm Hypersil ODS, 250 x 5mm id, HPLC Technology), fitted with a 55 x 5mm Lichroprep RP-18 pre-column (Merck). The HPLC (300/02 pumps model, Applied Chromatography Systems) was controlled by an Apple II computer linked to the HPLC via a Chromatography Interface Unit (Drew Scientific). The gradient elution programme was stored on floppy disc, loaded automatically via a disc-drive unit and monitored on a visual display unit (Kaga). Samples were continually monitored at a wavelength of 280nm by a UV

absorbance monitor (ACS model 750-11). The UV trace was recorded by an AR 55 Linear Recorder (Pye Unicam). The mobile phase consisted of acetonitrile (CH_3CN) and water (pH7 with TEAB). Triethylammonium bicarbonate (TEAB) was prepared by saturating a 2.5 mol dm^{-3} solution of triethylamine with carbon dioxide. Both solvents were filtered (Millipore 2 m pore size) and degassed before use. Individual cytokinin species were eluted using a linear gradient of $50cm^3$ acetonitrile dm^{-3} water (pH 7.0) to $200cm^3$ acetonitrile dm^{-3} water, (ie 5-20% acetonitrile) over 30 minutes at a flow rate of $2cm^3$ min^{-1}. Fractions were collected every minute by an LKB Ultrorac 700 fraction collector and submitted for bioassay. The HPLC columns were cleaned by washing with methanol before and after use and were equilibriated to the starting concentration of the eluting solvents for 15 minutes after washing and between sample runs. The cytokinin retention times were checked frequently with the aid of standards.

2.10.3 Cucumber_Cotyledon_Bioassay_for_Cytokinins

The cytokinin-like activity present was estimated by the cucumber cotyledon bioassay which depends upon the ability of cytokinins to regulate and stimulate the production of the chlorophyll in cut cucumber cotyledons. (Fletcher and McCullagh, 1971; Fletcher et al, 1973). The increase in chlorophyll production is proportional to the concentration of

cytokinin and thus this system can be used as a sensitive and rapid bioassay for cytokinins (Fletcher and McCullagh, 1971; Fletcher et al., 1982).

Cucumber (Cucumis sativus, L. var. Butchers Disease-Resisting; Clause UK) seeds were planted in washed moist vermiculite in plastic trays and germinated in the dark at 25 $^{\circ}$C for four days or until the hypocotyl was approximately 5cm long.

Two layers of Whatman (No 1) filter paper were placed in 45 x15mm plastic petri dishes. The samples were placed on the filter paper and then allowed to dry. A range of standards were prepared from 0 - 2000ng Zeatin, the standards were applied to the filter paper in methanol, which was allowed to evaporate. A 5mmol dm^{-3} KH$_2$PO$_4$ /40mmol dm^{-3} KCl solution was prepared and 2cm^3 added to each petri dish. At least two petri dishes were prepared for each standard.

In dim green light the cotyledons were removed, separated and placed adaxial surface down on the filter papers. Ten cotyledons were placed in each petri dish and care was taken to ensure random distribution between the petri dishes as some cotyledons are slightly larger than others.

The cotyledons were incubated in the dark for 12 hours at 25 $^{\circ}$C and then illuminated by fluorescent light (500 cd sr m^{-2}) for 3 hours. The chlorophyll was extracted from the cotyledons by removing them from the petri dishes, drying them on paper tissues and then placing them in 10cm^3 of dimethyl sulphoxide in test-tubes suspended in a water-bath at 70 $^{\circ}$C for 3 hours. The tubes were then vigorously shaken, the cotyledons were removed and the absorbance of the solution was recorded in a UV spectrophotometer (Pye Unicam SP1800) at 663 and 645 nm. The differences between the absorbances at these two wavelengths were then calculated and used to obtain a standard curve. The amount of Zeatin equivalent/sample was estimated from the standard curve.

2.11 Quantitative Analysis of Indole-3-Acetic Acid and Abscisic Acid

The extraction and estimation of indole-3-acetic acid, (IAA), was basically as reported by Blakesley et al., (1983); the technique was developed in these laboratories. The standard spectrophotofluorimetric assay (Stoessl and Venis, 1970; Knegt and Bruinsma, 1973) was modified by the inclusion of a high performance liquid chromatography step to separate the derivatised extract. The derivative, 2-methylindolo-2,3:3',4'-pyr-6-one, (2-MIP), is produced when IAA

reacts with acetic anhydride (AA) in the presence of an excess of trifluoroacetic acid (TFA). The fluorescence of 2-MIP can then be measured.

The extracted ABA was purified and then methylated with diazomethane prior to analysis by GC-ECD.

2.11.1 Purification_of_IAA_and_ABA

Samples of cell culture of 400 - 1000cm^3 were taken for IAA and ABA analysis. The cells were freed from medium by filtration under reduced pressure using a Buchner funnel and Whatman (No 1) filter paper. The sample was washed with 500cm^3 of ice-cold double distilled water. The fresh mass of the cells was recorded and the sample was frozen at -20°C. When frozen the cells were freeze-dried (Chemlab instruments), reweighed and stored at -20°C prior to analysis.

As far as possible all manipulations were performed in the cold (4°C) and in darkness or dim green light to prevent IAA breakdown.

To extract the IAA and ABA the cells were homogenised in a Wareing blender in 100 cm^3 of cold methanol. The sample was transferred to a 250cm^3 Erlenmeyer flask sealed with foil and agitated overnight on an orbital shaker in darkness at 4°C. Internal standards of 31,300 dpm (^{3}H) IAA (specific activity 0.98TBq mmol^{-1}) and 7,000 dpm (^{14}C) ABA (259 MBq mmol^{-1} were added.

These were obtained from the Radiochemical Centre, Amersham. The homogenate was filtered through Whatman No 1 filter paper under reduced pressure using a Buchner funnel. The sample was then washed with $20cm^3$ of methanol and the washing and filtrate were combined and concentrated to near dryness in a florentine Quickfit flask under reduced pressure at 30^oC using a Buchi rotary evaporator. The residue was taken up in $30cm^3$ of $0.01 \ mol \ dm^{-3}$ potassium ortho-phosphate (K_2HPO_4) buffer (pH 8) and washed once with half its volume of petroleum ether and once with half its volume of diethyl ether in separating flasks. The extract was adjusted to pH 3 with dilute hydrochloric acid and partitioned three times against half its volume of diethyl ether each time. The diethyl phases were combined, $0.5cm^3$ of $0.01 \ mol \ dm^{-3} \ K_2HPO_4$ buffer (pH 8) was added and the sample was then concentrated to near dryness under reduced pressure at 30^oC.

The residue was dissolved in $0.5cm^3$ of $0.01 \ mol \ dm^{-3}$ K_2HPO_4 buffer (pH 8) and was washed five times each time with an equal volume of buffer. The extract was then carefully applied to a 100 x 10mm column of insoluble polyvinyl pyrrolidone (PVP). The column was eluted by gravity flow with $0.01 \ mol \ dm^{-3} \ K_2HPO_4$ buffer (pH 8) and the first $150cm^3$ of eluate was collected. After acidification to pH 3 with concentrated HCl the eluate was extracted three times, each time with half its volume of diethyl ether. The diethyl ether phases were

combined and the water was removed from the extract by freezing and subsequent filtering with Whatman No 6 phase separator filter paper. The diethyl ether was then evaporated to dryness under reduced pressure at $30\,^{\circ}C$. The residue was taken up in $50mm^3$ diethyl ether and stored at $-20\,^{\circ}C$ in a sealed vial prior to further purification by high performance liquid chromatography (HPLC).

2.11.2 Preparative HPLC of IAA and ABA

The HPLC techniques employed were as described by Blakesley et al., (1984). The ethanolic extract was reduced to dryness under a stream of nitrogen gas and was then taken up in $50mm^3$ of methanol. The sample was injected via a Rheodyne valve and a $200mm^3$ injection loop onto a preparative $5\mu m$ Hypersil ODS column (150 x 22.5mm internal diameter) fitted with a 55 x 5mm pre-column packed with Hypersil ODS. The HPLC (Applied Chromatography Systems) was fitted with an Apple computer chromatography interface and was connected to a detector system consisting of a UV absorbance monitor (Model LC75, Perkin-Elmer) set at 254nm and a spectrophotofluorimeter (Model MPF-43A, Perkin-Elmer) set at an excitation wavelength of 280 +/- 10nm and an emission wavelength of 350 +/- 10nm. The technique of ion-pair reverse phase separation was utilized. The mobile phase was a gradient of $150-350cm^3$ methanol in a dm^3 of 0.01 mol dm^{-3} Tetraethyl ammonium chloride (TEA)/1.0m mol dm^{-3} phosphate buffer (pH 6.6), at a rate

of 10cm^3 min^{-1} for 20 minutes. The elution concentration was held at 350cm^3 dm^{-3} for 5 minutes before returning to 150cm^3 dm^{-3} at the rate of 20cm^3 minute^{-1}. The flow rate was maintained at 2cm^3 min^{-1}.

Standards of IAA, c-t-ABA and t-t-ABA were separated by ion-pair chromatography and their retention times were noted. The samples were then purified by this technique and the fraction with the same retention time as authentic IAA was collected, diluted with double redistilled water, acidified to pH 3 with dilute HCl and extracted three times, each time with half its volume of diethyl ether. The diethyl ether phases were then combined and the water was removed by freezing and filtering with a Whatman No 6 phase separator filter. The diethyl ether was evaporated to dryness at 30°C under reduced pressure using a silica gel vacuum line. The residue was taken up in 50mm^3 of methanol and stored in a sealed vial at -20°C prior to analysis.

The fraction with the same retention time as authentic c-t-ABA was collected, diluted with 20cm^3 double re-distilled water, acidified to pH 3 with dilute HCl and extracted three times, each time with half its volume of diethyl ether. The diethyl phases were combined and the water was removed as before. The residue was taken up in 50mm^3 of methanol and stored in a sealed vial at - 20°C prior to analysis by gas chromatography using an electron capture detector (GC-ECD).

2.11.3 Derivatization of IAA

This IAA estimation technique involves the HPLC separation of 2-methylindolo-2,3:3',4'-pyr-6-one(2-MIP), the derivative formed by the reaction of IAA with acetic anhydride (AA) in the presence of trifluoroacetic acid (TFA) (Blakesley et al., 1983). The samples were evaporated to dryness under a stream of nitrogen gas. When thoroughly dry the derivatization technique was initiated by the addition of 25mm^3 of a 1:1 mixture of AA-TFA. After agitation and a period of five minutes the mixture was separated by HPLC (Advanced Chromatography Systems).

2.11.4 HPLC Separation and Estimation of IAA

Immediately after derivatization the sample was subjected to analytical HPLC using a 5μm Hypersil ODS column (250 x 5mm internal diameter), fitted with a 55 x 5mm pre-column packed with Hypersil 5 ODS. The detection system utilized was a spectrophotofluorimeter set at an excitation wavelength of 445 +/- 10nm and an emission wavelength of 480 +/- 10nm. The 2-MIP was eluted by a gradient of 500cm^3 dm^{-3} - pure methanol in pH 3.5 water at a flow rate of 2cm^3 minute^{-1}. The elution concentration increased at a rate of 10cm^3 dm^{-3} min^{-1} for 5 minutes, 20cm^3 dm^{-3} min^{-1} for 10 minutes and 50cm^3 dm^{-3} min^{-1} for 5 minutes and was then held at pure methanol for 5 minutes before returning to 50cm^3 dm^{-3}. The fraction with the same retention time as 2-MIP was

collected and diluted with 10cm^3 of Lumagel scintillation fluid (Fisons). The recovery of (^{3}H) 2-MIP was determined using a scintillation counter (Packard Tricarb).

A calibration curve was obtained by plotting peak height against the amount of the derivative, 2-MIP. The amount of 2-MIP (m) was calculated from the following formula:

$$m = b/a \; (c + d)$$

where   a = the dpm (^{3}H) IAA added

b = the dpm recovered

c = the mass of the IAA standard

d = the mass of the (^{3}H) IAA.

From the calibration curve the quantity of IAA present in the original sample can be calculated. The amount of 2-MIP (m) can be obtained from the curve, a correction is made for the mass of (^{3}H) 2-MIP (n) and the recovery of IAA calculated thus:

$$\text{IAA in original sample} = (m - n)e/f$$

Where   e = (^{3}H) IAA initial dpm

f = (^{3}H) 2-MIP dpm.

2.11.5   Identification_of_ABA

The purified extract was methylated with ethereal diazomethane prior to analysis by GC-ECD. The diazomethane was prepared by dissolving 4g of N-methyl-

N-nitroso-p-toluene sulfonamide in 50cm^3 of diethyl ether over an ice bath. 2g of potassium hydroxide were then dissolved in 1cm^3 of double redistilled water and mixed with 20cm^3 of ethanol. The potassium hydroxide solution was slowly added to the diethyl ether which was then agitated for 15 minutes. The mixture was then gently heated and approximately 40cm^3 of ethereal diazomethane was redistilled and collected over an ice bath.

The extract was reduced to dryness under a stream of nitrogen gas in a gas chromatography "v-vial" (Pierce). Approximately 200mm^3 of diazomethane was added and the v-vial was sealed for 10 minutes. The diazomethane was removed under a stream of nitrogen gas and the procedure was repeated.

The dried extract was taken up in 10mm^3 of methanol prior to analysis by GC-ECD. The GLC (Pye Unicam, Series 104) was fitted with an OV17 1.5% Gas Chrom Q column (1.5 x 4.0mm internal diameter). The samples were injected at 200°C with a temperature gradient of 200 to 240°C rising at a rate of 2°C minute^{-1}. The injector temperature was 250°C and the detector 270°C. Nitrogen was used as the carrier gas. Mixed isomer ABA standards were injected before the sample analysis and the retention time was noted for c,t-ABA. 1mm^3 of

sample was then injected and the fraction and associated peak with the same retention time as the standard c,t-ABA, corresponded to endogenous ABA.

3.0 PLANT GROWTH REGULATORS AND THE CELL CYCLE

3.1 Introduction

Despite intensive research into the regulation of the cell cycle the mechanism is as yet little understood. It is very probable that the regulation is achieved by a complex interaction of numerous substances rather than by one simple regulatory factor. This can be assumed because various workers have been able to elucidate some correlations between levels of certain substances and physiological events but have been unable to attribute the complete regulatory process to such a singular substance. It is also known that the five major groups of plant growth regulators can exhibit synergistic and antagonistic affects on each other, and thus it would be naive to attempt to attribute cell cycle regulation to one group independently.

Plant cell suspension cultures provide a uniform system in which the correlative influences present in the intact plant are removed, and in which differential patterns of distribution of growth substances and nutrients are essentially eliminated, (Elliott et al,. 1977). Such cultures thus provide a good system for investigating the roles of the plant growth substances in cell cycle regulation.

In this section the roles of IAA and the cytokinins have been investigated in the regulation of the cell cycle of Acer pseudoplatanus cells grown in batch culture. The cells were cultured in a liquid medium containing 2,4-Dichlorophenoxyacetic acid at a concentration of 4.5 x 10^{-6} mol dm^{-3}. The complete composition of the culture medium is shown in Table 2.1 of Section 2.0. The affect of trigonelline, a reported G2 arrestor, is also investigated (Evans et al., 1979).

Both IAA and the cytokinins have been strongly implicated in the regulation of the cell cycle. There has been much research into the endogenous levels of IAA and some preliminary work on the cytokinins of cultured sycamore cells; much of this work has been carried out in our laboratories and is therefore particularly relevant, (Elliott et al., 1977; Moloney et al., 1983; Firby, 1985; Elliott et al., 1987).

3.1.1 Auxins

As noted earlier the Acer pseudoplatanus cells used throughout this study were grown in a medium containing 2,4-D, an auxin. Previous attempts to grow such cells in 2,4-D-free medium resulted in cell lysis and a reduction in cell numbers, (Street et al., 1968); this supported the theory that they were auxin dependent. However, further work has provided evidence that this strain of cells can habituate to grow successfully in

the absence of 2,4-D, (Hall 1979). Other workers have also concluded that sycamore suspension cultures are auxin dependent (Street et al., 1968; Simpkins et al., 1970).

Evidence has accumulated that this particular strain was capable of synthesizing IAA, even when growth occurred in 2,4-D containing medium, (Farrimond, 1978; Weston et al., 1978). Still further work provided evidence that IAA ia a natural product of these Acer pseudoplatanus cells, (Moloney et al., 1983).

Moloney (1979) observed that the uptake of added 2,4-D reached a peak on the second day of culture, during the lag phase of batch culture growth, and then declined. By the ninth day after transfer very little of the free acid was detected in the medium. Moloney thus concluded that regulation of growth by the extracellular 2,4-D concentration, (previously postulated by King in 1976), was unlikely. Moloney et al., (1983) suggested that the role of 2,4-D in this system may be to trigger the cell's metabolism during the lag phase, the cell may then become capable of synthesizing sufficient auxin in the form of IAA, for growth. Moloney (1979) and Robinson (1982) have unequivocally demonstrated by mass spectrometry the presence of IAA in both Acer pseudoplatanus cells and culture medium which has supported cell suspension culture growth.

Moloney (1979), Robinson (1982), Lewis (1983) and Firby
(1985) each noted a peak in the endogenous IAA levels at
approximately Day 3 of batch culture growth; that is,
close to, or at the first synchronous division. Firby
(1985) also reported a peak in endogenous IAA preceding
the period of DNA synthesis, and postulated that it
could act as a trigger or possible initiator for DNA
replication in the cell cycle.

Elliott et al., (1987) attempted to investigate further
the hypothesis that IAA may act as a trigger for
mitosis. A cold-induced synchronization technique was
employed and parallel increases in mitotic indices and
endogenous IAA levels were observed through several
induced synchronous divisions. Elliott concluded that
IAA does act as a trigger for mitosis. However, the
results from these experiments are not fully understood
as during some periods of cold incubation (10 ^{o}C)
elevated levels of IAA were observed which decreased
again prior to the restoration of the normal temperature
(25 ^{o}C). Elliott postulated that these elevated levels
may be attributable to a "shock response" by the cells
to the temperature change (which throws some doubt upon
the validity of this technique), or that some of the
cells were synthesizing IAA in preparation for the next
division, which would have occurred if they had been at
the optimum incubation temperature. Robinson (1982)
did investigate the IAA biosynthetic capacity of the
cells in asynchronous batch culture and observed an

increase in synthesis during the lag phase prior to the
increase in the IAA level coincident with the first
synchronous division, but then found that the synthesis
decreased and remained at a fairly constant level
throughout the rest of the growth cycle.

Robinson (1982) and Lewis (1983) both reported levels of
free IAA in batch culture medium which had supported
cell growth significantly higher than the intracellular
levels found per cm^3 of culture. Robinson observed
that the IAA in the medium of an asynchronous batch
culture reached a small peak during the first
synchronous division and then increased until Days 16-
18, (the onset of stationary phase), when the levels
declined. Later in the stationary phase the level was
seen to increase again. Lewis (1983) reported a very
similar trend, although in cells grown in low-nitrate
medium the extracellular IAA levels were substantially
lower. In contrast, the intracellular IAA levels did
not appear to be affected by nitrate starvation, despite
the fact that little cell division occurred. Lewis
proposed that in such cells the endogenous IAA is
responsible for promoting cell expansion. Although it
has been suggested that extracellular levels may be more
important in growth regulation than intracellular levels
(Weiler, 1981), Robinson (1982) provided contradictory
evidence as she found no uptake of labelled IAA from the
culture medium. One must remember that cell suspension
cultures are essentially closed systems and are unlike

the whole plant where IAA can be transported away from sites of synthesis. In a closed system the IAA may accumulate in the bathing media, (this would account for the high levels detected in used media), without having a regulatory affect on cell growth. Indeed, this high extracellular level may be detrimental to cell growth. Lewis (1983), realising the shortcomings of attempting to correlate plant growth substance levels with physiological events in asynchronous batch cultures, decided to concentrate further research on continuous cultures.

In a continuous culture, growth takes place under steady state conditions; that is, growth occurs at a constant rate and in a constant environment (Herbert et al., 1956). Lewis utilized the chemostat system, which is an open continuous culture where growth rate and cell density are held constant by a fixed rate of input of medium containing a growth limiting nutrient, in this case nitrate, and the removal of culture at an equal rate. A positive log-linear correlation was found between the intracellular IAA level and the specific growth rate. Such a correlation was not found for extracellular levels. By increasing or decreasing the nitrate concentration in the feed medium a new higher or lower steady state could be established. Such experiments are termed "step-up" or "step-down". Lewis observed that in a "step-up" situation the intracellular IAA level rose as the rate of cell division increased.

As the new steady state was approached the intracellular IAA levels per cell declined again until they reached a mean level similar to the level before the "step-up". In "step-down" experiments the IAA level was seen to decrease and then again re-established the original level. This suggests that IAA is either causing or is an effect of a change in growth rate.

Moloney (1979) obtained a relationship between absolute growth rate and the common logarithm of IAA levels in batch cultured cells. The IAA levels were expressed on a per cell basis and as Lewis (1983) pointed out, it would seem more likely that IAA levels per cell should be correlated with specific growth rate since this quantity takes into account the number of cells present at a given time. The absolute growth rate is merely an expression of the changing slope of the growth curve, with no allowance for the number of cells present.

In the light of this previous work one can conclude that the endogenous IAA of these Acer pseudoplatanus cells appears to be a trigger for mitosis or cytokinesis; it may be involved in cell expansion and is almost certainly connected with the specific growth rate of the cells. The extracellular levels in the culture medium appear to be of less significance and may only accumulate in the medium because the system is closed.

3.1.2 Cytokinins

There is a close association between the auxins and the
cytokinins. The cytokinins of Acer pseudoplatanus have
been investigated by both Robinson (1982) and Firby
(1985) in these laboratories. The Acer pseudoplatanus
cells used in these studies do not require a supply of
cytokinins to maintain growth in suspension culture.
Cells which do not require an exogenous supply of a
generally essential growth substance are invariably
found to be supplying the substance themselves by
endogenous synthesis. This would appear to be so with
this strain of Acer pseudoplatanus cells (Robinson,
1982; Firby, 1985).

Earlier research into the cytokinins of cultured
sycamore cells induced Mackenzie et al., (1972) to
propose the hypothesis that a critical minimum level of
intracellular cytokinin is built up within the cells by
an excess of endogenous synthesis over loss to the
culture medium and internal degradation. This critical
"trigger" level is necessary before cell division
proceeds. They formulated this hypothesis after
observing that in very low inoculum density cultures
cell division may never occur, or if it does, an
extended lag phase is present. They supplied cytokinins
to such cultures and found that this induced cytokinesis
in cultures that would otherwise not have divided and
that the extended lag phase cultures exhibited a
shortening of the lag phase. They noted that zeatin was

effective at producing these responses at much lower concentrations than the non-naturally occurring cytokinin, kinetin.

Mackenzie and Street (1972) investigated the endogenous cytokinins during the batch culture growth cycle of Acer pseudoplatanus cells. A rapid rise in cytokinin-like activity was observed in the cells during the lag phase which precedes active cell division. Later the cytokinin-like activity per cell progressively declined. Two cytokinin-like fractions, (active in the tobacco and soybean callus bioassays), were detected in the cells. Mackenzie and Street termed these "Cytokinin 1" and "Cytokinin 2" and tentatively suggested that the latter may be zeatin riboside based on its chromatographic properties.

Robinson (1982) further investigated the cytokinins of the batch cultured sycamore cells used in this present study. She utilized the Amaranthus bioassay and detected the presence of fractions of cytokinin-like activity which co-chromatographed with zeatin and zeatin riboside. Robinson also found that during the batch culture growth cycle the total endogenous cytokinin-like activity increased during the lag phase and reached a peak on Day 2. A second peak was observed during the linear phase when the cells were dividing asynchronously. This second peak was not as high, nor as sharp as the first; Robinson suggested that this

could be because when the cells are asynchronous the endogenous level per cell is reaching a maximum at slightly different times in each cell and therefore spreading the peak. Thus, during synchrony one might expect peaks to be high and sharp. These findings are not totally consistent with those of Mackenzie and Street (1972). Although both studies revealed a peak in the lag phase, Mackenzie and Street observed a progressive decline in activity throughout the rest of the growth cycle, whereas Robinson reported the second peak in activity during the linear phase. It is worth noting that the sampling regime employed by Robinson was more intense than that of Mackenzie and Street and thus it is possible that the latter workers may have missed the somewhat rapid fluctuations in endogenous levels detected by Robinson. Cytokinin activity was also detected in the medium by Robinson although the level was seen to remain relatively constant thoughout the batch culture growth cycle.

The intracellular and extracellular cytokinin-like activity has also been investigated in cold induced partially synchronized _Acer pseudoplatanus_ cells, (Robinson, 1982). The intracellular level was found to rise in parallel with the mitotic activity before cyto-kinesis, reinforcing the view that cytokinins are involved in mitosis and/or cytokinesis in these cells. The intracellular level declined during periods in the cold and remained low until reincubation at $25\,^{\circ}C$. The

extracellular levels showed very slight increases as the intracellular levels increased, however, Robinson did not consider these fluctuations to be significant. Throughout all of these investigations into the cytokinins of sycamore cells Robinson found no noticeable difference in the patterns of occurrence of the compounds which co-chromatographed with zeatin and zeatin riboside; when one was detected at high levels the other was too and vice versa.

Firby, (1985), extending Robinson's investigations reported a sharp peak in total cytokinin activity, (approximately 9×10^{-16} g zeatin eqvs cell^{-1}) 20 hours after inoculation. After this peak the cytokinin levels rapidly decreased and remained at a low level (approximately 3×10^{-16} g zeatin eqvs cell^{-1}) until rising to a second broader peak of approximately 7×10^{-16} g zeatin eqvs cell^{-1} at 65 hours. This second peak slightly preceded the peak in the mitotic index level. Individual cytokinin analysis revealed a compound which co-chromatographed with zeatin to be the main cytokinin-like activity present in the 20 hour peak. Zeatin-like activity was found to be consistently higher than the other cytokinins throughout the cell cycle. A second less distinct zeatin-like activity peak was seen at 65 hours. Despite being the predominant cytokinin-like activity found, the zeatin-like activity did not appear to be present until 15 hours after inoculation and also appeared to decline during

cytokinesis. Conversely, zeatin-riboside-like materials were detected soon after inoculation and from the onset of cytokinesis to the end of the cell cycle. A peak of zeatin-riboside-like activity was also found 55-65 hours after inoculation. The zeatin-riboside-like and zeatin-like activity appeared to fluctuate such that when one was at a high level the other was at a low level. Zeatin-riboside-like activity was at a maximum 5 hours after inoculation when no zeatin-like activity was detected. The glucoside-like activity, however, appeared to fluctuate in parallel with the zeatin-like activity with peaks at 20 hours and 65-70 hours, but was not found during the remainder of the cell cycle.

Firby deduced from these fluctuations in the various cytokinin-like substances present that zeatin and zeatin riboside probably have different functions in the regulation of the cell cycle. This is reflected in the inverse relationship of their fluctuations. O-glucoside activity was observed after the peak in zeatin-like activity at 65 hours and preceding the increase in zeatin riboside-like activity. Firby proposed that the conversion to the O-glucoside form prevented the metabolic breakdown of zeatin, or that the O-glucoside released free active cytokinin, (as zeatin-riboside), necessary for the ensuing cell cycle. Conversely, the zeatin-O-glucoside may itself be an active cytokinin; as it increased between 68-70 hours it may be associated with cytokinesis.

3.1.3 Trigonelline

The quest for natural cell division regulators led to the discovery of the compound trigonelline (Nicotinic acid N-methyl betaine). It has been identified in many species of plants and animals (Willeke et al., 1979; Beers, 1967). Trigonelline is often the most abundant molecule of the pyridine nucleotide metabolic pathway for the production of nicotinamide adenine dinucleotide (NAD), (Godavari and Waygood, 1973; Preiss and Handler, 1957). Thus, trigonelline may have a regulatory role in NAD biosynthesis.

Trigonelline has been found to be present in the cotyledons of Pisum sativum and is transported to the roots and shoots after germination. Tramontano and co-workers (1982) experimented with excised roots of Pisum sativum and reported that trigonelline promoted preferential cell arrest in G2 in 40% of all root cells during normal cell differentiation. In the absence of trigonelline the cell population arrested in G1. It was noted that this cell arrest was more closely correlated with the concentration of trigonelline in the culture medium than with the quantity/concentration of trigonelline in the roots. Similar G2 cell arrest has been observed in Glycine max and Phaseolus vulgaris (Evans and Tramontano, 1984).

Robinson (1982), working with the same strain of <u>Acer</u> <u>pseudoplatanus</u> cells as those used in this present study, incubated cells with a range of concentrations of trigonelline. She found that a concentration of 10^{-4} mol dm^{-3} stopped cell division without a large decrease in the number of non-viable cells. Robinson attempted to utilise this inhibitive ability of trigonelline to induce synchrony. Four day old cultures were incubated with this concentration for 24 hours under otherwise normal growth conditions, then freed from trigonelline by washing and transferring to fresh medium. A lag phase of two days followed by a 40% synchronous cell division occurred. The cultures then developed into asynchrony. No further analyses were performed to determine exactly where in the cell cycle the cells had been arrested. However, the observed shortened lag phase following the treatment and resuspension in fresh medium could be indicative of G2 arrest.

In this present study an attempt was made to inhibit cytokinesis by incubating cells from inoculation in medium containing a trigonelline concentration of 10^{-3} mol dm^{-3}. It was intended that plant growth regulator analysis could then be performed on the arrested cells to assist in the understanding of the plant cell cycle.

3.2 Experimental Methods

Large scale (21.0 dm^3) cultures were grown as described in Section 2.0 for the determination of batch culture parameters and for some of the cytokinin estimations and trigonelline treatments. Smaller, litre bottle cultures (490 cm^3) were grown as also described in Section 2.0 for the remainder of the cytokinin estimations and trigonelline treatments and the IAA analysis. In all cases 10 cm^3 samples were taken to assess cell number, dry mass, cell viability and mitotic index when required. The cytokinin estimations were made from samples of 470 cm^3 or 1000 cm^3. The IAA estimations were made from samples obtained from harvesting 490 cm^3 cultures, minus the 10 cm^3 samples for growth parameters. The samples were therefore approximately 480 cm^3, with some variations due to evaporation; the sample volume was routinely recorded. The cytokinin and IAA estimation techniques have been previously described in Section 2.0.

The pH of the culture medium was monitored using an autoclavable pH electrode present inside the enclosed large scale culture vessel and connected to a digital pH meter.

3.3 Results_and_Discussion

3.3.1 Growth_Parameters_in_Batch_Culture

The majority of the information available to date on the growth and metabolism of cultured cells has been obtained from batch cultured cells. In this system a known volume of inoculum is cultured in a known volume of nutrient medium with no addition of nutrients. The system is basically a closed system although homogenous culture samples can be removed aseptically, leaving the batch culture with the same cell density but a smaller culture volume. It is assumed that such homogenous sampling has a negligible effect on the growth of the culture as a whole.

The growth cycle of batch cultured cells can by described by a sigmoidal curve, (Figure 1.1). The cycle has five distinguishable phases of growth:

(i)   Lag Phase

(ii)   Exponential Phase

(iii) Linear Phase

(iv)   Growth Deceleration Phase

(v)   Stationary Phase

The state of the cells in each phase is described in more detail in Section 1.4. The transition of cells from one phase to another in the growth cycle is determined by many, mainly unelucidated, internal and external factors.

Much of the research in this area has been performed on various strains of Acer pseudoplatanus cell suspension cultures. Such cultures have revealed that after inoculation there is a critical lag phase where dry mass, mean cell volume and cell number remain relatively constant. This is followed by a limited period during which the increase in cell number is maintained at a high rate. The culture then decelerates in its growth rate and the mean cell volume increases, (Henshaw et al., 1966). Sycamore cells in suspension cultures have been analysed to determine their DNA distributions through the growth cycle. This has revealed that the cells accumulate in G1 during the stationary phase (Bayliss and Gould, 1974) and that the S, G2 and M periods are of a relatively constant time duration, whereas G1 is subject to some degree of variability (Gould et al., 1974). Firby, (1985) determined the duration of these phases for Acer pseudoplatanus cells in suspension culture. She noted a G1 period of 55 hours duration and by 65 hours the S period was complete. The G2 period lasted a further 7 hours with mitosis being complete 73 hours after the cells were transferred to fresh medium.

Acer pseudoplatanus cells have been maintained in suspension culture in this laboratory since 1971. The culture conditions have been detailed in Sections 2.2

and 2.3; subculture being carried out every 21 days. The growth parameters were estimated as described in Section 2.7.

Although the growth parameters of cultured cells have been well studied it is important when embarking on further investigations to ensure that the strain of cells under study still exhibit the same basic patterns of growth. It is always possible that variations in growth patterns may develop, which if undetected could hinder the progress of further research.

The changes in cell number during batch cultured growth of the cells used in this study are shown in Table 3.1 and are represented graphically in Figure 3.1. The cells produce the typical sigmoidal growth curve of Acer pseudoplatanus cells in batch cell suspension culture. The standard errors for the cell number counts are all below +/- 10% and many are below +/- 5%. As these errors are very small they have not been plotted on Figure 3.1.

Cell number counts provide a relatively rapid and accurate account of the growth of the culture. However, it must be noted that every culture is different, not least because it is impossible to inoculate every culture with exactly the same number of cells. Fortunately, the cell culture transfer regime employed in these laboratories has resulted in cultures

TABLE 3.1

<u>Acer</u> <u>pseudoplatanus</u> cell number changes during batch
culture growth

TIME (Hrs)	DAY	No of cells in 1 cm^3 of culture (x 10^3) +/- SE		% Error (+/-)
0	0	232.8 +/- 9.3	⎫	4.0
3		243.3 +/- 14.8	⎪	6.1
10		247.2 +/- 15.9	⎪	6.4
19		240.0 +/- 10.5	⎪	4.4
24	1	249.5 +/- 7.4	⎪	3.0
30		224.3 +/- 14.5	⎬ $\bar{x}$ = 239.8	6.5
42		232.2 +/- 14.5	⎪	6.2
48	2	225.9 +/- 15.8	⎪	7.0
60		252.8 +/- 7.1	⎪	2.8
66		232.8 +/- 14.7	⎪	6.3
70		257.0 +/- 9.3	⎭	3.6
72	3	433.5 +/- 13.8	⎫	3.2
75		426.7 +/- 11.7	⎬ $\bar{x}$ = 429.6	2.7
80		428.6 +/- 30.9	⎭	7.2
96	4	510.5 +/- 18.9		3.7
120	5	713.2 +/- 26.3		3.7
168	7	821.9 +/- 43.3		5.3
192	8	954.2 +/- 20.8		2.2
216	9	1104.2 +/- 70.0		6.3
264	11	1242.4 +/- 63.2		5.1
312	13	1508.2 +/- 44.1		2.9
360	15	1573.7 +/- 30.3		1.9
408	17	1649.0 +/- 45.5		2.8
456	19	1674.5 +/- 45.7		2.7
504	21	1694.2 +/- 67.0		4.0

Standard Error (SE) = +/- $\dfrac{\sigma n-1}{\sqrt{n}}$
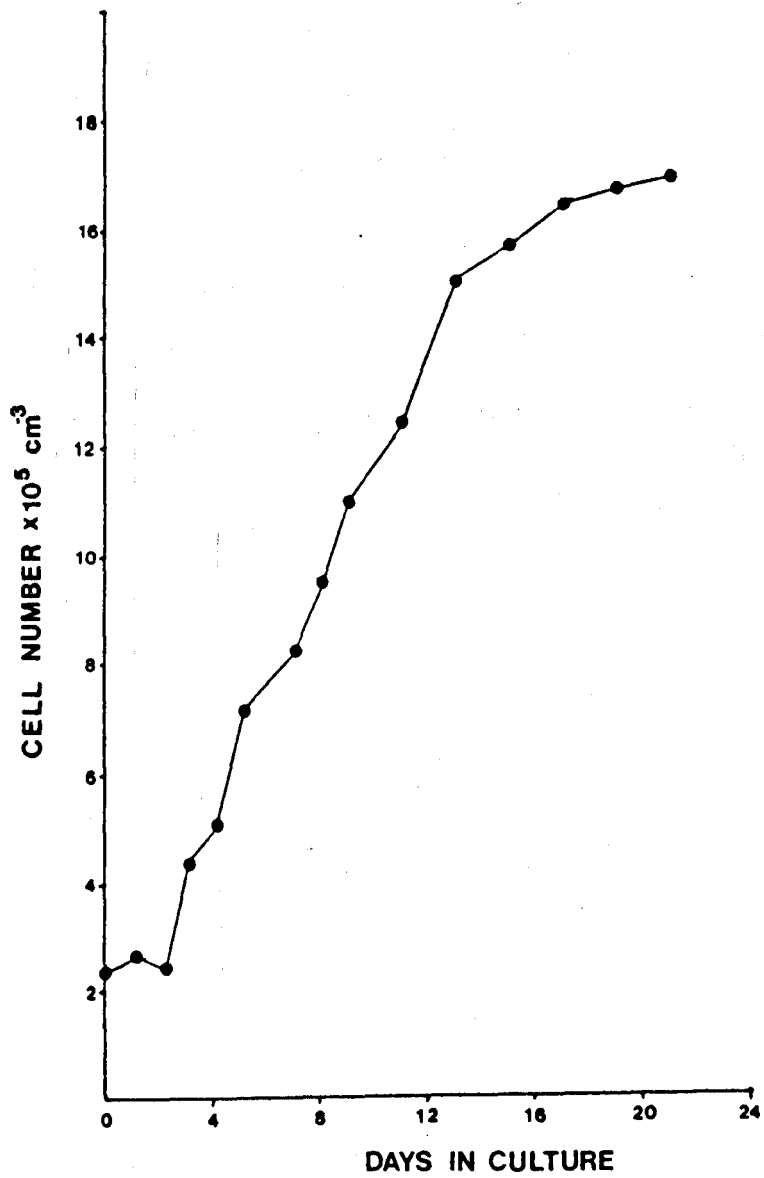
Figure 3.1

Acer pseudoplatanus cell number changes
during batch culture growth.

exhibiting predictable trends of growth behaviour. However, when comparing data from different batch cultures one must remember that by 72 hours after inoculation the majority of the cells in one culture may have reached mitosis, whereas the cells in another similar culture may not divide until 80 hours. It is therefore advisable to use the point at which cytokinesis occurs as a "marker" to compare trends between cultures rather than culture age per se. The lag, exponential and stationary phases can likewise be compared. This particular problem is especially acute when separate litre bottle cultures are harvested at different time points rather than samples being taken aseptically from one large batch culture, (Firby, 1985). As the cultures may be slightly out of step with each other, such a system is not suitable for close hourly sampling.

The changes in cell culture fresh and dry mass are shown in Table 3.2. Fresh mass is usually approximately ten times the dry mass of plant cell cultures but it is not a reliable parameter by which to monitor cell growth. Spuriously high fresh masses are often recorded preceding, during and after mitosis and during the exponential phase when there is considerable active cell division. This is probably due to the fact that the cells were noted to be more difficult to free of medium when they were actively dividing.

TABLE 3.2

Acer pseudoplatanus cell fresh and dry mass changes
during batch culture growth.

TIME (Hrs)	DAY	FRESH M (mgs/ml)	DRY M (mgs/ml)	DRY M OF 10^6 CELLS (mgs)
0	0	7.35	0.64	2.75
3		7.67	0.62	2.55
10		7.91	0.68	2.75
19		7.78	0.65	2.71
24	1	8.02	0.71	2.85
30		7.94	0.63	2.81
42		7.89	0.70	3.01
48	2	7.54	0.66	2.92
60		8.35	0.72	2.85
66		10.12	0.77	3.31
70		9.97	0.81	3.15
72	3	10.31	0.80	1.85
75		9.73	0.84	1.97
80		9.95	0.87	2.03
96	4	10.81	0.92	1.80
120	5	15.43	1.24	1.74
168	7	18.09	1.75	2.13
192	8	22.50	2.11	2.21
216	9	28.61	2.71	2.45
264	11	37.12	3.30	2.66
312	13	45.03	4.13	2.74
360	15	47.01	4.64	2.95
408	17	51.13	4.96	3.01
456	19	54.91	5.24	3.13
504	21	53.62	5.13	3.03
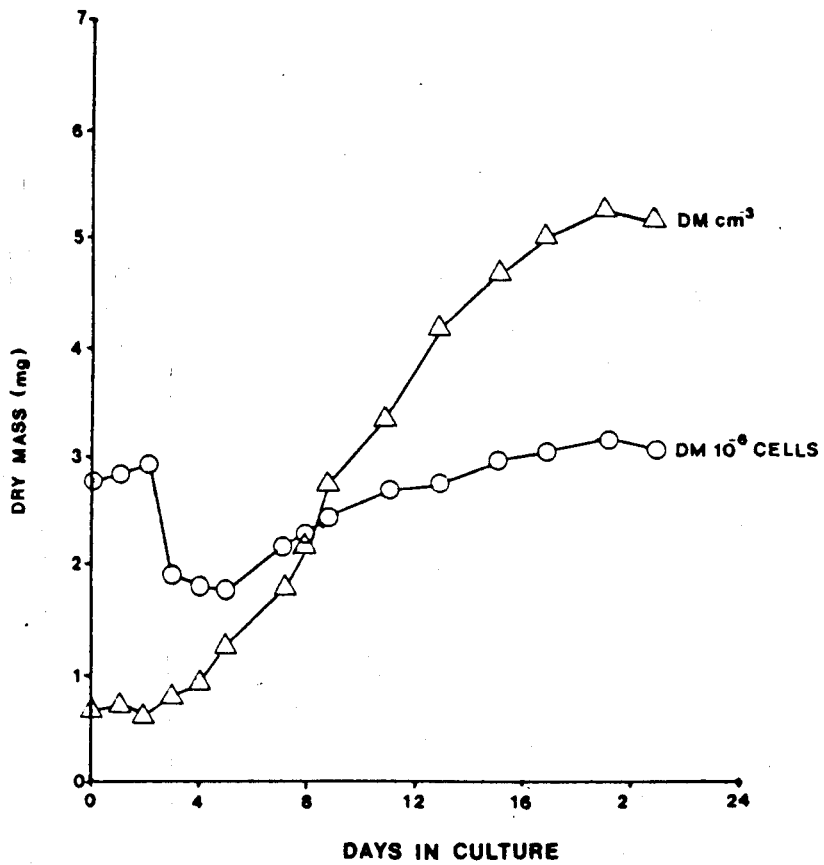
Figure 3.2

Acer pseudoplatanus cell dry mass changes
during batch culture growth.

Dry mass is a more reliable growth parameter and the data, expressed in mg cm^{-3} of culture and represented graphically in Figure 3.2, reflects the same sigmoidal pattern as for cell number. Figure 3.2 also shows dry mass plotted on a per cell basis. The cell dry mass rises very slightly before mitosis and then rapidly drops at the point of cytokinesis. The dry mass per cell then increases rapidly during the exponential phase and stabilises again by the stationary phase. The dry mass of 10^6 cells at Day 21 is similar to that at inoculation.

The mean cell volume (MCV) can be calculated from the packed cell volume (PCV) which is obtained by the centrifugation of a known volume of culture in a graduated centrifuge tube. PCV, expressed as a percentage, and MCV are shown in Table 3.3. MCV changes are also shown in Figure 3.3 and are similar to the cell dry mass changes previously discussed. At the point of cytokinesis the MCV drops rapidly as a high proportion of the cells divide over a relatively narrow time period, (approximately 3 hours), at the first synchronous division. As the culture becomes asynchronous the MCV gradually increases as the cells are at different stages of the cell cycle and are thus different sizes. As the rate of cell division declines

TABLE 3.3

<u>Acer</u> <u>pseudoplatanus</u> mean cell volume changes during
batch culture growth.

TIME (Hrs)	DAY	PCV (%)	CELL NO x 10^3 (per ml)	MCV Volume of 10^6 cells (cm^3)
0	0	3.0	232.8 +/- 9.3	0.129
3		3.0	243.3 +/- 14.8	0.123
10		3.0	247.2 +/- 15.9	0.121
19		3.0	240.0 +/- 10.5	0.125
24	1	3.0	249.5 +/- 7.4	0.120
30		3.0	224.3 +/- 14.5	0.134
42		3.0	232.2 +/- 14.5	0.129
48	2	3.0	225.9 +/- 15.8	0.133
60		3.5	252.8 +/- 7.1	0.138
66		3.5	232.8 +/- 14.7	0.150
70		4.0	257.0 +/- 9.3	0.156
72	3	4.0	433.5 +/- 13.8	0.092
75		4.0	426.7 +/- 11.7	0.094
80		4.5	428.6 +/- 30.9	0.105
96	4	5.0	510.5 +/- 18.9	0.098
120	5	6.0	713.2 +/- 26.3	0.084
168	7	6.5	821.9 +/- 43.3	0.079
192	8	8.0	954.2 +/- 20.8	0.084
216	9	10.0	1104.2 +/- 70.0	0.091
264	11	13.5	1242.3 +/- 63.2	0.109
312	13	17.0	1508.2 +/- 44.1	0.113
360	15	19.0	1573.7 +/- 30.3	0.121
408	17	21.0	1649.0 +/- 45.5	0.127
456	19	21.5	1674.5 +/- 45.7	0.128
504	21	22.0	1694.2 +/- 67.0	0.130
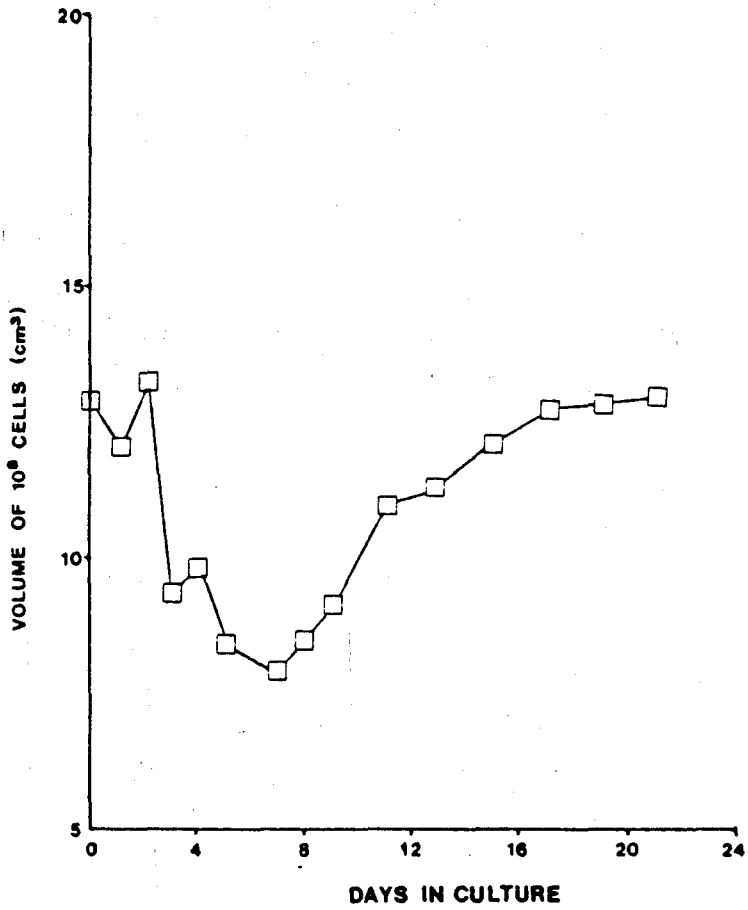
Figure 3.3

<u>Acer pseudoplatanus</u> mean cell volume changes
during batch culture growth.

In the stationary phase the MCV stabilizes at a maximum level similar to that at inoculation; this is a reflection of the large cells characteristic of the stationary phase.

All of the growth parameters calculated and expressed on a per cell basis ideally assume that the cell counts represent 100% viable cells. However, for various reasons cultures very rarely contain 100% viable cells. Non-viable cells present in an inoculum culture are passed on to the new culture during transfer and some cells may become non-viable during the actual transfer process, notably due to the heat from the flame. A number of cells die for "natural" reasons too, especially in the stationary phase when the cells are old and possibly toxic waste products accumulate in the bathing media. The routine cell counting procedure does not differentiate between live and dead cells and thus a cell viability test is performed using phenosafranin which stains the non-viable cells.

The percentages of non-viable cells present are shown in Table 3.4; the levels are mostly below 5%. Such levels are typical of cells in batch culture and are not considered to be significant. Cell viability is an excellent indicator of the "health" of a culture and is of particular use when the culture conditions are being

TABLE 3.4

<u>Acer</u> <u>pseudoplatanus</u> cell viability changes during batch culture growth.

TIME (Hrs)	DAY	NON-VIABLE CELLS (%)
0	0	4.4
3		3.6
10		3.0
19		4.2
24	1	4.6
30		3.4
42		5.0
48	2	5.8
60		2.8
66		5.2
70		3.6
72	3	4.0
75		3.8
80		5.4
96	4	3.6
120	5	4.8
168	7	5.2
192	8	3.8
216	9	4.2
264	11	5.6
312	13	3.6
360	15	6.0
408	17	5.0
456	19	4.6
504	21	4.0

altered in attempts to induce synchrony. A good synchronization technique should have little or no effect on cell viability.

Microdensitometry can be utilized to establish the degree of synchrony of a culture. Cells that are in mitosis can be visually identified and expressed as a percentage of the culture populations. As cells are only in mitosis for a relatively short time period (< 5 hours), asynchronous cultures only have approximately < 1% of cells in mitosis, whereas a synchronous culture would have a larger number of cells in mitosis at, or preceding the point of cytokinesis. Thus a peak in mitotic activity can be seen at a synchronous cytokinesis, usually of approximately 10%. The percentage of cells in mitosis during the first synchronous division of a batch culture is shown in Table 3.5, and is expressed graphically in Figure 3.4. Although the highest percentage attained was 6.47%, it is likely that approximately 75% of the cells in the culture would have divided within 5 hours of each other, (ie approximately 72 hours). The length of time a cell is in mitosis relative to the cell cycle is very small and thus the mitotic peak is small and not very cumulative. The peak in mitotic activity usually slightly precedes cytokinesis as in this case.

TABLE 3.5

<u>Acer</u> <u>pseudoplatanus</u> mitotic index changes during batch culture growth.

TIME (Hrs)	DAY	MITOTIC INDEX (%)
0	0	0.00
24	1	0.41
48	2	1.59
60		3.08
70		6.47
72	3	3.48
75		2.52
96	4	1.62
120	5	0.78
168	7	0.42
216	9	0.41
312	13	0.40
360	15	0.00
456	19	0.00
504	21	0.00
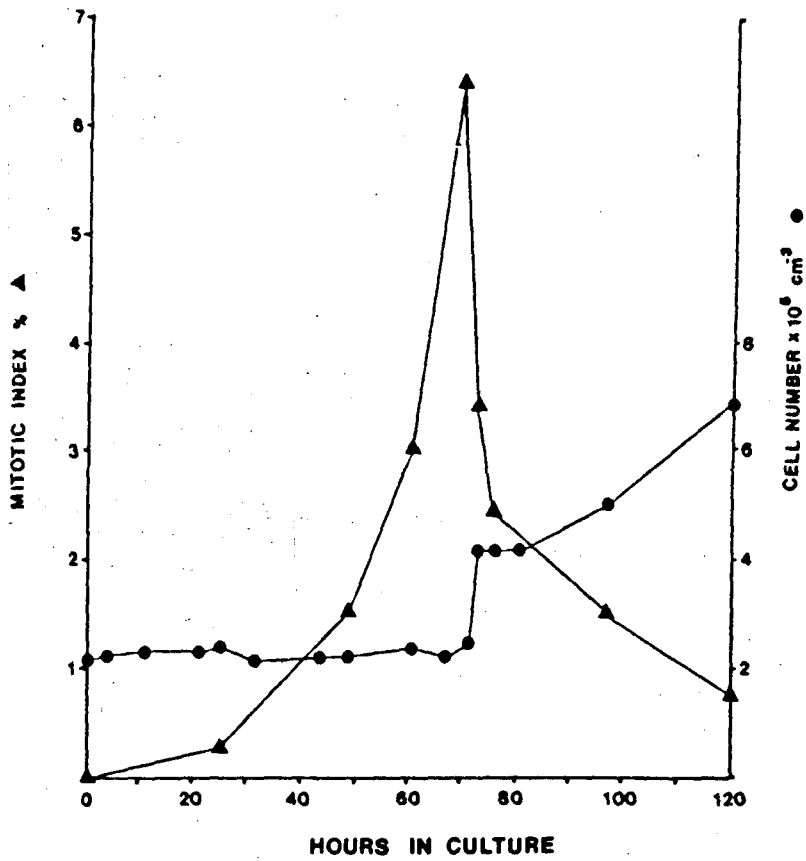
Figure 3.4

<u>Acer</u> <u>pseudoplatanus</u> mitotic index changes
during the first synchronous division of batch
culture growth.

Mitotic index alone is a poor index of synchrony (Everett et al., 1981), as other factors, notably changes in cell growth rate and death rate, (King and Street, 1977) can cause dramatic fluctuations in mitotic index.

It is advisable to use more than one parameter as an indicator of synchronous growth. Ideally these should at least include:

(i) Periodic approximately 100% increases in the cell number followed by periods of constant cell number.

(ii) Division periods occupying only a small fraction of the cell cycle.

(iii) Increases in the mitotic index preceding cell number increases.

Figure 3.4 shows the first division of a batch culture. The cell number is relatively stable after inoculation until 72 hours when there is a rapid step-like increase to another relatively stable level. As the culture develops asynchrony this pattern of step-like cell number increase is gradually lost. Table 3.1 shows the mean cell numbers of these levels of stable cell number as 2.398×10^{-5} cells cm^{-3} preceding cell division and 4.296×10^{-5} cells cm^{-3} after division. The degree of synchrony may be expressed as a percentage:

$$\text{Degree of Synchrony (\%)} = \frac{\bar{x} \text{ Post-division Cell No} - \bar{x} \text{ Pre-division Cell No}}{\bar{x} \text{ Pre-division Cell No}} \times 100$$

$$= \frac{4.296 \times 10^{-5} - 2.398 \times 10^{-5}}{2.398 \times 10^{-5}} \times 100$$

$$= 79.15\% \text{ synchrony}$$

This highly synchronous first division after subculture is typical of these <u>Acer pseudoplatanus</u> cells and is often utilized by cytologists to investigate the cell cycle and its regulation. The duration of the different stages of the cell cycle can be estimated by techniques such as microdensitometry which reveals the patterns in the DNA profiles of the cells. Figure 3.5 shows these profiles in histogram form with percentage of nuclei plotted against the microdensitometer values which are expressed in arbitrary DNA units.

All cell cycles appear to possess the potential for modifications which can result in daughter cells with duplicated or even halved nuclear complements, (Brown and Dyer, 1972). Such cells may be important in future cell development or differentiation, (Dyer, 1976). During the G1 phase each chromosome is present as a single chromatid and the DNA content of one such set is referred to as having a value C. Usually a G1 cell is diploid with a DNA value of 2C. During the S phase the chromosomes are replicated and the DNA content is doubled to 4C.

During anaphase the chromatids separate to produce two identical nuclei and the original G1 DNA content of 2C is re-established. Due to the cells' potential for states such as polyploidy, higher DNA content values than 4C can be achieved.

Estimations of the relative DNA contents of cell nuclei by microdensitometry can reveal the cell cycle stage of the cell. The colour intensity of plant nuclei stained with Feulgen stain is proportional to the DNA content and can be measured relatively accurately using a microdensitometer, (Bayliss and Gould, 1974). The DNA frequency distributions, (Figure 3.5), usually reveal two peaks which represent the nuclei in the 2C and 4C DNA content levels; that is, the G1 and G2 phases of the cell cycle. Theoretically the S-phase nuclei are those between the G1 and G2 peaks but often these two peaks merge into one another making S-phase estimations difficult, (King, 1980). As the cells approach the stationary phase of the batch culture growth curve the cellular DNA contents become larger, which is reflected in the right-hand broadening of the frequency histograms. This may be indicative of a change in the cellular metabolism from the previous active cell division to cell expansion and perhaps differentiation. Curtailed or shortened cell cycles have been found in differentiating meristematic tissues resulting in polyploidal cells with correspondingly higher DNA contents, (Nagl, 1974; 1975; Dyer, 1976). Referring to
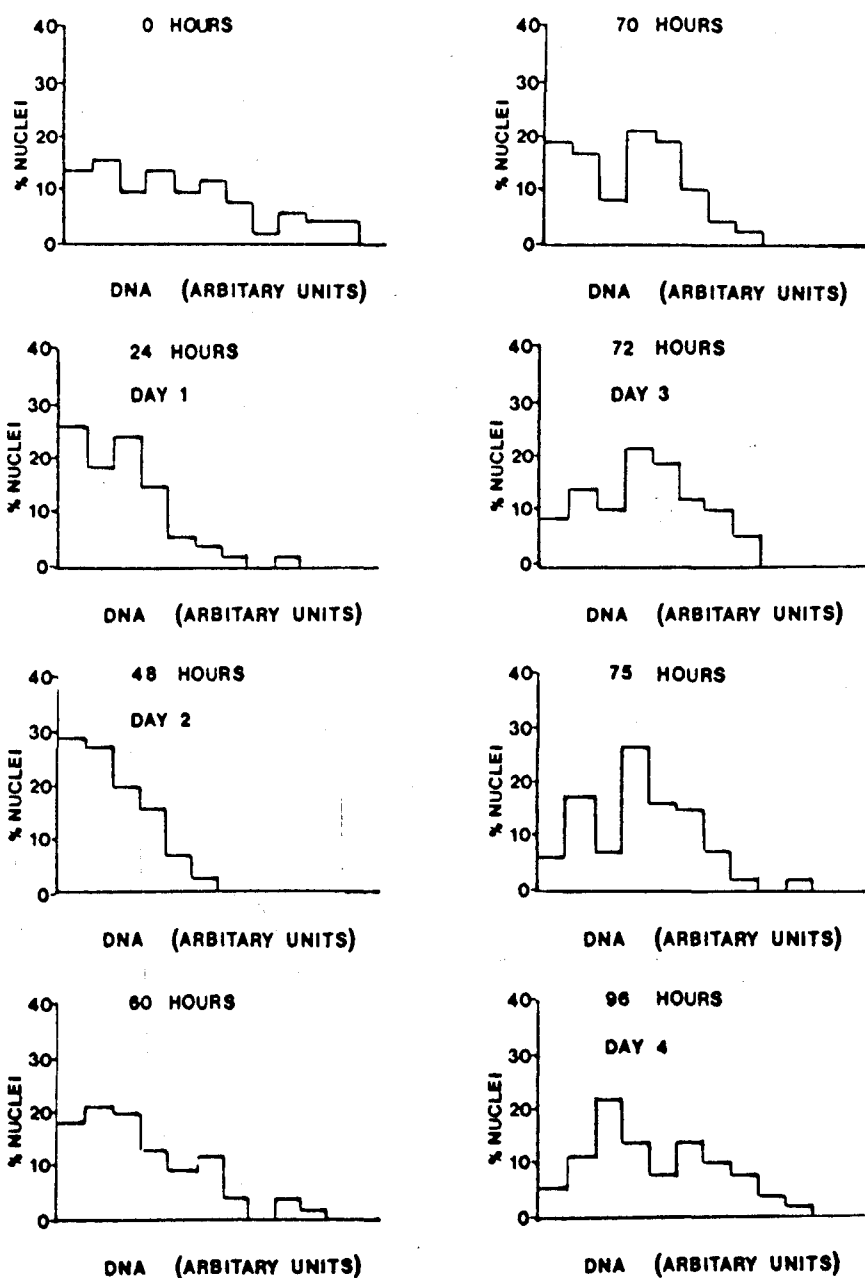
Figure 3.5

Frequency distributions of different DNA levels
in batch cultured Acer pseudoplatanus cells.

120 HOURS
DAY 6

360 HOURS
DAY 15

168 HOURS
DAY 7

456 HOURS
DAY 19

216 HOURS
DAY 9

504 HOURS
DAY 21

312 HOURS
DAY 13

DNA (ARBITARY UNITS)
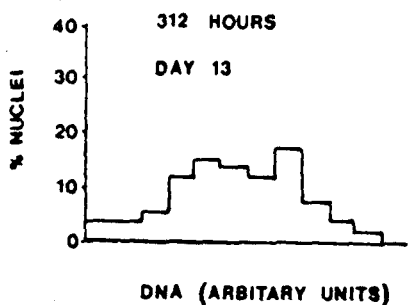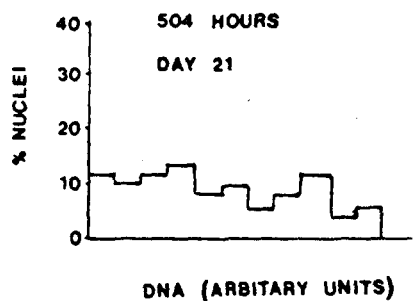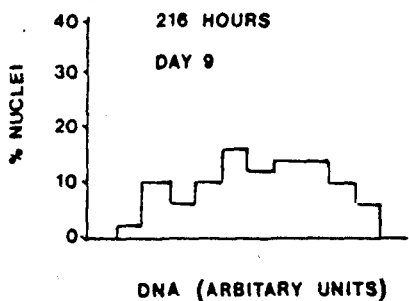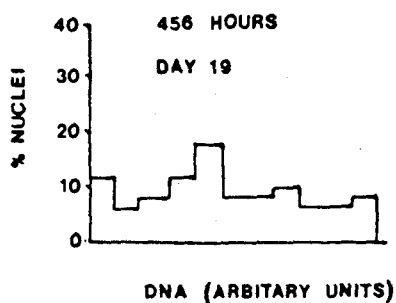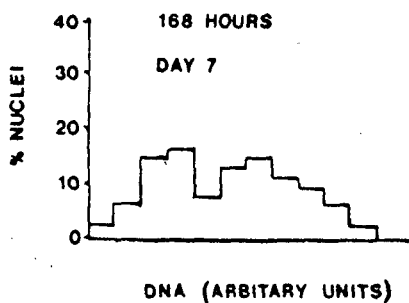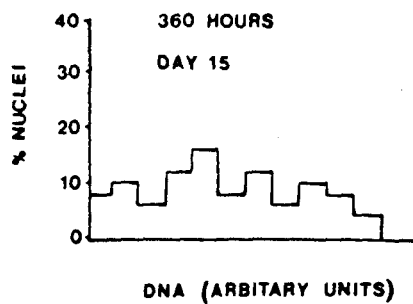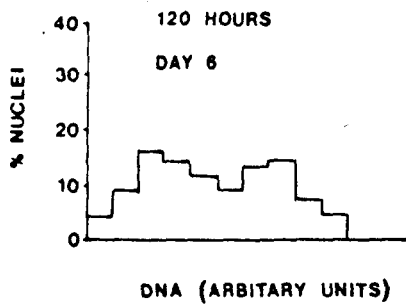
Figure 3.5 it can be seen that from Day 7 the DNA content begins to increase, probably because the cells are beginning to change from mitotic cycles to differentiating cycles. It is possible that at this stage the G2 trigger factor ceases to be effective and thus mitosis is not achieved.

The DNA profiles for 0 hours (inoculation), and Day 21 (transfer), are similar and the cells are relatively evenly distributed between what can be assumed from the work of Firby (1985) to be the G1 and G2 states. However, after inoculation the percentage of cells in the G2 state appears to decline resulting in a Day 2 profile of predominantly G1 cells despite the absence of any apparent cytokinesis. This phenomenon was also noted by Firby (1985), who subsequently analysed the actual DNA content per cell which was found to be 6.74pg at inoculation and only 4.68pg after 40 hours of culture.

At 60 hours the profiles show the G1 phase and also the apparent reappearance of the G2 phase. By 70 hours the G1 and G2 phases are clearly defined and by 72-75 hours, (close to cytokinesis), the cells are predominantly in the G2 state. These easily distinguishable G1 and G2 phases are typical of a synchronous culture. By 96 hours, when most of the cells have divided, the majority of the cells are in G1 again. There then follows a period of rapid asynchronous growth when the

G1 and G2 phases become less distinct. As the culture
develops into the stationary phase, which is
characterised by large cells with correspondingly higher
levels of DNA per nucleus, the profiles become broader
with the cells arresting both in the G1 and G2 phases.

A previous study of the timing of the cell cycle in
these Acer pseudoplatanus cells involving subculturing
cells of different ages (Firby, 1985), revealed a G1
phase of variable duration with the S, G2, M and D
(division) phases constituting a non-expandable block of
approximately 28 hours. Assuming the existence of a G0
phase before the G1, a constant cycle of 33 hours was
proposed with the cells spending variable time periods
within the G0 phase. The time spent in the G0/G1 phase
was not found to be proportional to the age of the
inoculum and this led to the hypothesis that there may
be a turnover of the DNA pool. It was proposed that the
non-proportional amount of time spent by 28 day
inoculated cells in the G1 phase could be due to their
previous loss of turnover DNA, on transfer new rounds of
DNA synthesis would be immediately initiated.
Initiation of DNA synthesis in such cells could occur
earlier than in 21 day transferred cells had they not
spent longer in the G0 compartment. The hypothesis
assumes that the longer cells are in the G0 the longer
they will take to return to the cell cycle. In the case
of 28 day old subculturing the DNA turnover is complete
before transfer, and thus the cells are primed for

further DNA synthesis. However, 21 day subcultures require a period of at least 40 hours before their DNA content has been sufficiently reduced and further synthesis can occur. 14 day subcultures spend a very short time in G0 and presumably they can enter and leave this compartment very quickly. This theory explains the apparent disappearance of the G2 phase preceding cytokinesis noted earlier in Figure 3.5.

The stationary phase cell DNA profiles reveal cell arrest in both the G1 and G2 phases. Carbohydrate starved cells are known to arrest in the G1 and G2 states; this relates to Van't Hof and Kovac's principal control points hypothesis (1972) which proposed G1 and G2 control points. Although stationary phase <u>Acer</u> <u>pseudoplatanus</u> cells are actually nitrate limited, (King, 1973), this hypothesis can still be considered.

21 day inoculum cells appear to be arrested in G1 and G2 but the proportion of cells in each phase is transient before the first mitosis, with the percentage of cells in G1 increasing and those in G2 decreasing. As these proportions change in the absence of cytokinesis it can be assumed that the G2 cells "leak" backwards through the cell cycle into the G1 state. As there appears to be no reversal of this leakage without cytokinesis occurring, it is proposed that the main control point is present in the G1 phase. Phases such as the G1, which exhibit variability in their duration, are considered to

be more likely to be phases controlled by trigger factors. This variability may be caused by the cells passing through this stage of the cell cycle at different rates or may be due to an arrest of variable length at a specific point (Prescott, 1976). Firby (1985) suggests that the control point in G2 may be a short "pause" in the cell cycle where the cells are awaiting a "trigger" before they continue in the cycle. If this trigger is not seen within a defined time period DNA turnover occurs and the cells reverse the cycle and move back to the G1 state.

In 26 day old cultures Firby (1985) noted that a "true" stationary phase existed when the majority of cells were in the G1 phase and no further changes were observed in the DNA frequency distributions until DNA synthesis commenced after subculture. This suggests that the G1 control point may have been released on transfer to fresh medium and thus the trigger factor, causing G1 arrest, may be a growth substance or nutrient deficiency, or, perhaps, a product block in an enzymic reaction which could not be released until new reactants were introduced. However, this does not fully explain why 21 day old inoculum cells continue to revert to the G1 phase on transfer to fresh medium. It is possible that these cells have become nutrient, growth substance or reactant-limited, and are already programmed to revert back to G1 after an unsuccessful attempt to continue through the cycle to mitosis. Thus, despite

transfer to fresh culture media, they must continue backwards to G1 before further normal progress in the cell cycle can be achieved. Whatever the precise mechanism of the one, two or even more control points, it would appear that the G1 control point is of high significance, not least because it occurs early in the cell cycle. In this present study the postulated roles of IAA and the cytokinins are considered as tentative G1 trigger factors.

3.3.2 Quantitative Analysis of Cytokinins

Much of the research into the cytokinins of cultured cells has been concerned with the effect of added cytokinins. As these sycamore cells are known to synthesize cytokinins, this study is concerned only with the variations in endogenous cytokinin levels with respect to the phases of the cell and batch growth cycles.

The total and individual cytokinins were estimated as described in Section 2.10. The ion-exchange step was included as when it was omitted in preliminary experiments the cytokinin-like activity appeared to be depressed. Duplicate samples of the same cells were analysed and a mean value of 4.55 ng zeatin equivalents g^{-1} dry mass was obtained without the ion-exchange step, as opposed to 14.5 ng zeatin equivalents g^{-1}, (approximately 3x), when this step was included.

It is probable that the ion-exchange column removes substances such as phenolics from the extracts which may cause inhibition in the cucumber cotyledon bioassay.

The cucumber cotyledon cytokinin bioassay was employed as Hosford (1984) evaluated a number of cytokinin bioassays in these laboratories and found this one to be relatively accurate over the required range, and also fairly rapid and practicable. The cytokinins cannot, however, be unequivocally identified by bioassay, nor can they be precisely quantified. Nevertheless, useful data can be obtained by assessing the total cytokinin-like activity present and by separating the individual cytokinins by HPLC. Both the total cytokinins and the materials which co-chromatograph with the individual cytokinins can be quantitatively expressed in terms of ng zeatin equivalents, this being the amount of zeatin required to elicit the same response as the active fraction in the bioassay.

After the chlorophyll has been extracted from the cotyledons the absorbance of the supernatant is measured at 663nm and 645nm. Using the equation:

Chlorophyll content (μg cm^{-3}) = (Absorbance at 663nm x 8.02)
+
(Absorbance at 645nm x 20.2)

(Arnon, 1949)

the chlorophyll levels in the supernatants can be estimated. A standard curve is then drawn of Log$_{10}$

ng zeatin against the chlorophyll concentration
(μg cm^{-3}), with the equation of the line being obtained
by the "best fit" method. Figure 3.6 shows a typical
standard curve obtained from serially diluted zeatin
standards. Zeatin is often used as a "typical
cytokinin" in bioassays to obtain standard curves. It
can be seen that the relationship between zeatin
concentration and chlorophyll content exhibits good
linearity with a regression coefficient, r, of 0.96.
Using this bioassay the other individual cytokinins can
be quantitatively estimated and represented in terms of
ng zeatin equivalents. However, it should be noted that
the individual cytokinins are unlikely to elicit the
same response in the bioassay as the zeatin standard
itself. In fact, Figure 3.7 shows the cucumber
cotyledon bioassay standard curves for kinetin and 6-
Benzyladenine, with regression coefficients, r, of 0.89
and 0.97 respectively. These cytokinins have induced
the production of relatively higher chlorophyll levels
than those obtained with the zeatin standards. Thus it
can be deduced that this particular bioassay may under-
estimate the actual levels of kinetin and 6-BAP present
when zeatin is used as a standard. Conversely, some
cytokinins may induce less chlorophyll production than
zeatin and thus over-estimates of levels may be made.
Hosford (1984), found zeatin riboside and dihydrozeatin
produced standard curves similar to zeatin, whereas
isopentenyl adenine and isopentenyl adenosine induced
less chlorophyll production. It must be remembered that
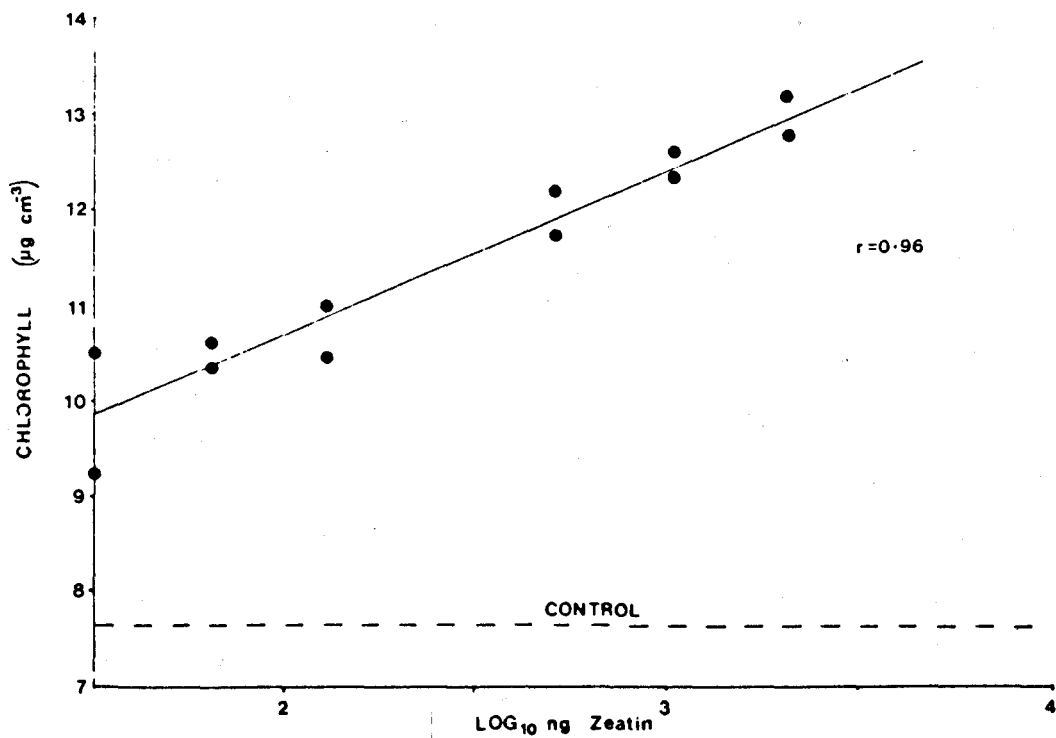
Figure 3.6

Typical cucumber cotyledon chlorophyll
retention bioassay zeatin standard curve.

the units used, (ng Z eqv), standardize the cytokinin activities to equivalent zeatin levels, but, as can be seen from Figure 3.7, these cannot be assumed to be the actual ngs present. When total cytokinin activities are being estimated synergistic effects may result in under or over-estimates of the actual cytokinin activity present. Despite these limitations, (which are inherent in the majority of bioassay systems), the standard curves do exhibit good linearity and the bioassay appears to be reproducible.

Thus, for assessing the basic patterns of cytokinin activity present this system is acceptable, although only tentative conclusions should be drawn from such data.

Scott et al., (1982), pointed out the limitations of cytokinin bioassays, which include their variability, susceptibility to interference and their lack of chemical specificity. High concentrations of organic salts, acidic and phenolic compounds, as well as the other plant growth substances, can also cause interference in cytokinin bioassays. It is therefore essential that the extraction procedure utilised removes such interfering compounds. The method used in this present study is detailed in Section 2.10.1. The extracts were suitably purified by passing through a Sephadex SP-C-25 (H^+) ion-exchange column and a Sep-Pak C18 cartridge. Total cytokinin activity was then
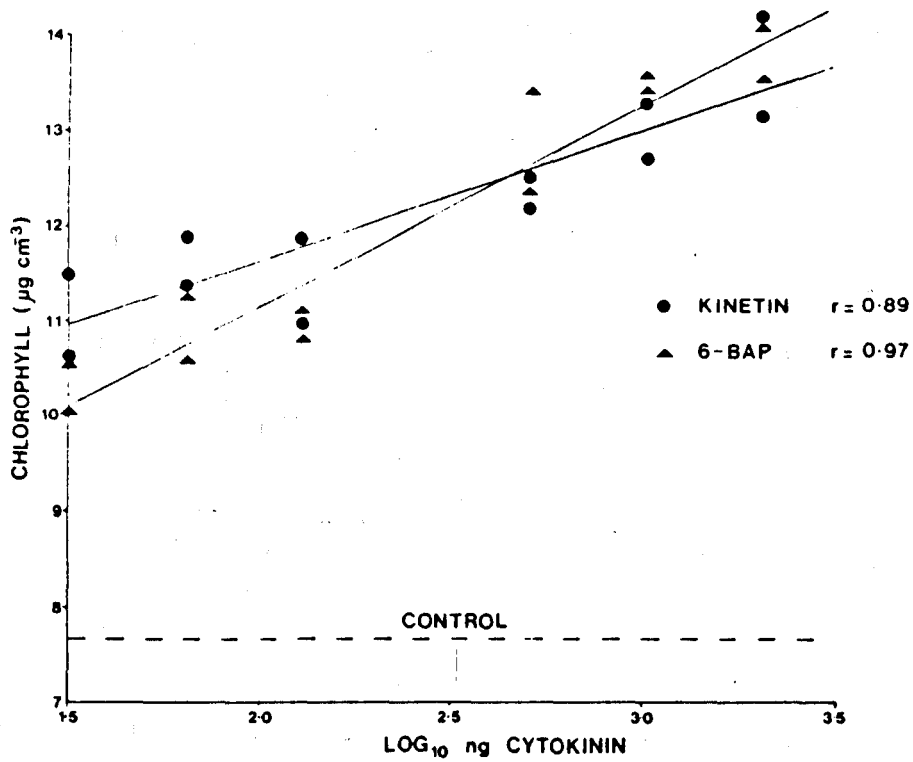
Figure 3.7

Cucumber cotyledon chlorophyll retention
bioassay standard curves for kinetin and 6-BAP.

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estimated by the cucumber cotyledon bioassay. The individual cytokinins were separated by analytical HPLC and the relevant fractions were separately bioassayed. The HPLC techniques employed achieved good peak resolution of the cytokinin standards (Figure 3.8). However, the extracts are not pure enough for the individual cytokinin peaks to be distinguished. When extracts are passed through the HPLC the baseline is not regained between peaks and thus peak height cannot be related to the cytokinin content of the cells. Nevertheless, the fractions relating to the retention times of the relevant standards were collected and the cytokinin activity present therein was assessed by bioassay.

The variations in the total endogenous cytokinin activity of the cells were investigated during the first 14 days of the growth cycle. One litre bottle containing 490cm^3 of culture was harvested for each time point, with 470cm^3 being used for the cytokinin analysis. The results are tabulated in Table 3.6 and are expressed both on a per cell and a per gram dry mass basis. The data are illustrated in Figures 3.9 and 3.10 respectively along with the cell number curve.
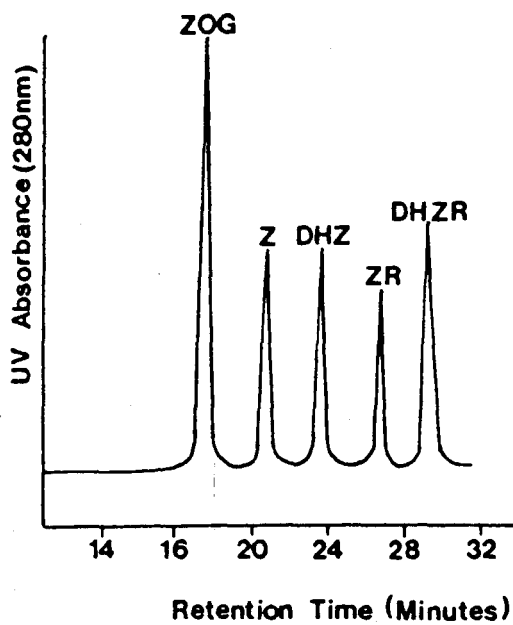
Figure 3.8

The separation of five cytokinin standards
by analytical HPLC using a column (250 x 5mm id)
of 5um Hypersil ODS.

After inoculation the total endogenous cytokinin activity present increased rapidly to a maximum level of 0.65 ng Z eqvs 10^{-6} cells, (222.2 ng Z eqvs g^{-1} DM) after 19 hours. The cytokinin activity then rapidly decreased to approximately a quarter of this high level before increasing slightly to a broader peak, between 43-55 hours after inoculation, of approximately 0.2 ng eqvs 10^{-6} cells. At the point of cytokinesis (67 hours), the activity level fluctuated down to 0.07 ng Z eqvs 10^{-6} cells, but had increased to 0.18 ng Z eqvs 10^{-6} cells three hours later. After cell division the cytokinin-like activity gradually declined and stabilized at a base level of approximately 0.1 ng Z eqvs 10^{-6} cells.

TABLE 3.6

Changes in cell number, dry mass and total cytokinin activity through the first 14 days of the batch growth cycle of _Acer pseudoplatanus_ cells in suspension culture.

Culture Age (Hrs)	Cell Number x 10^{-3} cm^{-3}	Dry Mass (mg cm^{-3})	Total Cytokinin Activity	
			ng Z eqvs 10^{-6} cells	ng Z eqvs g^{-1} DM
3	287.8 +/- 18.5	0.85	0.24	82.4
19	309.8 +/- 34.2	0.90	0.65	222.2
24	281.0 +/- 21.0	1.20	0.28	66.7
27	314.0 +/- 33.5	1.23	0.16	40.0
43	340.5 +/- 22.7	1.05	0.23	76.2
47	359.5 +/- 22.8	1.21	0.18	49.6
55	354.3 +/- 14.3	1.29	0.20	54.3
67	602.3 +/- 20.0	1.39	0.07	28.8
70	599.0 +/- 9.6	1.48	0.18	74.3
80	570.0 +/- 28.6	1.39	0.12	50.4
103	581.9 +/- 31.2	2.14	0.14	37.4
164	795.0 +/- 45.6	3.60	0.09	19.4
307	967.1 +/- 39.7	6.71	0.11	16.4
331	1136.8 +/- 64.9	6.58	0.11	19.8
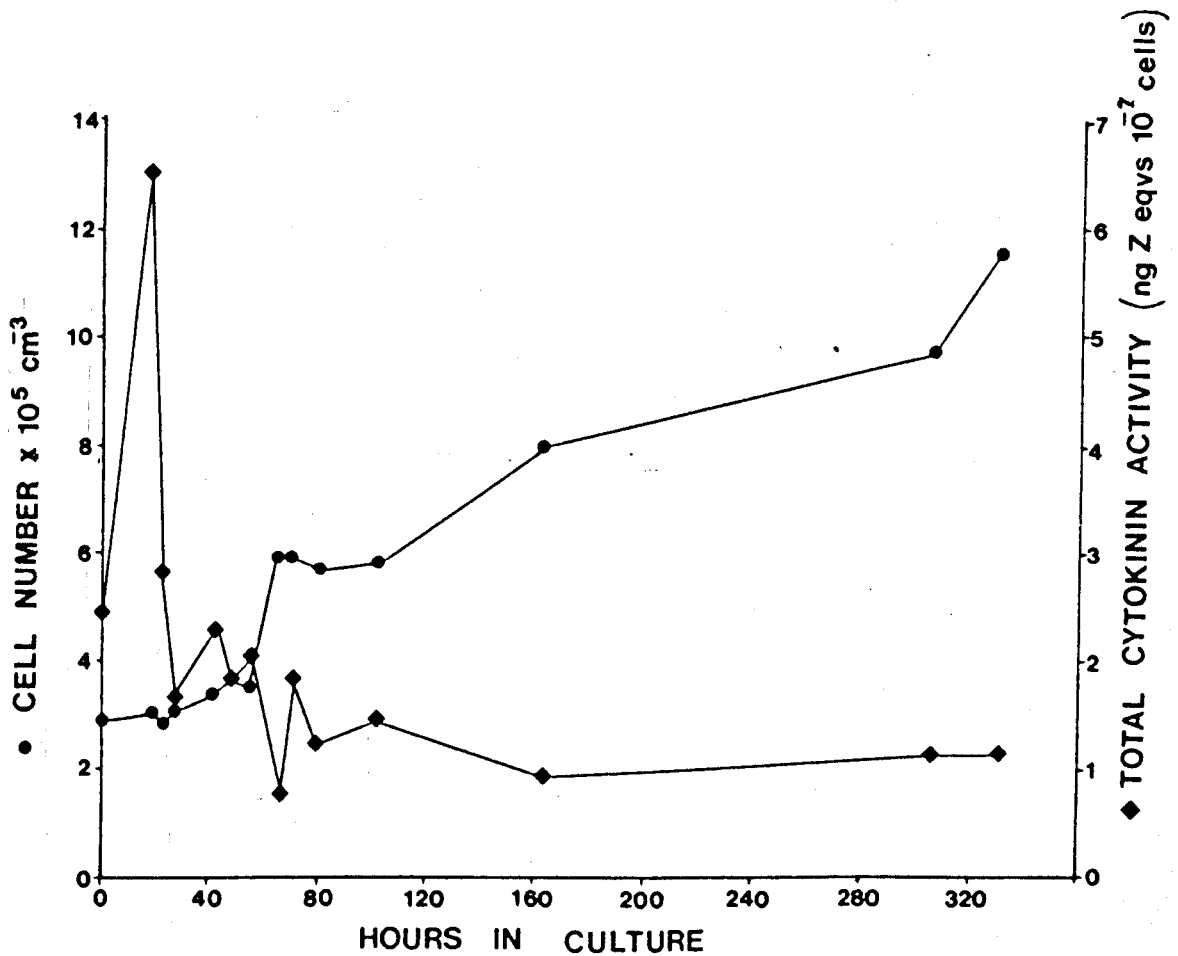
Figure 3.9

Changes in the endogenous total cytokinin
activity (ng Z eqvs 10^{-7} cells) during the
first 14 days of the batch growth cycle of
Acer pseudoplatanus cells.
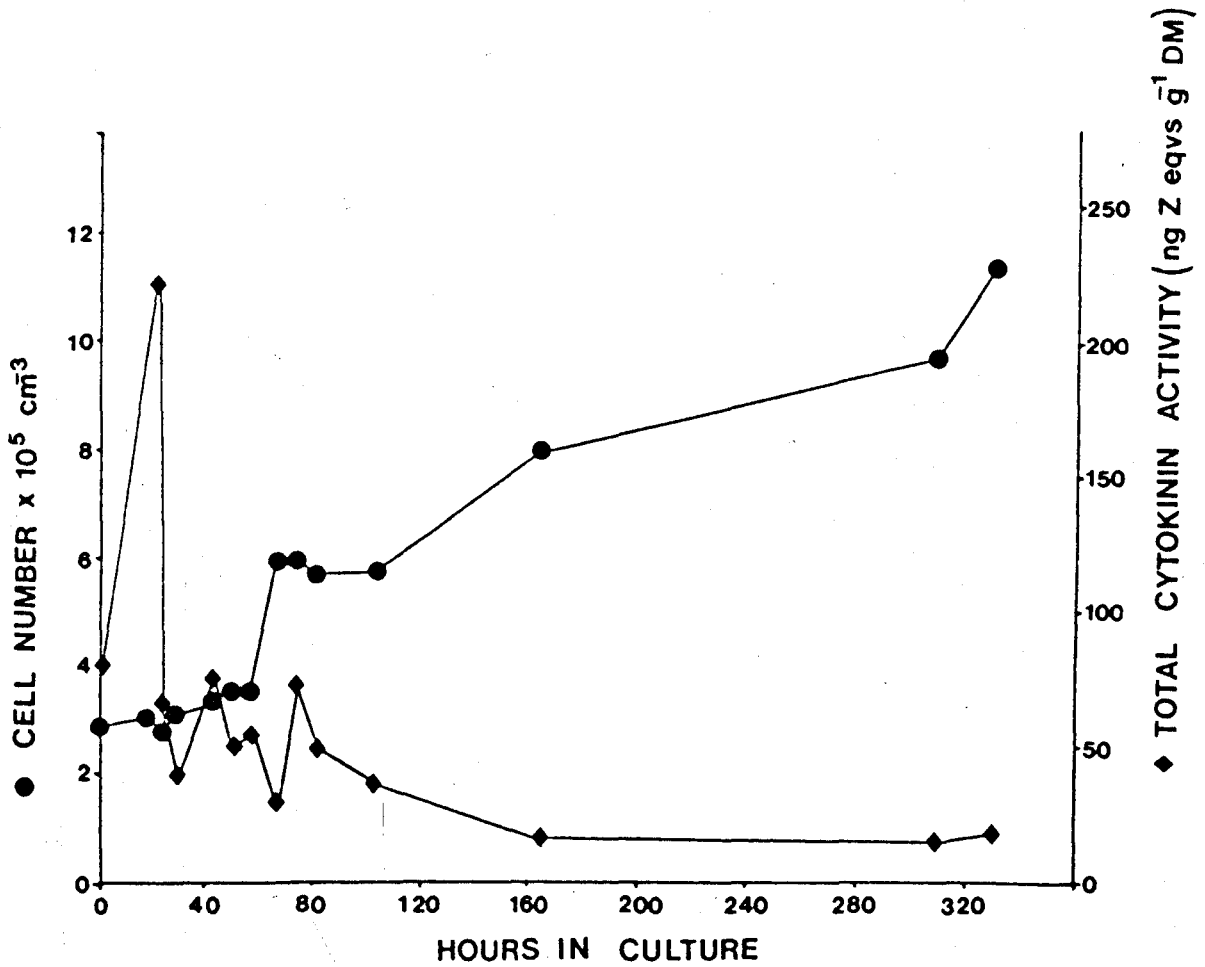
Figure 3.10

Changes in endogenous total cytokinin
activity (ng Z eqvs g^{-1} DM) during the first
14 days of the batch growth cycle of Acer
pseudoplatanus cells.

As already noted in Section 1.1.2, the previous investigations into the cytokinins of Acer pseudoplatanus cells by Mackenzie and Street (1972), Robinson (1982) and Firby (1985), did not produce completely compatible results. This can probably be explained to some extent by differences in the extraction techniques employed and also by the different bioassay systems utilized. Mackenzie and Street (1972) used the Nicotiana tabacum callus bioassay, as described by Miller (1963), to assess the levels of their Cytokinin 1 through the batch culture cycle, and expressed their cytokinin-like activity in terms of kinetin equivalents. Robinson (1982), employed a modified version of the Amaranthus betacyanin bioassay (Biddington and Thomas, 1973), and expressed her cytokinin-like activity in terms of BA equivalents. However, the extraction techniques and bioassay system employed in this study are identical to those used by Firby (1985) in these laboratories and as such these two sets of data can be compared more meaningfully.

Firby (1985), only investigated the first 80 hours of batch culture growth, but the other workers all looked at the full growth cycle. Mackenzie and Street found an inoculation level of approximately 0.2 ng K eqvs 10^{-6} cells of the predominant Cytokinin 1 and a peak of approximately 0.38 ng K eqvs 10^{-6} cells associated with cytokinesis at Day 4. They then found the activity progressively declined to a base level of approximately

0.1 ng K eqvs 10^{-6} cells. This present experiment did not have a zero time point but the value at 3 hours after inoculation is similar to Mackenzie and Street's inoculation level. However, they did not detect a 19 hour peak as their sampling regime was not as frequent as the one used in this study. The peak they associated with cell division was higher than that obtained here and closer to cytokinesis.

Robinson (1982) reported a higher total cytokinin activity inoculation level of approximately 0.6ng BA eqv 10^{-6} cells. Again the sample points were not close enough to detect any 19 hour peak, but a peak in activity of approximately 0.95ng BA eqv 10^{-6} cells was found between 60-70 hours. Unlike the trend seen here or that reported by Mackenzie and Street (1972), Robinson (1982) found the cytokinin-like activity level remained high during the linear phase of growth before progressively declining in the stationary phase. The results of this experiment do not reveal this final peak. In cold-induced synchronous cultures, Robinson (1982) reported an increase in activity to peaks of approximately 0.9ng BA eqv 10^{-6} cells preceding each cytokinesis and a decline to levels of approximately 0.1ng BA eqvs 10^{-6} cells during periods of cold treatment after division.

As previously mentioned the results of Firby (1985) should be more comparable to the present data. Firby's results reveal the same basic trend, with the 19 hour peak (20 hour in this case due to a different sampling timetable), being the predominant common characteristic. Firby did not sample at inoculation either, and therefore this timepoint cannot be directly compared, but her 5 hour sample (0.27ng Z eqvs 10^{-6} cells) was similar to the 3 hour level obtained here. Both 20 hour peak activity levels reported by Firby were approximately 0.9ng Z eqvs 10^{-6} cells, one of these being obtained by the addition of the activity levels of four different cytokinins. The 65 hour peaks reported by Firby were approximately 0.7ng Z eqvs 10^{-6} cells in both cases.

Clearly, even when the same techniques are employed with the same cells, identical results are not achieved. Such variations may be attributed to some extent to differences between cultures and unequal losses in the extractions. It is important to emphasise the point that trends in activity levels are far more important than actual levels when bioassay results are being analysed. Another factor to be considered with the results from this particular experiment is that each sample point was obtained from different litre bottle cultures rather than from one large culture, and thus the data may be distorted because some cultures will grow at a faster rate than others. This point is of

particular significance when the activity levels are rapidly changing, as they are likely to do during a synchronous first division when almost every cell in the culture is at the same point in the cell cycle and they are thus producing and ceasing to produce substances simultaneously. Thus, if one culture is out of step with another, spurious fluctuations may be observed. This problem is less acute as the cells develop asynchrony because the peaks and troughs of activity are averaged out by the cells being at different points in their cycles anyway, and thus a base level develops. As the culture moves towards the stationary phase cell division declines and thus the cells probably produce less cytokinin anyway; this trend is apparent from the results of all the previous researchers discussed here.

Because of the problems involved with the close sampling of litre bottles and the difficulty in interpreting data from cells exhibiting asynchronous growth, further experiments involved the use of large scale 21 litre batch cultures and the first division period only was investigated.

Two 21 litre batch cultures were sampled through the first synchronous division. One of these cultures was sampled regularly and 1000cm^3 of culture was analysed at each time point for total cytokinin activity. The second culture was inoculated simultaneously and less regular samples of 2000cm^3 were taken. These samples

were analysed for zeatin (Z), zeatin riboside (ZR), dihydrozeatin (DHZ) and zeatin-O-glucoside (Z-O-G) activity. The cucumber cotyledon bioassay is inaccurate at levels < 30ng Z eqvs per sample, and thus 2000cm^3 samples were taken for the individual cytokinin estimations to ensure that some activity was detected when the extracts were fractionated. The results from these two batch cultures are tabulated in Tables 3.7, 3.8 and 3.9, and are illustrated in Figures 3.11, 3.12 and 3.13. The majority of the cells of both cultures underwent cytokinesis at 72 hours and therefore the two cultures can be regarded as comparable.

The inoculation levels of 0.17 and 0.2ng Z eqvs 10^{-6} cells are similar to those obtained by Mackenzie and Street (1972) for Cytokinin 1, Firby's 5 hour sample level (0.27 ng Z eqvs 10^{-6} cells) and the previous 3 hour level obtained. However, all of these inoculation levels are below the 0.6ng BA eqv 10^{-6} cells reported by Robinson (1982).

The total 19 hour peaks obtained from both cultures (ie the total of the four individual cytokinins in the case of the second culture) were identical, with an activity level of 0.46ng Z eqvs 10^{-6} cells. However, this value is below that obtained in the previous experiment, (0.65ng Z eqv 10^{-6} cells) and far lower than Firby's levels which were approximately 0.9ng Z eqvs 10^{-6} cells in both cases.

TABLE 3.7

Changes in cell number, dry mass and total cytokinin activity through the first division of <u>Acer pseudoplatanus</u> cells grown in batch cell suspension culture.

Culture Age (Hrs)	Cell Number $\times 10^{-3}$ cm^{-3}	Dry Mass mg cm^{-3}	Total Cytokinin Activity	
			ng Z eqvs 10^{-6} cells	ng Z eqvs g^{-1} DM
0	232.8 +/- 9.3	0.64	0.17	62.5
3	243.2 +/- 14.8	0.62	0.12	48.4
10	247.2 +/- 15.9	0.68	0.20	73.5
19	240.0 +/- 10.5	0.65	0.46	169.2
24	249.5 +/- 7.4	0.71	0.20	70.4
30	224.3 +/- 14.5	0.63	0.18	63.5
42	232.2 +/- 14.5	0.70	0.13	42.9
48	225.9 +/- 15.8	0.66	0.22	75.8
60	252.8 +/- 7.1	0.72	0.24	83.3
66	232.8 +/- 14.7	0.77	0.26	77.9
70	257.0 +/- 9.3	0.81	0.16	49.4
72	433.5 +/- 13.8	0.80	0.09	50.0
75	426.7 +/- 11.7	0.84	0.09	47.6
80	428.6 +/- 30.9	0.87	0.07	34.5
96	510.5 +/- 18.9	0.92	0.06	32.6
120	713.2 +/- 26.3	1.24	0.04	24.2
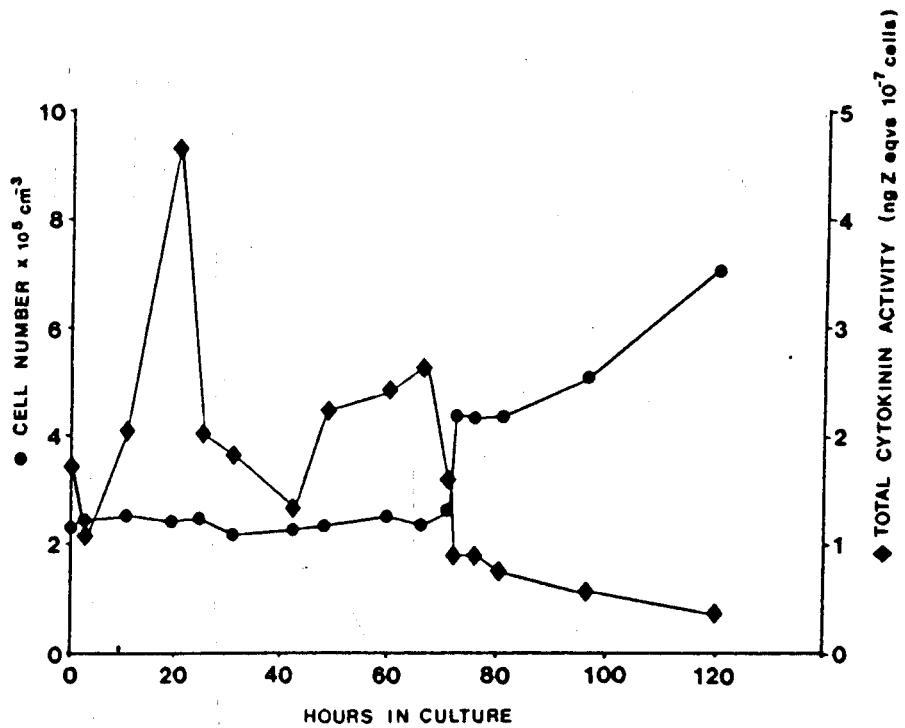
Figure 3.11

Changes in the endogenous total cytokinin activity (ng Z eqvs 10^{-7} cells) of _Acer_ _pseudoplatanus_ cells during the cell cycle.

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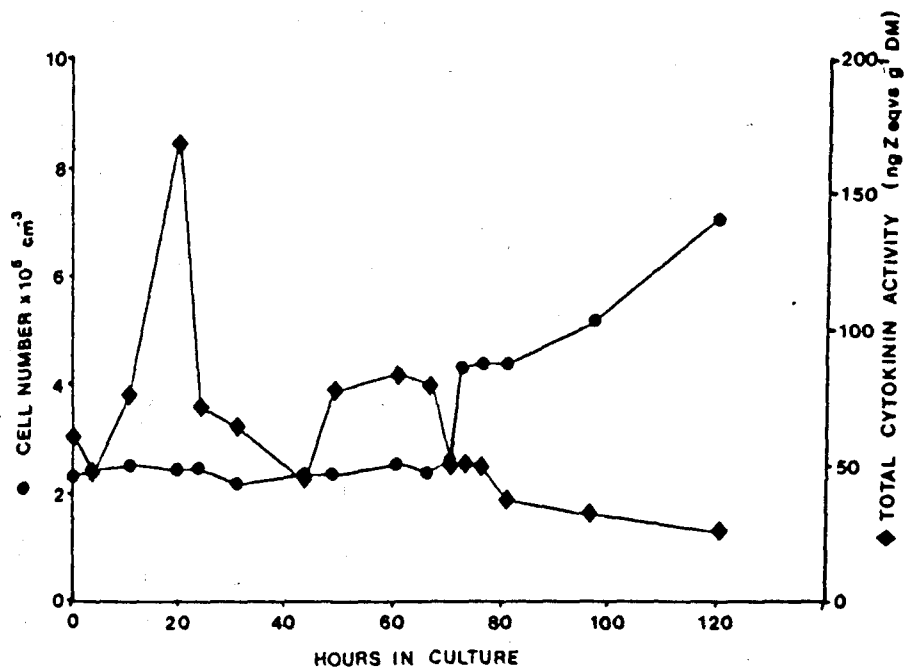
Figure 3.12

Changes in the endogenous total cytokinin
activity (ng Z eqvs g^{-1} DM) of <u>Acer</u>
<u>pseudoplatanus</u> cells during the cell cycle.

TABLE 3.8

Changes in the activity levels of four endogenous cytokinins (ng Z eqvs 10^{-6} cells) of <u>Acer pseudoplatanus</u> cells during the cell cycle.

Culture Age (Hours)	Cell Number $\times 10^{-3}$ cm^{-3}	Cytokinin activity (ng Z eqvs 10^{-6} cells)				
		Z	ZR	DHZ	Z-O-G	TOTAL
0	246.1 +/- 10.1	0.12	0.08	-	-	0.20
19	237.7 +/- 9.8	0.22	0.08	0.09	0.07	0.46
30	251.6 +/- 7.2	0.14	0.06	-	-	0.20
66	243.5 +/- 11.8	0.28	0.09	-	-	0.37
70	239.9 +/- 13.4	0.18	-	-	0.07	0.25
72	450.2 +/- 15.3	0.09	-	-	-	0.09
75	434.8 +/- 11.6	0.09	-	-	-	0.09
96	471.3 +/- 12.2	0.04	0.04	-	-	0.08
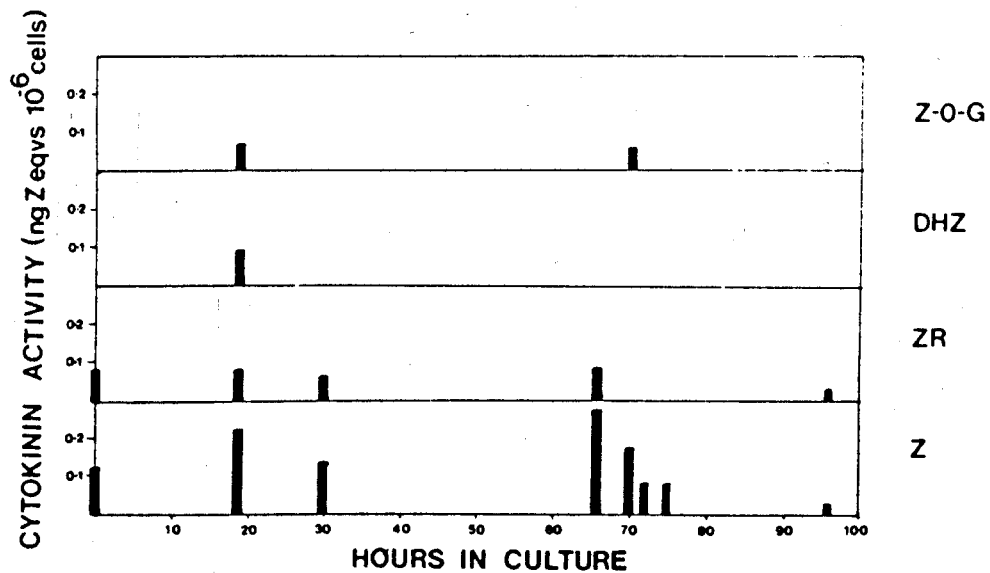
Figure 3.13

Changes in the activity levels of four
endogenous cytokinins (ng Z eqvs 10^{-6} cells)
of <u>Acer pseudoplatanus</u> cells during the
cell cycle.

TABLE 3.9

Changes in the activity levels of four endogenous cytokinins (ng Z eqvs g^{-1} DM) of <u>Acer</u> <u>pseudoplatanus</u> cells during the cell cycle.

Culture Age (Hours)	Dry Mass (mg cm^{-3})	Cytokinin Activity (ng Z eqvs g^{-1} DM)				
		Z	ZR	DHZ	Z-O-G	TOTAL
0	0.63	47.3	32.1	-	-	79.4
19	0.64	81.9	31.5	32.7	25.8	171.9
30	0.65	53.0	23.9	-	-	76.9
66	0.71	96.2	30.6	-	-	126.8
70	0.78	55.6	-	-	21.3	76.9
72	0.79	50.6	-	-	-	50.6
75	0.81	49.4	-	-	-	49.4
96	0.88	21.5	24.0	-	-	45.5

The sampling time points were concentrated close to the expected point of cytokinesis in an attempt to clarify the relative position of the second peak in cytokinin activity. In both cultures the second peak achieved its maximum level at 66 hours, producing levels of 0.26 and 0.37ng Z eqvs 10^{-6} cells. These levels are comparable to those of Mackenzie and Street (1972) and with the previous experiment, if one assumes that the peak of activity seen was distorted by the "litre bottle effect". However, they are far lower than the peaks of approximately 0.95ng BA eqvs 10^{-6} cells and 0.7ng Z eqvs 10^{-6} cells reported by Robinson (1982) and Firby (1985) respectively.

The baseline levels during the stationary phase were again in the region of 0.1ng Z eqvs 10^{-6} cells which is consistent with all the previous results.

Thus, it appears that there are two pronounced peaks in total cytokinin-like activity during the cell cycle, one 19-20 hours after inoculation and a second slightly preceding cytokinesis, and therefore at approximately 65-70 hours depending on the culture. The differences in the actual levels between experiments might also be explained by the apparent sharpness of the first peak, some samples may produce lower levels at this time point simply because the actual peak in endogenous cytokinin-like activity occurred merely an hour or so beforehand or indeed had not yet occurred.

This argument can be applied for the whole culture period and as such a recommendation for future work would be that at least hourly samples should be taken, as the levels can fluctuate so rapidly. Unfortunately, the bioassay system used here requires at least 400cm^3 of culture to be sampled at each time point to ensure that at least the total activity is above the detectable 30ng level. This would mean that immensely large culture volumes would be necessary and also the risk of culture infection would be increased because of the large number of samples taken. If a more sensitive assay system were employed, for example RIA, then smaller sample volumes may be adequate.

Although total cytokinin activity is undoubtedly important it is likely that specific cytokinins have specific roles during the cell cycle. The data obtained from the second 21 litre batch culture (Tables 3.8, 3.9 and Figure 3.13), reveal zeatin to be the main cytokinin activity present, being the predominant constituent of the 19 and 66 hour peaks. Zeatin riboside-like activity was found to be present at a relatively constant level until cytokinesis, after which none was detected until 96 hours after sub-culture. Dihydrozeatin was only found to be present within the 19 hour peak and zeatin-O-glucoside was found only within the two peaks of activity.

Robinson (1982) detected materials which co-chromatographed with both endogenous zeatin and zeatin riboside and discovered no noticeable difference in their patterns of occurrence. Firby (1985) investigated the endogenous levels of zeatin, zeatin riboside, zeatin-O-glucoside and zeatin-9-glucoside. She noted that the zeatin and zeatin riboside-like activity levels appeared to fluctuate such that when one was at a maximum the other was at a minimum and vice versa; this phenomenon was not seen in the present experiment, although similar results were obtained in preliminary experiments. In both investigations zeatin-like activity was found to be consistently higher than the other cytokinins estimated. However, Firby did not detect the presence of zeatin until 15 hours after inoculation, nor was any zeatin activity detected after the peak associated with cytokinesis. Conversely, high levels of zeatin riboside were detected at these low zeatin stages in the cell cycle. In this present study zeatin was detected throughout the cell cycle although levels were low at the above mentioned times. In both studies zeatin-O-glucoside was found during the two peaks in activity; Firby also investigated zeatin-9-glucoside which was only found during the 20 hour peak.

These data require careful consideration as there are again differences between the two sets of results. However, it is likely to be significant that all of the cytokinins in both cases were detected in the 19-20 hour

activity peak, implying that this is an important stage in the cell cycle. Also, zeatin was consistently found to be the major component of the 65-66 hour peak, implying an involvement in cytokinesis. It is probable that all of the cytokinins analysed, and others not investigated here, have independent and/or synergistic effects in the cell cycle. It must be remembered that any apparent absence of activity at a time point may simply be a reflection of the lack of sensitivity of the bioassay. Another factor to note at this point is that the different cytokinins may have different active concentration ranges, so that a low concentration for one cytokinin may be an optimal peak concentration for another.

Interpretation of the levels of the independent cytokinins detected is difficult because the functional significance of the various forms and the control of their interconversions is not fully understood (Letham and Palni, 1983). The concentrations normally present in the whole plant are dynamic and are regulated by enzymatic interconversions, degradation, compartmentation and transportation. In whole plants there are many reports of cytokinins being found in phloem and xylem exudates (Van Staden and Davey, 1979), but in closed non-differentiated cell suspension cultures such a system cannot exist. This is substantiated by the fact that higher endogenous levels of cytokinins have been detected in <u>Beta vulgaris</u> cells maintained in

suspension culture than in the whole plant (Hosford, 1984). Cells in suspension culture can only remove cytokinins non-metabolically by allowing them to accumulate in the bathing media. Robinson (1982), noted that the level in the medium remained relatively low and constant throughout the growth cycle despite endogenous fluctuations. It would therefore appear that leaching out into the media is not the primary mechanism for lowering the concentrations of unwanted endogenous cytokinins and thus it is likely that some degradation, interconversion or compartmentation must occur. However, the fact that the level in the culture medium may be low compared to the cellular levels must not lead one to disregard such levels as insignificant. Mackenzie and Street (1972), pointed out that because the PCV of their _Acer pseudoplatanus_ cultures was approximately 20% of the culture volume, it followed that 50% of the cytokinin activity of a 17 day culture may be in the bathing medium. Media levels were not assessed in this study, but future work may reveal some media level fluctuations especially if close sampling was concentrated around the 19 hour peak of endogenous activity. However, due to the dilution factor any rise in activity would be less noticeable than it is in the cells.

The levels of zeatin, zeatin riboside, zeatin-O-glucoside and dihydrozeatin may fluctuate during the cell cycle due to their interconversion. It is believed

that there is interconversion between the free bases, nucleosides and nucleotides (Letham and Palni, 1983), and it is apparent that there is a general relationship between the form in which a cytokinin is present and its relative activity. Thus it is conceivable that cytokinins can be converted to more or less active forms depending on the plant's requirements at a specific time.

It is generally accepted that the glucosides are storage forms of cytokinins which are less active than the free bases. Thus the functional level of cytokinin activity is temporarily lowered but the potential for the restoration by interconversion is still present if necessary. The O-glucosides are relatively stable and are known to accumulate in mature leaves; it is possible that the plant's ability to produce glucosides increases with age, (Wareing et al., 1977; Palmer et al., 1981a) as they are almost undetectable in young leaves (Henson, 1978). The O-glucosides are the main metabolites of both exogenously applied and endogenous cytokinins (Letham et al., 1982). The free base, zeatin, is released on hydrolysis by the enzymic action of β-glucosidase (Letham and Palni, 1983). There is much confirmatory evidence that the O-glucosides are storage forms of both the free bases and ribosides. For example, during cytokinin accumulation the increase in the O-glucoside metabolite activity is known to be noticeably more than that of the bases and ribosides

(Summons et al., 1980; Palmer et al., 1981b). Also, there is evidence that applied O-glucosides convert to the free cytokinin bases and ribosides. During stages of plant growth and development where active cytokinins are believed to be involved the levels of the O-glucosides have been seen to decline rapidly, implying a possible conversion to the active forms (Smith and Van Staden, 1978; Palmer et al., 1981b). Van Staden and Dimalla (1978), noted that the decrease in O-glucoside levels concurrent with the breaking of dormancy in potato tubers was reflected in increases in the activity of a compound which cochromatographed with zeatin riboside. When root to shoot transport is impeded glucosides are known to accumulate in the roots; thus, in the whole plant the roots may provide a cytokinin store in the form of low-activity glucosides which can be converted to highly active cytokinin forms and transported to the required sites (Van Staden and Dimalla, 1977; Van Staden and Smith, 1978).

The formation of 7- and 9-glucosides, alanine conjugates and side chain cleavage to adenine and its derivatives may also provide a mechanism for lowering the physiological level of cytokinin activity in plant tissues (Letham and Palni, 1983). The 7- and 9-glucosides have enhanced metabolic stability and are far less active than their corresponding free bases. It is thought that they are not actually active forms of cytokinin. They are resistant to hydrolysis by

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β-glucosidase and are believed to be "bound" or deactivated forms which are produced to lower the plant's physiological cytokinin level, perhaps by cellular compartmentation (Letham and Palni, 1983).

The cytokinins are known to be present in both the xylem and phloem of plants (Skene, 1975; Letham, 1978; Goodwin et al., 1978; Van Staden, 1976a,b). Translocation in the phloem is thought to be bi-directional and Van Staden has proposed that in woody plants there may be recycling of the cytokinins in autumn. The ribosides are thought to be the predominant translocated form of cytokinin in the xylem (Goodwin et al., 1978; Letham, 1978).

Research into the nucleotides was initially hindered because of high losses from methanol extraction techniques and also their susceptibility to hydrolysis by non-specific plant phosphatase (Bieleski, 1964; Horgan, 1978). Recently the advent of sophisticated extraction and estimation techniques for nucleotides using HPLC systems (Scott and Horgan, 1982) has facilitated research in this area. However, their specific roles are still little understood although they are believed to be associated with cytokinin uptake and transport across membranes (Laloue et al., 1977) and may be important translocated forms of cytokinin (Skene, 1975; Letham, 1978). They have also been implicated in biosynthetic pathways (Horgan et al., 1981).

The free base is believed to be the main active form of cytokinin (Letham and Palni, 1983), and the regulation of the interconversions between the storage (O-glucoside) forms and the active free base is thought to be enzymic. Whitty and Hall (1974), reported the presence of an oxidase-like enzyme in <u>Zea mays</u> kernels which was capable of cleaving isopentenyl-type side chains. The saturated side chains were unaffected by this enzyme. This phenomenon may explain the known stability of dihydrozeatin O-β-D-glucoside (DHZOG) relative to zeatin-O-β-glucoside (ZOG) (Wareing <u>et al</u>., 1977). The interconversion processes are undoubtedly very complex and are as yet little understood. It would seem though that the different cytokinin forms do have specific roles in the whole plant and that their interconversion is important in transportation, storage and in maintaining the required levels of active cytokinins during growth and development.

In summary, the individual cytokinin levels estimated in this study follow the same basic patterns as those obtained by previous workers with the exception that Z and ZR were not found to fluctuate inversely with each other as reported by Firby (1985). It appears that zeatin is the predominant endogenous cytokinin present in these cells with peaks in activity at 19 and 66 hours after inoculation. It is therefore tentatively concluded that zeatin is the main active cytokinin in this system. Although the levels of ZR, (a possible

translocatable form of cytokinin with the potential for conversion to Z) are relatively constant throughout the cell cycle, there is an absence of ZR-like activity during the latter hours of the broad 66 hour Z peak which may be a reflection of conversion to the free base. Z-O-G, a possible storage form of cytokinin, was only detected during the first peak and slightly after the second peak of total cytokinin activity. This may be because the endogenous levels present at other stages of the cell cycle were too low to be detected by this bioassay. If Z-O-G is a storage form one might have expected the levels to have dropped as the levels of ZR and Z increase, and _vice versa_. However, this does not seem to be the case here, although during the second broad peak in total activity the Z and ZR increases preceded the Z-O-G increase, possibly implying that the Z is being converted either directly from Z or via ZR to Z-O-G possibly to lower the physiologically active level of cytokinin or to prevent Z breakdown. Later in the cell cycle, (96 hours), the ZR level increased again and there was an absence of Z-O-G, (or undetectable levels), possibly due to a reversal of this conversion process. No Z-O-G was detected at inoculation where one might have expected to see stored cytokinin in the old inoculum cells. However, leaf tissues are thought to be the major site of cytokinin glucosylation and therefore as these cells were extracted from cambial tissue care must be taken when interpreting results. These cells may not be capable of efficient cytokinin glucosylation

and may rely more on direct synthesis of cytokinins than interconversion. The DHZ was only detected during the first 20 hour peak and may be a means of lowering the physiological level of Z,or the DHZ may have a specific role itself at this stage in the cell cycle.

Only tentative conclusions can be drawn from the data obtained here as bioassay results are only able to reveal trends in activity levels rather than accurate quantification of definite growth substances. Ideally this work should be repeated using an HPLC system and the growth substances should be unequivocally identified by GC-MS. The extraction losses were not estimated here but they are assumed to be equal, at least for each cytokinin estimated. There do appear to be fluctuations between the different cytokinin forms but more sample points with a more sensitive estimation system are needed before any unequivical conclusions can be drawn. Nevertheless, the underlying trends in all of the results presented in this section on cytokinins are of peaks in activity at approximately 19 and 66 hours after inoculation. This trend was also found by Firby (1985) and Elliott et al., (1986). However, Hosford's sugar beet cells produced an initial peak in activity at 19 hours but the second peak in this case was at approximately 50 hours. In these cells cytokinesis occurred earlier (55 hours) than in sycamore cells (72 hours), and this substantiates the link between this second peak and mitosis and/or cytokinesis. In both

cases there is a similar interval of approximately 5-6 hours between this peak in activity and the onset of cytokinesis.

Several workers have reported peaks of cytokinin-like activity coincident or slightly preceding active cell division in cell suspension cultures. In Acer pseudoplatanus cells this has been noted by Mackenzie and Street (1972), Robinson (1982), who reported successive peaks corresponding to cold-induced partially synchronous divisions, and Firby (1985). In tobacco cell suspension cultures Nishinari and Syono (1980) reported elevated endogenous cytokinin-like activity which paralleled the increase in the mitotic activity. In sugar beet cell suspension cultures, Hosford (1984), reported increased levels of cytokinin associated with the period of rapid cell division.

Cytokinins have always been regarded as being linked in some way to cell division in the whole plant (Section 1.1.2). The fact that this association is also prevalent in cell suspension cultures is encouraging in the light of criticism of interpretation of results from closed cell suspension cultures. The advantage of using such cultures as opposed to whole plants in this kind of research is that they exhibit a high degree of first division synchrony and are also amenable to various

forms of induction synchrony. Thus the timing of the peaks in cytokinin-like activity can be relatively accurately estimated with respect to the various stages of the cell cycle.

Cell cycle time variations are generally believed to be associated with the expandability of the G0/G1 phases, (Section 1.5). The sycamore cells used in this present study usually have a cell cycle time of approximately 73-75 hours. The G0/G1 phases occupy more than half of the cycle time period with the onset of DNA synthesis, (the S phase), usually occurring approximately 45 hours after inoculation. The S phase is complete after a period of approximately 12.5 hours (approx 57.5 hours after inoculation). The G2 phase is far shorter than the G1, occupying about a tenth of the cell cycle (7.5 hours) and thus mitosis is usually initiated 65 hours after transfer. The remaining 8-10 hours of the cycle are concerned with mitosis and cytokinesis; as these two processes are relatively rapid it is difficult to differentiate between them in the time scale. Generally mitosis is complete by 70 hours and the cells have divided by 73-75 hours, the cycle then proceeds once more. Thus, to determine whether a metabolic event, such as a peak in growth regulator activity, is associated with mitosis or cytokinesis is not always straight forward. In this case the 66 hour cytokinin activity peak is coincident with the onset of mitosis, however, it may also be associated with

cytokinesis as it precedes the division and any peak closely preceding a division would have to occur near to or during mitosis. Zeatin and zeatin riboside are the main components of the 66 hour peak, however, by 70 hours no ZR activity was detected but Z activity was still present along with some zeatin-O-glucoside activity. It may be tentatively concluded that Z and ZR are associated with mitosis but ZR is not necessary for the process of cytokinesis, whereas Z and Z-O-G are. For reasons discussed in detail earlier in this section, these conclusions must be considered with caution.

The cytokinin-like activity detected appeared to increase gradually (with some fluctuations) from about 40 hours after inoculation before reaching a maximum before cytokinesis. This is the period during which the cells synthesize their DNA in preparation for mitosis. Firby, (1985) found zeatin to be the predominant cytokinin during this period, but suggested that as the peak did not actually precede, but was coincident with DNA synthesis, the two events may be unconnected.

The acute 19 hour peak in cytokinin-like activity is less obviously linked with the cell cycle. It occurs during the G1 phase, or, if one considers the existence of a G0 phase and a defined G1 phase of approximately 25 hours (as postulated by Firby in 1985), at the end of the G0 and the beginning of the G1 phase.

An analysis of the total protein present in these sycamore cells (Firby, 1985) revealed plateaux levels at 20 and 40 hours and another increase preceding cell division at 65 hours. These three stages of the cell cycle were where the most cytokinin-like activity was found both in this present study and in previous investigations. The cytokinins are believed to regulate the cell cycle by controlling the rates of protein synthesis. Thus, by regulating the amount and type of proteins present the cytokinins may trigger further complex steps which allow the completion of the cell cycle.

The exact process of protein synthesis regulation and the other subsequent trigger factors involved are not fully known. Experiments involving cytokinin-dependent cultured soybean cells have shown that on the addition of zeatin there is rapid polyribosome formation preceding cytokinesis, (Fosket and Short, 1973; Short et al., 1974). As the polyribosome content is an indicator of the protein synthesis potential, (Beevers and Poulson, 1972; Payne et al., 1971; Travis and Key, 1971) this experimental evidence led to the conclusion that the cytokinins regulate the cell cycle by an effect on the rate of protein synthesis (Short et al., 1974). However, the association between the cytokinins and the induction of protein or polyribosome synthesis was questioned by Fosket and co-workers (1977). They noted that when soybean cells were transferred to fresh

medium, polyribosome synthesis was induced regardless of whether or not the new medium contained cytokinin , but the cells lacking cytokinin failed to divide. The hypothesis that cell division may be regulated by specific proteins was proposed when it was noted that such cytokinin treatments altered the spectrum of proteins synthesized, (Fosket et al., 1977). After cytokinin application specific proteins have been found to be synthesized in soybean cells (Fosket et al., 1977; Tepfer and Fosket, 1978) and tobacco cells (Jouanneau, 1970). It is believed that these specific proteins are rate-limiting for cytokinesis (Fosket and Tepfer, 1978). Polyribosome formation may be brought about by an effect of cytokinins on protein synthesis at the translational level. Cytokinins may stimulate protein synthesis by inducing the conversion of untranslated mRNA into polysomes (Short et al., 1974; Tepfer and Fosket, 1978). Ralph and co-workers (1980) proposed that cytokinins may influence mRNA selection by affecting the distribution of ions in plant cells, if certain mRNAs are preferentially utilized under different ionic conditions. There is evidence that the cytokinins affect cation availability (Ralph et al., 1976; Fosket et al., 1977) and it is believed that ions and ion fluxes are involved in the regulation of protein synthesis (Carrasco and Smith, 1976; Pollock and Fischer, 1976) and the progression of cells through the mitotic cycle (Pardee et al., 1978; Koch and Leffert, 1979).

Thus, cytokinins may regulate the cell cycle by altering ion fluxes which regulate specific protein synthesis essential for the continuation of the cell cycle.

The pattern of the changes in the levels of the endogenous cytokinin-like activity present during the first synchronous cell division of Acer pseudoplatanus cells in suspension culture is consistent between the different cultures investigated. The actual levels vary depending on the sample time point and the limitations of the estimation technique. The role of cytokinins as G2 regulators is well documented and paralleled cytokinin and mitotic activity levels (Short and Torrey, 1972a,b; Nishinari and Syono, 1980) have implicated the cytokinins as mitotic regulators. The close proximity of mitosis to cytokinesis complicates the research into the regulation of the cell cycle, but as cytokinin-dependent cultures exposed to a cytokinin starvation regime exhibit G2 arrest this implies a G2 regulatory point. The approximately 66 hour peak in activity, at or before mitosis and/or cytokinesis may induce the synthesis of specific proteins necessary for one or both of the processes to proceed. In this study the cytokinin levels appeared to peak at or before mitosis, but as noted earlier this does not rule out the possibility of this peak actually regulating cytokinesis directly. The 19 hour peak in activity is relatively sharp and could easily be missed by a less intensive sampling regime. It is very likely to be associated

with protein synthesis and polyribosome formation and as these are pre-requisites for cell division, this is also a possible point in the cell cycle where the cytokinins have a regulatory role. The period of protein synthesis approximately 40 hours after sub-culture, is also reflected by cytokinin increases and thus the cytokinins probably have an essential role at this period also. The preliminary study of the changes in the levels of the four different cytokinins investigated here reveal dynamic profiles implying rapid interconversion and possibly degradation, export and compartmentization. It is likely that there are other cytokinins present in these cells each with different roles to play at the various regulatory points tentatively outlined here.

3.3.3 Quantitative_Analysis_of_the_Endogenous_IAA_Levels of_Acer_pseudoplatanus_cells

Although these Acer pseudoplatanus cells are routinely grown in a culture medium containing the auxin 2,4-D, they are capable of synthesizing IAA (Moloney, 1979; Robinson, 1982; Farrimond, 1978; Weston et al., 1978). The previous investigations in these laboratories into the endogenous IAA of sycamore cells have revealed elevated levels at Day 3 of batch culture growth; that is, close to or coincident with the first synchronous division. A 45 hour peak in IAA levels was also observed by Firby in 1985. This preceded DNA synthesis and thus led to Firby making the tentative conclusion

that endogenous IAA may act as a trigger or possible initiator for DNA replication in the _Acer pseudoplatanus_ cell cycle.

In this study the endogenous IAA was extracted and estimated essentially as described by Blakesley et al., (1983) (Section 2.11). This method involved the simultaneous extraction of IAA and ABA; the growth substances were then separated during the ion-pair reverse phase chromatography purification step. Good separation of authentic IAA, c,t-ABA and t,t-ABA was obtained by ion-pair reverse phase chromatography (Figure 3.14). An HPLC chromatogram showing the purification of an _Acer pseudoplatanus_ extract can be seen in Figure 3.15. Although as expected, the chromatograms reveal the presence of other unwanted substances, the peaks relating to IAA, t,t-ABA and c,t ABA are visible. These relevant fractions were collected from the HPLC in separate vials. The IAA was derivatized to 2-methylindolo-2,3:3',4'-pyr-6-one (2 MIP) as described in Section 2.11.3 and was then subjected to analytical HPLC (Section 2.11.4). The fluorescence trace obtained after the analytical HPLC of a derivatized IAA standard is shown in Figure 3.16. The peak obtained is sharp and the baseline is very stable; this facilitates the accuracy of peak height measurements. A typical fluorescence trace obtained after the analytical HPLC of a derivatized purified _Acer pseudoplatanus_ cell sample is shown in Figure 3.17. In
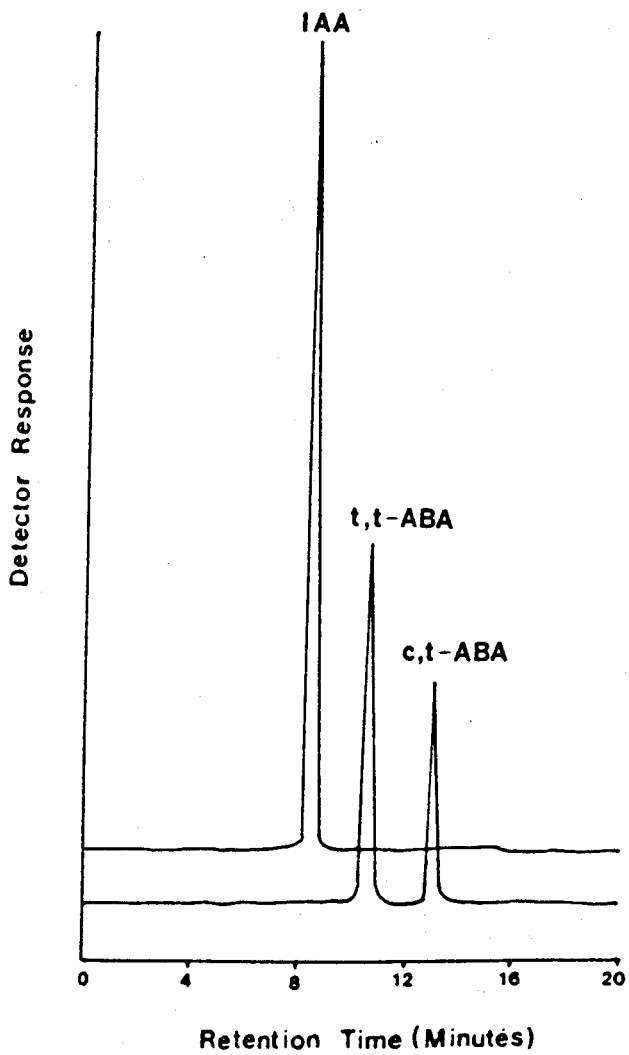
Figure 3.14

HPLC chromatogram of IAA, t,t-ABA and c,t-ABA
standards separated by ion pair reverse phase
chromatography.
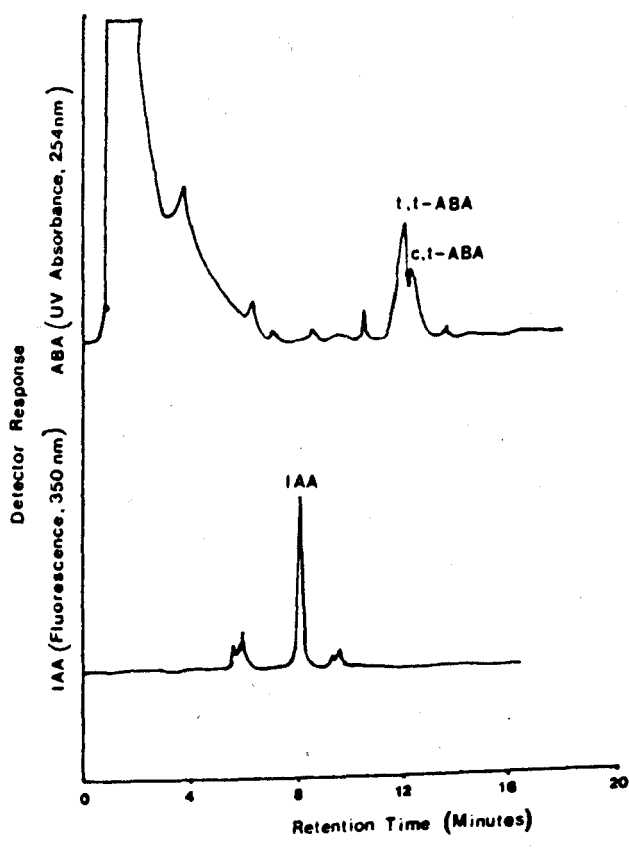
Figure 3.15

HPLC chromatogram obtained by subjecting
purified <u>Acer pseudoplatanus</u> cells to
preparative HPLC.
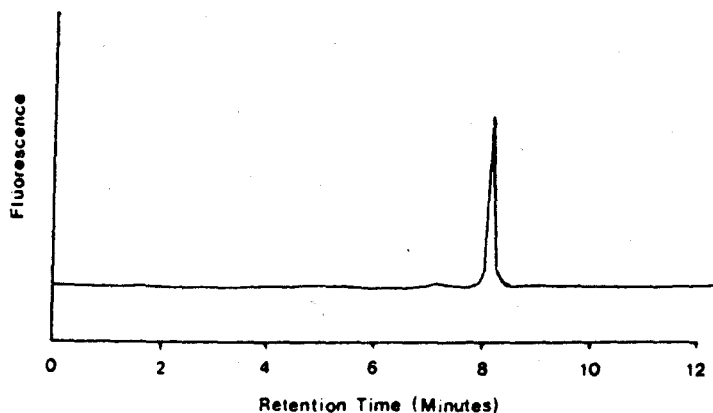
Figure 3.16

Analytical HPLC chromatogram of 2-MIP produced
by derivatization of IAA with acetic anhydride
- TFA.
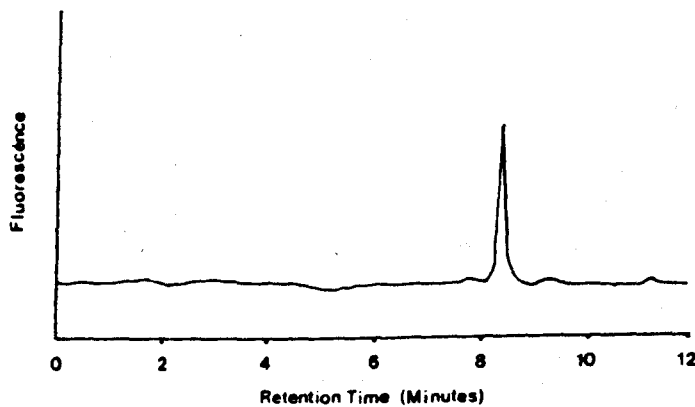
Figure 3.17

Analytical HPLC chromatogram of purified
Acer pseudoplatanus cells following
derivatization with acetic-anhydride - TFA.

this case the peak is very slightly broader and there are small baseline fluctuations but a reasonably accurate peak height measurement can still be obtained.

The relevant fraction was again collected from the HPLC in order that the amount of radioactivity present could be estimated in a liquid scintillation counter. During the extraction procedure there is a degree of IAA breakdown along with the inevitable losses which occur during the numerous transfers of the extract from one receptacle to another. There are also likely to be losses at the derivatization stage. As the amount of radioactivity in the original internal standard is known, the percentage recovery can be calculated. However, there is a further complication in that the breakdown products of ^{3}H IAA are also likely to be radioactive and may elute off the HPLC column close to the IAA. This would be a real problem if the IAA eluted slowly off the column producing a broad peak. In this case sharp IAA peaks were always obtained and thus this phenomenon was considered to produce a negligible affect on the radioactivity recoveries.

A standard calibration curve was drawn by plotting peak height against the amount of the derivative 2-MIP, (Figure 3.18). There is a linear relationship between peak height and the quantity of 2-MIP. The amount of IAA present in the original sample was then calculated as described in Section 2.11.4.
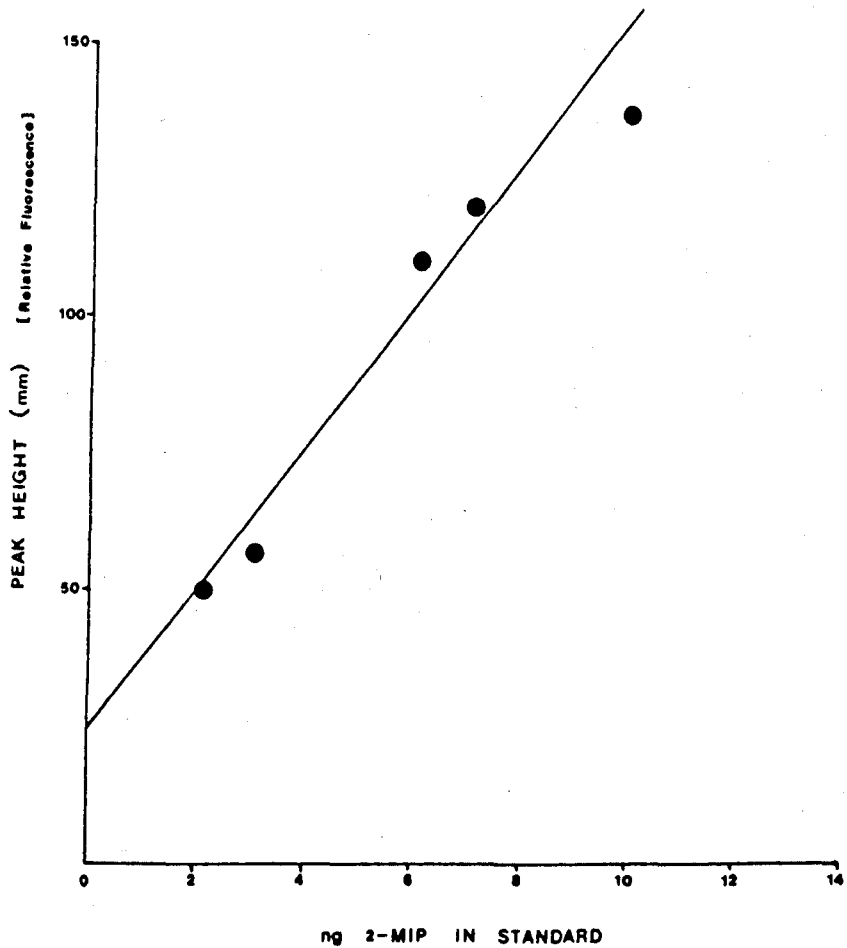
Figure 3.18

Calibration curve for 2-MIP standard obtained
by derivatizing (3H) IAA with acetic anhydride
- TFA and subjecting the derivative to
analytical HPLC.
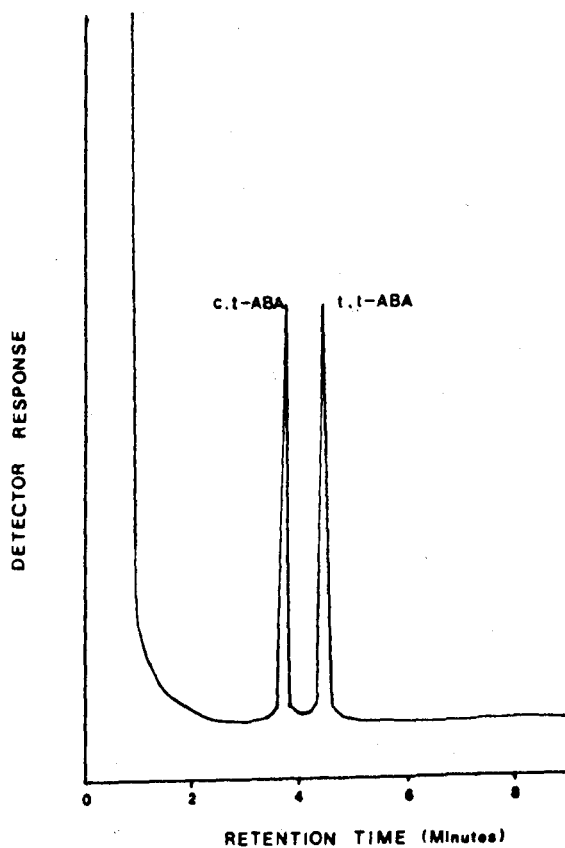
Figure 3.19

GC-ECD chromatogram showing separation of the methyl esters of c,t-ABA and t,t-ABA.
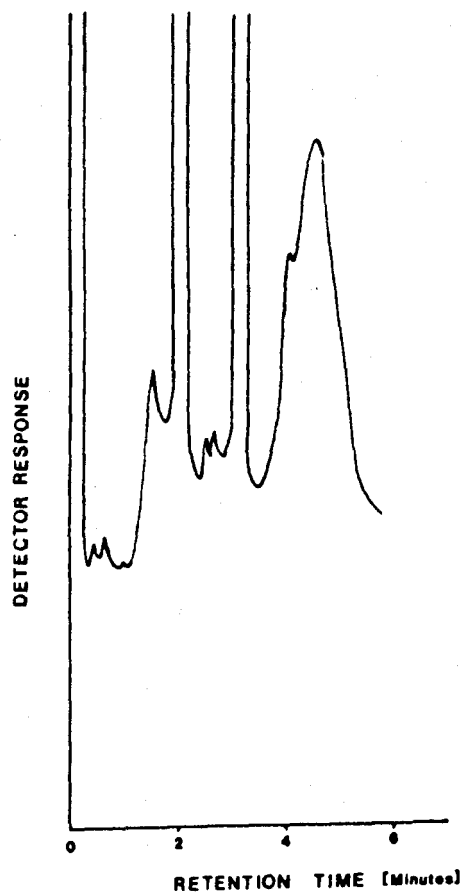
Figure 3.20

GC-ECD chromatogram of purified Acer
pseudoplatanus cells.

The ABA fraction was then subjected to GC-ECD. Good separation of c,t and t,t-ABA standards was obtained (Figure 3.19) but unfortunately the chromatograms obtained from the sample fractions revealed the necessity for further purification (Figure 3.20). However, there was not time available in this present study for any further ABA extractions.

The endogenous IAA levels of <u>Acer pseudoplatanus</u> cells maintained in batch culture have been comprehensively investigated in these laboratories. Similar trends have been revealed by several workers with high levels being observed around mitosis. The exact levels, despite sophisticated estimation techniques, did however reveal some variability. This may be accounted for by rapid fluctuations in levels. Therefore, in this present study, only the first synchronous division after transfer was investigated, with the sample points being concentrated around mitosis. One litre bottle, containing 490cm^3 of culture, was harvested for each time point with 480cm^3 being used for the actual IAA analysis.

The results are tabulated in Table 3.10 and are expressed on a per cell and a per gram dry mass basis in Figures 3.21 and 3.22 respectively. The experiment was also repeated with similar time points; the corresponding data are tabulated in Table 3.11 and illustrated in Figures 3.23 and 3.24.

In the first experiment (Figures 3.21 and 3.22) an inoculation level of 3.14 ng 10^{-6} cells (690.5 ng g^{-1} DM) was found. After 24 hours of culture the IAA level increased to 4.86 ng 10^{-6} cells (1401.0 ng g^{-1} DM), but the level then rapidly decreased to a level of 0.49 ng 10^{-6} cells (197.8 ng g^{-1} DM) by 42 hours. A sharp rise to a level of 1.87 ng 10^{-6} cells (576.2 ng g^{-1} DM) was observed at 72 hours and the levels then declined. Cytokinesis occurred between 92-97 hours after inoculation, the corresponding increase in the cell number was reflected by another increase in the IAA levels to 1.38 ng 10^{-6} cells (598 ng g^{-1} DM).

In the second experiment (Figures 3.23 and 3.24) the inoculation level was 6.96 ng 10^{-6} cells (2342.3 ng g^{-1} DM), more than twice that found previously. This level then rapidly declined to 1.94 ng 10^{-6} cells (689.6 ng g^{-1} DM) by 24 hours and continued to decrease at a slower rate until stabilizing at a level of approximately 0.6 ng 10^{-6} cells (200 ng g^{-1} DM) between 67-73 hours after inoculation. Cytokinesis occurred between 77-91 hours and this was reflected by an increase to a level of 0.86 ng 10^{-6} cells (445.1 ng g^{-1} DM) by 91 hours.

Despite differences in the timing of the fluctuations in the IAA levels between the two experiments the range of levels is similar. Both experiments involved the use of separate litre bottle batch cultures which were harvested at each time point. As discussed previously

Table 3.10

Changes in cell number, dry mass and endogenous IAA levels through the first division of *Acer pseudoplatanus* cells in suspension culture.

| Culture Age (Hrs) | Cell Number x 10^{-3} cm^{-3} | Dry Mass (mg cm^{-3}) | IAA levels |
			ng g^{-1} DM	ng 10^{-6} cells
0	242.6 +/- 11.8	1.10	690.5	3.14
24	276.4 +/- 8.5	0.96	1401.0	4.86
42	283.6 +/- 10.2	0.71	197.8	0.49
66	291.7 +/- 6.9	0.96	192.6	0.63
72	327.4 +/- 10.4	1.06	576.2	1.87
76	269.8 +/- 13.5	1.06	279.4	1.10
89	342.1 +/- 13.9	1.08	240.4	0.76
92	319.3 +/- 16.9	1.17	79.1	0.29
97	478.7 +/- 31.5	1.10	598.0	1.38

Table 3.11

Changes in cell number, dry mass and endogenous IAA levels through the first division of *Acer pseudoplatanus* cells in suspension culture.

| Culture Age (Hrs) | Cell Number x 10^{-3} cm^{-3} | Dry Mass (mg cm^{-3}) | IAA levels |
			ng g^{-1} DM	ng 10^{-6} cells
0	273.4 +/- 11.5	0.81	2342.3	6.96
24	296.0 +/- 18.1	0.83	689.6	1.94
43	331.4 +/- 9.8	0.71	462.0	0.99
67	331.7 +/- 5.0	1.00	202.7	0.61
73	354.3 +/- 8.1	1.15	192.7	0.62
77	331.0 +/- 11.6	0.77	344.8	0.80
91	665.7 +/- 24.2	1.29	445.1	0.86

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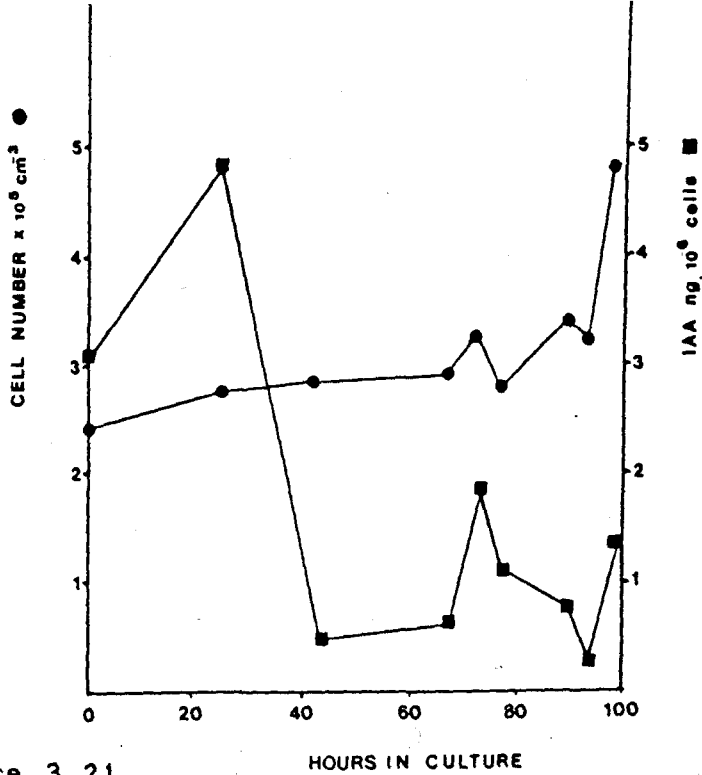
Figure 3.21

Changes in the endogenous IAA levels
(ng 10^{-6} cells) of <u>Acer</u> <u>pseudoplatanus</u> cells
during the cell cycle.
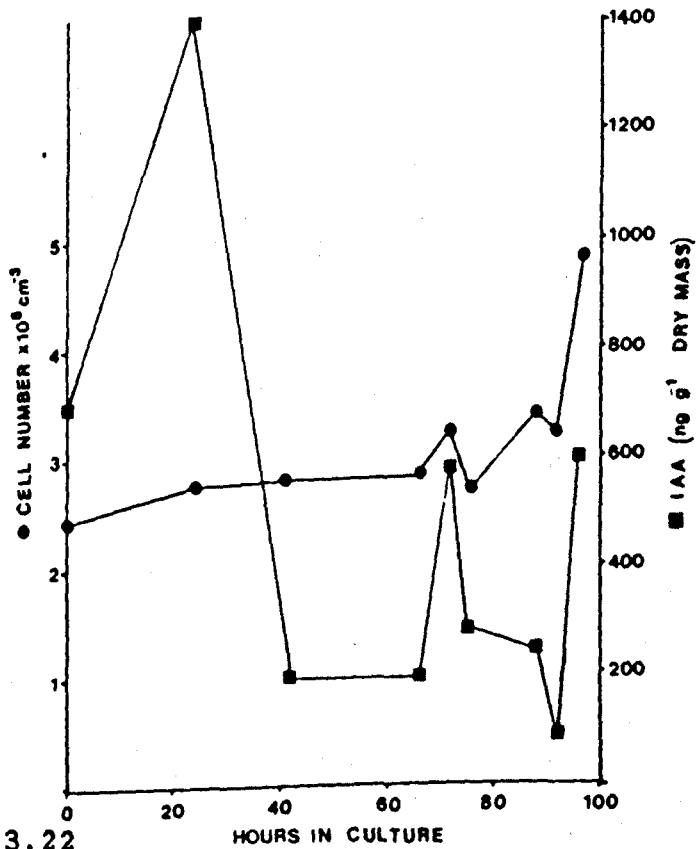
Figure 3.22

Changes in the endogenous IAA levels
(ng g^{-1} DM) of <u>Acer</u> <u>pseudoplatanus</u> cells
during the cell cycle.
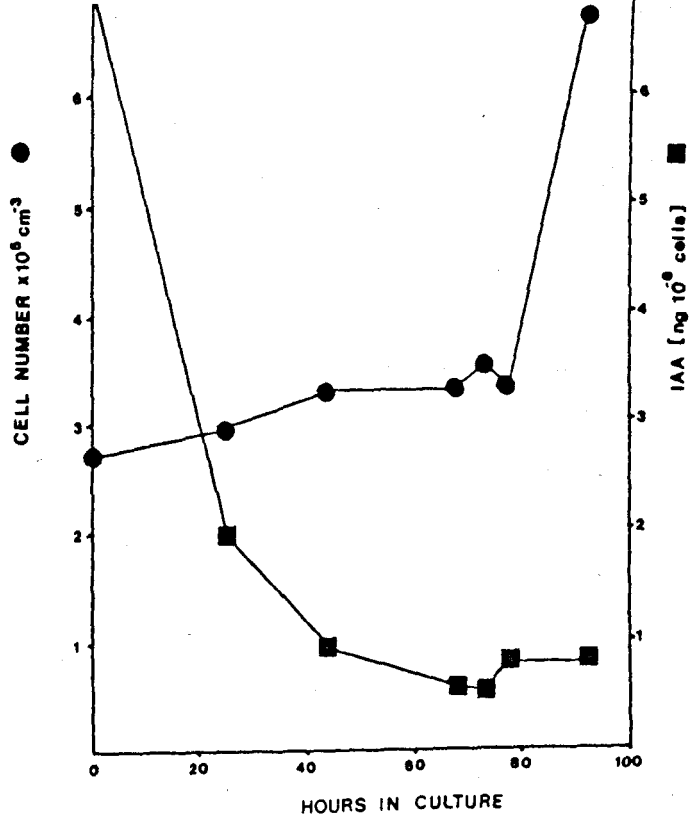
Figure 3.23

Changes in the endogenous IAA levels

(ng 10^{-6} cells) of <u>Acer</u> <u>pseudoplatanus</u> cells
during the cell cycle.
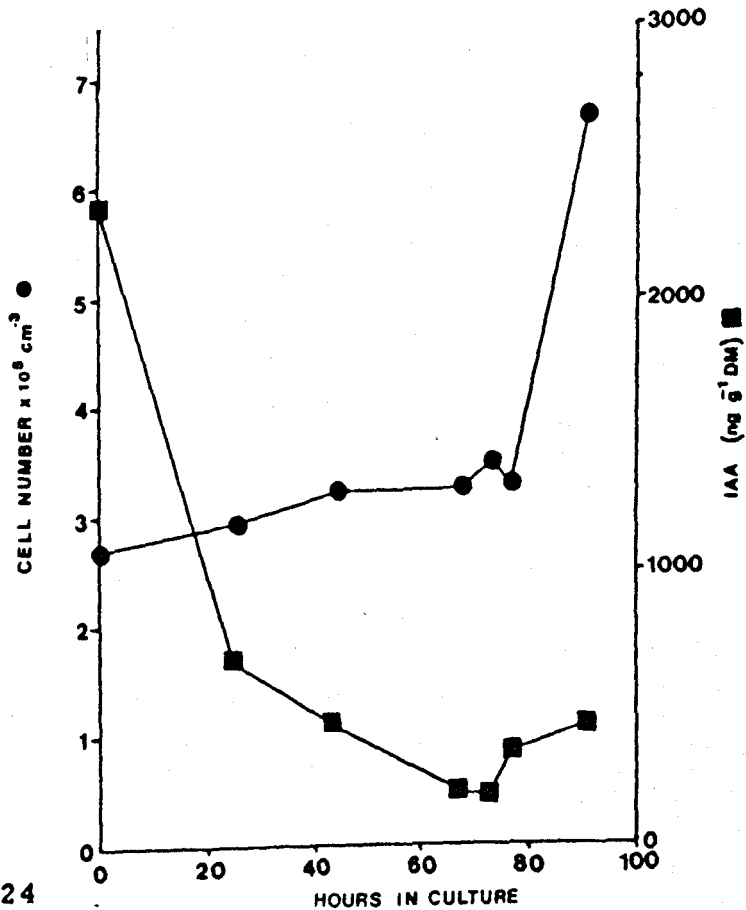
Figure 3.24

Changes in the endogenous IAA levels

(ng g^{-1} DM) of <u>Acer</u> <u>pseudoplatanus</u> cells
during the cell cycle.

In this section, this can lead to distorted trends. For example, in the first IAA experiment cytokinesis occurred relatively late, but if one of the cultures harvested at an earlier timepoint had been allowed to proceed to cytokinesis it may have occurred earlier. Nevertheless, there are similarities in the general patterns between the two sets of cultures. High inoculation levels followed by a rapid decline and then a smaller increase associated with mitosis and/or cytokinesis were common to both sets of data.

The previous workers in these laboratories all reported elevated IAA levels associated with mitosis or cytokinesis. Firby (1985) also observed an earlier peak in activity at 45 hours after sub-culture which she tentatively concluded may act as a trigger or possible initiator for DNA replication. This peak was not found in this present study. Even though the trends reported by these previous workers are all similar, there are large differences between the ranges of the levels found. Robinson (1982) reported an inoculation level of 0.3 ng 10^{-6} cells, Lewis (1983) found 0.55 ng 10^{-6} cells and Firby (1985) found a 5 hour level of 0.119 ng 10^{-6} cells. Although there is some variability between these figures it is not as significant as the difference between them and the inoculation values obtained here of 3.14 ng 10^{-6} cells and 6.96 ng 10^{-6} cells. The high level associated with mitosis was found to be 0.77 ng 10^{-6} cells by Lewis and 0.406 ng 10^{-6} cells by Firby.

The coincident levels in this present study were between
0.8-1.87 ng 10^{-6} cells. However, Robinson (1982)
reported IAA levels associated with mitosis as high as
7.5 ng 10^{-6} cells, which is similar to the very high
inoculation level reported here.

The differences between the ranges found may be
attributable to differences in the extraction techniques
utilized. This is unlikely to be the reason however, as
the methods employed were all similar and reliable and
the losses were accurately estimated with the aid of
radio-labelled standards. Another possible explanation
is that the cells may have been subtly modified over the
years that they have been maintained in suspension
culture. Such changes may result in different actual
endogenous IAA levels existing, or they may result in
other physiological or metabolic changes which affect
the actual amounts of IAA extracted from the cells.
This may explain why the basic trends observed are
similar but the actual levels are different.

The high inoculation levels reported here were not found
by any of the previous workers. An interesting
observation is that Lewis and Robinson, who examined the
IAA levels through the full batch culture growth cycle,
reported that the IAA levels declined as the cells
entered the stationary phase. It is therefore difficult
to explain how such high levels could be found here in
lag phase cells which are essentially sub-cultured

stationary phase cells. One possible explanation is that, as previously mentioned, these experiments may not be directly comparable with those of previous workers and had stationary phase cells been investigated here elevated levels might also have been found. It is unlikely that the cells could synthesize IAA instantaneously as would have to be the case if direct synthesis was the source of these high inoculation levels. It is possible that the endogenous IAA is compartmentalized and only becomes extractable for some reason on inoculation. Another explanation may be that due to pH and medium constituent concentration changes on transfer to fresh medium some of the exogenous IAA present in the inoculum medium may enter the cells. Many of the fluctuations in the levels were relatively rapid, occurring over a matter of hours and thus a peak observed by one worker may be completely missed by another and vice versa. However, this particular point is unlikely to explain the high inoculation levels.

The peak in IAA associated with mitosis and/or cytokinesis was found consistently in all the IAA analyses discussed here and it is therefore considered to be significant. It is difficult to connect any of the other fluctuations with stages of the cell cycle. There is evidence with other plant systems of a mitotic role for IAA; it is generally considered to be a cell division factor although the exact mechanism by which it acts is not clear. Das and co-workers (1956) found that

IAA when present with kinetin induced mitosis and subsequently cytokinesis in excised tobacco pith tissue. In the absence of IAA no mitosis occurred and in the absence of kinetin some mitosis occurred but only a few of the cells successfully divided. Thus, IAA does not singularly regulate cell division in this system. Other research has implicated IAA in the initiation of DNA synthesis in tobacco pith tissue (Patau et al., 1957; Naylor et al., 1954). Cooke and Meyer (1981) demonstrated that auxin was essential at the beginning of the culture period to induce mitosis in tobacco mesophyll protoplasts. They found that cytokinins were necessary later in the culture period for the development of the mitotic apparatus (Meyer and Cooke, 1979). It is generally believed that auxins stimulate DNA polymerase activity and DNA synthesis, both pre-requisites for mitosis (Yeoman and Mitchell, 1970; Simard, 1971; Jouanneau and Tandeau de Marsac, 1973).

Further substantiating evidence for IAA's mitotic and cell division role has been obtained from work involving synchronized cultures. Close correlations have been observed between IAA levels and mitotic indices. In synchronously dividing Chlorella cells the levels of endogenous IAA per cell doubled prior to cytokinesis (Grotbeck and Vance, 1972). In synchronized tobacco cell cultures the cellular IAA levels were found to fluctuate in parallel with the number of cells in mitosis (Nishinari and Yamaki, 1976). Robinson (1982),

working with the cells used in this present study observed four consecutive cold-induced partially synchronous divisions and found that the IAA per cell increased prior to each division in parallel with the mitotic indices.

As discussed in Section 3.1.1, IAA is also believed to have a role in cell expansion and to be directly related to the specific growth rate of these Acer pseudoplatanus cells.

The initiation of cytokinesis by IAA may be due to a direct affect on cell cycle regulation or the affect may be more general in that IAA is needed for other metabolic processes which are themselves pre-requisites but not necessarily regulators of cell division. It is difficult to differentiate between direct and indirect regulatory roles and there is much apparently conflicting evidence in the literature. Some researchers believe that auxin acts specifically in the G1 phase (Yeoman and Mitchell, 1970; Nishi et al., 1977), whereas others believe its role in the cell cycle is more general (Péaud-Lenoël, 1977; Everett et al., 1981). Gamburg (1982) noted that some cell cultures require auxin to remain viable as well as for cell division and this may obscure auxin's specific role in the mitotic cycle.

The system studied here is also complicated by the presence of the auxin 2,4-D. Moloney (1979) suggested that 2,4-D may act as a metabolic trigger during the lag phase which is essential before the cell's begin to synthesize their own auxin in the form of IAA. Another factor to consider is the presence of IAA in the bathing medium (Robinson, 1982; Lewis, 1983) which may affect the cells and could potentially be taken back into the cells.

Because mitosis and cytokinesis occupy short periods of the cell cycle it is possible that even though IAA levels increase in parallel with mitotic indices they are actually more directly linked with the ensuing cytokinesis. It is possible that IAA is involved in the progression of cells through the stages of mitosis and cytokinesis, although in some plants or plant systems or in the presence of other growth regulators it may only be essential for one of the two processes. Firby (1985) noted that the peak in IAA occurred slightly later than mitosis and postulated that IAA may be more directly associated with cytokinesis in this system. As mitosis occurs rapidly and IAA fluctuations also appear to be very rapid close sampling around this period is necesary in any further studies, preferably in synchronized cultures.

Although Robinson did find that IAA increases

paralleled mitotic index increases in cold-induced Acer

pseudoplatanus cultures other unexplained IAA

fluctuations did occur somewhat randomly which may have

been artefacts of the synchronization regime employed

(Elliott et al., 1987). In Section 4.3.3 IAA levels are

investigated in cells synchronized by a nitrate/

phosphate feeding regime.

3.3.4 Effect of Trigonelline treatments on Acer pseudoplatanus cells

To facilitate the investigations into the role of the

plant growth substances in the regulation of the cell

cycle, an attempt was made to arrest the cells in the

G2 state. It was intended that further investigations

would reveal any differences in the patterns of the

levels of growth substances present when compared with

the data obtained from untreated cells.

Cell arrest had been achieved by Robinson after

incubation with the alleged G2 arrestor, trigonelline

(Robinson, 1982). In this present study cells were

incubated from inoculation in medium containing a

trigonelline concentration of 10^{-3} mol dm^{-3}. A large

scale 21 dm^3 batch culture was used for this experiment

and was sampled regularly, along with a control culture,

for cell density and viability. The cell density data

are presented in Table 3.12 and Figure 3.25 and the cell

viability data in Table 3.13. It can be seen that there

is very little difference between the two growth curves,
and the trigonelline culture did exhibit cytokinesis
despite the presence of the alleged G2 arrestor. The
cell viability data reveal that slightly more of the
cells present were non-viable in the treated culture but
the levels were always below 10% and often below 5% and
are thus not considered to be significant.

The concentration of trigonelline used in this
experiment was actually higher than that employed by
Robinson and it was therefore difficult to understand
why cytokinesis had continued unimpeded. In order to
confirm the inhibitive property of this substance
duplicate 490 cm^3 litre bottle cultures containing a
range of trigonelline concentrations were inoculated.
The cell number data from these cultures are presented
in Table 3.14. In all cases cytokinesis occurred
despite the presence of trigonelline. The cell density
levels at 177 hours were slightly higher in the control
cultures but this is probably due to the slightly higher
cell viability in these cultures. The data obtained
previously by Robinson (1982) are shown in Figure 3.26.
It can be seen that as the trigonelline concentration
was increased the amount of growth declined so that with
a concentration of 10^{-4} mol dm^{-3} no growth occurred.
However, referring back to Table 3.14 it can be seen
that growth did occur at this concentration in this
study. The essential difference between these two
experiments appears to be that Robinson added

Table 3.12

Acer pseudoplatanus cell number changes for cells
cultured in normal medium and with added trigonelline
(10^{-3} mol dm^{-3})

| TIME | CELL NUMBER x $10^{-3} cm^{-3}$ +/- SE | |
(Hours)	CONTROL	TRIGONELLINE (10^{-3} mol dm^{-3})
0	258.0 +/- 22.1	289.5 +/- 10.0
19	259.3 +/- 12.9	265.6 +/- 27.4
47	243.6 +/- 12.4	236.4 +/- 16.6
58	210.2 +/- 16.0	252.1 +/- 26.6
65	235.1 +/- 25.5	251.1 +/- 16.7
73	268.8 +/- 18.7	233.5 +/- 13.2
80	326.1 +/- 37.3	260.0 +/- 14.8
90	354.6 +/- 26.8	306.5 +/- 19.0
102	372.6 +/- 13.4	281.9 +/- 10.8
129	433.9 +/- 43.1	387.0 +/- 33.5
140	395.5 +/- 32.6	425.3 +/- 30.5
188	413.6 +/- 31.6	356.9 +/- 31.3
260	550.1 +/- 69.1	450.9 +/- 64.0
308	751.8 +/- 32.1	859.9 +/- 42.2
361	833.7 +/- 36.4	993.5 +/- 73.7
429	968.6 +/- 81.5	1267.8 +/- 156.8
500	1465.6 +/- 75.5	1296.0 +/- 130.1
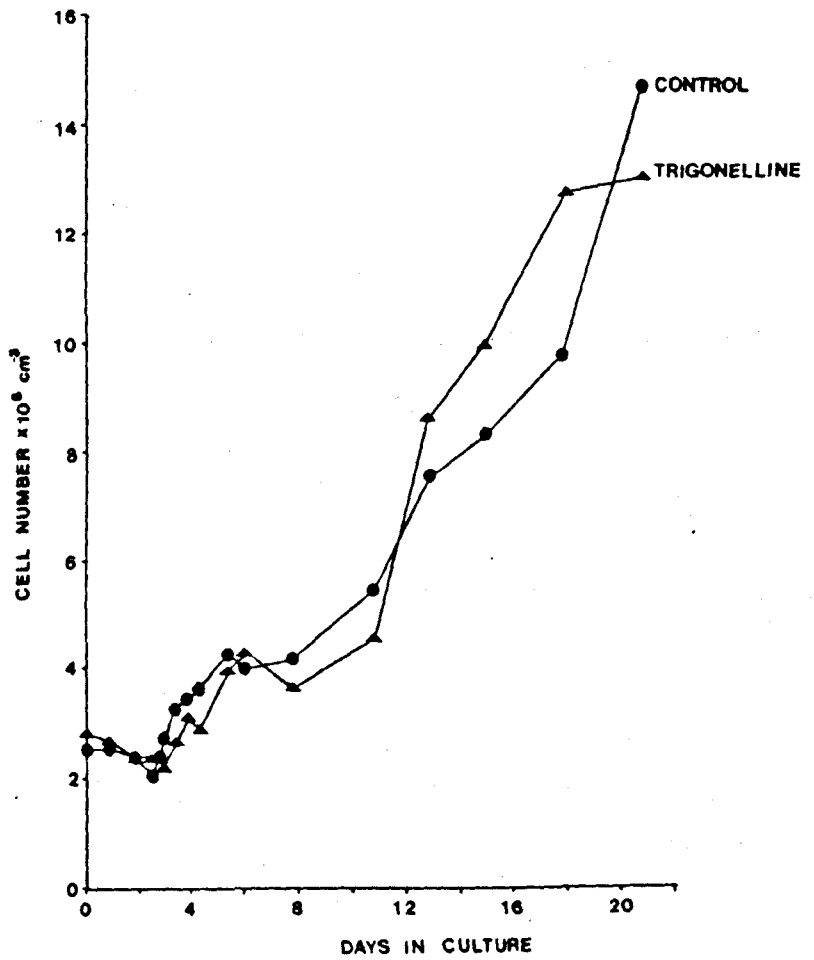
Figure 3.25

<u>Acer pseudoplatanus</u> cell number changes
for cells cultured in normal medium and with
added trigonelline $(10^{-3}$ mol dm$^{-3})$.

Table 3.13

Acer pseudoplatanus cell viability changes for cells
cultured in normal medium and with added trigonelline
(10^{-3} mol dm^{-3})

TIME	% NON-VIABLE CELLS	
(Hours)	CONTROL	TRIGONELLINE (10^{-3} mol dm^{-3})
19	2.3	3.6
58	1.7	4.7
73	1.8	6.6
90	1.6	7.2
140	2.0	3.8
188	4.2	8.8
260	7.6	6.6
308	3.8	4.8
361	5.2	4.8
429	5.2	7.2
500	4.0	3.4

Table 3.14

Acer pseudoplatanus cell number changes for cells cultured under a range of trigonelline concentrations.

TIME (Hrs)	CELL NUMBER x 10^{-3} cm^{-3} +/- SE				
	CONTROLS	10^{-4} mol dm^{-3}	5×10^{-4} mol dm^{-3}	10^{-3} mol dm^{-3}	5×10^{-4} mol dm^{-3}
0	290.8+/-23.0	276.4+/-24.8	296.0+/-21.0	256.7+/-16.3	244.3+/-21.7
	253.4+/-27.1	332.7+/-20.0	273.1+/-20.2	265.9+/-15.6	243.0+/-14.1
81	336.6+/- 9.1	286.8+/-18.7	307.8+/-31.2	315.0+/-20.4	329.4+/-14.3
	362.8+/-32.4	344.5+/-24.8	287.5+/-25.1	323.5+/-27.7	309.1+/-12.5
129	329.4+/-23.5	323.5+/-19.2	279.0+/-14.1	459.1+/-14.6	440.7+/-36.7
	355.6+/-26.7	294.7+/-30.0	332.7+/-32.3	336.6+/-21.1	386.4+/-31.9
177	575.0+/-41.8	525.9+/-31.3	480.0+/-13.0	466.9+/-15.3	470.2+/-26.3
	602.5+/-57.4	552.7+/-48.2	468.2+/-23.5	447.9+/-43.2	421.1+/-28.0

Duplicate cultures were maintained for each trigonelline concentration.
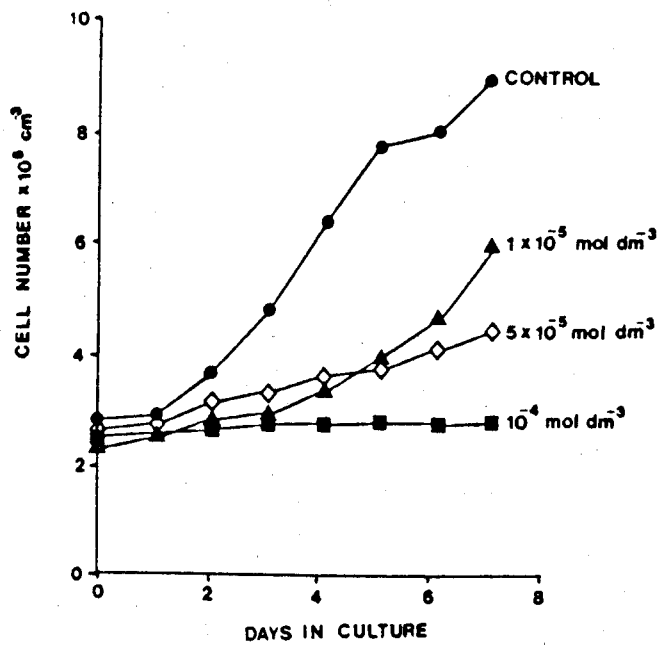
Figure 3.26

Changes in Acer pseudoplatanus cell number
at different concentrations of trigonelline
(Robinson, 1982).

trigonelline to the medium by filter sterilization after pH adjustment and autoclaving, whereas in this study trigonelline was added with the other solid medium constituent prior to pH adjustment and autoclaving. It was noted that trigonelline makes the medium more acidic and this may explain why Robinson found inhibition. Another possibility is that trigonelline may break down during the process of autoclaving, although this is unlikely because the HPLC retention time before and after autoclaving (in double distilled water) was the same. It is possible however, that when autoclaved in the presence of the other medium constituents a caramel effect occurs.

The effect of medium pH on cell culture growth is investigated in Section 5.3.1. The inhibition of growth caused by acidic medium is illustrated in Figure 5.1 and is similar to that found by Robinson (Figure 3.26) for a range of trigonelline concentrations. It is unclear from the literature which of the previous workers investigating cell arrest by trigonelline treatments have taken into account the effect of trigonelline on medium pH. It is likely that trigonelline does have cell arresting properties in some instances but in this case the effect seems to be largely caused by an alteration to the medium pH.

3.4 Conclusions

In this section the growth parameters of <u>Acer</u> <u>pseudoplatanus</u> cells in batch cell suspension culture were investigated. The cells were found to exhibit reproducible growth patterns with cytokinesis usually occurring 70-75 hours after sub-culture with some cultures dividing earlier or later due to a variable lag phase. The first division was found to be highly synchronous with up to 79% of the cells dividing within a few hours of each other.

The endogenous levels of IAA and the cytokinins were investigated in an attempt to ascertain their roles in the <u>Acer</u> <u>pseudoplatanus</u> cell cycle by correlating any observed fluctuations with known physiological or metabolic events during the first synchronous division.

The total endogenous cytokinin levels were found to increase to peaks of activity at 20 and between 65-70 hours after sub-culture. Some variations in the actual levels were found but the general trends were found consistently. Zeatin-like activity was the main cytokinin detected, being the predominant constituent of the two peaks. Zeatin riboside-like activity was found to be present at a relatively constant level up until cytokinesis after which none was detected until 96 hours after sub-culture. Zeatin-O-glucoside was found only within the two peaks of activity and dihydrozeatin was found only within the 19 hour peak in activity. As

Robinson (1982) detected low and constant levels of
cytokinin activity in used media it is likely that
leaching out into the media is not the primary mechanism
for lowering the concentrations of unwanted endogenous
cytokinins. This evidence along with the apparent rapid
changes in levels of the different endogenous cytokinins
implies that some degradation, interconversion or
compartmentation may take place. Cytokinin form,
activity and stability appear to be related and it is
therefore probable that during the cell cycle the
cytokinins may be interconverted to meet the specific
needs of the cell at different cell cycle stages.

The 19 hour cytokinin peak and the increasing levels
from 40 hours culminating in the 65-70 hour peak are
likely to be connected with metabolic events. Firby
(1985) reported elevated total protein levels at these
time points and it is postulated that the rate of
synthesis of such proteins is regulated by the
cytokinins (Short et al., 1974). It is believed that
these specific proteins are rate-limiting for
cytokinesis (Fosket and Tepfer, 1978). Cytokinins may
stimulate protein synthesis by inducing the conversion
of untranslated mRNA into polysomes (Short et al., 1974;
Tepfer and Fosket, 1978).

The role of cytokinins as G2 regulators is well
documented and paralleled cytokinin and mitotic activity
levels (Short and Torrey, 1972a,b; Nishinari and Syono,

1980) have implicated the cytokinins as mitotic regulators. The cytokinin levels present before cytokinesis may induce DNA synthesis necessary for mitosis and specific proteins which trigger further events in the cell cycle.

The cytokinins may regulate the cell cycle both in the G1 and G2 phases. Different cytokinins may act as regulators at different stages in the cycle or they may exert their affect synergistically amongst themselves or with the other growth regulators.

The IAA levels were found to increase close to mitosis and/or cytokinesis implying that IAA may have a regulatory role at this point although the exact mechanism of action is not fully understood. Firby (1985) also reported a peak in endogenous IAA preceding the period of DNA synthesis (45 hours) and postulated that it could act as a trigger or possible initiator for DNA replication in the cell cycle. Generally it is believed that auxins stimulate DNA polymerase activity and DNA synthesis, both pre-requisites for mitosis (Yeoman and Mitchell, 1970).

IAA appears to be involved in several processes throughout the duration of the cell cycle including early protein synthesis, DNA synthesis, mitosis and cell division. However, the differences in the levels obtained by various workers imply that either the levels

fluctuate very rapidly or perhaps the cells have adapted to being maintained in suspension culture and are actually producing different amounts of IAA than were found previously. This is particularly noticeable in this study at the point of inoculation when very high levels were obtained which are difficult to interpret. It is unlikely that stationary phase cells contain such levels or that the IAA could be synthesized so quickly on inoculation. One possibility is that the cells may take in the IAA from the used media transferred on sub-culture, although even this process is unlikely to be rapid enough to account for the high levels observed.

Future work in this area should include an intensive sampling time-table from a single culture of both IAA and the cytokinins. Both of these growth regulators appear to fluctuate rapidly during the cell cycle and therefore the system of harvesting separate cultures for each time point is inappropriate. The first synchronous division may not be representative as the cells are under stress during and after the transfer, and the levels obtained may be artefacts of this stress situation. Thus, the levels should be investigated during synchronized divisions induced by other techniques, if the same patterns of growth regulator levels are found it is less likely that they are artefacts and more likely that they are actually involved in the regulation of the cell cycle.

The experiments involving the use of the alleged G2 arrestor, trigonelline (Tramontano et al., 1982) have been included in this section because it was originally intended that the growth regulator levels would be estimated in G2 arrested cells. Previous work in these laboratories produced data showing complete cell arrest in cells cultured at a concentration of 10^{-4} mol dm^{-3} trigonelline (Robinson, 1982) and an inhibition of culture growth at lower concentrations. On release from trigonelline treatment (10^{-4} mol dm^{-3}) by washing and transfer to fresh medium, a shortened two day lag phase occurred. This shortened lag phase is indicative of a G2 arrest.

However, in these investigations no cell arrest occurred and it has thus been concluded that the effect observed by Robinson may have been a reflection of the pH status of the medium. Trigonelline considerably increases the acidity of the culture medium, and this was not accounted for by Robinson, who added trigonelline by filter sterilization after pH correction and autoclaving. Indeed, the cell number data presented in Figure 5.1 for cells cultured in a range of acidic mediums are similar to those obtained by Robinson for a range of trigonelline treatments (Figure 3.26). It is therefore tentatively concluded that in this case the inhibitor, trigonelline, produced its effect largely through acidifying the culture medium. This theory could be substantiated by performing microdensitometric

analysis of cells arrested by trigonelline (added by filter sterilization) and pH. It would be interesting to determine whether or not the cells would be arrested in the G2 state as proposed by Robinson, or in the more usual G1 state. The effect of medium pH on <u>Acer pseudoplatanus</u> cells is discussed in more detail in Section 5.0.

4.0 PRODUCTION OF SYNCHRONIZED CULTURES

4.1 Introduction

Batch cell suspension cultures provide a system in which cells can be grown rapidly under sterile conditions. In a well established cell line, such as the Acer pseudoplatanus cultures used in this present study, there is little cell aggregation and thus the cultures can be regarded as being relatively homogeneous. Virtually every cell is bathed by the same surrounding medium and is influenced by identical external factors; this facilitates work involving correlations between cellular endogenous plant growth regulator levels and cell cycle events.

Although the first division after sub-culture is routinely >75% synchronous (that is, the majority of the cells divide within a matter of hours of each other), batch cultures rapidly develop asynchrony. The cell's mitotic cycles and biosynthetic pathways become out of step with other cells in the culture and thus precise correlations between metabolic events, endogenous concentrations and physiological changes are not possible. For this reason much of the research into the cell cycle has been concentrated on the first highly synchronous division. It is believed that during transfer the cells are exposed to stress and arrest in their cell cycles at the same stage. When they recover from the transfer to fresh medium their cell cycles are

in step with one another. Research involving this first
division can be criticized because the process of
transfer to fresh medium involves a multitude of
different physical and chemical alterations to the
cell's environment. The actual synchronization may be
induced by one or all of these changes, and by the same
argument, any fluctuations in the substances synthesized
by the cells may be artefacts of the process of sub-
culture and not specifically related to the cell cycle.

The ideal system would be a culture that exhibited a
high degree of synchrony with successive divisions
achieved with as little interference as possible to the
cell's usual cultural environment. Unfortunately,
naturally occurring cell division synchrony is not
common amongst the higher plants and thus most of the
synchronously dividing populations studied have been
induced by manipulation. There are two synchronization
techniques routinely employed, these are selection
synchrony and induction synchrony (Mitchison, 1971).

Selection synchrony involves the physical separation of
cells at a specific stage in the cell cycle from the
rest of the cell population. This is difficult to
achieve under sterile conditions and also requires the
easy detection and separation of the cells at specific
stages. This technique has been utilized with the
micro-organism _Schizosaccharemyces pombe_ which exhibits
a linear relationship between size and the stage of the

cell in the cycle (Mitchison and Vincent, 1965). Another inherent problem with this method of synchronization is that it would be very time-consuming to obtain a large population of synchronized cells.

Induction methods of synchrony are more numerous and widespread than selection methods but they have the disadvantage of the introduction of physical and/or chemical variables to the system which may alter the cell's usual behaviour. Thus, elevated levels of, for example, growth regulators at a particular point in the cell cycle may be a result of this interference rather than the amplification of specific peaks caused by the cells cycling simultaneously. Induction synchrony is achieved by blocking the cell cycle at a specific point, either by the addition of a chemical or by an alteration of the physical environment (Prescott, 1976). The cells tend to accumulate at a similar stage in the cell cycle. When the blockage is overcome, either by the release of the inhibitor or the addition of an essential substance, a limited number of synchronous cell divisions occur. Various plant cell suspension cultures have been induced to divide synchronously by a number of induction techniques. Synchrony inducers employed include inhibitors of DNA synthesis, inhibitors of mitosis and starvation/regrowth procedures. Induced synchrony has been achieved by cold treatments (Okamura et al., 1973; Robinson, 1982), warm treatments (Fujitsu et al., 1972), anoxia and aeration cycles (Constabel et al., 1974),

regimes of pre-starvation and a light/dark pre-treatment
(Nishinari and Yamaki, 1976), periods of specific
starvation followed by the reintroduction of auxin
(Yeoman and Aitchison, 1976; Nishi et al., 1977),
cytokinin (Jouanneau, 1971; Jouanneau and Tandeau de
Marsac, 1973) and phosphate (Komamine et al., 1978). A
starvation/regrowth treatment for Acer pseudoplatanus
cell suspension cultures was achieved in 1973, (King
et al., 1973; King and Street, 1973). This regime
produced five consecutive synchronous cell divisions and
nitrate was reported to be the limiting factor, (King
et al., 1974; Gould and Street, 1975).

Induced synchronous cell suspension cultures still
possess some of the limitations of the batch cultures,
notably that waste products and leached substances
cannot be removed. Thus data obtained from such systems
should still be considered tentatively. However, if the
same biosynthetic patterns through the cell cycle are
detected in cells under different synchronization
regimes it is likely that the data obtained can be
realistically related back to the whole plant situation.

It is important to define exactly what is meant by a
"synchronous culture". Such cultures exhibit periodic
increases in the number of cells, with the increase at
each division being close to 100%. Between these
divisions periods of constant cell number occur.

Synchrony is usually expressed as a percentage and is calculated as follows:

$$\frac{\bar{x}\ \text{Post Division Cell Number} - \bar{x}\ \text{Pre-Division Cell Number}}{\bar{x}\ \text{Pre-Division Cell Number}} \times 100$$

The mean post and pre-division cell numbers should be used for accuracy.

The degree of synchrony (%) is the main demonstrator of the synchronous state but other indicators are increases in the mitotic index preceding cytokinesis and short division periods relative to the cell cycle time (Firby et al., in preparation). In the past periodic fluctuation in mitotic index levels was considered to be substantiating evidence for the existence of synchrony (Jouanneau, 1971; Constabel et al., 1977). However, mitotic index levels may be affected by changes in the growth rate, the population of cells and the cell death rate (King and Street, 1977). Thus mitotic index alone is not a good index of synchrony (Everett et al., 1981). Other synchrony indices have been suggested for plant cell suspension cultures, for example, Scherbaums synchrony index (Gould and Street, 1975).

In this present study the dependence of Acer pseudoplatanus cells in suspension culture on the nutrients nitrate and phosphate, was utilized to develop a synchronization technique. The standard culture medium is a modified version of Stuart and Street's

medium (1969) containing 7.05 m mol dm^{-3} $NaNO_3$ and 0.85 m mol dm^{-3} NaH_2PO_4. In batch culture <u>Acer pseudoplatanus</u> cells have been found to utilize the nitrogen source supplied and become nitrogen-limited (King <u>et al</u>., 1973; Young, 1973; Jessup and Fowler, 1976). Phosphate is also depleted from <u>Acer pseudoplatanus</u> culture medium at approximately the same rate as nitrate (Wilson, 1976). After the development of a reliable synchronization technique it was utilized to ascertain the endogenous IAA levels through an induced synchronous division. The system was also semi-automated with the aid of a Commodore-PET computer.

4.2 <u>Experimental Methods</u>

Litre bottle cultures ($490cm^3$) were grown as described in Section 2.0, but with sodium nitrate concentrations of 3.5, 5.0, 7.0, 10.0 and 14.0 m mol dm^{-3} to confirm that the cells were nitrogen limited. Triplicate cultures were used for each concentration. The nitrate concentration of the medium was monitored at intervals using ion-selective electrodes (Orion Research Inc, Cambridge, Mass.). The electrode was calibrated against a range of sodium nitrate concentrations prepared in <u>Acer pseudoplatanus</u> culture medium (Figure 4.5).

Large scale cultures containing $9.0dm^3$ of medium and $1.5dm^3$ of inoculum were used for the synchrony induction experiments at the routine concentration (7.05 m mol dm^{-3} $NaNO_3$). Litre bottle ($490cm^3$) cultures were used

for the experiments involving the induction of synchrony at 3.5, 5.0, 10.0 and 14.0 m mol dm^{-3} NaNO$_3$. Large scale (21.0dm^3) cultures were used for the development of an automated synchronization system. An autoclavable pH electrode was placed inside the enclosed culture vessel and was connected to a digital pH meter (Corning) or a computer interface. An alcohol sterilized ion-selective nitrate electrode and a reference electrode (Orion Research Inc) were placed in a specially modified sealed glass tube incorporated into the culture vessel system. The electrodes were fed with culture through silicone rubber tubes pumped by a Watson-Marlow flow inducer. The pH and nitrate electrodes were connected to an interface box and were monitored by a Commodore-PET computer.

Synchrony was induced in all cases by the addition of NaNO$_3$ and NaH$_2$PO$_4$.2H$_2$O in solution after each cell division to reestablish the original nitrate concentration. Since phosphate is depleted at approximately the same rate a proportional amount of phosphate was added (Wilson, 1976). In the case of the litre bottle cultures this addition was made via a 2 ml auto-claved automatic pipetting unit (A R Horwell). The required amounts of NaNO$_3$/NaH$_2$PO$_4$ solution were made up so that only approximately 1 ml was needed on each addition. With the larger cultures a concentrated NaNO$_3$/NaH$_2$PO$_4$ solution was attached to the culture vessel before autoclaving. In the manual cultures this

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adaption also included a graduated glass tube so that known volumes could be added on the release of a gate clip. In the automated system the solution was added gradually by slow turning of a flow inducer controlled by the computer, whilst the nitrate levels were constantly monitored via ion selective electrodes.

Cellular nitrate concentrations were estimated as follows. $150cm^3$ of culture was filtered through glass-fibre paper (GF/C Whatman) and the cells were washed free of medium with nitrate-free _Acer pseudoplatanus_ medium. The washed cells were resuspended in $15cm^3$ of nitrate-free medium and incubated in a boiling water bath for 30 minutes with occasional agitation. The supernatant and cells were then quantitatively transferred to a Potter glass/glass homogenizer and homogenized (20 strokes at full speed). The homogenate was centrifuged to remove cell debris, and the supernatant was made up to $25cm^3$. The nitrate concentration was then determined using ion-selective electrodes.

4.3 Results_and_Discussion

4.3.1 Nitrate_as_a_Limiting_Nutrient

Despite being surrounded by plentiful amounts of nitrogen in the air, higher plants are by themselves unable to utilize this supply. They obtain their nitrogen in a combined form such as ammonia or nitrate.

Some plants have overcome this potential problem by forming symbiotic relationships with nitrogen fixing bacteria such as the _Rhizobium_ species. These micro-organisms fix nitrogen from the air and synthesize ammonia. In such symbiotic relationships the plant provides the bacteria with essential carbohydrates and the bacteria supplies the plant with nitrogen in the form of ammonia (Hawker and Linton, 1979).

More usually plants obtain nitrogen from the soil and water in which they grow. Most of this nitrogen is released upon the decay of organic matter in the form of ammonia. Nitrifying bacteria quickly oxidize this ammonia to nitrate which is then taken up by plants. Plants utilize ammonia directly mainly to synthesize essential amino acids. Nitrate, however, has to be reduced back to ammonia or a derivative before it too can be utilized by the plant and converted into amino acids or other nitrogenous products. The reduction of nitrate to ammonia initially involves the reduction of nitrate (NO^{3-}) to nitrite (NO^{2-}), a reaction catalyzed by the flavo-protein enzyme complex, nitrate reductase. The nitrite is then reduced to hyponitrite ($N_2O_2^{2-}$) by nitrite reductase, this is reduced to hydroxylamine (NH_2OH) by hyponitrite reductase and hydroxylamine is then reduced to ammonia.

It may seem inefficient that in nature ammonia is quickly oxidized to nitrate, which must then be reduced back again to ammonia before it is utilized in plant biosynthesis. However, this process does have advantages to the plant, for example, nitrate is a more stable storage form than ammonia and is also far less toxic (Conn and Stumpf, 1976).

The nitrogen of ammonia is primarily utilized by the plant in the formation of glutamic acid. Glutamic acid is formed by the following reversible reaction which is catalyzed by glutamic dehydrogenase:

$$\propto - \text{ketoglutaric acid} + NH_3 + NADH + H^+$$

$$\rightleftharpoons$$

$$\text{Glutamic acid} + NAD^+ + H_2O$$
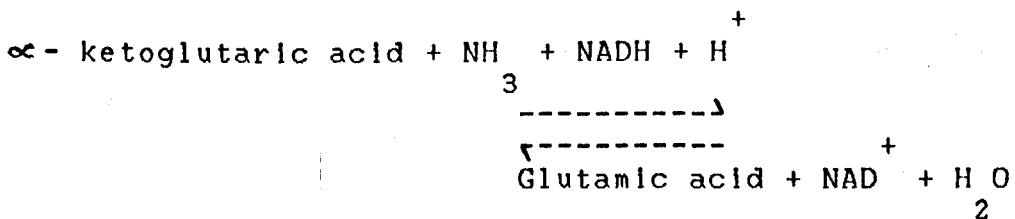
Other amino acids may be formed by the transfer of the amino-group of glutamic acid to other carbon skeletons, a process known as transamination. Thus, transfer can take place from glutamic acid to pyruvic acid, another intermediate in the plant's metabolic cycles, giving a new amino acid, alanine, and producing more $\propto$-ketoglutaric acid. These reactions are readily reversible and are the basis of the synthesis of many different amino acids. The coupling of glutamate synthesis with transamination is represented schematically overleaf:

Amino $RCHNH_2CO_2H$ ⟶ α-ketoglutaric   ⟶ $NADH + H^+ + NH_3$
acid   acid

Keto $RCOCO_2H$ Glutamic acid $NAD^+ + H_2O$
acid

            Transaminases   Glutamic
                                           dehydrogenase
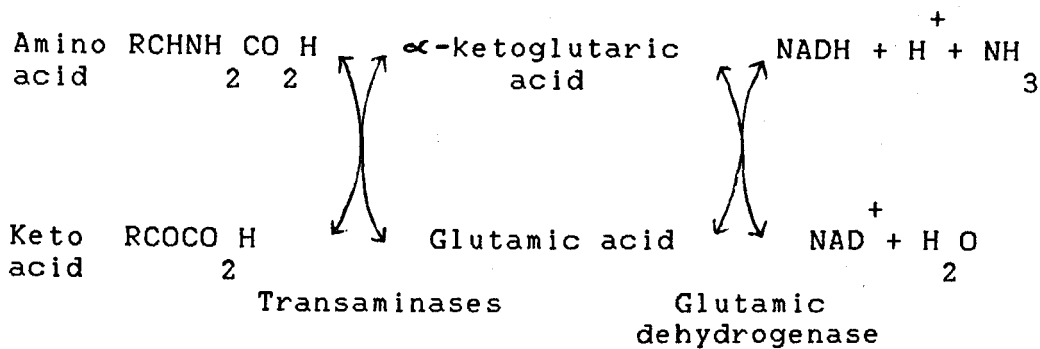
                                       (Conn and Stumpf, 1976)

Glutamic acid and its amide, glutamine, are the primary products of inorganic nitrogen assimilation and have central roles in intermediary nitrogen metabolism (Stewart et al., 1980). Amides are essentially amino acids with an extra $-NH_2$ group attached on an acidic group and are often formed when the amount of ammonia available is in excess of requirements. They are probably a nitrogen storage mechanism which overcomes the problem of storing toxic free ammonia whilst providing nitrogen in a form readily available for synthesis. Amides can accumulate to high levels in cells, especially when protein reserves are being degraded. Asparagine often accumulates in germinating seedlings. The amides may also be utilized in the synthesis of certain proteins. ATP is required for the formation of amides, and as the reaction which results in amide production is essentially reversible, the amides are a potential energy store.

Another group of nitrogenous compounds essential as building blocks for large informational molecules are the nitrogen bases. Nucleotides are formed by the addition of pentose sugar and phosphate to these nitrogen bases. The nucleotides themselves are the building blocks for the nucleic acids.

Various workers have implicated nitrogen as a limiting nutrient in batch cultured Acer pseudoplatanus cells. As it appears to play such a central metabolic role it is likely that if it is limiting, growth would be quickly affected. Young (1973) reported that nitrate and urea, (an alternative nitrogen source) when supplied in the culture medium were depleted after only seven days of incubation. When the supplied nitrate concentration was doubled the final cell number of the batch cultured cells approximately doubled and there was an increase in the total biomass. Jessup and Fowler (1976) observed similar results and proposed that there is a proportional relationship between cell yield and media nitrogen concentration. They also supplied Acer pseudoplatanus cells with either nitrate or glutamate as their only nitrogen source and noted that although the cells grew well with each source there were differences in their growth patterns.

The sycamore cells used in this present study were originally cultured with urea as an additional nitrogen source to that of sodium nitrate. However, when urea

was later omitted from the culture medium cell growth appeared unaffected and thus nitrate alone was supplied in this study.

In order to confirm this apparent nitrogen limitation in the line of _Acer pseudoplatanus_ cells investigated here, they were batch cultured in a range of sodium nitrate concentrations for 21 days. The standard sodium nitrate concentration is 7.0 m mol dm^{-3}. The range investigated included 3.5, 5.0, 7.0, 10.0 and 14.0 m mol dm^{-3}. The cell numbers and dry masses cm^{-3} were monitored during the batch culture growth cycle and the results are tabulated in Tables 4.1 and 4.2 respectively and illustrated in Figures 4.1 and 4.3 respectively.

Table 4.1

Changes in Cell Number $(x\ 10^{-3}\ cm^{-3})$ with Culture Age at a range of sodium nitrate medium concentrations

DAY	Sodium nitrate $(m\ mol\ dm^{-3})$		
	3.5	5.0	7.0 (Control)
0	239.2 +/- 17.15	248.1 +/- 2.61	232.0 +/- 10.37
2	233.7 +/- 15.36	245.6 +/- 8.81	228.9 +/- 9.07
4	358.2 +/- 12.65	409.0 +/- 7.46	406.2 +/- 7.77
6	369.6 +/- 8.15	417.1 +/- 4.76	412.6 +/- 10.46
8	559.7 +/- 15.70	579.3 +/- 15.07	715.1 +/- 23.80
12	638.1 +/- 22.55	928.6 +/- 31.95	1138.2 +/- 14.40
16	900.2 +/- 29.94	1498.8 +/- 96.05	1633.7 +/- 23.65
21	1119.0 +/- 53.42	1696.1 +/- 56.8	1952.0 +/- 106.28

DAY	Sodium nitrate $(m\ mol\ dm^{-3})$	
	10.0	14.0
0	247.7 +/- 9.51	244.8 +/- 10.65
2	241.3 +/- 11.51	249.3 +/- 8.96
4	407.8 +/- 9.85	423.8 +/- 8.86
6	671.2 +/- 23.24	749.2 +/- 19.68
8	760.8 +/- 20.68	932.7 +/- 7.90
12	1304.5 +/- 21.63	1563.9 +/- 46.26
16	1914.8 +/- 33.06	2279.0 +/- 15.09
21	2797.2 +/- 53.84	3170.5 +/- 54.34

The data are the mean values +/- Standard Errors obtained from three separate cultures at each nutrient concentration.
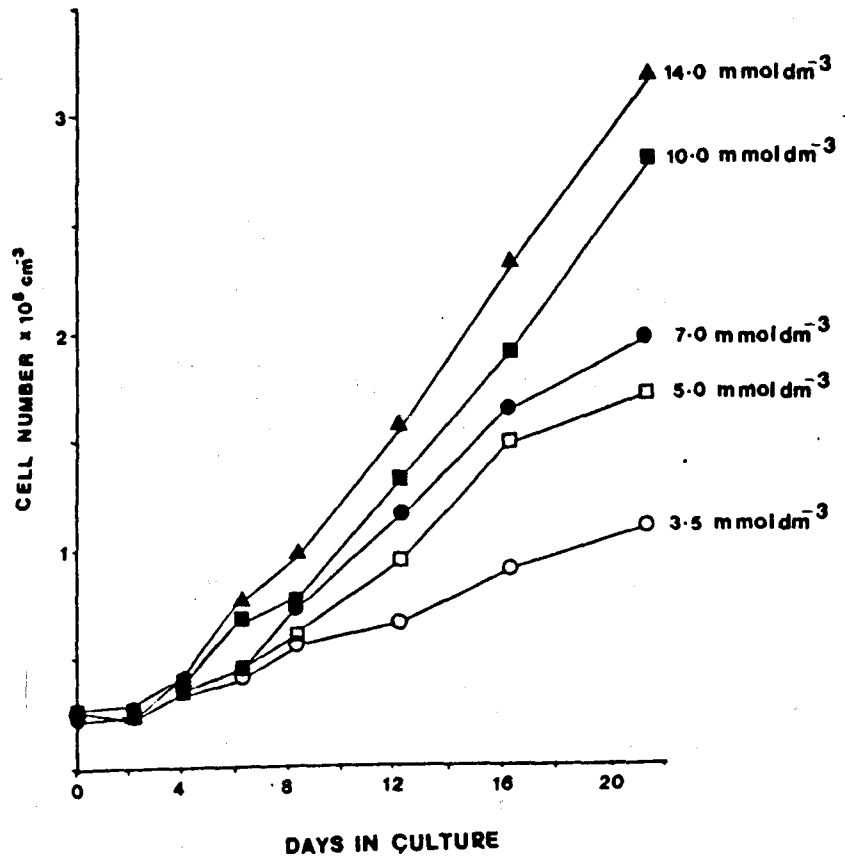
Figure 4.1

Changes in _Acer pseudoplatanus_ cell number at different medium nitrate concentrations.

All of the cultures were inoculated with similar densities of cells but the final cell numbers achieved after 21 days of batch culture growth varied considerably. The final cell numbers increased with the concentration of nitrate supplied, the lowest concentration (3.5 mmol dm^{-3}) produced a figure of 1119.0 +/- 53.42 x 10 cm^{-3} and the highest concentration (14.0 mmol dm^{-3}) produced 3170.5 +/- 54.34 x 10^{-3} cells cm^{-3}. Thus, a threefold increase in cell number was achieved by a fourfold increase in the nitrate concentration of the bathing media. The control cultures (7.0 mmol dm^{-3}) produced a final cell number of 1952.0 +/- 106.28 x 10^{-3} cm^{-3}, twice the level achieved by half the concentration. Figure 4.2 shows the changes in the final cell numbers plotted against the nitrate concentration. Lewis (1983) plotted a similar graph for a range of nitrate concentrations below the control level and found there was a high positive correlation between the two variables. In this case any linearity appears to be lost above a concentration of 10m mol dm^{-3}. Firby (1985) noted that the final cell number achieved in an 18m mol dm^{-3} nitrate culture was similar to that of a 10m mol dm^{-3} nitrate culture. Thus, although increases in the nitrate concentration result in increased final cell yields, the relationship is not infinite. In this case the highest cell number was produced by the 14m mol dm^{-3} culture but it is possible that higher levels may be attained in, for example, 12m mol dm^{-3} cultures. The very high nitrate concentrations
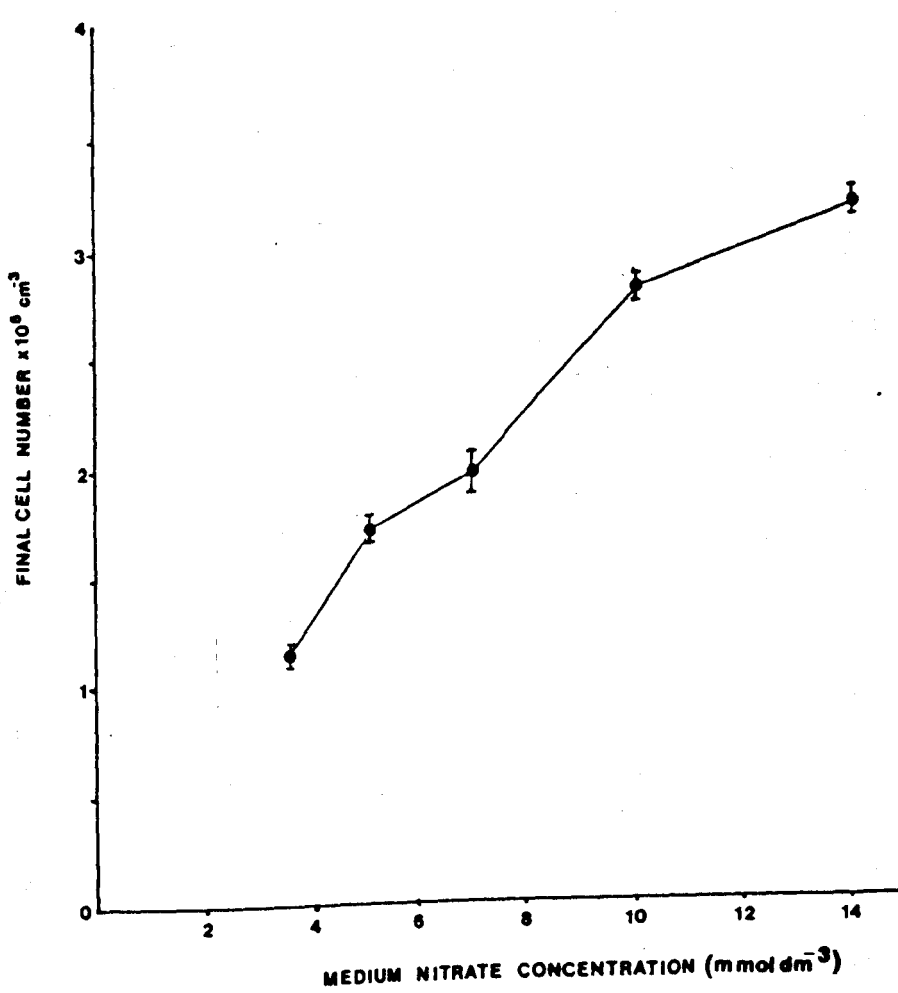
Figure 4.2

Changes in Acer pseudoplatanus final cell
numbers   at   different   medium   nitrate
concentrations.

appear to inhibit rather than enhance growth. This may be due to the increased levels of sodium in the media as the nitrate was added in the form of sodium nitrate. Sodium may have a toxic affect on the cells. This possible problem may be alleviated in future studies by the use of potassium nitrate as potassium is the major cation present in the normal medium.

A similar apparent inhibition of growth at high nitrate concentrations is revealed by the changes in dry mass accumulation over the culture period (Figure 4.3). The highest final dry mass yield was produced by the 10.0 m mol dm^{-3} culture (7.82 +/- 0.23 mg cm^{-3}) but this value was only marginally above that of the control (7.48 +/- 0.20 mg cm^{-3}) and the 14.0m mol dm^{-3} culture (7.35 +/- 0.14 mg cm^{-3}). The final growth parameter data are summarised in Table 4.3 where the dry mass is also expressed on a per cell basis. Figure 4.4 illustrates these dry mass data graphically. Between the nitrate concentrations of 3.5 and 7.0m mol dm^{-3} there is a semi-linear increase in final dry mass per cm^3 of culture with increasing nitrate concentration. However, the increase from 7.0 to 10.0m mol dm^{-3} produced very little affect on the final dry mass per cm^3 of culture and in the 14.0m mol dm^{-3} cultures a decline in the final dry mass per cm^3 was observed.

The dry mass per cell declined with increasing nitrate concentrations. This was also reported by Jessop and Fowler (1976). This may be due to the fact that more of the cells are actively dividing at the higher nitrate concentrations and therefore there is a larger proportion of small cells, rather than the larger cells characteristic of the stationary phase. Referring back to Figure 4.1 it can be seen that the typical sigmoidal cell number curve discussed in Section 3.0 is apparent in the 3.5, 5.0 and 7.0m mol dm^{-3} sodium nitrate cultures. The 10.0 and 14.0m mol dm^{-3} cultures however, do not appear to have entered a true stationary phase and thus active cell division is still taking place.

The cellular and media nitrate levels were monitored as described previously with ion-selective electrodes. It was anticipated that the nitrate would be depleted from the medium and accumulate in the cells. However, in preliminary experiments non-reproducible data were obtained with decreases followed by increases in the nitrate concentrations in the medium implying export of nitrate from the cells. Further work revealed this phenomenon to be an artefact of the monitoring technique employed. Although the standard calibration curve obtained by plotting the log of the nitrate concentration against the relative mV reading exhibits good linearity (Figure 4.5), recalibration was found to be necessary after each sample time point. This problem was particularly acute when the recommended

<u>Table 4.2</u>

Changes in Dry Mass (mg cm^{-3}) at Different Medium Nitrate Concentrations

DAY	Sodium nitrate (m mol dm-3)		
	3.5	5.0	7.0
0	0.82 +/- 0.13	0.94 +/- 0.11	0.87 +/- 0.19
2	0.79 +/- 0.08	1.07 +/- 0.07	1.16 +/- 0.15
4	0.90 +/- 0.06	1.23 +/- 0.07	1.38 +/- 0.11
6	1.29 +/- 0.09	1.89 +/- 0.09	2.27 +/- 0.11
8	1.72 +/- 0.08	2.34 +/- 0.13	2.91 +/- 0.16
12	2.80 +/- 0.17	4.71 +/- 0.17	6.13 +/- 0.12
16	3.77 +/- 0.17	5.37 +/- 0.16	6.91 +/- 0.18
21	4.51 +/- 0.27	6.06 +/- 0.20	7.48 +/- 0.20

DAY	Sodium nitrate (m mol dm-3)	
	10.0	14.0
0	0.83 +/- 0.13	0.88 +/- 0.17
2	1.22 +/- 0.13	1.17 +/- 0.12
4	2.28 +/- 0.16	2.03 +/- 0.09
6	2.48 +/- 0.18	2.92 +/- 0.12
8	3.17 +/- 0.21	3.13 +/- 0.12
12	5.67 +/- 0.11	5.51 +/- 0.12
16	7.16 +/- 0.23	6.95 +/- 0.17
21	7.82 +/- 0.23	7.35 +/- 0.14

The data are the mean values +/- Standard Errors obtained from three separate cultures at each nutrient concentration.
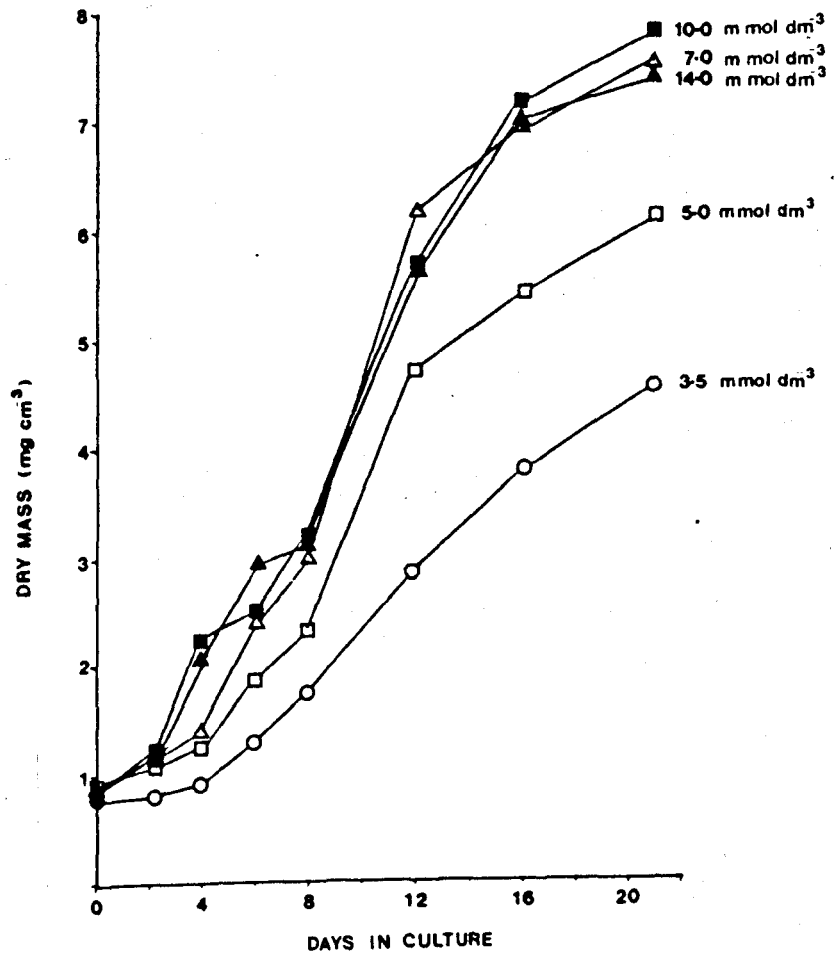
Figure 4.3

Changes in _Acer pseudoplatanus_ dry mass at different medium nitrate concentrations.

Table 4.3

Changes in the Final Cell Number and Dry Mass at Different Nitrate Concentrations

Sodium Nitrate m mol dm^{-3}	Final Cell Number x 10^{-3} cm^{-3}	Final Dry Mass mg cm^{-3}	ng cell^{-1}
3.5	1119.0 +/- 53.42	4.51 +/- 0.27	4.03
5.0	1696.1 +/- 56.80	6.06 +/- 0.20	3.57
7.0	1952.0 +/- 106.28	7.48 +/- 0.20	3.83
10.0	2797.2 +/- 53.84	7.82 +/- 0.23	2.80
14.0	3170.5 +/- 54.34	7.35 +/- 0.14	2.32
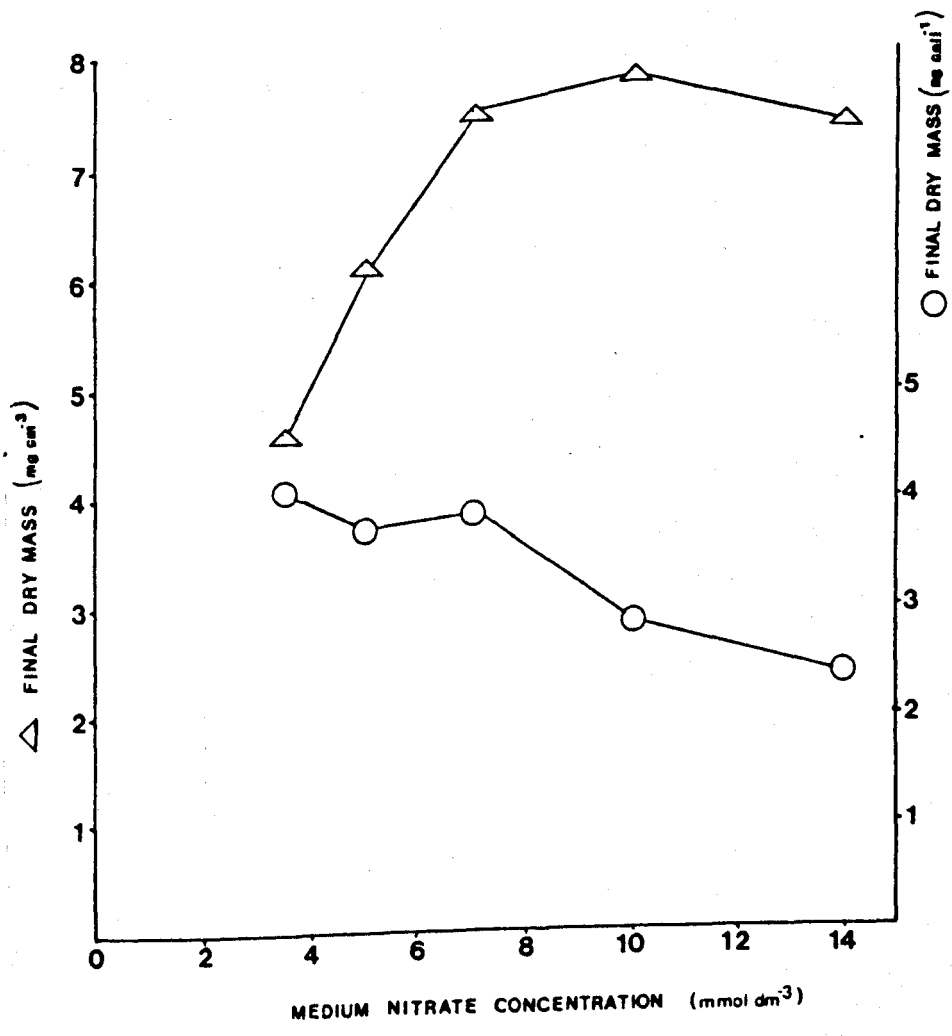
Figure 4.4

Changes in *Acer pseudoplatanus* final dry mass at different medium nitrate concentrations.

reference electrode was used. This electrode requires
to be filled with reference filling solutions which can
evaporate and thus shift the calibration curve. Ideally
the reference solution should be changed daily and the
electrode recalibrated with nitrate standards. Besides
being time consuming this system of daily recalibration
was not suitable for incorporation into a culture
vessel, as described later in this section, and the warm
environment of the growth room caused rapid evaporation.
Therefore a glass reference electrode (Orion) was used
which proved to be reliable and required little
recalibration. When the preliminary experiments were
repeated using this glass reference electrode the
previous spurious fluctuations were not found and the
medium nitrate levels were seen to gradually decline
(Table 4.5) whilst the cellular nitrate levels increased
(Table 4.6). The cellular nitrate levels were only
estimated in the control cultures.

Another problem associated with the use of ion-selective
electrodes for nitrate concentration estimations is the
interference of other ions. In the culture medium used
here the ion most likely to interfere is chloride. In
order to ascertain the extent of this chloride ion
interference the electrodes were calibrated against the
normal nitrate standards which were routinely made up in
culture medium and then the readings were obtained from
nitrate standards in media containing 50% and 0% of the
usual media chloride concentrations. The results are
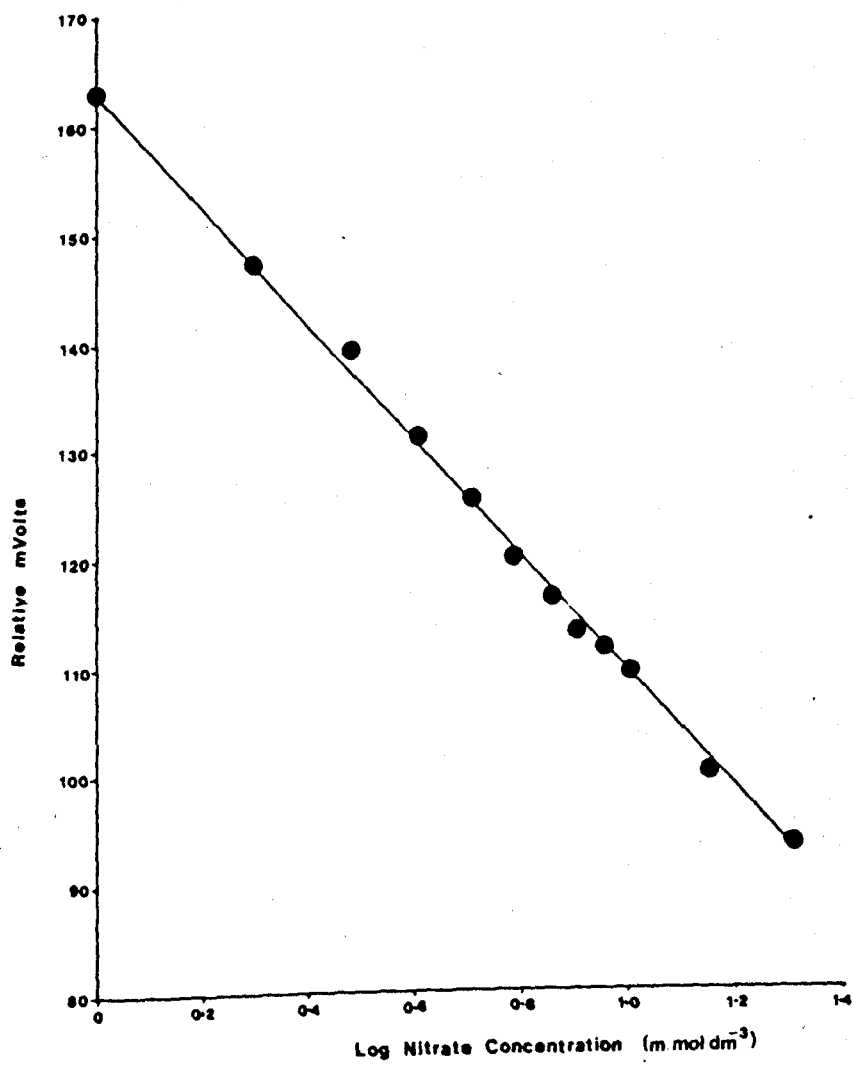
Figure 4.5

Nitrate calibration curve for _Acer_
pseudoplatanus culture medium.

shown in Table 4.4. When 50% of the usual chloride
concentration was present the calibration readings were
found to be almost identical to the original readings.
However, when no chloride was present higher mV levels
for 0 and 1 mmol NO_3 were obtained implying lower
nitrate levels. Thus at very low nitrate concentrations
the presence of chloride ions may lead to slight over-
estimates of the nitrate levels. This interference was
not considered to be significant.

The nitrate concentrations of the media throughout the
duration of the culture cycle are shown in Table 4.5 for
cells grown in a range of initial nitrate concen-
trations. The data are illustrated graphically in Figure
4.6. The standard errors are all <10% except when the
means were between 0-1 mmol dm^{-3} when the error is high.
This is due to the fact that the nitrate electrode
calibration curve cannot be accurately used to estimate
nitrate levels below 1 mmol dm^{-3} and such levels were
therefore assumed to be zero. The high standard error
occurs because in such cases one or more of the three
cultures has an assumed zero nitrate concentration
whilst the remaining culture(s) has a value greater than
or equal to 1.0 mmol dm^{-3}.

Table 4.4

The effect of chloride ions on nitrate electrode calibration

Nitrate Concentration (mmol dm^{-3})	Relative mV		
	100% Cl$^-$	50% Cl$^-$	0% Cl$^-$
0	200	200	211
1	161	161	166
2	146	146	145
3	137	137	136
4	130	130	129
5	124	125	124
6	120	120	120
7	115	117	116
8	112	113	113
9	111	111	110
10	108	108	108

Table 4.5

Medium nitrate depletion by <u>Acer pseudoplatanus</u> cells cultured at a range of initial nitrate concentrations.

Time	Medium Nitrate (mmol dm^{-3})				
(Days)	3.5	5.0	7.0	10.0	14.0
0	3.44+/-0.12	4.64+/-0.06	6.61+/-0.15	9.77+/-0.23	13.65+/-0.22
2	2.51+/-0.12	2.89+/-0.20	5.89+/-0.14	8.45+/-0.11	12.74+/-0.21
4	-	1.06+/-0.05	4.04+/-0.05	5.25+/-0.12	11.13+/-0.15
6	-	-	0.37+/-0.64	1.87+/-0.08	2.97+/-0.08
8	-	-	-	-	0.69+/-0.60
12	-	-	-	-	-
16	-	-	-	-	-
21	-	-	-	-	-

The data are the mean values +/- Standard Errors obtained from three separate cultures at each nutrient concentration.
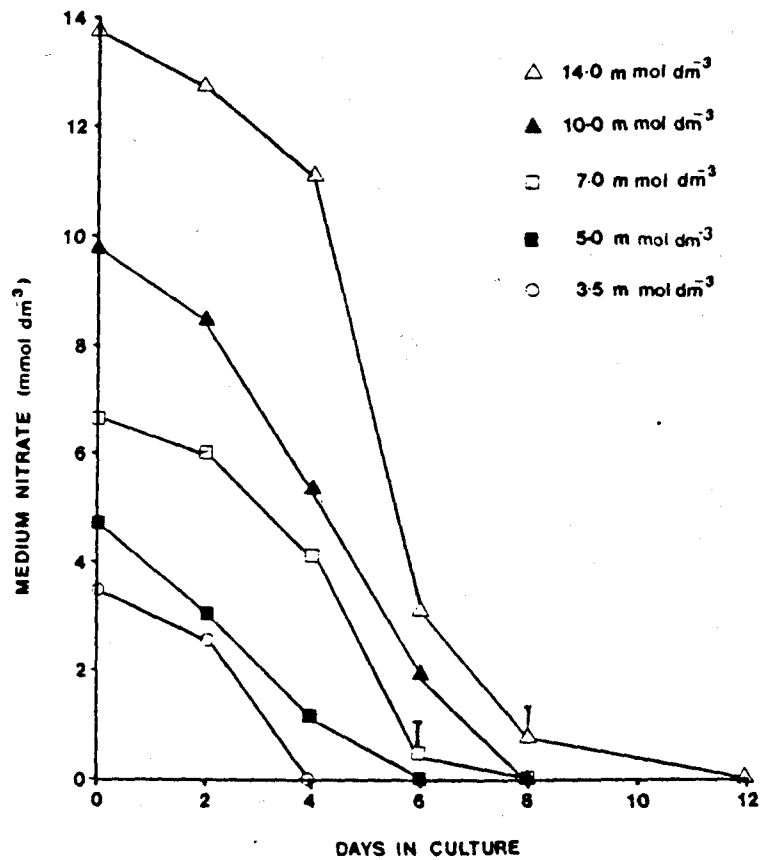
Figure 4.6

Medium nitrate depletion by _Acer pseudoplatanus_
cells cultured at a range of initial nitrate
concentrations.

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The medium nitrate was depleted by Day 4 in the 3.5 mmol dm^{-3} cultures, Day 6 in the 5.0 mmol dm^{-3} cultures, between Days 6-8 in the 7.0 mmol dm^{-3} cultures, by Day 8 in the 10.0 mmol dm^{-3} cultures and between Days 9-12 in the 14.0 mmol dm^{-3} cultures. In all of the cultures the rate of nitrate depletion was lowest between Days 0-2 whilst the cells are in the lag phase. The rate of depletion then increased resulting in the early complete depletion of nitrate in the two lowest concentrated cultures. During the period of rapid cell division, commencing at approximately Day 3, there is a correspondingly rapid decrease in the medium nitrate levels in the 7.0, 10.0 and 14.0 mmol dm^{-3} cultures. This depletion was most rapid in the 14.0 mmol dm^{-3} cultures where most cell division was occuring. Similar results were obtained by Jessup and Fowler (1976) who also worked with sycamore cells.

Referring back to Figure 4.1 it will be remembered that the growth rate as represented by cell density was higher at higher initial nitrate levels. The fact that nitrate is relatively rapidly depleted from the media in the low nitrate cultures (3.5 and 5.0 mmol dm^{-3}) may explain why low growth rates were observed in these cultures. Conversely, in the high nitrate cultures (10.0 and 14.0 mmol dm^{-3}) nitrate depletion occurred over a longer time period and growth rates were higher. Thus, nitrate may be a growth limiting factor.

However, depletion of medium nitrate does not necessarily mean that the cells themselves are nitrate-limited. The cells may accumulate nitrate in a store for future utilisation. In order to determine the fate of the depleted medium nitrate cellular nitrate levels were estimated in the control cultures as described previously. The results are tabulated in Table 4.6 and illustrated graphically in Figure 4.7. The medium nitrate concentration is also replotted in Figure 4.7. The nitrate levels detected in the cells were relatively low and thus, as discussed earlier, the standard errors are high. Nevertheless the cellular nitrate levels increased after sub-culture to a maximum level by Day 6. This increase correlated with the depletion of nitrate from the medium. The cellular nitrate level then declined rapidly during the exponential phase of batch culture growth.

Firby (1985) indirectly investigated the activity of the inducible enzyme nitrate reductase by estimating the production of nitrite within the cells. Nitrate reductase occupies a likely regulatory position in the plant's nitrogen metabolic pathway as it controls the first basic step, the reduction of nitrate to nitrite. After sub-culture an increase in nitrate reductase activity was noted after 25 hours. A peak in activity was reached between Day 4 and 5 of culture, but the activity level then rapidly declined. Thus, the peak in nitrate reductase activity occurred before the peak in

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cellular nitrate observed here and the subsequent rapid decline in the level of enzyme activity paralleled the high rate of nitrate depletion from the medium. Firby concluded that the nitrate reductase activity appeared to correlate more closely with the nitrate concentration of the medium than the levels of nitrate within the cells. Similar patterns of fluctuating cellular nitrate, medium nitrate and nitrate reductase activity levels have been reported by Filner (1966) in _Nicotiana tabacum_ cell suspension cultures and also by Young (1973) in other lines of _Acer pseudoplatanus_ cells.

Nitrate reductase, like most enzymes, is known to have an optimum working pH range. This has been reported _in vitro_ as between 6.0-7.0 (Evans and Nason, 1953; Prakash and Naik, 1982). The pH of the culture medium is initially within this range (approximately 6.4) but during the batch culture growth cycle the pH levels were found to fluctuate significantly (Table 4.6). However, it is unlikely that such pH fluctuations would occur within the cells (Raven, 1980) and thus the activity of the nitrate reductase is likely to be unaffected by these media pH changes. Nevertheless, the media pH changes may be an indirect affect of nitrate uptake by the cells. The medium pH and cellular nitrate patterns are illustrated together in Figure 4.8. The pH of the medium was routinely adjusted to 6.4 before being autoclaved. After autoclaving the medium was usually

<u>Table 4.6</u>

Changes in medium and cellular nitrate concentrations and medium pH levels in <u>Acer</u> <u>pseudoplatanus</u> cells cultured at the usual nitrate concentration $(7.0 \text{ mmol dm}^{-3})$.

Time (Days)	Medium Nitrate mmol dm^{-3}	Cellular Nitrate mmol dm^{-3}	Medium pH
0	6.61+/-0.15	-	6.23+/-0.12
2	5.89+/-0.14	0.69+/-0.60	6.50+/-0.10
4	4.04+/-0.05	1.09+/-0.08	7.10+/-0.10
6	0.37+/-0.64	1.75+/-0.08	7.10+/-0.26
8	-	1.70+/-0.11	6.77+/-0.25
12	-	0.67+/-0.58	6.00+/-0.10
16	-	-	5.87+/-0.11
21	-	-	6.00+/-0.10

The data are the mean values +/- Standard Errors obtained from three separate cultures at each nutrient concentration.
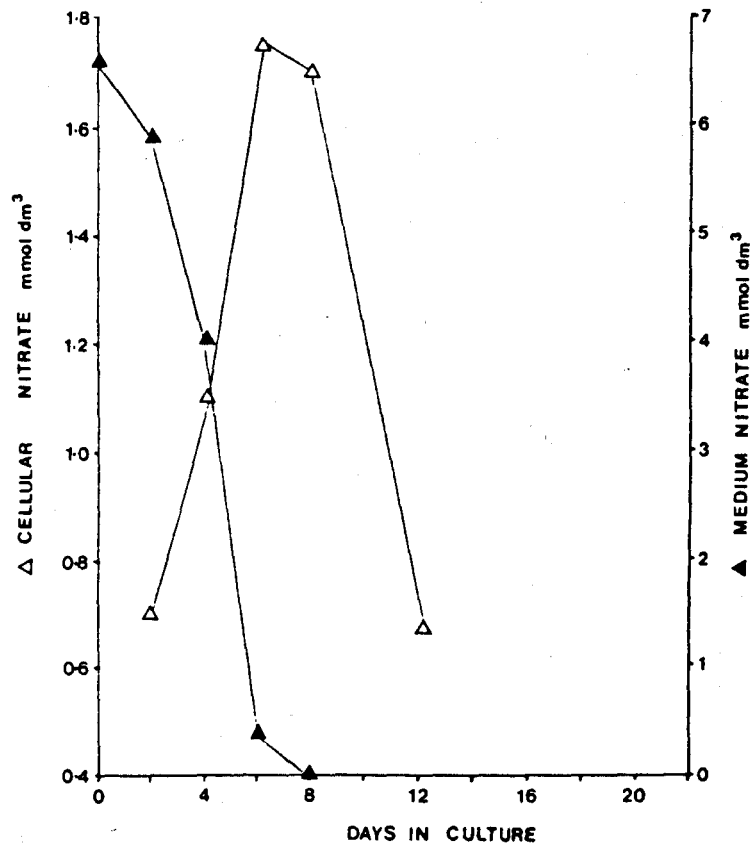
Figure 4.7

Changes in the medium nitrate and cellular
nitrate concentrations in Acer pseudoplatanus
cells cultured at the usual nitrate
concentration (7.0m mol dm^{-3}).
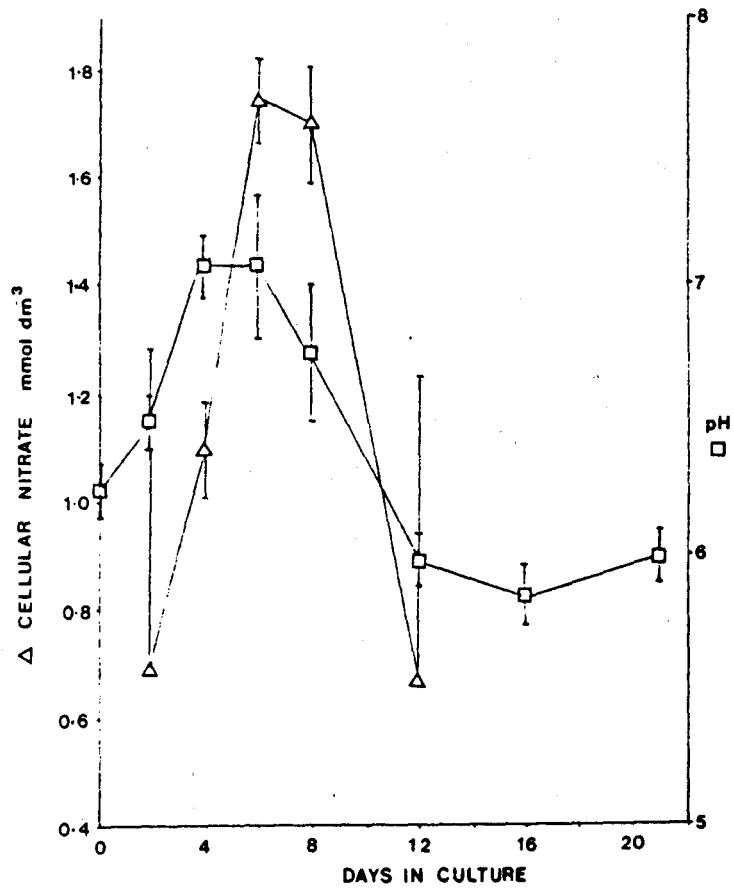
Figure 4.8

Changes in medium pH and cellular nitrate concentrations in Acer pseudoplatanus cells cultured at the control nitrate concentrations (7.0m mol dm^{-3}).

found to be slightly more acidic and after inoculation with stationary phase cells it was found to be 6.23+/-0.12. The medium pH then rapidly increased until maximum values of 7.1+/-0.1 and 7.1+/-0.26 were achieved between Days 4-6. The pH levels then declined to a value of 5.87+/-0.11 by Day 16 and then increased to a final Day 21 value of 6.00+/-0.10. The rise and fall in the medium pH levels slightly preceded the rise and fall in the cellular nitrate levels. The increase in the alkalinity of the medium may be due to the efflux of OH^- from the cells as they take up the NO_3^- ions. As the culture developed into an asynchronous state a rapid decline in the pH was observed. This is probably a reflection of a build-up of acidic waste by-products in the medium as it is a closed system, and the cessation of any OH^- efflux from the cells due to the depletion of medium nitrate. Another possibility is that the cells may develop different biosynthetic pathways when growth is limited, notably those that result in the production of secondary products. The presence of such substances is likely to affect the pH of the culture medium.

The initiation of secondary metabolism has been linked with the soluble nitrogen content of the medium and the level of auxin in the culture (Phillips and Henshaw, 1977). As the cells enter the stationary phase they become nitrate-limited and thus their metabolic pathways are switched from those of primary metabolism, which are predominantly associated with cytokinesis, to those of

secondary metabolism which are generally concerned with the synthesis of secondary products. In sycamore cell suspension cultures phenolics have been reported as the main secondary product (Westcott and Henshaw, 1976). Firby (1985) observed a decline in the amount of extractable cellular protein in Acer pseudoplatanus suspension cultures as nitrate became limiting. Thus, it appears that as nitrate becomes limited the cells are unable to perform the essential metabolic processes required for successful cytokinesis, such as protein synthesis. Coincident with the decline in nitrate availability and the subsequent decrease in primary metabolism is an increase in the activity of the enzyme phenylalanine ammonia-lyase (PAL). This enzyme catalyzes the first step in the phenylpropanoid metabolic pathway which results in the synthesis of phenolics. Westcott and Henshaw (1976) noted increased PAL activity and phenolic production in sycamore cells as nitrogen became a limiting factor. Phillips and Henshaw (1977) proposed that the switch from protein synthesis to phenolic synthesis may be controlled by competition for the common precursor, phenylalanine. As nitrate limitation causes the cessation of primary metabolism more phenylalanine becomes available for secondary product synthesis.

However, there is evidence in the literature of phenolic synthesis completely unrelated to nitrate availability (Davies, 1972a). Phosphate is also known to be depleted

from Acer pseudoplatanus suspension culture medium and
at approximately the same rate as nitrate (Wilson,
1976). It is possible that in certain plant culture
systems a situation may develop whereby nitrate is not
limiting but phosphate is and thus primary metabolism is
impeded and secondary products are produced regardless
of the fact that nitrate is plentiful. This would
explain the previously mentioned instance observed by
Davies (1972a). Thus it would seem likely that any
shortage of essential substrates for primary metabolism
will lead to secondary product accumulation provided
that all the essentials are available for this secondary
synthesis. In the case of Acer pseudoplatanus cell
suspension cultures nitrate and phosphate are the
predominant growth limiting substances (Young, 1973;
Wilson, 1976).

As mentioned earlier the initiation of secondary
metabolism has also been linked with the level of auxin
in the culture (Phillips and Henshaw, 1977). Westcott
and Henshaw (1976) investigated this relationship by
reducing the 2,4-D concentration in the medium of
sycamore cells in suspension culture. They noted a
marked increase in the synthesis of phenolics with
little or no effect on the growth rate. King (1976)
also reported increased levels of phenolics in sycamore
cells after a reduction in 2,4-D. Conversely, Davies
(1972b) experimented by supplying 2,4-D to rose cultures
in the mid-exponential phase. In this case phenolic

synthesis was inhibited but the inhibition of the activity of the enzyme PAL was less significant, implying that the effect is seen at some other point in the metabolic pathway. This phenomenon may be again explained by the availability of the substrate, phenylalanine. Phillips and Henshaw (1977) reported that the addition of 2,4-D to stationary phase cell suspension cultures resulted in an increase in protein turnover which was coincident with the inhibition of phenolics synthesis. Presumably these cells did not revert back to true primary metabolic pathways such as increasing net protein synthesis because they were nitrate and possibly phosphate limited. However, the 2,4-D appeared to stimulate protein synthesis in the form of protein turnover and thus inhibited secondary product synthesis because of competition for phenylalanine.

As with most areas of plant growth regulator research it is unlikely that the switch from primary to secondary metabolism is controlled by one growth regulator independently. Ethylene is known to be produced at a high rate by <u>Acer pseudoplatanus</u> cells in early stationary phase (Mackenzie and Street, 1970; Constabel et al., 1977). This gaseous plant growth substance has been reported to have an inhibitory affect on PAL activity and thus phenolic synthesis (Westcott, 1976). It is likely that 2,4-D and ethylene and possibly other plant growth regulators interact together resulting in

the overall control of secondary product synthesis. Indeed, 2,4-D has been found to enhance ethylene production in _Acer pseudoplatanus_ cells (Mackenzie and Street, 1970).

The availability of nitrate and/or phosphate appears to play a crucial role in both the population dynamics of batch cell suspension cultures and in their choice of metabolic pathways. The evidence from the data presented here and those reported in the literature supports the hypothesis that as nutrients become limiting the cells divert from primary metabolic pathways resulting in cytokinesis, to secondary metabolic pathways resulting in the synthesis of secondary products. This switch in metabolic pathways may explain why batch cell suspension cultures initiate in a synchronous state but soon develop asynchrony. A prime aim in this present study was to develop a reliable method of inducing synchrony without causing traumatic changes to the cells normal cultural environment. Thus, a system was developed whereby the cells were prevented from becoming nutrient limited by a nutrient feeding mechanism in the hope that they would maintain the synchronous state characteristic of the first division after subculture.

4.3.2 Inducement of synchrony by nitrate and phosphate feeding

The results of the experiments performed in Section 4.3.1 revealed that nitrate and phosphate do limit growth in batch cell suspension culture. However, this problem cannot necessarily simply be overcome by substantially increasing the amount of nitrate and/or phosphate available to the cells from the outset of subculture because the cells appear to grow optimally at a nitrate concentration between 7-10mmol dm^{-3}. That is, not much higher than the usual medium nitrate level of 7mmol dm^{-3}. Another possibility would be to inoculate less cells into the usual medium, thus producing a culture with more nitrate available per cell. This system was utilised by King and Street (1973) who worked with another strain of Acer pseudoplatanus cells in suspension culture. They produced five consecutive relatively synchronous divisions by this technique. However, this method is not ideal for research into cellular plant growth regulator levels because the cell dry mass yields of samples are low as the cell density is low. Growth regulators are often present at very low concentrations and thus an ideal synchronization technique would produce cultures with high cell yields. Another inherent problem with this method is that many cultures fail to grow when inoculated at very low densities, and indeed, previous workers in these laboratories could not synchronize this strain of cells in this manner.

Although phosphate and nitrate are known to be depleted from the medium by Day 7 and 8 respectively, (Wilson, 1976; Young, 1973; Firby, 1985; Figure 4.6), it appears that nitrate is the primary growth limiting nutrient. Wilson (1976) observed that in cultures containing more than half the standard phosphate concentration the final cell density achieved was not related to the initial phosphate concentration. However, at lower phosphate concentrations the final cell density was found to be proportional to the initial phosphate concentration. Thus, in the higher phosphate cultures something other than phosphate was limiting growth and it is believed that this factor is nitrate. The data obtained by Lewis (1983) in these laboratories are compatible with this hypothesis. She noted that although the final cell number obtained by cells grown in medium containing half the standard phosphate concentration was similar to that attained by cells grown in half the standard nitrate concentration, it appeared that at lower phosphate concentrations more growth occurred than with equivalent amounts of nitrate.

Firby (1985; Firby, O'Sullivan, Leach and Elliott, in preparation) also noted that nitrate appears to actually limit growth more than phosphate in these cultures. Preliminary experiments in the development of the synchronization technique employed in this study involved adding nitrate or phosphate or nitrate and

phosphate to cultures after cytokinesis. The addition of nitrate alone after each division produced three consecutive synchronous divisions; these being 72.0%, 74.1% and 57.0% synchronous. By the fourth division the synchrony level decreased to 47.0% and since this is less than 50% the culture had essentially become asynchronous. The addition of phosphate alone resulted in a decline in synchrony from that obtained on subculture (76.6%) to levels of 49.3%, 35.9% and 28.0%, which are similar to those produced by control asynchronous batch cultures. However, when nitrate and phosphate were added after each successive cytokinesis, levels of synchrony were achieved of 82.9%, 80.0% and 76.0%, all of which were higher than the 74.2% synchronous division obtained on subculture. In all cases the amount of nitrate and phosphate added was sufficient to re-establish the standard concentrations in the medium. Nitrate was measured with an ion-selective electrode after sampling and phosphate was assumed to be depleted at an equivalent rate.

This method of induction synchrony proved to be very reliable, especially when the cells were grown in a single large volumed batch culture. However, when this method was applied to a number of cultures grown in separate litre bottles and harvested at different time points, problems were encountered. As discussed previously, all cell suspension cultures are slightly different and as a result they divide at slightly

different times.   This method of synchrony relies upon
the   fact   that   the   nutrients are   added   as   soon   as
possible after cytokineses.   However, when a time point
culture   is   harvested it may have reached the stage   of
cytokinesis   and   thus   nutrients   are   added   to   the
remaining   cultures,   the cells of which may or may   not
have   divided.   As Figure 4.15   illustrates,   in   these
cases   synchrony   was   lost   and some   of   the   cultures
developed   into asynchrony possibly because some of   the
cells had switched from primary to secondary   metabolism
due to the lower availability of nutrients.

The   time   staggering of cytokinesis   between   different
cultures,   although often slight,   can sometimes produce
misleadingly low synchrony levels when cell numbers from
duplicate   large   batch cultures   are   averaged.   Thus,
where   synchronous   data are presented from large   batch
cultures,   only   one culture is considered although   the
trends have been shown to be reproducible.

A typical synchronous cell number curve obtained by this
method   is   illustrated in Figure 4.9 and the   data   are
tabulated in Table 4.7.   A control curve is also plotted
in Figure 4.9.   Cell number estimations were made   more
regularly   close to the expected point of cytokinesis in
order   that the nutrient feed could be added as soon   as
possible   after   the   cell   division.   The   percentage
synchrony of each division was found to be high   (98.4%,
83.1%   and   59.0%).   After each successive   synchronous

Table 4.7

Acer pseudoplatanus cell number changes during
nitrate/phosphate induced synchronization

TIME (Hrs)	DAY	No of cells in 1 cm^3 of culture (x 10^{-3}) +/- SE		% Error (+/-)
0	0	249.2 +/- 8.9		3.6
3		245.3 +/- 9.1		3.7
24	1	238.1 +/- 10.1		4.2
48	2	239.7 +/- 12.2	$\bar{x}$ = 244.8	5.1
60		246.6 +/- 9.9		4.0
66		249.2 +/- 7.7		3.1
70		245.3 +/- 13.1	68.9%	5.3
72	3	402.1 +/- 10.2	synchronous	2.5
75		405.7 +/- 7.7		1.9
96	4	419.1 +/- 10.3		2.5
120	5	404.1 +/- 7.9		2.0
130		420.1 +/- 9.3	$\bar{x}$ = 413.4	2.2
139		403.1 +/- 6.2		1.5
142		411.6 +/- 8.0		1.9
144	6	441.7 +/- 10.2	98.4%	2.3
147		785.2 +/- 10.4		1.3
168	7	820.6 +/- 16.3		2.0
192	8	805.5 +/- 21.1		2.6
202		783.2 +/- 23.7		3.0
211		797.0 +/- 14.2	$\bar{x}$ = 820.1	1.8
214		794.4 +/- 19.5		2.5
216	9	835.6 +/- 18.8		2.2
218		939.1 +/- 16.2	83.1%	1.7
220		1460.4 +/- 58.2		4.0
240	10	1439.4 +/- 43.4		3.0
264	11	1464.3 +/- 52.1		3.6
274		1521.9 +/- 37.3		2.5
283		1533.7 +/- 61.4	$\bar{x}$ = 1501.5	4.0
286		1481.3 +/- 22.2		1.5
288	12	1515.4 +/- 42.6		2.8
290		1595.2 +/- 45.5	59%	2.9
292		2027.5 +/- 68.7		3.4
293		2417.8 +/- 103.7		4.3
294		2483.0 +/- 101.0	$\bar{x}$ = 2388.9	4.1
312	13	2535.7 +/- 84.3		3.3
316	14	2480.7 +/- 68.6		2.8

Standard Error (SE) = +/- $\dfrac{\sigma n - 1}{\sqrt{n}}$
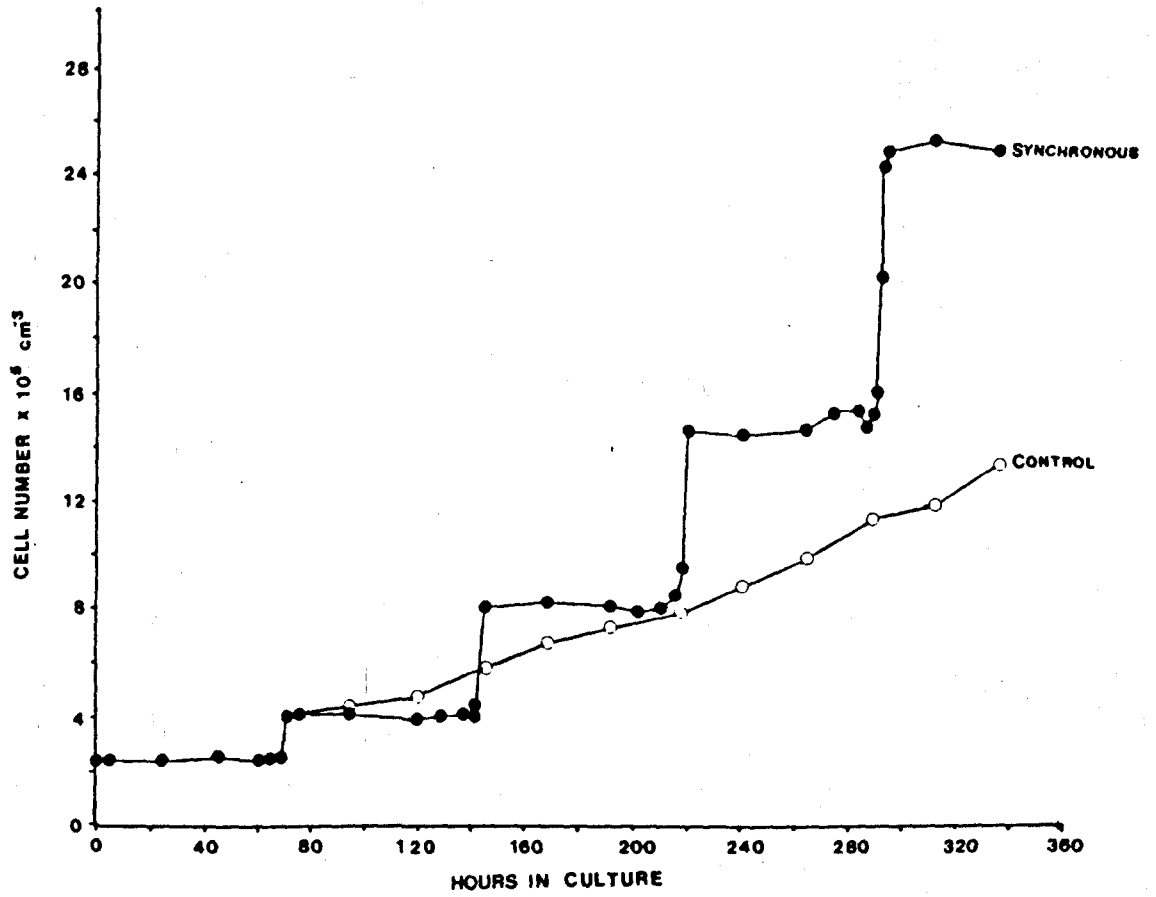
Figure 4.9

Acer pseudoplatanus cell number changes
during nitrate/phosphate induced synchrony.

division periods of constant cell number were observed, typical of a culture exhibiting a high degree of synchrony. The process of cell division occurred over a time range of approximately 10 hours which is a relatively small fraction of the 72 hour cell cycle. The mitotic index levels obtained from the first induced synchronous cell cycle are tabulated in Table 4.8 and illustrated in Figure 4.10. A peak in mitotic activity was seen preceding cytokinesis. Thus, all of the salient features to confirm the synchronous state outlined previously were found to be present with this induction technique.

Figure 4.9 illustrates the typical step-like pattern of a synchronous cell number curve. Each cell division exhibits a high degree of synchrony and the period of time between each division is approximately equal. This implies that the majority of the cells in the culture are involved in primary metabolism and that they are dividing after an optimal time period for the culture conditions. Conversely, the cells in the control culture become nutrient limited and a large proportion of the population shift from primary to secondary metabolism. Thus, there is less active cell division in the control culture and as a result the final cell density is lower than that achieved by the synchronous culture.

<u>Table 4.8</u>

<u>Acer pseudoplatanus</u> mitotic index changes during the first synchronous division after subculture and a subsequent nitrate/phosphate induced synchronous division

----

TIME (Hrs)	DAY	CELL NUMBER ($\times 10^{-3}$ cm^{-3})	MITOTIC INDEX (%)
0	0	249.2	0.6
24	1	238.1	0.6
48	2	239.7	1.6
60		246.6	5.2
66		249.2	9.8
70		245.3	4.4
72	3	402.1	3.0
96	4	419.1	1.6
120	5	404.1	1.4
130		420.1	2.8
139		403.1	7.2
142		411.6	10.2
144	6	441.7	6.6
147		785.2	4.4
168	7	820.6	1.8
192	8	805.5	1.6
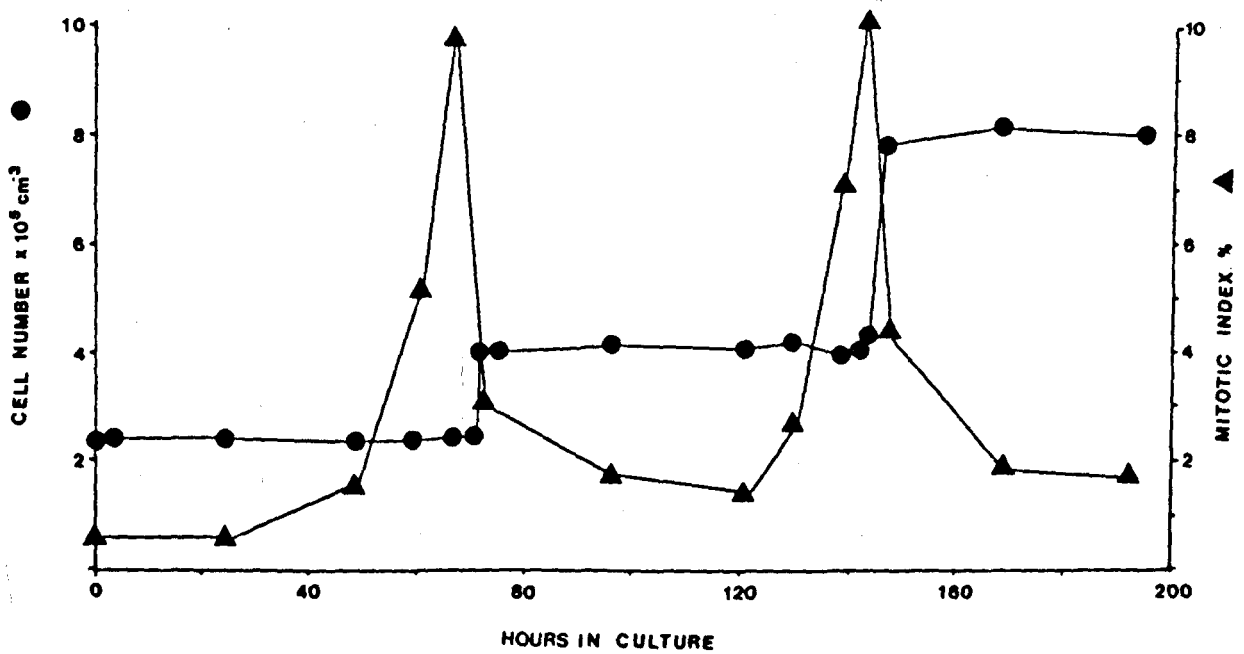
Figure 4.10

Acer pseudoplatanus mitotic index changes
during the first synchronous division after sub-
culture and a subsequent nitrate/phosphate
induced synchronous division.

The dry mass per cell, mean cell volume and cell via-
bility data obtained from the first induced synchronous
division are tabulated in Table 4.9 along with the data
from the first synchronous division on subculture.   The
dry mass and cell volume data are illustrated in
Figure 4.11.   These two parameters produced similar
patterns to those obtained on subculture implying that
the method of synchrony did not cause a noticeable
disruption to the cell's growth patterns.   The cell
viability data revealed that the inducement technique
caused little or no affect on cell viability.

Having established a reliable synchronization technique
with standard cell cycle periods it was decided to
investigate whether the cells' growth rates could be
increased or decreased by providing more or less nitrate
and phosphate in the feed.   An attempt was made to
synchronize cultures with initial nitrate (and
correspondingly phosphate) concentrations of 3.5mmol
dm^{-3} and 14.0mmol dm^{-3}.   The results are tabulated in
Table 4.10 and illustrated in Figure 4.12.   In each case
the nutrient feed was supplied after cytokinesis to re-
establish the inoculum concentration.   The 3.5mmol dm^{-3}
culture produced the usual first division synchrony
(65.6%) but the synchronous state was not maintained and
the culture then developed into asynchrony presumably
because the cells were nutrient limited.   The 14.0mmol
dm^{-3} culture produced a first synchronous division by
Day 2.

Table 4.9

Acer pseudoplatanus dry mass (ng cell^{-1}), mean cell volume (MCV) and cell viability changes during nitrate/phosphate induced synchrony

TIME (Hrs)	DAY	CELL NUMBER (x 10^{-3} cm^{-3})	DRY MASS (ng cell^{-1})	MCV (cm^3)	CELL VIABILITY (% non-viable)
0	0	249.2	3.41	0.120	3.6
24	1	238.1	3.86	0.126	4.4
48	2	239.7	4.17	0.125	5.3
60		246.6	4.58	0.142	5.5
66		249.2	4.78	0.140	6.1
70		245.3	4.97	0.163	4.2
72	3	402.1	3.01	0.095	4.5
96	4	419.1	3.32	0.107	5.1
120	5	404.1	4.06	0.124	6.1
130		420.1	4.28	0.131	5.8
139		403.1	4.89	0.136	3.9
142		411.6	4.91	0.146	4.1
144	6	441.7	4.60	0.136	5.2
147		785.2	2.61	0.076	6.7
168	7	820.6	2.84	0.085	4.4
192	8	805.5	3.18	0.099	4.8
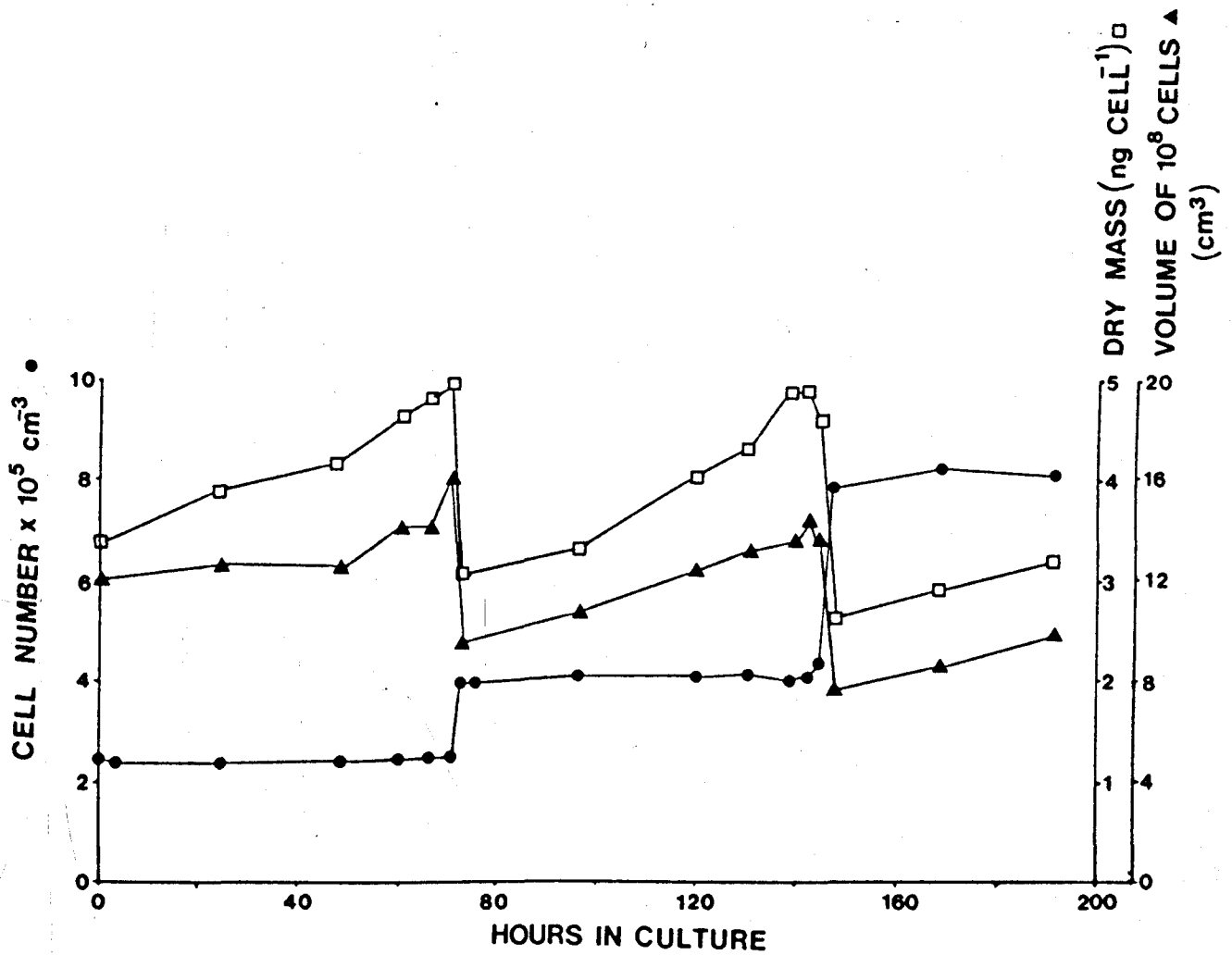
Figure 4.11

Acer pseudoplatanus mean cell volume and dry mass (ng cell^{-1}) changes during nitrate/ phosphate induced synchrony.

Table 4.10

<u>Acer pseudoplatanus</u> cell number changes during nitrate/ phosphate induced synchrony in cultures maintained at nitrate concentrations of 3.5 and 14.0 mmol dm^{-3} (and corresponding phosphate concentrations).

TIME (Hrs)	DAY	CELL NUMBER x 10^{-3} cm^{-3} +/- SE			
		3.5 mmol dm^{-3}		14.0 mmol dm^{-3}	
0	0	259.7 +/- 7.9		244.9 +/- 8.6	
24	1	263.3 +/- 7.7		246.9 +/- 8.9	$\bar{x}$ = 264.9
43			$\bar{x}$ = 263.6	302.9 +/- 10.0	73.5%
48	2	254.4 +/- 7.0		424.7 +/- 12.3	
70		277.0 +/- 9.4	65.6%		$\bar{x}$ = 459.6
72	3	396.2 +/- 12.2		434.8 +/- 9.6	
93			$\bar{x}$ = 436.6	519.3 +/- 12.4	93.4%
96	4	414.5 +/- 9.9		863.1 +/- 21.1	
120	5	499.0 +/- 12.1		869.0 +/- 28.1	
139		604.8 +/- 16.7			$\bar{x}$ = 888.7
140				933.9 +/- 25.8	
142		630.6 +/- 15.2			77.7%
144	6	632.0 +/- 16.8		1511.5 +/- 31.6	
147		647.7 +/- 18.2			
151		660.8 +/- 19.5			
168	7	687.6 +/- 13.1		1549.4 +/- 14.3	
172		698.1 +/- 13.2			$\bar{x}$ = 1579.0
188				1586.1 +/- 22.2	
192	8	710.5 +/- 17.8		1549.4 +/- 37.4	
210				1698.8 +/- 36.2	73.0%
212				2544.9 +/- 36.5	
216	9	747.2 +/- 16.1		2783.2 +/- 37.8	$\bar{x}$ = 2732.2
240	10	791.7 +/- 14.2		2868.4 +/- 38.9	

Standard Error (SE) = +/- $\dfrac{\sigma n - 1}{\sqrt{n}}$

Figure 4.12   _Acer pseudoplatanus_ cell number changes during nitrate/phosphate induced synchrony in cultures maintained at nitrate concentrations of 3.5 and 14.0m mol dm^{-3} (and corresponding phosphate concentrations).
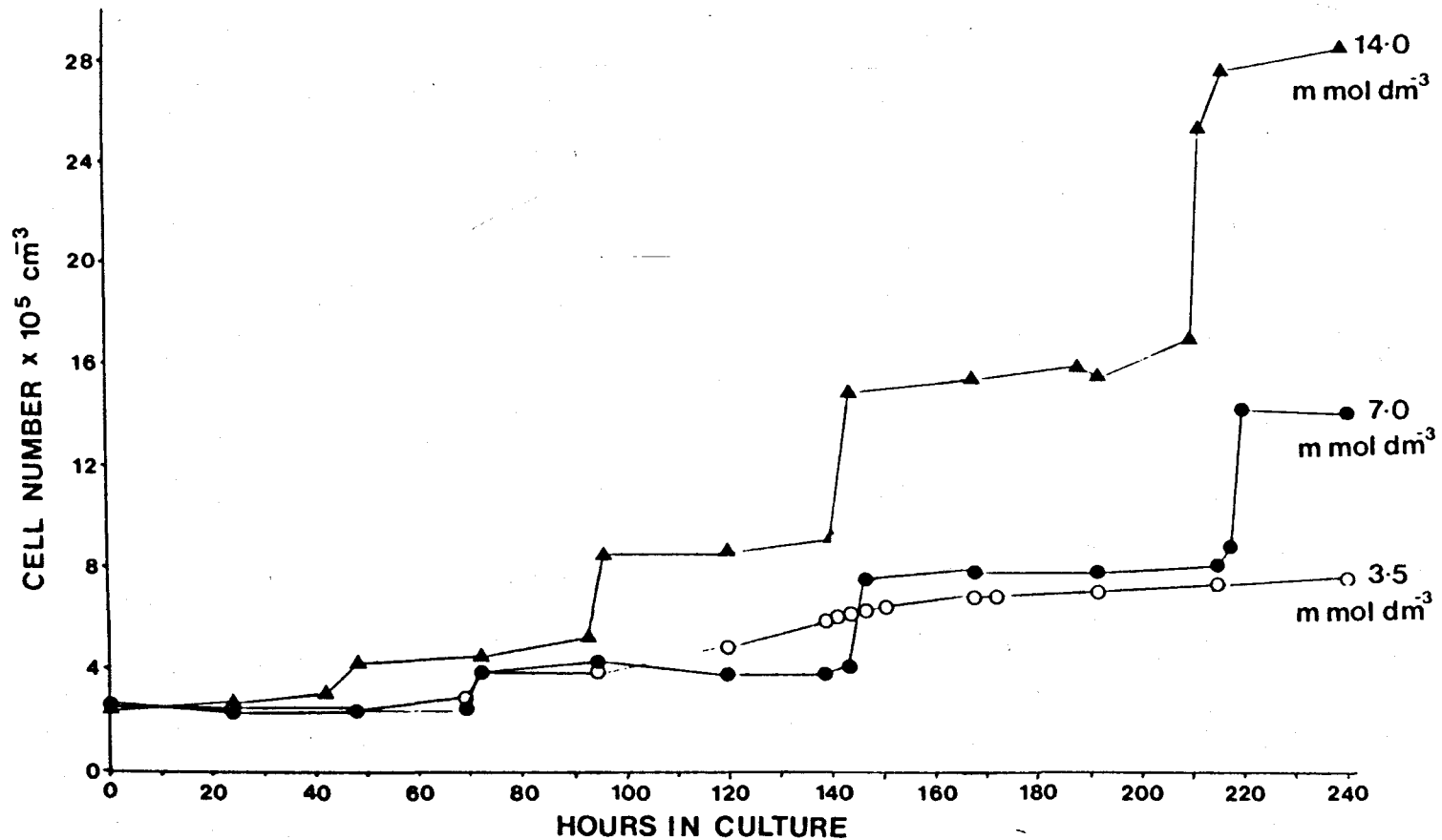
which implies that the cell culture growth rate was higher than that of cells maintained in control (7.0mmol dm^{-3}) or 3.5mmol dm^{-3} cultures which divided after three days. This high growth rate was maintained through two further highly synchronous divisions (93.4% and 77.7%). The growth rate then declined to that of the control cultures but remained highly synchronous (73.0%). Thus 14.0mmol dm^{-3} nitrate and phosphate feeding produced rapid highly synchronous divisions but after three synchronous divisions the growth rate decreased possibly because nitrate, phosphate or another factor became growth limiting.

Earlier work presented in this section revealed that in control batch cultures the medium pH became more alkaline as the medium nitrate decreased and the cellular nitrate level increased. These parallel fluctuations may be caused by a direct or indirect relationship between these parameters or they may be coincidental. To investigate this phenomenon further the medium pH levels were monitored during induced synchrony in the cultures maintained at 3.5mmol dm^{-3}, 7.0mmol dm^{-3} and 14.0mmol dm^{-3} nitrate (and corresponding phosphate levels). The results are tabulated in Table 4.11 and illustrated in Figure 4.13. Firby (1985) had previously noted that in unsynchronized cultures maintained at nitrate (and corresponding phosphate) concentrations of 3.5 and 14.0mmol dm^{-3} the medium pH levels increased on inoculation and remained slightly alkaline until the

Table 4.11

Acer pseudoplatanus medium pH changes during nitrate/ phosphate induced synchrony in cultures maintained at nitrate concentrations of 3.5, 7.0 and 14.0 mmol dm^{-3} (and corresponding phosphate concentrations).

TIME (Hrs)	DAY	pH		
		3.5 mmol dm^{-3}	7.0 mmol dm^{-3}	14.0 mmol dm^{-3}
0	0	6.20	6.15	6.20
24	1	6.45	6.35	6.50
43				6.70
48	2	6.70	6.55	6.75
50				6.75
70		6.95	6.85	
72	3	6.95	6.85	6.85
73		6.95	6.85	
93				7.00
96	4	7.00	7.00	7.05
98				7.05
120	5	7.10	7.10	7.15
139		7.25	7.20	
140				7.25
142		7.25		
144	6	7.25	7.25	7.25
147		7.30	7.30	
151		7.30	7.30	
168	7	7.10	7.35	7.30
172		7.05		
188				7.20
192	8	7.00	7.30	7.25
210				7.20
212				7.20
216	9	6.95	7.15	7.15
220			7.15	
222			7.15	
240	10	6.90	7.00	7.10

**Figure 4.13** <u>Acer pseudoplatanus</u> medium pH changes during nitrate/phosphate induced synchrony in cultures maintained at nitrate concentrations of 3.5, 7.0 and 14.0m mol dm-3 (and corresponding phosphate concentrations).

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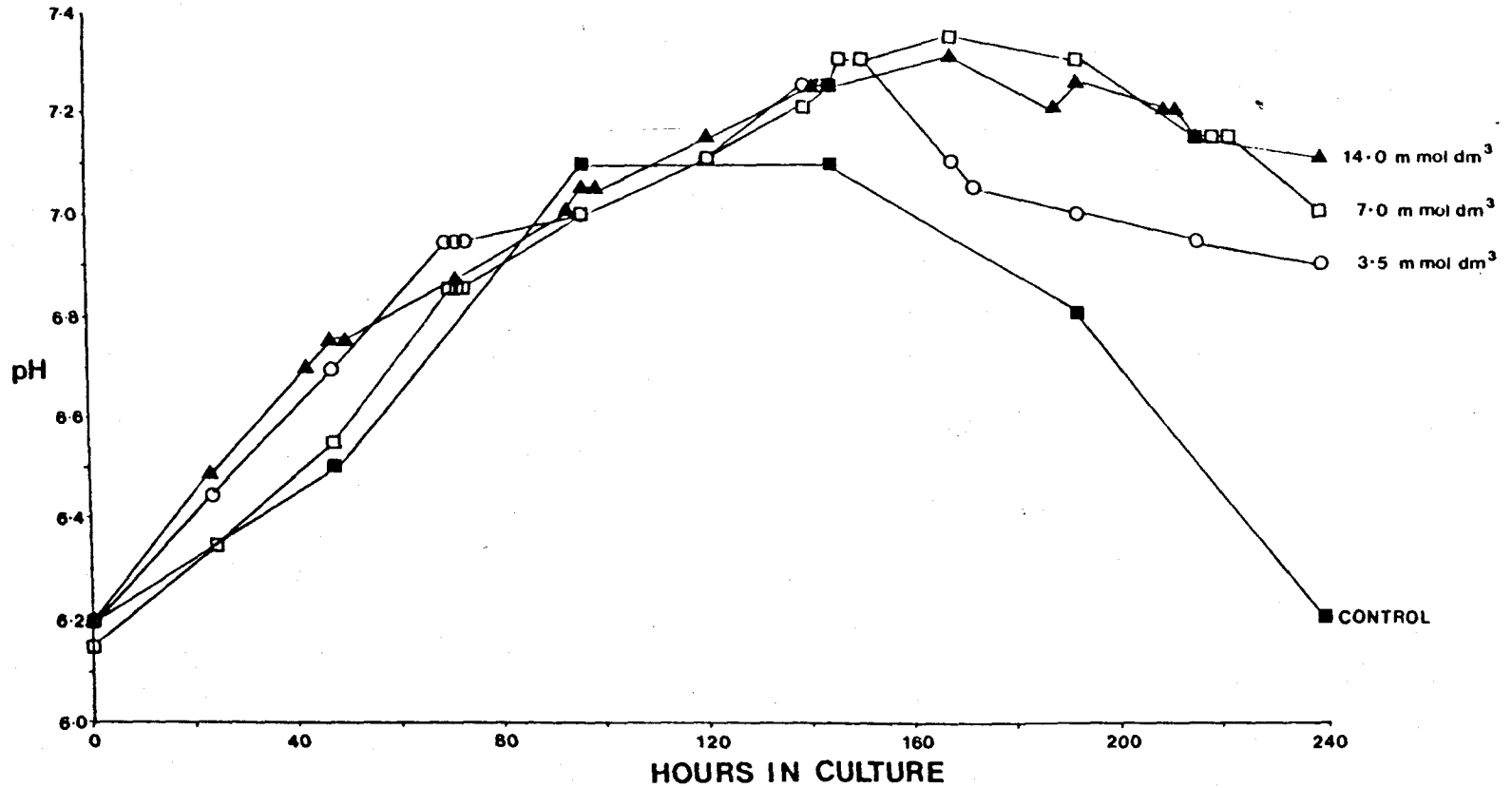
medium nitrate was depleted. This nutrient depletion occurred earlier in the 3.5mmol dm^{-3} cultures and later in the 14.0mmol dm^{-3} cultures and the maximum pH levels achieved, before the medium became more acidic, reflected this pattern. The degree of medium alkalinity also appeared to be related to the nutrient status; the more nutrient available the higher the pH value achieved. Thus, in cultures maintained at half the standard nitrate and phosphate concentrations the nitrate was depleted by Day 4 when the pH achieved a maximum level of 7.20. In the control cultures the nitrate was virtually depleted by Day 6 when the maximum pH level of 7.35 was recorded. In the cultures maintained at double the standard nutrient concentration the highest pH value of 7.40 was found at Day 7 with the nitrate being completely depleted by Day 8.

Figure 4.13 illustrates the data obtained from synchronized cultures maintained at the three different nitrate and corresponding phosphate concentrations. On inoculation the pH levels appeared to increase as found earlier in batch culture. The addition of the nutrients appeared to produce little or no affect on the medium pH. The pH stabilized at a level between 7.2-7.4 before eventually steadily declining in all three cultures. This decline occurred later and was less rapid than that observed earlier in control asynchronous batch cultures (control curve in Figure 4.13). Table 4.12 shows the corresponding medium nitrate levels and it appears that

in all cases the timing of complete medium nitrate depletion between feeds is associated with the decline in medium pH. This may be a reflection of some of the cells becoming nitrate limited and thus switching from primary to secondary metabolic pathways. Further comparable data is tabulated in Table 4.14 and illustrated in Figure 4.15. Here the synchronous state was not maintained due to the late addition of the second nitrate feed. It is assumed that as nitrate became less plentiful asynchrony developed and the medium pH declined.

It seems likely that synchrony is achieved in these cultures by overcoming the shortage of the growth-limiting nutrients nitrate and phosphate. The medium alkalinity associated with these synchronously dividing cells may merely be a side effect of the uptake of nitrate into the cells. Conversely, the pH increase may be due to another process associated with cytokinesis or it may be a pre-requisite for cytokinesis. Future work involving maintaining cultures in the apparently optimal alkaline state in an attempt to induce synchrony would reveal whether or not the pH status of the medium does have any direct affect on synchrony. The fact that the pH reaches a maximum when the most nitrate has been taken up by the cells implies that the corresponding OH^- efflux may be the causal factor.

Table 4.12

Acer pseudoplatanus medium nitrate concentrations during nitrate/phosphate induced synchrony in cultures maintained at nitrate concentrations of 3.5, 7.0 and 14.0 mmol dm^{-3} (and corresponding phosphate concentrations).

TIME (Hrs)	DAY	Medium Nitrate (mmol dm^{-3}) 3.5	7.0	14.0
0	0	3.41	6.75	14.05
24	1	3.13	6.11	13.26
43				12.24
48	2	2.65	5.82	-->13.80
50				13.33
70		1.42	4.59	
72	3	-->3.54	-->6.88	12.52
73		3.50	6.84	
93				10.26
96	4	2.64	5.22	-->13.90
98				13.83
120	5	0.93	3.57	11.02
139		0.00	2.29	
140				6.42
142		0.00		
144	6	0.00	2.00	6.34
147		-->3.45	-->6.61	-->13.78
151		3.42	6.60	
168	7	1.73	3.50	9.72
172		1.70		
188				3.63
192	8	0.00	0.00	3.28
210				0.52
212				0.00
216	9	-->3.30	-->7.51	-->13.58
220			7.41	
222				
240	10	0.00	2.13	8.69

Arrows indicate nutrient additions

4.3.3 Estimation of IAA levels in nitrate/phosphate induced synchronous-cultures

As discussed previously (Section 3.3.3) the endogenous IAA of these Acer pseudoplatanus cells has been investigated comprehensively in these laboratories. Despite variations in actual levels the basic trend has consistently revealed a peak in endogenous IAA levels coincident with mitosis during the first highly synchronous cytokinesis associated with subculture. A 45 hour peak tentatively attributed to DNA synthesis was also reported by Firby (1985). In the case of this present study unaccountably high inoculation levels were also observed.

Unfortunately, data obtained from the first synchronous division on subculture may be unrepresentative because the process of inoculation is very traumatic, both physically and chemically. However, further substantiating evidence was provided by Robinson (1982) who partially synchronized the cells by a cold shock system. She observed four consecutive cold-induced partially synchronous divisions and found that the IAA per cell increased prior to each division in parallel with the mitotic indices. Unfortunately, other unexplained IAA fluctuations were also noted which occurred somewhat randomly and may have been artefacts of the synchronization technique employed.

The nitrate/phosphate feeding method of inducing synchrony developed here (Firby, O'Sullivan et al., in preparation) produced better synchrony than the cold-treatment method employed by Robinson and was considered to be less intrusive. Thus, it was decided to investigate the endogenous IAA levels during nitrate/phosphate induced synchronous cell divisions. The data obtained are tabulated in Table 4.13 and illustrated in Figure 4.14 alongside the cell number data. One litre bottle containing $490cm^3$ of culture was harvested for each time point with $480cm^3$ being used for the actual IAA analysis. These data have been presented previously up until the point of cytokinesis in Table 3.11. Figure 4.14 reveals that after the initially high inoculation level followed by the subsequent decline and ensuing small peak associated with mitosis the endogenous IAA levels decreased and maintained a baseline level of between 0-1ng 10^{-6} cells despite the fact that the cells were exhibiting highly synchronous divisions. No subsequent peaks associated with mitosis were observed. This pattern was found to be reproducible and it was concluded that the previous peaks in IAA levels coincident with mitotic activity may have been artefacts of the process of subculture and the cold shock mechanism. When the majority of the cells were actively dividing they were seen to maintain a relatively constant endogenous IAA level of approximately 1ng 10^{-6} cells. This level may be biochemically significant to the cells.

Table_4.13

Changes in cell number, dry mass and endogenous IAA levels during nitrate/phosphate induced synchronization.

TIME	CELL NUMBER	DRY MASS	IAA LEVELS	
	-3 -3	-3	-1	-6
(Hours)	x 10 cm	(mg cm)	ng g DM	ng 10 cells

TIME	CELL NUMBER	DRY MASS	IAA LEVELS ng g DM	IAA LEVELS ng 10 cells
0	273.4 +/- 11.5	0.81	2342.3	6.96
24	296.0 +/- 18.1	0.83	689.6	1.94
43	331.4 +/- 9.8	0.71	462.0	0.99
67	331.7 +/- 5.0	1.00	202.7	0.61
73	354.3 +/- 8.1	1.15	192.7	0.62
77	331.0 +/- 11.6	0.77	344.8	0.80
91	665.7 +/- 24.2	1.29	445.1	0.86
94	617.2 +/- 8.4	1.60	180.1	0.47
122	657.6 +/- 18.0	1.69	374.0	0.96
136	662.4 +/- 24.7	2.10	174.1	0.55
143	669.3 +/- 14.9	2.04	62.0	0.19
163	714.8 +/- 31.8	2.58	0.0	0.00
184	711.9 +/- 13.7	2.98	47.9	0.20
190	1316.0 +/- 76.0	4.13	131.8	0.41
208	1275.0 +/- 67.5	3.98	303.7	0.95
232	1437.5 +/- 76.5	4.52	120.2	0.38
256	1317.0 +/- 45.0	5.81	187.3	0.83
262	1411.9 +/- 59.8	6.60	82.8	0.39
330	2205 +/- 40.8	7.88	48.1	0.17
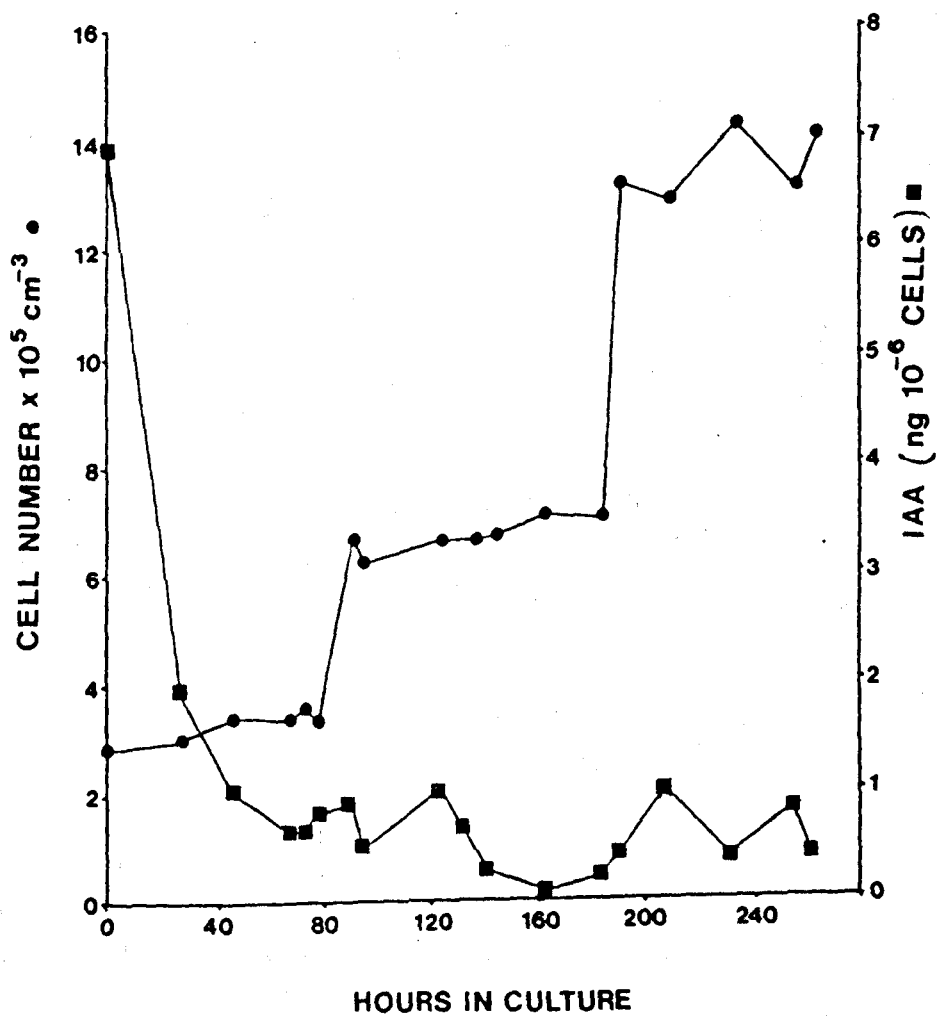
Figure 4.14

<u>Acer pseudoplatanus</u> endogenous IAA changes
during nitrate/phosphate induced synchronization.

4.3.4 Development of an automated synchronization system

As mentioned previously synchrony was not always maintained, particularly when an attempt was made to synchronize a number of cultures based on the timing of cytokinesis in a representative culture. The cell number data obtained from such an experiment are tabulated in Table 4.14 and illustrated in Figure 4.15. Although the synchronous state was initially achieved it was lost between the third division and fourth division. This situation did not always develop but it did require that large numbers of separately harvested cultures needed to be used with each experiment to ensure that sufficient cell number estimations could be made to ascertain a mean division time. This made the technique time consuming and also increased the risk of infection. The only real advantage of this technique is that, provided enough cultures are initially inoculated, large sample volumes can be harvested.

Because of the problems encountered in this area it was decided to concentrate further work on large scale batch cultures. However, even in these cultures synchrony could also be lost if the manual addition of nutrient feed was not supplied within several hours after cytokinesis. It was therefore necessary to develop an automated system to ensure that on the occasions when a culture divides a few hours earlier or later than expected the nutrient feed is added automatically and the synchronous state is maintained.

If a culture system is to be successfully automated the culture vessel itself must facilitate the use of various monitoring techniques whilst maintaining good sterile homogenous growth conditions. Wilson and co-workers (1971) developed a basic large scale batch culture unit which is relatively easily adapted for automation. Their culture system consisted of a wide necked reaction flask sealed with a reaction flask lid. The culture was agitated with the aid of a stirrer bar and a magnetic stirrer plate and was aerated with filtered, piped compressed air. An adaptor was constructed so that the air inlet and outlet used the same port on the vessel lid. Sampling was performed manually by the temporary closure of the air outlet line. This produces a positive pressure in the culture vessel and forces a volume of culture into the sample container, the line to which can be sealed with a gate clip to prevent contamination of the culture when the sample is drained from the sample container. This system could be automatically sampled through a stainless steel needle valve controlled by a solenoid. The sample of known volume is emptied into a tube containing a known volume of chromium trioxide solution. A turntable could position different tubes to receive each sample.

Table 4.14

Acer pseudoplatanus cell number changes during nitrate/phosphate induced synchronization. (Due to late nutrient addition the synchronous state was not maintained.)

TIME	CELL NUMBER	MEDIUM
	-3 -3	pH
(Hours)	x 10 cm +/- SE	

TIME (Hours)	CELL NUMBER x 10⁻³ cm⁻³ +/- SE	MEDIUM pH
0	237.1 +/- 6.37	6.20
26	249.1 +/- 5.75	6.25
43	257.4 +/- 8.13	6.50
67	300.6 +/- 13.22	6.80
73	--> 427.6 +/- 11.63	6.90
79	460.4 +/- 11.57	6.90
91	494.1 +/- 32.06	7.00
120	430.9 +/- 23.98	7.15
136	441.6 +/- 19.17	7.20
147	735.4 +/- 24.31	7.20
160	743.9 +/- 15.95	7.15
167	760.0 +/- 23.86	7.20
184	748.9 +/- 19.96	7.20
208	1380.2 +/- 27.49	7.00
214	1405.0 +/- 19.66	7.05
283	1767.0 +/- 71.85	6.50
333	1845.0 +/- 51.62	6.30

Arrow indicates nutrient addition.
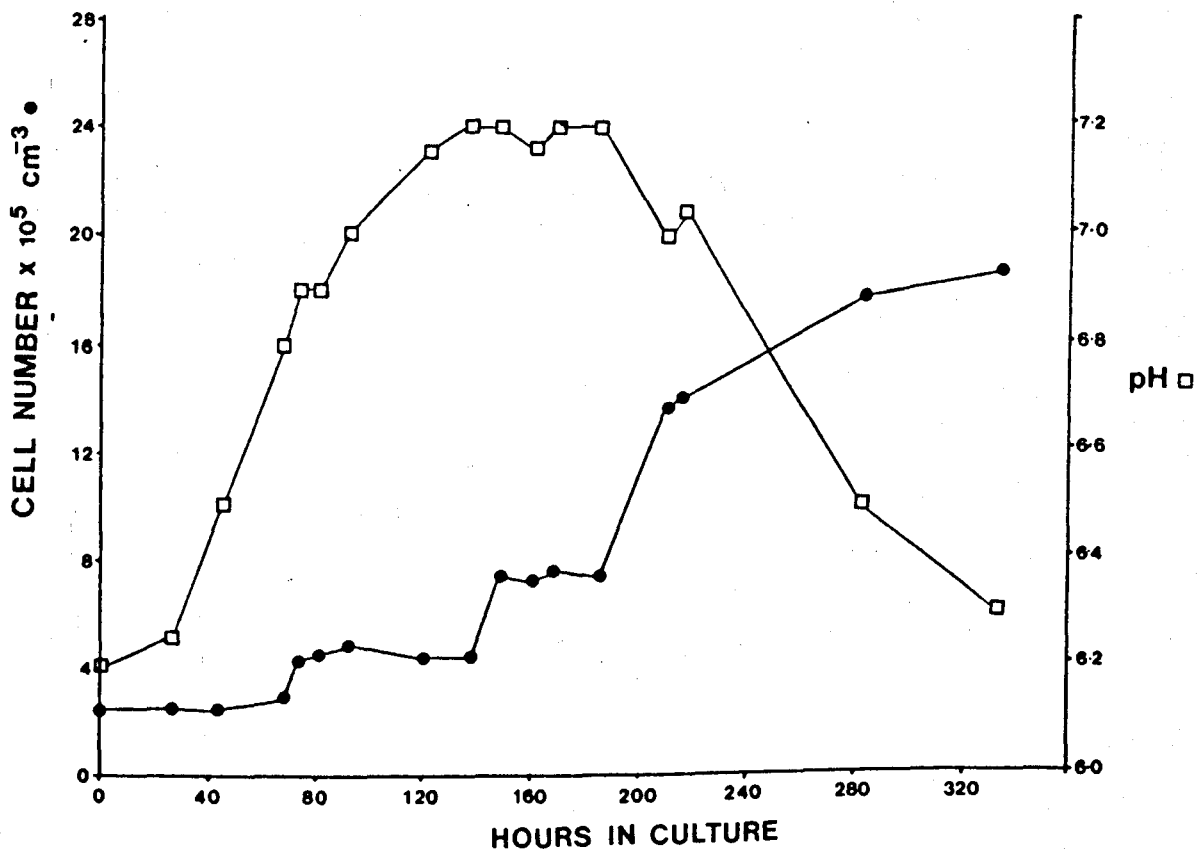
Figure 4.15

Acer pseudoplatanus cell number and medium
pH changes during nitrate/phosphate induced
synchronization. (Due to late nutrient addition
the synchronous state was not maintained).

The system developed here uses the same basic principles of Wilson and co-workers (1971) but with modifications to include pH and non-autoclavable nitrate electrodes within the culture system, measurement of the culture's absorbance to facilitate the detection of cytokinesis, and a reservoir of nitrate/phosphate feed.

A wide necked Quickfit borosilicate glass reaction vessel of 10dm^3 capacity was used as a culture vessel. The culture vessel was sealed by a Quickfit reaction flask lid which was secured by a wire clip. A plastic-coated stirrer bar was placed in the culture vessel which was then balanced on a cork ring on a magnetic stirrer plate set at 200 rpm. The culture vessel was situated in the growth room at 25°C and under constant illumination as described in Section 2.0.

The basic design of the culture vessel is illustrated in Figure 4.16. The culture was aerated with compressed air from a Tecalemit compressor (Roborough, Plymouth, Devon). The air was sterilized before entering the culture vessel by passage through two microflow miniature line filters (Microflow Ltd, Fleet, Hants) arranged in series. The air was then dispersed near the bottom of the culture vessel by a sintered aerator. The same lid port was also utilized for the air outlet which was filled with a non-absorbent cotton wool filter. Condensation was prevented in this air outlet filter by purpose built heating wires.

A second port on the vessel lid combined as inoculation and sample lines. A Quickfit cone was extended with a glass tube so that it nearly reached the bottom of the culture vessel. The upper end of the cone was attached by transparent silicon rubber tubing to a Quickfit socket which was filled with non-absorbent cotton wool and covered with aluminium foil prior to autoclaving. The inoculation cells were grown in aspirator bottles on a shaker. A Quickfit cone was attached by silicone rubber tubing to a tubule at the bottom of the aspirator bottle. The cone was also filled with non-absorbent cotton wool and foil covered. The cells were prevented from leaking into the cotton wool by a gate clip. On inoculation the cone from the inoculation culture bottle was joined to the socket on the culture vessel under absolute ethanol in a large crystallizing dish. The gate clips were released and the inoculation culture flowed under gravity into the fresh medium. The gate clips were then closed.

The sample line was joined to the inoculation line with a glass "T-junction" tube. To prevent the inoculant flowing towards the sample vessel the line was closed behind the junction with a gate clip. During manual sampling the air outlet was temporarily closed with a gate clip. After a few seconds had elapsed to allow the build up of a slight positive pressure within the culture vessel the gate clip on the silicone rubber tube to the sample vessel was released whilst the gate clip
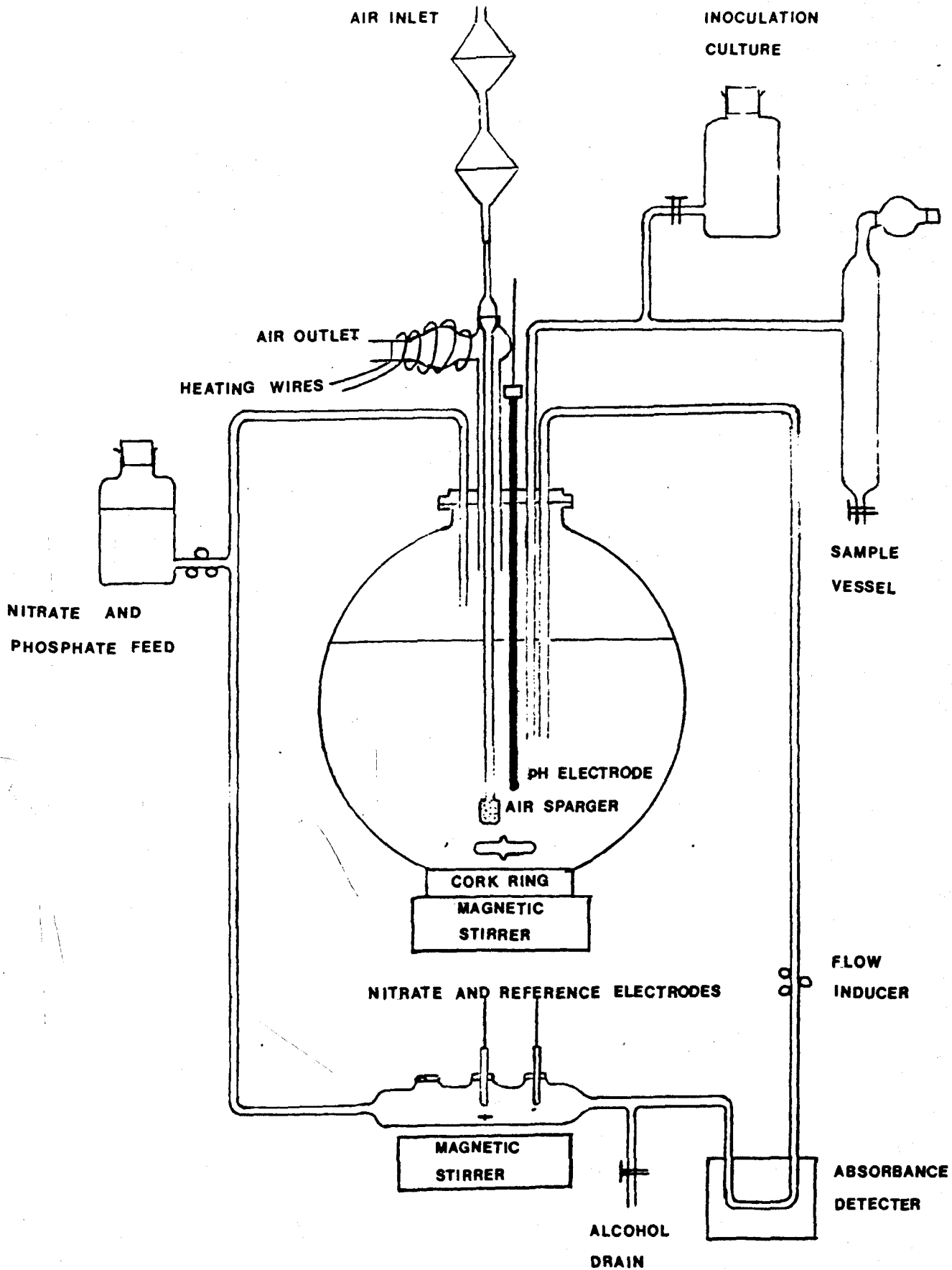
Figure 4.16

Diagram of culture vessel used for automated
synchronization.

on the inoculation line remained closed. The culture flowed into the sample vessel until the required volume had been obtained. The gate clip was then re-fastened and the air outlet line was released. The culture sample was then drained from the sample vessel upon the release of a gate clip placed on a piece of silicone rubber tubing attached to a tubule at the base of the sample vessel. The top of the sample vessel was designed like the air outlet on the culture vessel to enable air to enter the vessel as the sample was removed. The tubing at the base of the sample vessel was then quickly dipped in absolute ethanol and the gate clip was closed.

One of the lid ports was specially adapted in order that an autoclavable pH electrode could be placed in the culture vessel. A Quickfit screw seal was used to hold the electrode and to ensure a complete seal.

Another lid port contained a Quickfit cone with an extended glass tube that nearly reached the bottom of the culture. This was positioned furthest from the air sparger to lessen the chance of bubbles entering the line and thus causing spurious absorbance readings. The culture was pumped up the glass tube and along a silicone rubber tube by a Watson Marlow flow inducer. The piece of tubing was moved along slightly each day to ensure that the flow inducer did not wear a hole through the tubing. The rubber tubing was attached to a purpose

made glass "U-tube" which was placed in a wooden housing containing a red light emitting diode on one side of the tube, and on the other side a photodiode. This was contained in a light proof box and was attached to a power supply.

The other end of the glass U-tube was attached by silicone rubber tubing to a Quickfit cone which was joined to a Quickfit socket attached by a short piece of silicone rubber tubing to a tubule on a purpose built electrode holding vessel. This vessel possessed three electrode entry points identical to the one in the main vessel lid used for the autoclavable pH electrode. This part of the apparatus was not autoclaved as the nitrate electrode used was non-autoclavable. The electrode vessel was attached at both ends to the main culture system by Quickfit cone and socket joints. In the main culture system these corresponding joints were filled with non-absorbent cotton wool, foil covered and secured with gate clips prior to autoclaving. The nitrate and glass reference electrodes were placed in the electrode vessel and secured as the pH electrode. The two lengths of silicone rubber tubing attached to the Quickfit cone and socket were secured with gate clips close to the joint. A drain outlet was attached by a glass "T-junction" tube between one joint and the electrode vessel; this was also secured with a gate clip. A small magnetic stirrer bar was also placed in the electrode vessel. The electrode vessel was then

completely filled with absolute ethanol through the third redundant electrode port. To ensure that no potentially contaminating air was incorporated into the vessel the port was secured with a screw topped lid under ethanol. The whole was left for half an hour by which time it was assumed to be sterile. The electrode vessel was then incorporated into the main autoclaved culture system. The vessel was supported by clamps in a crystallizing dish containing sufficient ethanol to completely submerse the Quickfit joints. After several minutes had elapsed and all visible air bubbles in the joints had been displaced by the ethanol the joint gate clips were released. The foil was removed from the corresponding autoclaved joints and they were also immersed in the ethanol. The cotton wool was removed and all the air was displaced before the respective cones and sockets were joined under ethanol and secured with wire springs to their glass hooks. The gate clip on the return line (that is, the tube not immersed in the culture medium) was released to enable the drainage of the absolute ethanol on the release of the gate clip on the drain outlet tube. The electrode vessel was tilted vertically to facilitate the complete drainage. The drain outlet was then quickly resecured with a gate clip. The electrode vessel was placed above a magnetic stirrer plate to prevent the cells settling out. A litre bottle containing the concentrated nitrate and phosphate feed was attached by a tubule, silicone rubber

tubing and a glass "T-junction" to the return line. A Watson-Marlow flow inducer was placed on the feed line to pump the feed into the culture as required.

Before autoclaving the main culture vessel care was taken to ensure all open connections were plugged with non-absorbent cotton wool and covered with aluminium foil. All tubes which reached below the medium level were closed with gate clips outside the vessel and the outlet tap was left open. Inoculation took place in the growth room when the medium temperature had dropped to 25 ^{o}C.

The pH, nitrate and reference electrodes and the absorbance detector were all attached to a computer interface box and were frequently monitored and the data recorded on a cassette tape. The flow inducer on the nitrate and phosphate feed line was also controlled by the computer and could be switched on for a few seconds at a time to re-establish the original nitrate concentration when the nitrate present had decreased to the level usually found at cytokinesis and the other factors monitored indicated that cytokinesis had occurred.

With the advent of biotechnology the control of cultures by computers has developed considerably. Computers can continuously monitor various parameters thus detecting rapid fluctuations and can store the data in an easily

accessible form. The computer can also be programmed to switch on or off valves and vary flow rates and can therefore completely control complex culture systems. This precise control has led to major advances in the industrial field where large scale cultures are employed which require accurate monitoring and control if the system is to be economically viable.

In this present study a CBM PET computer was used in conjunction with an interface unit designed by Dr D A Armitage in the School of Chemistry at Leicester Polytechnic. The system was originally developed to control continuous cell suspension cultures, (Lewis, 1983), but was intentionally designed to be flexible enough to function in different modes of operation, for example, the induction of synchronized cultures.

The CBM PET computer uses BASIC (Beginners All-purpose Symbolic Instruction Code) which is a relatively easily learnt high level computer language. It has a visual display unit (VDU) which in this system can enable the user to see the current monitored parameters. At the rear of the computer two connections are situated which enable the user to interface to external devices. These are the userport and the IEEE port. The userport is a 24 way connector although normally only 12 of these are used. Eight of these lines (PAO-7) are programmable, bi-directional input/output lines. CA1 is an input

"handshake" line for lines PAO-7 which can also be used
for other edge sensitive inputs. CB2 is another similar
line which can be utilised for input or output.

The IEEE port is an international standard parallel
interface for both input and output.   This port can be
utilised to output data for example, to a printer. With
the necessary equipment this port could have been used
for all of the interfacing required here.   However, as
this equipment was not available it was decided to
utilise the userport for the control lines because it is
ideally suited to switching applications, and the IEEE
port as the main data input source.   Thus, the userport
can set a channel to a required function and the IEEE
port can read the value. This enables the handshaking
lines to be employed as further input/output lines or
control lines.   By utilising both ports a very flexible
interfacing system can be obtained which is ideal for
controlling a cell culture system.   The interface unit
used possessed the following functions: analogue input,
analogue output, a relay unit and buffered inputs.

The analogue input consisted of four channels in the
range +/- 3.999 V. The required channel was selected by
the userport using bits 0, 1, 2 and 3 to determine the
source of the analogue input.   The value was then
displayed on an IEEE digital voltmeter (also designed by

Dr Armitage). The analogue input function was utilised to monitor the absorbance or transmittance of the cell suspension.

The analogue output consisted of an 8-bit digital to analogue converter driven by the parallel userport and controlled by the CB2 line of the userport. An output voltage of between 0-2.55 V was available from the module. This signal could be amplified by a circuit board situated inside a Watson-Marlow flow inducer to 0-34 V in order to control the pump speed. The analogue output could also be used to operate a chart recorder if required.

The relay unit consisted of four independent relay switches with the capability of switching up to 240 V AC supply. The principle of operation is similar to that for the analogue output. The relay unit was used to switch on/off valves or flow inducers.

The buffered inputs enabled the monitoring of pH and nitrate levels by ion-selective electrodes. The buffered inputs could also be used in other applications where an input impedence of greater than 10^{12} ohms is required. The system employed a field effect transistor input buffer stage for each electrode. The buffering was essential because the electrodes measure a potential difference between a reference and an ion-selective electrode. The field effect transistor did not draw any

significant current from the electrode and thus a true voltage could be measured. The data obtained by the buffered inputs was also displayed on the IEEE DVM.

A more comprehensive account of these interface functions has been written by Lewis (1983) along with explanatory circuit diagrams.

A computer program (TORAB2) enables the user to manually select the input channel to be read or the setting of the output voltage. By using this program the user can ensure that the system is operating correctly and that all of the external devices are in working order. A listing of the program TORAB-2 is shown on the following page (Figure 4.17). When the program is run the computer screen displays a set of five options. These are terminate (T), analogue output (O), relay switches (R), analogue input (A) or buffered input (B). The operator selects the appropriate key for the required function and the computer moves to the relevant part of the program. The terminate option ends the program.

If the analogue output function is chosen the user is asked to input a value, termed AA, between 0 and 255 (line 210). The appropriate subroutine, lines 10000-10030 (listed in Figure 4.18) is then performed by the computer. The inputted value for AA is then stored on the userport data lines.

```
10 GOSUB 10400:A$=" "
20 PRINT"CLRCDCD OPTIONS AVAILABLE CDCD"
25 PRINT"CD     RVS T RVSO TERMINATE"
30 PRINT"CD     RVS O RVSO ANALOGUE OUTPUT"
40 PRINT"CD     RVS R RVSO RELAY SWITCHES"
50 PRINT"CD     RVS A RVSO ANALOGUE INPUT"
60 PRINT"CD     RVS B RVSO BUFFERED INPUT"
70 O$="TORAB":IF A$<>" " THEN 90
80 GET A$:IF A$=" "THEN 80
90 FORI=1TO5:IF A$=MID$(O$,I,1) THEN 110
100 NEXT I :GOTO 80
110 ON I GOTO 9999,200,300,400,500
200 PRINT"CDCD ANALOGUE OUTPUT MODE"
210 INPUT"CD WHAT VALUE(0-255)";AA
220 GOSUB 10000:GOTO 600
300 PRINT"CDCD RELAY SWITCH MODE"
310 PRINT"CD INDICATE ON OR OFF CDCD"
320  FOR  I=0TO3:PRINT"CU  RELAY";I,"     CRCRCRCRCR";::INPUT
A$:R%(I)=0:IF A$="ON"THENR%(I)=1
330 NEXT I:RR=0:FOR I=0TO3:RR=RR+2EXP(4+I)*R%(I):NEXT I
340 GOSUB 10000:GOTO 600
400 PRINT"CDCD ANALOGUE INPUT MODE"
410 INPUT"CD CHANNEL NO. (0-3)";XX
420  GOSUB  10200:PRINT"CUCRCRCRCRCRCRCRCRCRCRCR
CLCLCLCLCLCLCLCLCLCLCL";XX;"READS";VV
430 GOTO 600
500 PRINT"CDCD BUFFERED INPUT MODE"
510 INPUT"CD CHANNEL NO. (0-3)";XX
520  GOSUB  10300:PRINT"CUCRCRCRCRCRCRCRCRCRCRCR
CLCLCLCLCLCLCLCLCLCLCL";XX;"READS";VV
530 GOTO 600
600 PRINT"CD PRESS ANY KEY FOR OPTION PAGE"
610 GET A$:IF A$=" "THEN 610
620 GOTO 20
9999 END
10000 REM ANALOGUE OUTPUT AND RELAY ROUTINE
     THIS IS IDENTICAL TO THE ROUTINE IN KRISTPROG
```

The BASIC programming symbols have been amended slightly to
enable printing on a letter quality printer. Thus CLR = clear
screen , CD = cursor down , CU = cursor up , CL = cursor left ,
CR = cursor right , EXP = exponentiate , RVS = reverse screen ,
RVSO = reverse screen off.

Figure 4.17   Listing of computer program "TORAB-2".

```
1 DEF FNP2(X)=INT(100*X+0.5)/100:CR$=CHR$(13)
5 GOSUB 10400
10 INPUT"CLRCDCDCDCDCDCD TIME OF DAY: 24HR FORMAT";T$:D=0:TI$=T$
20 OPEN 2,1,1,"KRISTINE":PRINT"CLR";:GOSUB 900
30 IF MID$(TI$,3,2)="25" THEN 40
35 GOTO 30
40 H=0:H2=0:XX=3:FORI=1TO100:GOSUB 10300
50 H=H+VV:H2=H2+VV*VV:NEXTI
60 H2=FNP2(SQR((H2-H*H/100)/99)/100):H=FNP2(H/10000)
70 N=0:N2=0:XX=1:FORI=1TO100:GOSUB 10300
80 N=N+VV:N2=N2+VV*VV:NEXTI
90 N2=FNP2(SQR((N2-N*N/100)/99)/100):N=FNP2(N/10000)
100 T=0:T2=0:XX=0:FORI=1TO100:GOSUB 10200
110 T=T+VV:T2=T2+VV*VV:NEXTI
120 T2=FNP2(SQR((T2-T*T/100)/99)/100):T=FNP2(T/10000)
130 PRINT#2,D;CR$;TI$;CR$;H;CR$;H2;CR$;N;CR$;N2;CR$;T;CR$;T2
140 IF LEFT$(TI$,2)="00" THEN GOSUB 900
150 PRINT D;TAB(4);LEFT$(TI$,4);TAB(12);H;TAB(22);N;
160 PRINT TAB(32);T
170 GET T$:IF T$=" " THEN 30
180 IF T$= CHR$(160) THEN CLOSE2:END
190 GOTO 30
900 LET D=D+1
910 PRINT" DAY TIME         pH              NITRATE        TURB"
920 RETURN
10000 REM ANALOGUE OUTPUT AND RELAY ROUTINE
10010 POKE 59471,AA:POKE 59468,238
10020 POKE 59471,RR:POKE 59468,206
10030 RETURN
10100 REM D.V.M. ROUTINE
10110 OPEN 1,16:INPUT#1,VV$:VV=VAL(VV$):CLOSE1
10120 RETURN
10200 REM ANALOGUE INPUT ROUTINE
10210 POKE 59471,XXOR8AND11:TS=TI
10215 IF TI-TS<60 THEN 10215
10220 GOSUB 10110:RETURN
10300 REM BUFFERED ANALOGUE INPUT ROUTINE
10310 POKE 59471,XXOR4AND7:TS=TI
10315 IF TI-TS<60 THEN 10315
10320 GOSUB 10110:RETURN
10400 REM INITIALISE DEVICE VARIABLES
10410 POKE 59459,255:POKE 59468,206
10420 AA=0:XX=AA:VV=AA:RR=AA:DIM R%(3)
10430 RETURN
```

Figure 4.18   Listing of computer program "KRISTPROG".

If the relay function is chosen the user inputs whether each relay is required to be on or off and then the same subroutine is executed.

If the analogue input option is chosen the user is asked to input the channel to be read (line 410). The appropriate subroutine (lines 10200-10220) is then executed by the computer. The computer's internal crystal controlled clock is used to bring about a one second delay before a reading is taken by the IEEE DVM. The buffered analogue input procedure works similarly.

Having designed the suitable culture vessel described earlier preliminary experiments were performed with manually controlled cultures, the nitrate and phosphate feed being stored in a graduated vessel with the required volume being added by the manual release of a gate clip. The computer programme TORAB2 was utilised and it was found that a few minor modifications to the interface unit were required by Dr Armitage as the nitrate and pH electrodes produced different readings to those obtained by remote sampling.

A computer program was written (Figure 4.18) which enabled completely automatic frequent monitoring of the culture's absorbance, pH and nitrate levels. A hundred readings were taken for each time point and the means and standard deviations were calculated by the computer. Unfortunately due to the time delay involved whilst the

interface was being repaired which was coincident with the relocation of the research laboratories, it was not possible to fully automate the system. Therefore, the flow inducer on the feedline was controlled by manual input to the computer whilst the nitrate electrode was connected to a voltmeter. However, with the interface repaired and the insertion of the appropriate feedback subroutine into the program, the feed supply could easily be completely automated and nutrients could be added when the time, nutrient status, pH and absorbance all indicate that cytokinesis has occurred.

4.4 Conclusions

In this section an induction synchrony technique was developed based on the availability of the growth-limiting nutrients nitrate and phosphate.

In batch culture Acer pseudoplatanus cells have been reported to be nitrogen and phosphate limited (King et al., 1973; Young, 1973; Jessup and Fowler, 1976; Wilson, 1976; Firby, 1985). Young (1973) reported that nitrate and urea (an alternative nitrogen source) were depleted from the culture medium after only seven days of incubation. It was also noted that when the supplied nitrate concentrations were doubled the final cell number of the batch cultured cells approximately doubled and there was an increase in the total biomass. Jessup

and Fowler (1976) observed similar results and hypothesised that there is a proportional relationship between cell yield and media nitrogen concentration.

The sycamore cells studied in this present investigation were originally cultured with urea as an additional nitrogen source to that of sodium nitrate. However, when urea was later omitted from the culture medium cell growth appeared unaffected and thus nitrate alone was supplied in this study. The cells were routinely cultured in a standard medium containing sodium nitrate at a concentration of 7.0 mmol dm^{-3}.

In order to confirm that this strain of sycamore cells was nitrate limited and to investigate the affect on their growth of more than the standard nitrate concentration the cells were cultured in a range of nitrate concentrations. These included 3.5, 5.0, 7.0, 10.0 and 14.0 mmol dm^{-3} sodium nitrate. It was found that the final cell densities increased with the concentration of nitrate supplied. Figure 4.2 shows the changes in the final cell numbers plotted against the nitrate concentration. Lewis (1983) plotted a similar graph for a range of nitrate concentrations below the control level and found that a high positive linear correlation existed between the two variables. In this case any linearity appeared to be lost above a concentration of 10mmol dm^{-3} sodium nitrate. Firby (1985) noted that the final cell number achieved in an

18 mmol dm^{-3} culture was similar to that of a 10mmol dm^{-3} nitrate culture. Thus the linear relationship is not infinite and at very high nitrate concentrations growth is actually inhibited. This inhibition may be a reflection of the increased levels of sodium in such cultures and it is suggested that future work involving high nitrate levels should provide nitrate in the form of potassium nitrate.

The dry mass per cell was found to decline with increasing nitrate concentration. This was also reported by Jessop and Fowler (1976). This may be a reflection of the high number of cells actively dividing when nitrate is in plentiful supply. Figure 4.1 illustrates the cell number curves for cells cultured in the range of nitrate concentrations. The cultures maintained at 10.0mmol dm^{-3} and 14.0mmol dm^{-3} sodium nitrate did not appear to enter true stationary phases (characterized by larger cells) during the normal culture period presumably because they had not completely depleted their nutrient resources.

The medium nitrate levels in these cultures were monitored with an ion-selective electrode during the batch culture growth cycle. The medium was found to be depleted of nitrate by Day 4 in the 3.5mmol dm^{-3} cultures, Day 6 in the 5.0mmol dm^{-3} cultures, between Days 6-8 in the 7.0mmol dm^{-3} cultures, by Day 8 in the 10.0mmol dm^{-3} cultures and between Days 9-12 in the

14.0mmol dm^{-3} cultures. Thus, the medium nitrate was eventually completely depleted in all of the cultures which is further evidence that it may be a limiting factor.

The depletion of medium nitrate does not however, necessarily mean that the cells are completely nitrate limited. The cells may themselves store nitrate for future utilisation. Thus, cellular nitrate levels were estimated in the control cultures. The cellular levels were found to increase after sub-culture to a maximum level by Day 6. This increase correlated with the depletion of nitrate from the medium. The levels were then found to decline rapidly during the exponential phase.

Thus, there is substantial evidence both here and in the literature that on transfer to fresh medium _Acer_ _pseudoplatanus_ cells take up nitrate and accumulate an intracellular nitrate "store". As the cells enter the exponential phase which is characterized by active cell division this cellular nitrate concentration rapidly declines, presumably because it is utilised in essential primary metabolic pathways. It is proposed that the cells then become nitrate limited, active cell division declines and they enter the stationary phase.

Wilson (1976) reported that phosphate is also depleted from <u>Acer</u> <u>pseudoplatanus</u> culture medium at approximately the same rate as nitrate. Lewis (1983) noted that phosphate can also be growth-limiting to these sycamore cells, but the inhibition of growth in low phosphate cultures was less significant than that observed in low nitrate cultures. Preliminary experiments in the development of the synchronization technique employed in this study involved adding nitrate, phosphate or nitrate and phosphate to cultures after cytokinesis. Cultures fed with extra nitrate produced three consecutive synchronous divisions whereas the cultures fed with phosphate were asynchronous like the controls. However, cultures fed with nitrate and phosphate produced three highly synchronous divisions. Thus, nitrate and phosphate do limit the growth of these cells in batch culture but the effect of phosphate is less marked.

As sycamore cells become nutrient limited and enter the stationary phase they are known to switch their metabolic pathways from those of primary metabolism, which are predominantly associated with cytokinesis, to those of secondary metabolism which are generally involved in the synthesis of secondary products. In sycamore cell suspension cultures phenolics have been reported as the predominant secondary products (Westcott and Henshaw, 1976). The initiation of secondary metabolism has been linked with the soluble nitrogen content of the medium and the level of auxin in the

culture (Phillips and Henshaw, 1977). Firby (1985) noted a decline in the amount of extractable cellular protein as nitrate became limiting in sycamore cells.

Coincident with the decline in nitrate and phosphate availability and the subsequent decrease in primary metabolism is the increase in the activity of the enzyme phenylalanine ammonia-lyase (PAL). This enzyme catalyzes the first step in the phenylpropanoid metabolic pathway which results in the synthesis of phenolics. Westcott and Henshaw (1976) observed increased PAL activity and phenolic production in sycamore cells as nitrogen became limiting. It is believed that the switch from protein synthesis to phenolic synthesis is controlled by substrate competition for the common precursor, phenylalanine (Phillips and Henshaw, 1977).

The initiation of secondary metabolism has also been linked with the level of auxin in the culture (Phillips and Henshaw, 1977). They reported that the addition of 2,4-D to stationary phase cultures resulted in increased protein turnover which was coincident with the inhibition of the synthesis of phenolics. Presumably these cells did not revert back to true primary metabolism because they were nutrient limited but competition for phenylalanine during protein turnover prevented further secondary product synthesis. The other plant growth regulators may also play a role in

the switch from primary to secondary metabolism. Ethylene has been found to have an inhibitory effect on PAL activity and thus phenolic synthesis (Westcott, 1976).

Nitrate and/or phosphate availability appears to play an important role in both the growth of cell suspension cultures and their choice of metabolic pathways. It seems likely that as nutrients become limiting the cells divert from primary metabolic pathways resulting in cytokinesis, to secondary pathways resulting in secondary product synthesis. It was proposed that this phenomenon may explain why batch cell suspension cells quickly develop into asynchronous growth.

As was expected, experiments involving the addition of nitrate and phosphate after each subsequent cell division resulted in highly synchronous cultures. It is believed that such cultures were sustained in primary metabolic pathways. After each successive synchronous division there followed periods of constant cell number. The process of cell division occupied a relatively small fraction of the cell cycle (10 hours of the 72 hour cycle) and mitotic indices revealed a peak in mitotic activity preceding the induced synchronous division. All of these factors help to confirm the existence of the synchronous state. The dry mass per cell, mean cell volume and cell viability data during induced synchrony produced similar patterns to those obtained from the

first division after subculture. Thus it is thought
that the induction method is not too disruptive to the
cell's normal cultural environment and therefore the
risk of spurious artefacts of the system are low.

As this synchronization technique proved to be reliable
with consistent cell cycle times, a successful attempt
was made to increase the cell cycle time by providing
twice the standard nitrate and phosphate concentration
both initially and in the post-division feeds. However,
when half the standard nutrient supply was maintained
the synchronous state was rapidly lost presumably
because some of the cells became nutrient limited. It
is possible that the cell cycle time could be reduced if
the cells were maintained at a nutrient concentration
somewhere between the 3.5mmol-7.0mmol dm^{-3} sodium
nitrate levels.

The medium pH was monitored throughout both control
batch culture growth cycles and synchronized cultures at
different growth rates. In the control batch culture
the medium pH became more alkaline as the medium nitrate
decreased and the cellular nitrate level increased.
This may be due to the efflux of OH^- by the cells as
they took up the nitrate. As the culture developed into
the asynchronous state a rapid decline in the pH was
observed. This may have been due to the cessation of
any OH^- efflux from the cells as they were no longer
taking up nitrate from the medium or it may be a

reflection of the advent of secondary synthesis and the accumulation of acidic waste or secondary products in the closed system. In the synchronized cultures the medium pH was found to increase and remain high until the medium was eventually depleted of the supplied nitrate between additions and the cells developed into asynchrony. The decline in pH occurred earlier in the low nitrate fed cultures. The maximum medium pH level achieved was similar in all three cultures, although slightly higher in the culture maintained at 7.0 mmol dm^{-3}.

It can be tentatively concluded that <u>Acer pseudoplatanus</u> cells in batch suspension culture become nitrate and phosphate limited and thus switch from primary to secondary metabolism and in so doing become asynchronous. On transfer to fresh medium with plentiful nutrients nitrate-starved stationary phase cells are induced to divide synchronously, but this first division synchrony is soon lost as the nutrients become scarce. When the nutrients are supplied in higher concentrations the growth rate increases and when supplied in lower concentrations the growth rate declines. The addition of nitrate and phosphate after cytokinesis prevents the cells becoming nutrient-limited and maintains the synchronous state. When this induction technique is applied to cells cultured in medium containing twice the standard nitrate and phosphate concentrations highly synchronized cell

divisions with shorter cell cycles are produced. Thus, it seems that when the nutrients are present at an optimal concentration throughout the culture period growth is essentially unlimited and as all the cells progress through their cycles unhindered they remain in synchrony with one another.

It is unclear whether the changes which occur in the medium pH during batch and synchronized growth are a reflection of the nutrient status of the medium or some other undefined factor. It would be interesting to investigate whether batch cultured cells would still develop into asynchrony if their pH was maintained at the apparently optimal level (approximately 7.2) for cytokinesis despite the nutrient availability. In Section 5.0 the intracellular pH levels are investigated during batch and synchronized cycles.

The automated synchronization system developed in this section proved to be very reliable. The system is very flexible and can easily be modified for other culture systems or to monitor other parameters. It can provide very frequent monitoring of parameters such as pH and nitrate levels without the need for time consuming sampling by the researcher. The system could be adapted to include a Coulter counter and provided a relatively non-clumping cell line was used accurate cell number data could be obtained. The automatic addition of the nitrate and phosphate feed when the monitored parameters

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indicate that cytokinesis has occurred or is imminent would overcome the problem of losing the synchronous state when a culture divides slightly earlier or later than expected or at an inconvenient time.

There are a number of manipulations to the system based on the nutrient and pH status of the medium that could be performed with ease by this automated system and would provide additional useful information about cell cycles. These include maintaining the cells at constant nutrient levels throughout the whole culture period by the continuous addition of nutrients at the depletion rate. In this case one might expect even more highly synchronous rapid cell cycles because the cells would never become nitrate or phosphate limited. The nutrient concentrations could also be increased or decreased during the culture period and the pH level could also be maintained constant. Such experiments would increase the understanding of cell cycle regulation especially if the levels of the plant growth regulators in such cultures were also investigated.

The endogenous IAA levels through the synchronized divisions fluctuated very little. It had been expected that the peak in IAA activity observed coincident with mitosis in the synchronous division after subculture would occur with each subsequent induced division. These peaks had also been observed by Robinson (1982) who induced synchrony by a cold shock treatment. As the

IAA level appeared to remain relatively constant in
these actively dividing highly synchronous cultures it
is tentatively concluded that the previously observed
IAA peaks were artefacts caused by the shock of
subculture and the cold treatment. The data obtained
from this study imply that during active synchronised
divisions the endogenous IAA level remains at a constant
level which may have some biological significance. It
is possible that this level may be optimal for an IAA
receptor or some other factor and that on subculture the
cells synthesize IAA until this base level is achieved.
Once achieved cytokinesis can ensue. The high IAA
levels produced when the cells are exposed to a shock
may be unrelated to cytokinesis. This hypothesis is
investigated further in the following section.

Unfortunately, time did not allow further investigations
into the endogenous cytokinins during induced synchrony.
It would be interesting to determine whether or not the
peaks in cytokinin-like activity observed coincident
with the first synchronous division are also present
during induced synchrony.

5.0 EXTRACELLULAR AND INTRACELLULAR pH VARIATIONS DURING THE CELL CYCLE

5.1 Introduction

The _Acer pseudoplatanus_ cell suspension cultures used in this present study were routinely grown in a culture medium adjusted to pH 6.4 using 1 mol dm^{-3} KOH, as advised by Stuart and Street (1969). The experiments performed in the previous section revealed that on inoculation the medium pH decreased slightly due to the more acidic nature of the inoculum. In asynchronous batch cultures there then followed an increase in the alkalinity of the medium as the cells underwent rapid semi-synchronous cell division. As the culture developed into asynchrony the medium pH rapidly became more acidic (Figure 4.8). Most of the basic suspension culture media are very lightly buffered and considerable pH changes can occur in response to the introduction of the inoculum and its subsequent growth (Street, 1973b). The solute uptake ability of cells is very dependent on the pH status of the bathing medium. When an originally acidic medium (pH 4.8-5.4) rapidly becomes more neutral it is often necessary to incorporate ethylenediamine-tetraacetate (EDTA) to maintain the availability of iron and other metal ions (Ferguson, Street and David, 1958; Sheat, Fletcher and Street, 1959; Klein and Manos, 1960). In this study iron is provided in the form of NaFe (EDTA) (Stuart and Street, 1969).

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Further work in Section 4.0 involving the measurement of medium pH during nitrate/phosphate feed induced synchronization revealed that the medium pH maintained a near neutral level whilst the cells were dividing and nitrate was plentiful. However, the decline in medium nitrate was closely paralleled by the eventual decline in medium pH (Figure 4.13). It was postulated that this correlation may be partially related to the efflux of OH^- ions by the cells as nitrate is taken up and/or the accumulation in the medium of acidic waste and secondary products by the cells as they become nitrate-limited and enter secondary metabolic pathways.

As discussed in the previous section, it is unlikely that the dramatic pH fluctuations observed in the bathing medium would also occur within the cells (Raven, 1980). During the cell cycle various processes occur which would cause considerable intracellular pH changes if a regulatory process did not exist. It is believed that there is an active intracellular regulatory system which can off-set acid/base imbalances that would occur, for example, during metabolism or solute uptake (Roberts et al, 1980). The cytoplasmic pH is believed to be very important in processes such as polar transport, proton extrusion and enzymic activities.

There has been very little research into the cytoplasmic pH levels of the small cells typical of the higher plants. These cells tend to have large acidic vacuoles

which hinder the analysis of the smaller cytoplasmic area. The _Acer pseudoplatanus_ cells used in this present study are also highly vacuolated with 90% of the cell volume believed to be vacuolar (Bligny and Douce, 1976). The main results in this area have been obtained with animal cells (Gadian et al., 1979), bacteria (Shulman et al., 1977), yeast (Navon et al., 1979) and algae (Elgavish et al., 1980). However, various workers have utilized the technique of ^{31}P nuclear magnetic resonance (NMR) to estimate the vacuolar and cytoplasmic pH of plant cells. Roberts and co-workers (1980) used this technique to estimate the pH status of maize root tips, and a number of different plants in suspension culture have also been investigated by NMR (Martin et al., 1982).

Nuclei capable of exhibiting NMR are those which possess an odd number of protons. All nuclei possess spin as do electrons; nuclei with odd mass numbers have spins with values which are odd multiples of 0.5. Nuclei with even mass numbers have spins that are even multiples of 0.5. Because the nuclei are spinning with a positive charge they possess a magnetic moment which is always parallel to the spin angular momentum vector. Under the influence of a magnetic field the spins align either with or against the field producing distinct energy levels. At suitable radio frequencies and under powerful magnetic fields energy is adsorbed and the

nuclei jump from one energy level to another. It is this effect that is known as nuclear magnetic resonance.

These energy level transitions are induced by an oscillating magnetic field applied to a sample in a test tube. When the frequency of the oscillating field equals the transition frequency the transition occurs and the corresponding oscillation in the field induces a voltage oscillation in a receiver coil which can be amplified and detected. Each different kind of proton in a molecule can be distinctively split and identified by NMR. This is because the different protons have slightly different magnetic environments and thus slightly different resonant frequencies. It is for this reason that NMR is often used to identify structural units containing hydrogen nuclei. The effective field at a nucleus depends not only on the applied field but also on the shielding effect of the extranuclear electrons. For example, if ethanol (C_2H_5OH) is analysed by this method, separate proton resonance lines are obtained for the hydrogen nuclei found in the $-CH_3$, $>CH_2$ and OH groups and the 3:2:1 ratio is also apparent.

The estimation of pH values by ^{31}P-NMR depends upon the "chemical shift" of the intracellular ^{31}P resonance with pH. This is due to the fact that the environment surrounding the nucleus has a small but definitely measurable effect on the field sensed by the nucleus.

The chemical shift is expressed relative to a standard substance and calibration curves are prepared for the purpose (Figure 5.4). Unfortunately, pH changes are not the only alterations to the nuclei's environment that cause chemical shifts to the resonance effect. For example, if the ionic composition of the vacuolar and cytoplasmic compartments are different (Martin et al, 1982) other chemical shifts would be induced and thus the pH-based calibration curve would be less accurate. As there are many such instances during the metabolic processes the intracellular pH levels estimated by this technique cannot yet be regarded as definitive, but it is likely that the relative trends revealed are representative.

As mentioned previously this technique has been used to investigate the different pH levels maintained in the cytoplasm and the vacuoles of cells. The detection of the cytoplasmic and vacuolar peaks from an NMR spectrum depend upon the detection of different pools of phosphate. ^{31}P is a particularly useful nucleus to monitor because of its central role in cell metabolism (Rebielle et al, 1983). It is involved in most metabolic energy transductions, acts as an intermediate in the synthesis of numerous metabolites and complex molecules and it regulates the activity of many enzymic reactions and metabolic sequences such as respiration, glycolysis, photosynthesis and starch hydrolysis. There are two main Pi containing compartments in the plant

cell, the vacuole and the cytoplasm. Martin and co-workers (1982) working with Acer pseudoplatanus cell suspension cultures found two main peaks of Pi corresponding to two major intracellular pools with a mean pH around 5.5 and 7.0. They tentatively assigned these according to Roberts and co-workers (1980) to the vacuolar and cytoplasmic pools respectively.

The technique of NMR spectroscopy is now well established as a non-invasive method of studying intracellular metabolic changes in vivo. The proper interpretation of NMR spectra can yield information on not only intracellular pH but also intracellular compartmentation, membrane transport phenomena and metabolite concentrations, interconversions and fluxes (Kime et al, 1982b; Gadian et al, 1979). With the development of efficient circulatory systems the plant tissue can be maintained in a healthy condition over time periods of several hours (Lee and Ratcliffe, 1983). Thus, metabolic variations can be monitored during, for example, cytokinesis.

Martin and co-workers (1982) studied the NMR spectra of Acer pseudoplatanus cells under aerobic and anaerobic conditions in the NMR tube. For their analyses they removed Mn^{2+} and phosphate from the extracellular medium as these can cause interference and a masking of the intracellular phosphate pool peaks. They investigated aerobic and anaerobic cells and observed large

differences in their glucose-6-phosphate content, cytoplasmic inorganic pools of phosphate and their cytoplasmic pH. The G-6-P concentration was found to be markedly higher in the aerated cells, and increases in this sugar phosphate were correlated with decreases in the cytoplasmic inorganic pools of phosphate. The vacuolar pH was not affected by aeration but the cytoplasmic pH was found to be higher in the aerated cells.

Martin and co-workers (1982) pointed out that their analyses must be considered as a first approach to the problems of cytoplasmic pH and Pi pools in the sense that the pH differences and the distribution of Pi between cytosol and the different organelles are not known. The inner mitochondrial membrane acts as an insulating barrier for protons but it has been shown that the pH in the matrix may sometimes be altered by changes in the medium pH (Neuburger and Douce, 1980). It is therefore likely that the pH of cell organelles is dependent on the cytoplasmic pH. The cytoplasmic pH changes observed under aerobic and anaerobic conditions may be due to the removal of CO_2 as the O_2 was bubbled into the system, or changes in the cytoplasmic Mg^{2+} concentration resulting in a chemical shift of the calibration curve.

Intracellular pH changes are likely to play an important role in metabolic processes such as the regulation of cytokinesis which has been investigated in this present study. Although no definite conclusions can be drawn from the fluctuations in the levels of the endogenous plant growth regulators investigated here, it is possible that the distribution and/or activity of such substances could be affected by intracellular pH variations. Leguay and Guern (1975) demonstrated that the distribution of the auxin 2,4-D is largely dependent upon the intra and extracellular pH levels. Increases in internal pH of 2,4-D limited cells are known to induce cytokinesis as 2,4-D diffuses into the cells (Kurkdjian et al, 1978). In this section the vacuolar, cytoplasmic and extracellular pH levels were investigated to test the hypothesis that a threshold level of auxin is essential within the cells before cytokinesis can ensue. This level may be affected by the pH status of the cells. The NMR data obtained were also utilized to establish any metabolic changes which may occur during synchronized cell division and batch culture growth.

5.2 Experimental_Methods

Litre bottle cultures (490 cm^3) were grown as described in Section 2.0, but with the medium pH adjusted from the usual level of 6.4 to 2, 4 and 5 to investigate the effect of external pH on cell density. The medium pH

was also measured at each sample time point in order to determine any fluctuations during the batch culture growth cycle.

Litre bottle cultures ($490 \ cm^3$) were grown to obtain cells for the experiments involving the estimation of intracellular pH and metabolites by ^{31}P NMR. Using a spectrometer (Brucker CPX 200) operating at 101.27 MHz for ^{31}P it is possible to resolve and assign peaks to the acidic vacuolar compartment and to the slightly alkaline cytoplasm. The NMR conditions were as described previously (Kime et al., 1982b) with the addition of a circulatory system to maintain the cells in a healthy condition during the time course of the experiment (Lee and Ratcliffe, 1983). The cells were routinely circulated with the medium from which they had been harvested at the relevant cell/volume ratio in order to eliminate the effect of introducing fresh medium or dilution. The temperature in the probe head was maintained at $25 \ ^oC$ (normal culture temperature) to simulate normal growth conditions. Cell samples were taken at regular intervals from shaken cultures of the same age to determine if and when cytokinesis occurred. Cell density determinations were made at the initiation and termination of the experiment to determine that cytokinesis had actually taken place in the NMR tube. The pH of the circulating medium was also monitored in parallel with a control shaken culture. The pH profiles

were found to be similar and cytokinesis did occur in the NMR tube, thus it is assumed that the timescale of cultural events is comparable for circulated and shaken cells.

Synchrony was induced as described in Section 4.0 and the intracellular pH and metabolite variations were estimated in such cultures by ^{31}P NMR.

5.3 Results_and_Discussion

5.3.1 Medium_pH_and_Growth

The Acer pseudoplatanus cell suspension cultures used in this present study were routinely grown in a culture medium adjusted to pH 6.4 using 1 mol dm^{-3} KOH prior to autoclaving, as advised by Stuart and Street (1969). By Day 21 the medium pH becomes more acidic (approximately 6.0), possibly due to the accumulation of waste and secondary products. On transfer the acidic nature of the inoculum results in a slight decrease in the medium pH which is then superceded in batch cultures by a marked increase in the alkalinity of the medium as the cells undergo rapid cell division. As asynchrony develops due to competition for limiting nutrients the medium becomes acidic again until the Day 21 level is established.

In nitrate/phosphate feed induced synchronized cultures the medium pH maintained a near neutral level whilst the cells were dividing and the nutrients were plentiful. However, the decline in medium nitrate between feeds was closely paralleled by the eventual decline in medium pH. This correlation may be due to the efflux of OH^- ions from the cells during nitrate uptake and/or the accumulation of secondary products in the system as nitrate becomes limiting and a proportion of the cells begin to divide asynchronously.

The _Acer_ _pseudoplatanus_ cells used in this present study have been maintained in the same culture conditions for many years and it is likely that they have adapted to these conditions as best they can. Such predictable cell suspension cultures lend themselves to investigations involving controlled variables. In order to establish whether the medium pH level coincident with the exponential phase in asynchronous cultures and the synchronous state (approximately 7.0) is a specific level associated with this rapid cytokinesis or merely a reflection of an increase due to OH^- efflux and secondary product accumulation the following experiment was performed. Cells were inoculated into medium adjusted to pH 2, pH 4 and pH 5. The cell densities were estimated through the batch cell cycle and the data are tabulated in Table 5.1 and illustrated in Figure 5.1. All of the cultures were inoculated with similar cell densities. The highly acidic pH 2 culture appeared

to exhibit no growth and the culture changed from the usual pale yellow colour to almost white. Unfortunately, cell viability estimations were not undertaken but it is likely that these culture conditions were too severe for the cells and the culture became non-viable. The cells initiated in medium pHs 4 and 5 did exhibit growth although they experienced prolonged lag phases (approximately 7 days). The culture growth rates then increased although the final cell densities were not as high as in the control culture, and the level achieved by the pH 5 culture was higher than that in the pH 4 culture.

The medium pH levels were monitored in these cultures during the batch culture cycle and the data are tabulated in Table 5.2 and illustrated in Figure 5.2. In the culture maintained at pH 2 where no growth occurred the medium pH remained relatively constant at the level of 2.3 attained after inoculation. In the other two cultures the medium pH levels increased such that by Day 10 the pH 5 culture had increased to pH 7.0, and by Day 15 the pH 4 culture had increased to pH 6.8. These levels then declined and by Day 21 they were very similar to the level of pH 6.0 in the control culture.

Table 5.1

Changes in cell number during batch culture cycle for
Acer pseudoplatanus cells cultured in a range of medium
pHs.

TIME (Days)	CELL NUMBER x 10^{-3} cm^{-3} +/- SE			
	CONTROL	pH 2	pH 4	pH 5
0	242.3 +/- 9.3	244.9 +/- 9.5	229.5 +/- 10.3	232.5 +/- 13.6
7	814.0 +/- 20.5	238.4 +/- 8.8	328.4 +/- 9.0	394.9 +/- 7.4
10	1124.4 +/- 17.5	228.2 +/- 7.1	476.8 +/- 8.8	689.6 +/- 21.0
15	1439.4 +/- 63.2	250.8 +/- 11.6	812.7 +/- 14.1	1055.7 +/- 26.5
21	1587.4 +/- 22.0	261.0 +/- 11.8	994.8 +/- 17.9	1306.5 +/- 15.2

Table 5.2

Changes in medium pH during the batch culture cycle for
Acer pseudoplatanus cells cultured in a range of medium
pHs.

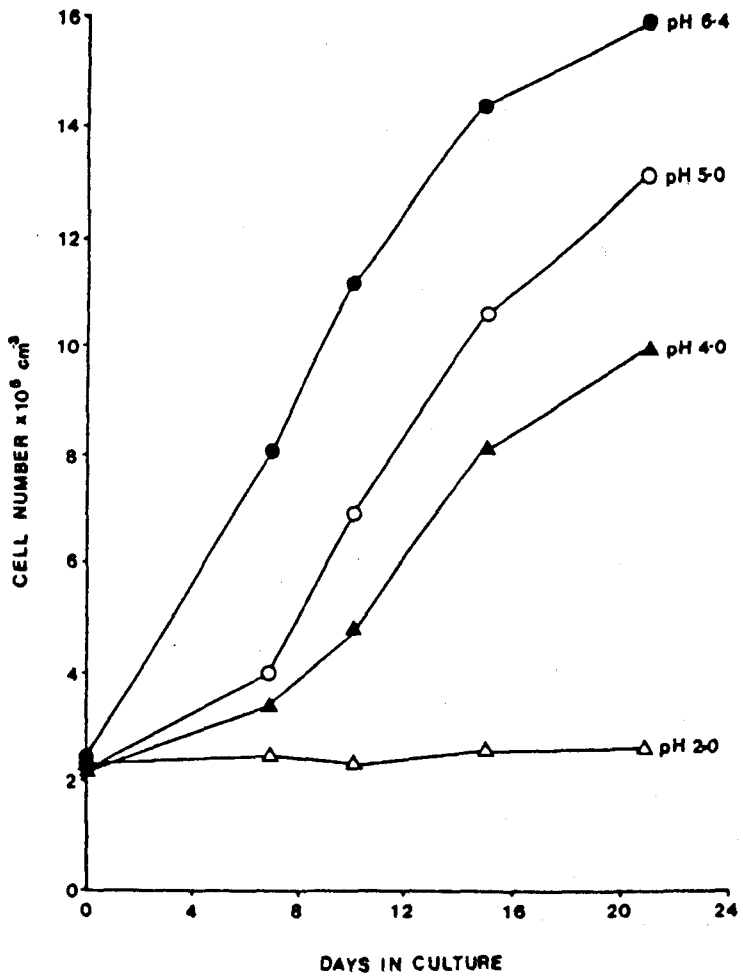| TIME (Days)	pH			
	CONTROL	pH 2	pH 3	pH 5
0	6.20	2.30	5.00	5.60
7	7.10	2.20	5.70	6.30
10	6.60	2.35	6.20	7.00
15	6.00	2.35	6.80	7.10
21	6.00	2.30	6.10	5.90

Figure 5.1

Changes in cell number during batch culture
cycle for <u>Acer pseudoplatanus</u> cells
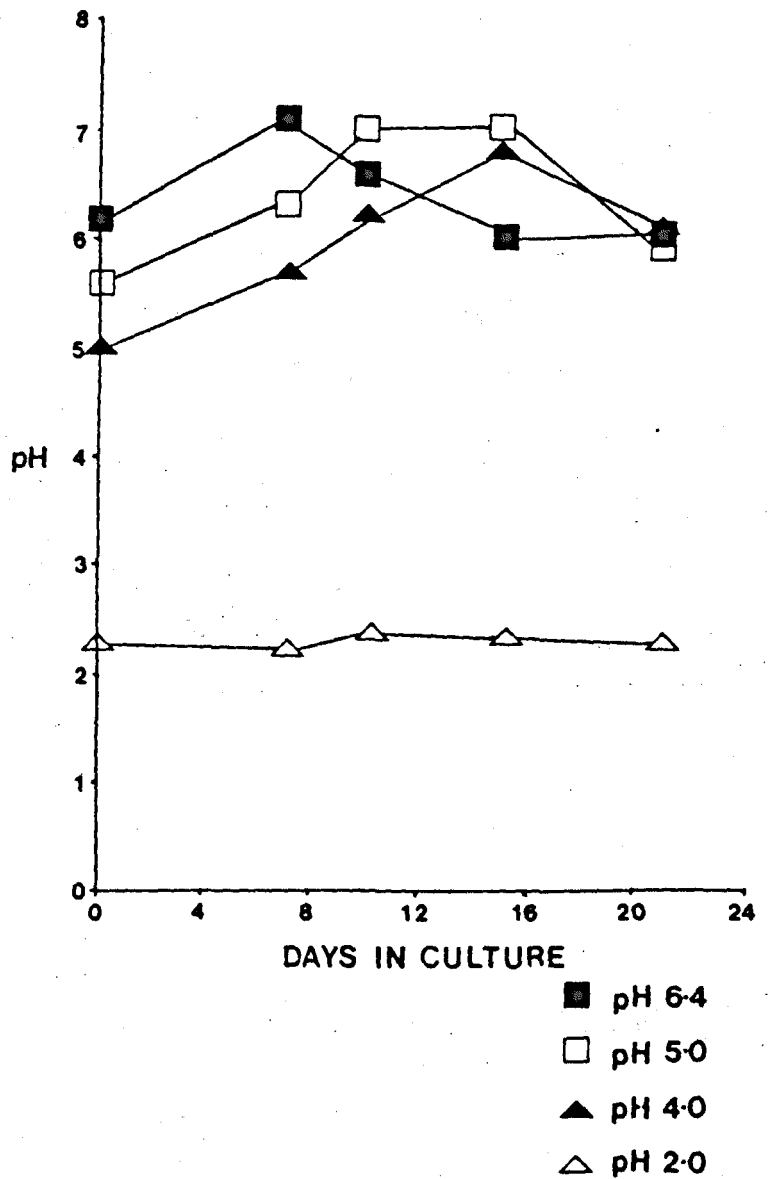cultured in a range of medium pHs.

Figure 5.2

Changes in medium pH during the batch culture
cycle for <u>Acer pseudoplatanus</u> cells cultured
in a range of medium pHs.

It is possible that the cells do require a specific external medium pH before they can exhibit cytokinesis; this would explain the extended lag phases in the acidic cultures. However, in a normal sub-culture situation control cultures exhibit a first synchronous division after approximately 72 hours when the medium pH is approximately 6.6. In this experiment the time points were quite staggered but it appears that by Day 10 cytokinesis had occurred in the pH 4 and pH 5 cultures when their medium pH levels were 6.2 and 7.0 respectively. Thus, if a specific pH level is essential before cytokinesis it is likely to be approximately equal to 6.2 which is coincidentally the level usually attained after sub-culture. As the growth curves and pH profiles of the pH 4 and pH 5 cultures are essentially both shifted due to extended lag phases it is postulated that the nature of the pH profile is largely attributable to the nitrate status of the culture medium. In more acidic conditions the cells would find it more difficult to take up nitrate which would result in the extended lag phases. The eventual increase in pH is likely to be due both to nitrate uptake and other metabolic processes. The subsequent decline in medium pH as the cells develop into asynchrony could again be due to the cessation of OH^- efflux as nitrate becomes less abundant and the production and accumulation of

acidic waste and secondary products. This hypothesis points to the nitrate content of cells as an important regulatory trigger for cytokinesis with the medium pH being a reflection of this.

5.3.2   Intracellular_pH_and_the_Growth_Cycle

NMR spectroscopy is a non-invasive technique which allows the researcher to investigate the molecular changes undergone during metabolic processes. Another important application of ^{31}P NMR is the determination of intracellular pH in living systems using the pH dependence of the chemical shift of the inorganic-phosphate (Pi) resonance. In plant tissue containing a significant cytoplasmic fraction, the same pH dependence leads to the characteristic appearance of two Pi resonances, corresponding to the cytoplasmic and vacuolar Pi. Thus the vacuolar and cytoplasmic pH can be estimated (Hughes et al, 1983).

The interpretation of ^{31}P NMR spectra can be complicated because of the large number of phosphorus compounds involved in the various metabolic pathways. An important distinguishing feature between compounds is their degree of chemical shift (δ) from a known reference standard. Figure 5.3 illustrates the ^{31}P NMR spectra obtained from a number of common phosphorus metabolite standards both individually and as a mixture. It can be seen that the peaks obtained are quite
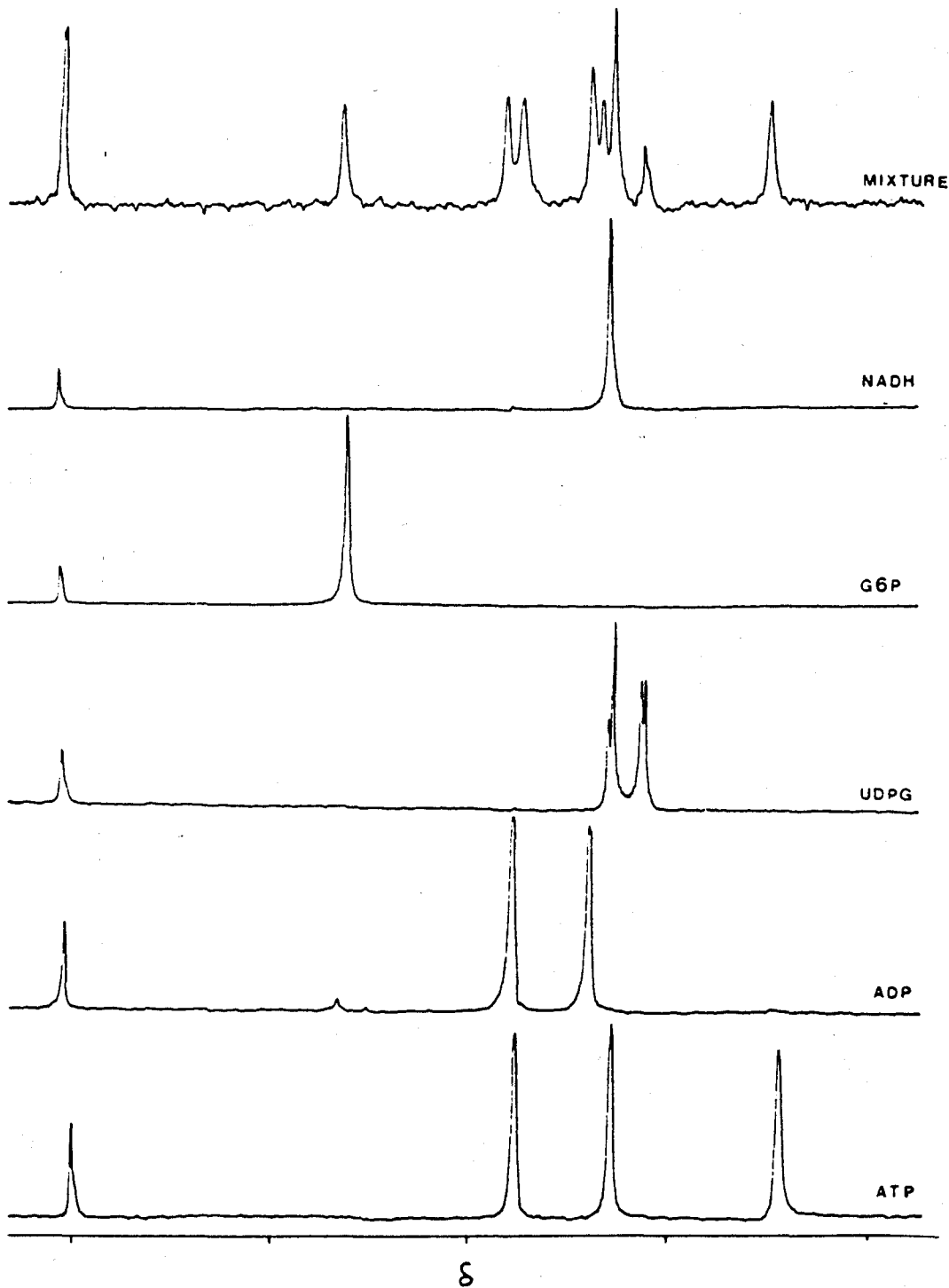
MIXTURE

NADH

G6P

UDPG

ADP

ATP

δ

Figure 5.3

31
P-NMR spectra for various standards.

distinguishable.   However,   when   plant   tissue   is
subjected   to   [31] P NMR spectroscopy the spectra   obtained
are not so easily interpreted as there is often overlap
between the resonances.   This makes it impossible to
complete   the   assignment   of   the   resonances   to   the
corresponding phosphorus compound without some knowledge
of   the   composition   of the sample.   This problem is
especially acute in tissue samples because the large
natural line widths cause a corresponding decrease   in
the   resolution of the spectrum.   It is therefore
relatively straightforward to identify   [31] P intensity
from sugar monophosphates or nucleoside triphosphates,
but it is more difficult to prove that an observed
resonance comes from, for example, glucose-6-phosphate
or uridine triphosphate (Kime et al, 1982b).

Ideally, the assignment of a tissue spectrum should be
reinforced by other analyses of the composition of the
tissue. Kime and co-workers (1982b) split the assignment
procedure into three steps, (i) assignment to classes
of phosphorus compound on the basis of the observed
chemical shift values; (ii) assignment to particular
phosphorus compounds on the basis of a characteristic
spectral property or a reported analysis of the tissue;
(iii) confirmation of the assignment by further
experiments,   eg by monitoring the spectrum while
perturbing the tissue, by spectroscopic analysis of a
tissue extract, or by some other physical or chemical
analysis.   In this study the resonances were assigned to

corresponding phosphorus compounds with the assistance of Mr S Bociek who has much experience in this field. This assignment was supported by the levels of known metabolites reported in the literature to be present in _Acer_ _pseudoplatanus_ cells at various stages of the batch cell cycle.

Although the use of ^{31}P NMR in this study revealed information on the metabolites present, the principal aim was to establish the intracellular pH levels during the batch and cell cycles. As described previously, the estimation of pH values by ^{31}P NMR depends upon the "chemical shift" of the intracellular ^{31}P resonance with pH changes. The chemical shift is expressed relative to a standard substance (dimethylene phosphoric acid) and a calibration curve is then drawn (Figure 5.4). The chemical shift is measured from the spectra and the corresponding pH value can then be read off the calibration curve.

A preliminary experiment was performed initially to establish whether or not the cytoplasmic and vacuolar phosphate pools would produce distinguishable peaks. The spectra obtained from 3, 11 and 23 day old _Acer_ _pseudoplatanus_ cells are shown in Figure 5.5. The peaks corresponding to the vacuole and the cytoplasm can be clearly seen amongst the other peaks in the spectra. Another peak was tentatively assigned to glucose-6-
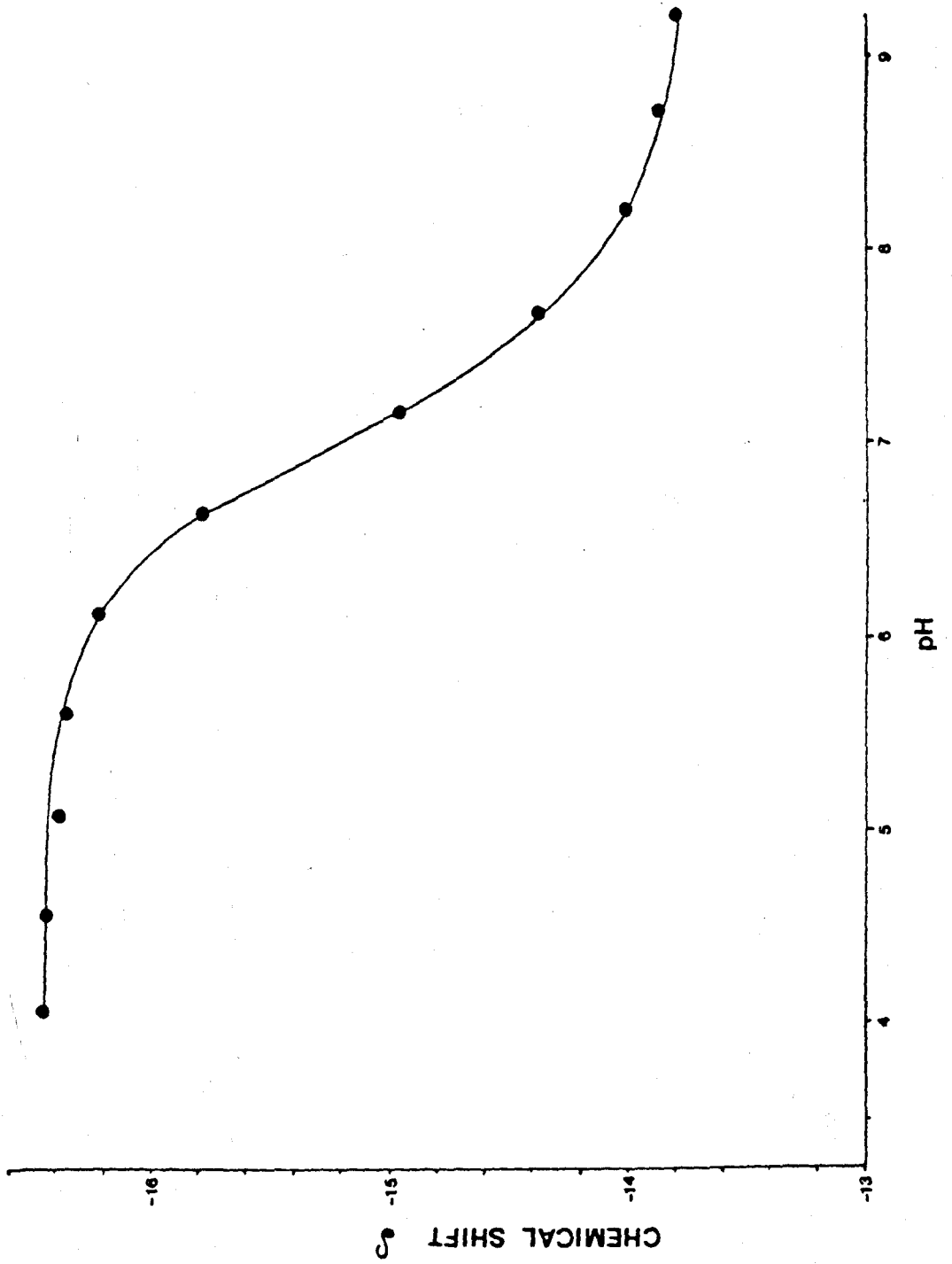
Figure 5.4

31
P-NMR standard curve.

phosphate and other peaks are thought to be produced by NTPs. The relative level of G-6-P increased over the culture period whilst the NTP levels appeared to decline.

The vacuolar and cytoplasmic pH levels were estimated from the calibration curve and the levels obtained are tabulated in Table 5.3 and illustrated in Figure 5.6 alongside extracellular data. The vacuolar pH was found to be lower than the cytoplasmic pH with the mean values being similar to those of 5.5 and 7.0 reported by Martin and co-workers (1980), who also worked with Acer pseudoplatanus cell suspension cultures. Despite the rapid fluctuation in the extracellular pH during the exponential phase, little variation was observed in the cytoplasmic and vacuolar levels although it must be noted that few sample points were taken. Both compartments became more acidic during the growth cycle; the vacuole became relatively more acidic than the cytoplasm, possibly due to the accumulation of acidic waste products. The relatively small variation in the cytoplasmic pH is probably a reflection of the buffering capacity of the cytoplasm which is important for the cell's metabolism. The extracellular pH was found to be at a level between the cytoplasmic and vacuolar levels implying that the pH of the medium does not have an

Figure
5.5
31
P-NMR spectra for 3, 11 and 23 day old
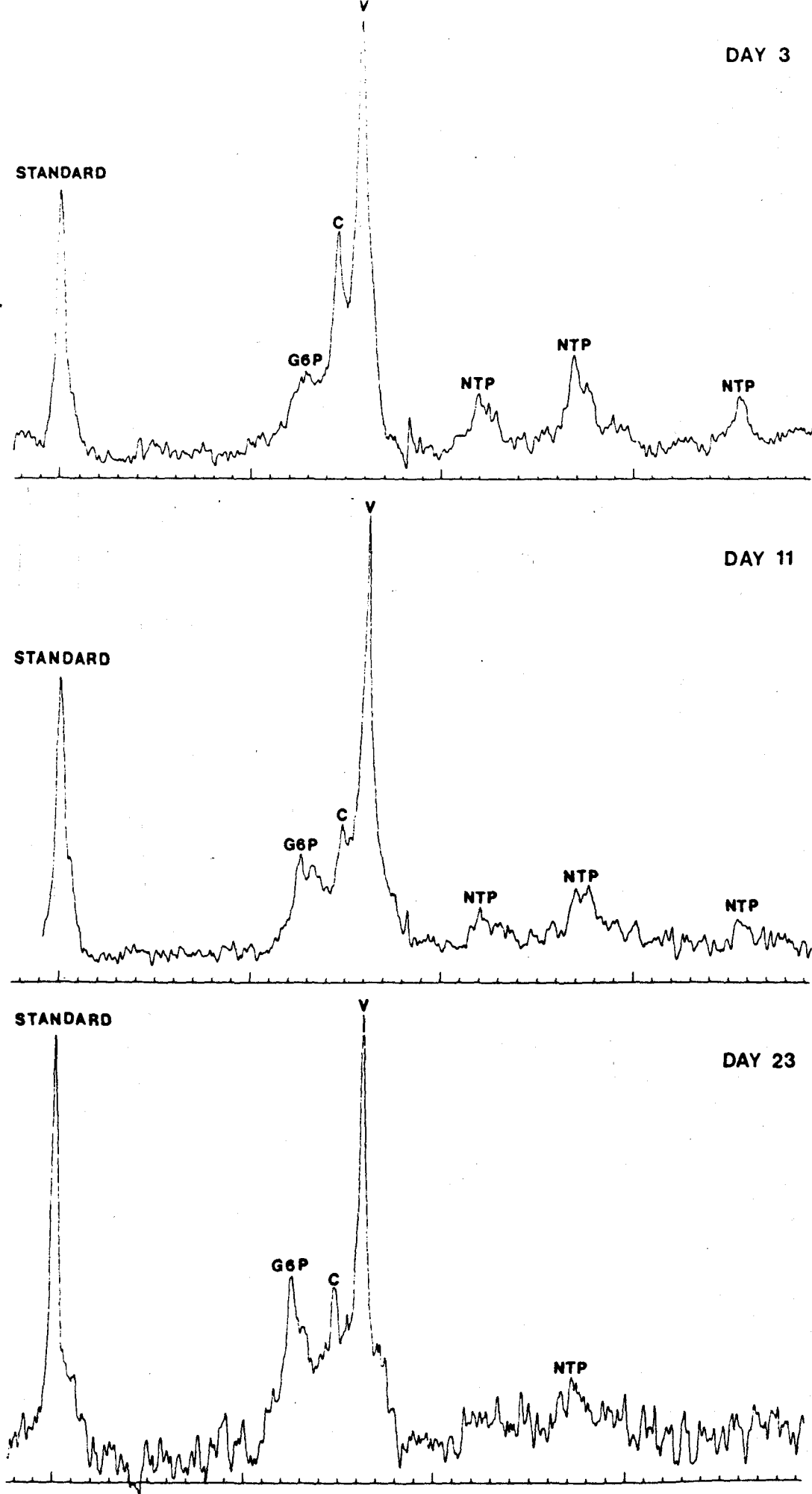_Acer pseudoplatanus_ cells showing vacuolar
(V) and cytoplasmic (C) pool peaks.

Table 5.3

Intracellular pH levels through batch growth cycle.

| TIME | pH | |
(Days)	CYTOPLASMIC	VACUOLAR
3	7.25	6.30
11	7.10	6.00
23	7.00	5.50
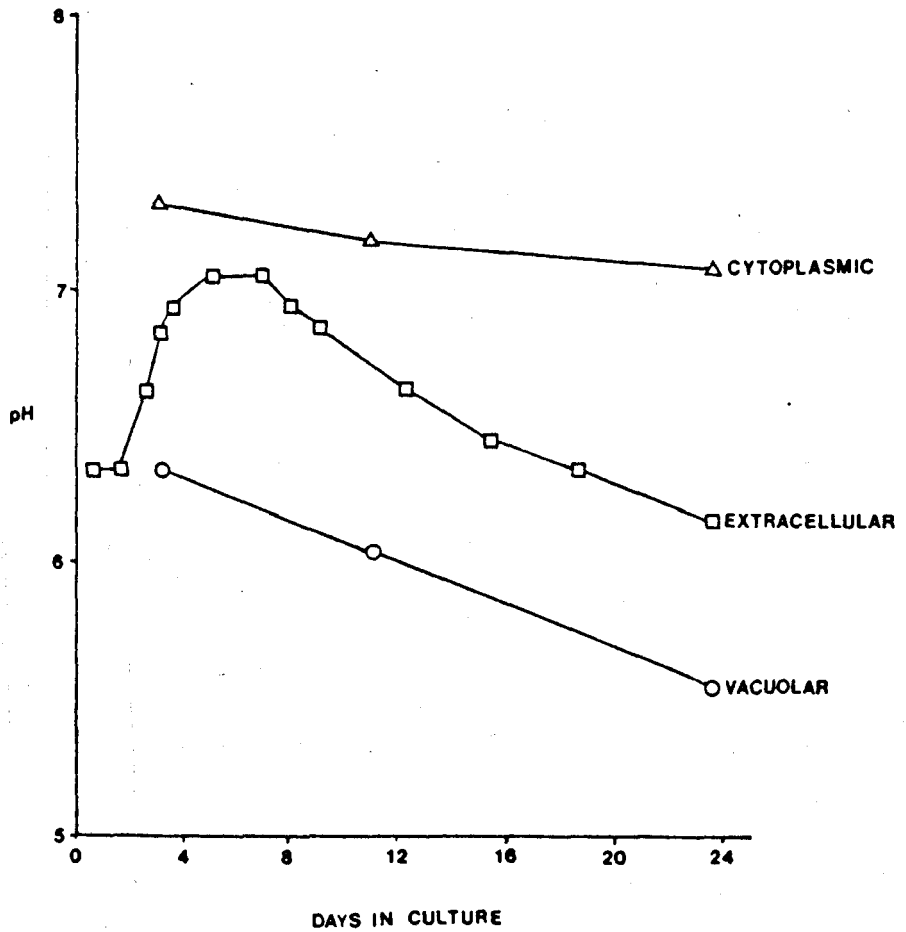
Figure 5.6

Extracellular and intracellular pH levels
through Acer pseudoplatanus batch growth
cycle.

over-riding effect on the intracellular pH levels. Clearly further experimentation was required with more sample points to establish whether any other fluctuations in the pH of these two compartments occurred.

In order to investigate the intracellular pH and metabolic changes at close time intervals use was made of the circulatory system (Lee and Ratcliffe, 1983) described previously. This system also facilitated simulation of events such as transfer into fresh medium.

5.3.2.1 Transfer Simulation

21 day old Acer pseudoplatanus cells were placed in the NMR tube and were circulated with the medium in which they had been cultured. The spectra revealed no distinguishable cytoplasmic peak, only a vacuolar peak which corresponded to a pH of 6.1 (Figure 5.7). The old medium was then replaced with fresh medium at the appropriate 1:6 ratio to simulate the conditions when cells are transferred on inoculation. After only 17.5 minutes the cytoplasmic Pi peak became distinguishable with a corresponding pH value of 6.85 (Figure 5.8). The cytoplasmic pH increased to approximately 7.0 and then remained relatively stable. The large number of scans obtained revealed little else of interpretable significance until the appearance of G-6-P after 12.25 hours (Figure 5.9). The results obtained from this experiment agree with previous workers (Martin

Figure 5.7

31
  P-NMR   spectra   for   21   day   old   Acer
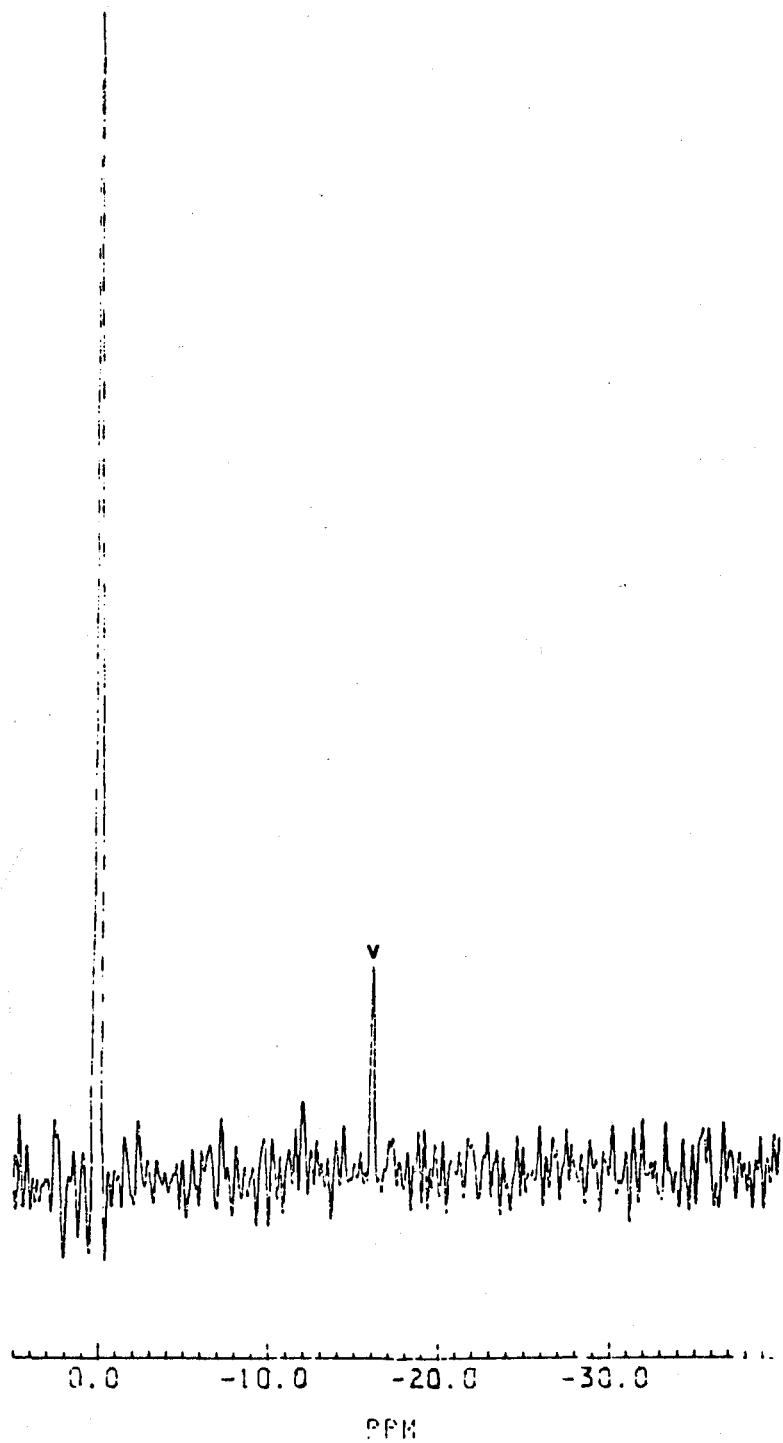pseudoplatanus cells.

Figure 5.8

31
 P-NMR spectra for <u>Acer</u> <u>pseudoplatanus</u>
cells 17.5 minutes after simulated transfer.
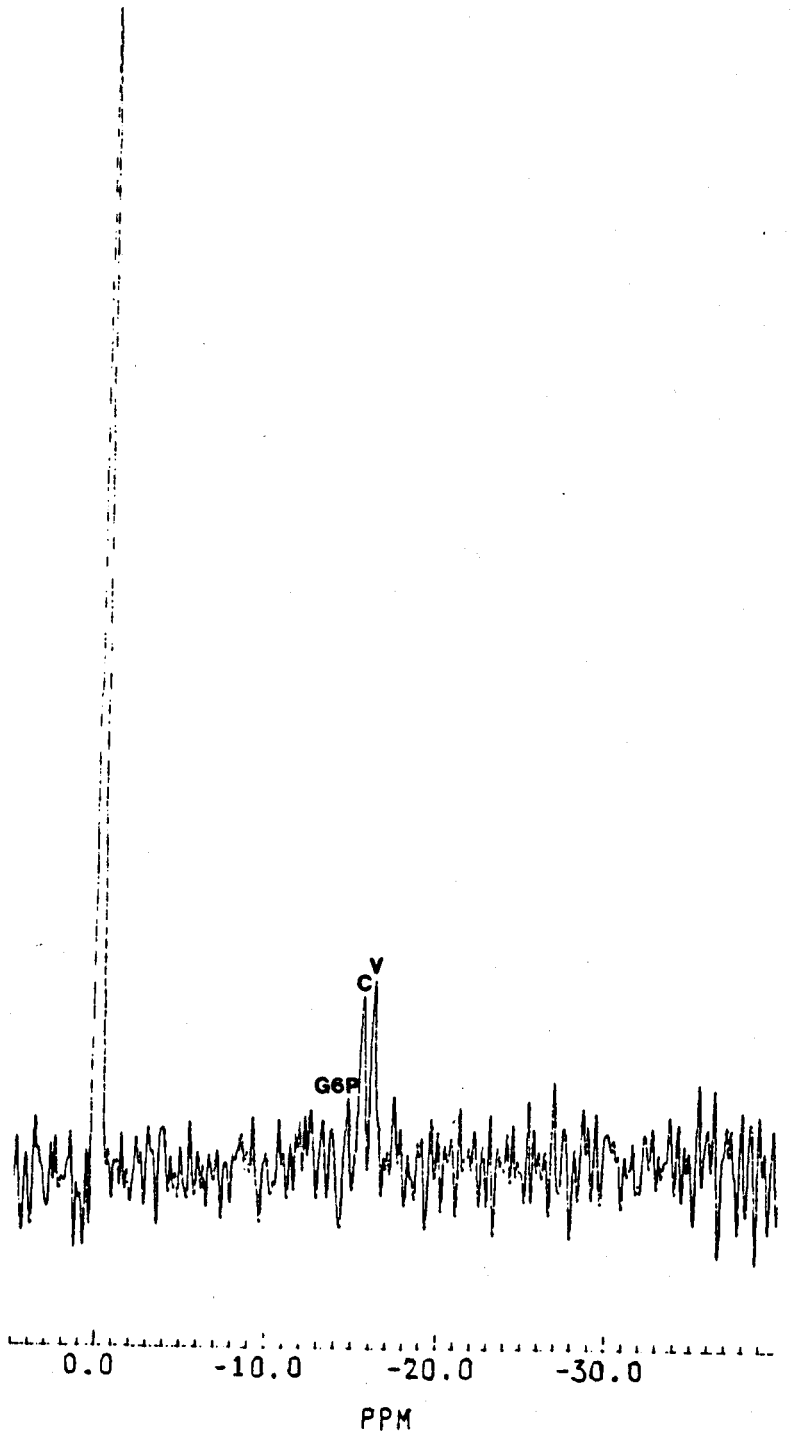
Figure 5.9

31
   P-NMR spectra for Acer pseudoplatanus
cells 12.25 hours after simulated transfer.

et al, 1982; Rebielle et al, 1982, 1983) who found that at low external concentrations Pi is accumulated in the cytoplasm. The cytoplasmic peak then began to move upfield (negative direction) reflecting acidification of the cytoplasm. In the 18 hour 57 minute scan (Figure 5.10) the cytoplasmic and vacuolar resonance peaks are virtually coincident with a pH value of 6.3. This cytoplasmic acidification may be due to the uncoupling action of compounds such as 2,4-D and dinitrophenol (Hughes et al, 1983; Kime et al, 1982a).

5.3.2.2   First Division Synchrony

Acer pseudoplatanus cells which had not yet exhibited a first synchronous division after sub-culture were placed in the NMR tube. The cells were scanned as they underwent a 78% synchronous division which occurred over a period of four hours. The cytoplasmic pH was found to be approximately 7.25 and the vacuolar pH was very stable at a level of 6.1. The spectra obtained 73 hours after inoculation (Figure 5.11) revealed the presence of G-6-P and NTPs. The relative level of cytoplasmic Pi to vacuolar Pi was seen to increase. As the cells entered the exponential phase the spectra remained essentially unchanged. An interesting resonance peak was found with a chemical shift value of -15.6 ppm. This peak was only evident when the cells were actively dividing.
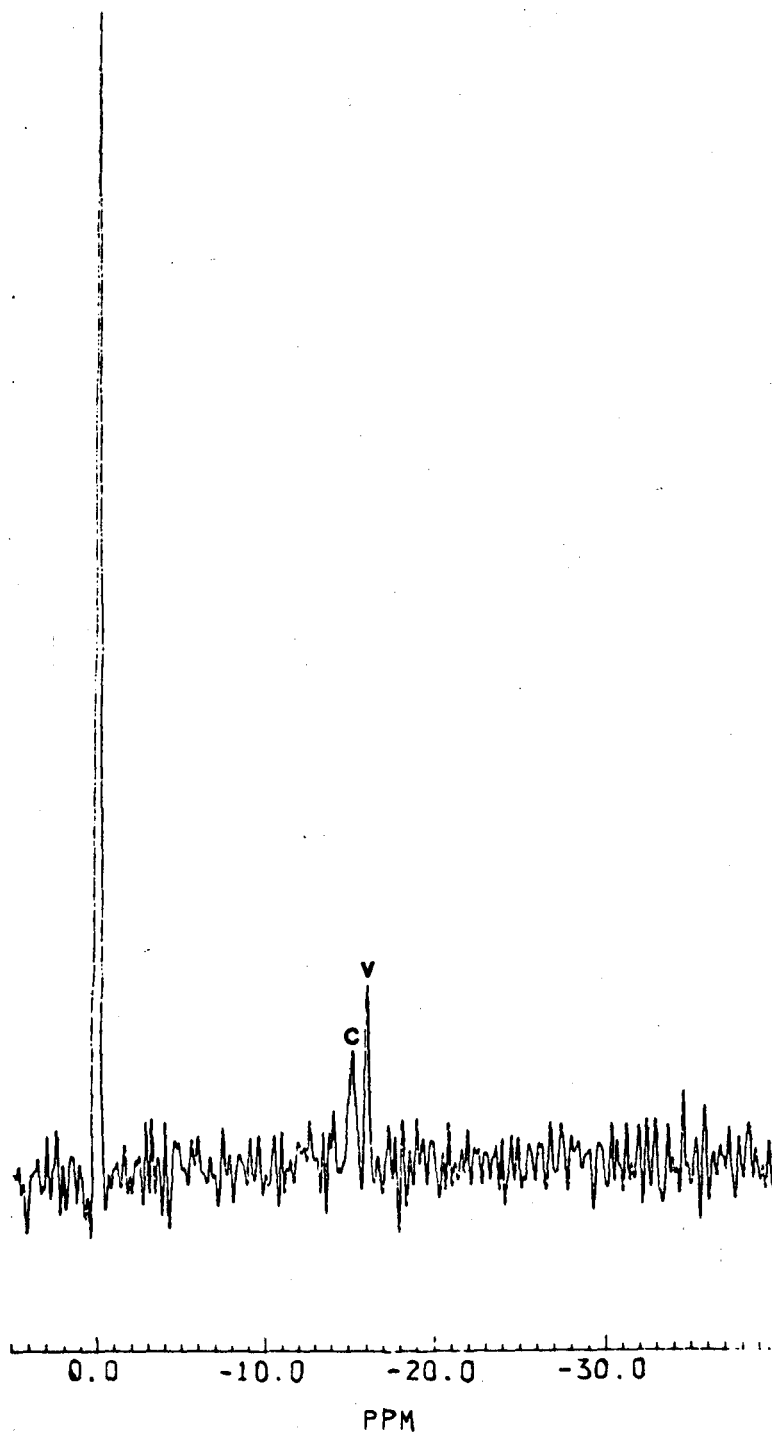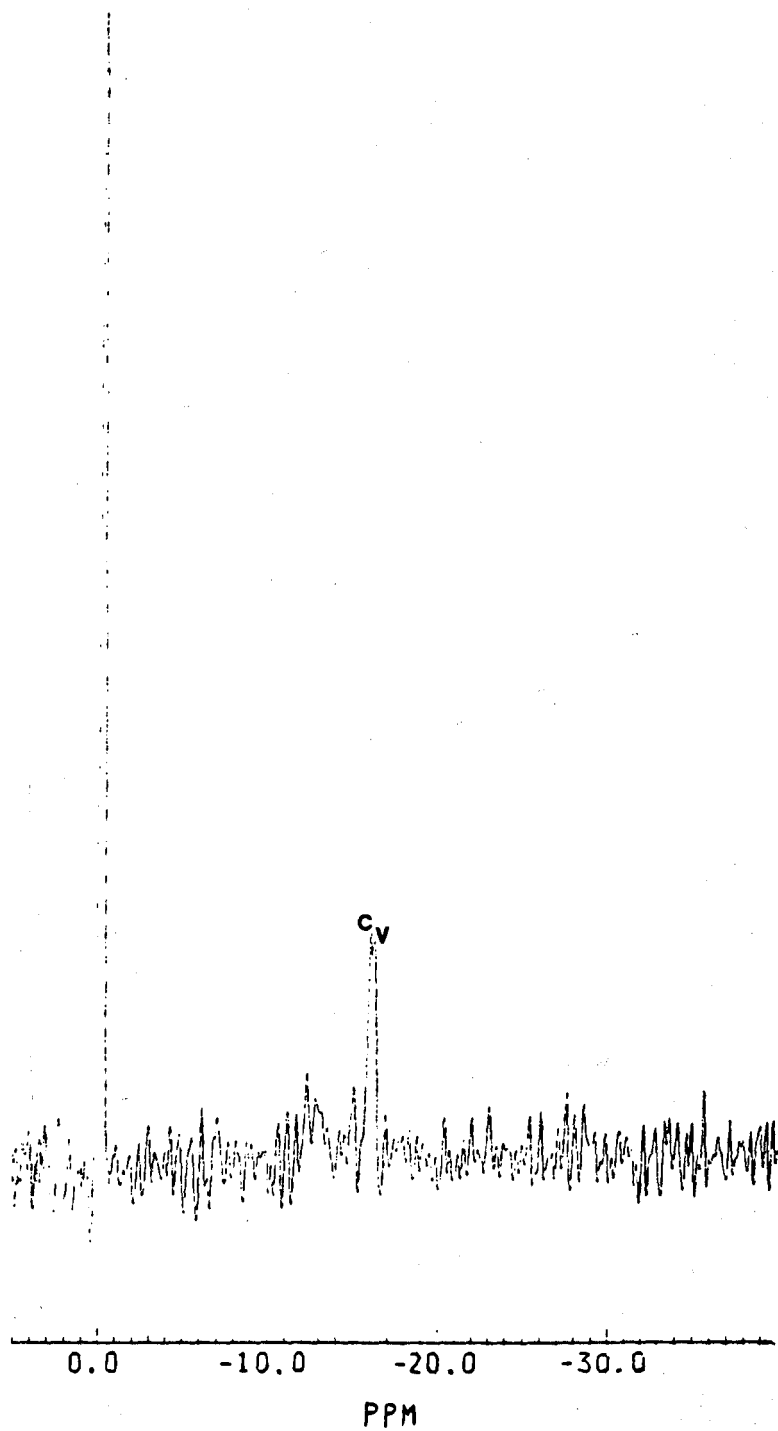
Figure 5.10

31
P-NMR spectra for <u>Acer pseudoplatanus</u>
cells 18 hour 57 minutes after simulated
transfer.
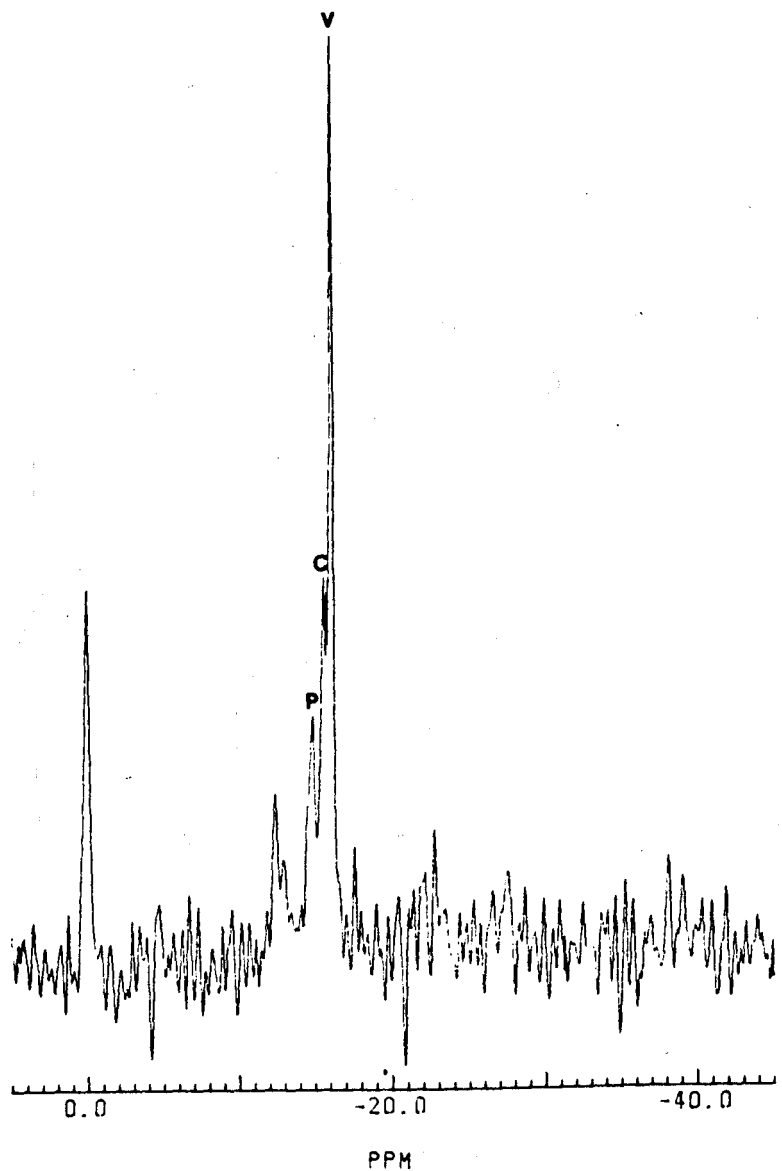
Figure 5.11

P-NMR spectra for Acer pseudoplatanus
cells undergoing first division showing the
resonance peak of a putative phosphodiester.

The peak corresponds to that of a phosphodiester, possibly glycerol-phospho-glycerol (G-P-G) (Ratcliffe, personal communication). Unfortunately, very little research has been directed at the phosphodiesters although their presence may be indicative of rapid membrane synthesis.

5.3.2.3 Batch_Culture

The intracellular pH fluctuations during the batch culture cycle were investigated in more detail than in the previously mentioned preliminary experiment. More data were obtained by employing the circulatory system and by using more cultures.

The vacuolar signal was found to be evident throughout the culture period and varied little from an average level of approximately 6.1. This is consistently higher than the vacuolar levels of approximately 5.7 previously reported (Rebielle et al., 1982; Kime et al., 1982a; Hughes et al., 1983) and the 23 Day level of 5.5 obtained in the preliminary experiment. These differences are probably due to differences between the cell types investigated and, in the case of the preliminary results, the effect of maintaining the cells in a healthier condition in this latter experiment by the use of the circulatory system. However, Martin and co-workers (1982) reported no affect on the vacuolar pH

by aeration despite an increase in the cytoplasmic pH. Thus, the increased pH level may be a reflection of other optimal conditions such as nutrient and other medium constituent availability.

The cytoplasmic pH was found to be more variable than the vacuolar pH. In dividing cells the cytoplasmic pH was found to be 7.5-7.6 and this level was maintained until the end of the active growth period (Day 8-9). The cytoplasmic pH then declined rapidly until a level of 7.0 was reached by Day 13. The ratio of cytoplasmic to vacuolar Pi also changed during the growth period reaching parity in 3-6 day old cells, favouring the cytoplasmic pH at Day 8 and then declining until no detectable cytoplasmic resonance was found in stationary phase cells.

5.3.2.4 Nitrate_Synchronised_Division

Acer pseudoplatanus cells were synchronized as described in Section 4.0 by the addition of a nutrient feed and were then placed in the NMR tube prior to the expected induced division. The spectra obtained (Figure 5.12) revealed characteristically high cytoplasmic pH levels of 7.5-7.6 whilst the vacuolar pH remained relatively stable at 6.1. The signals from G-6-P and NTP were found to be prominent but their relative levels appeared to remain constant. The ratio of cytoplasmic Pi to
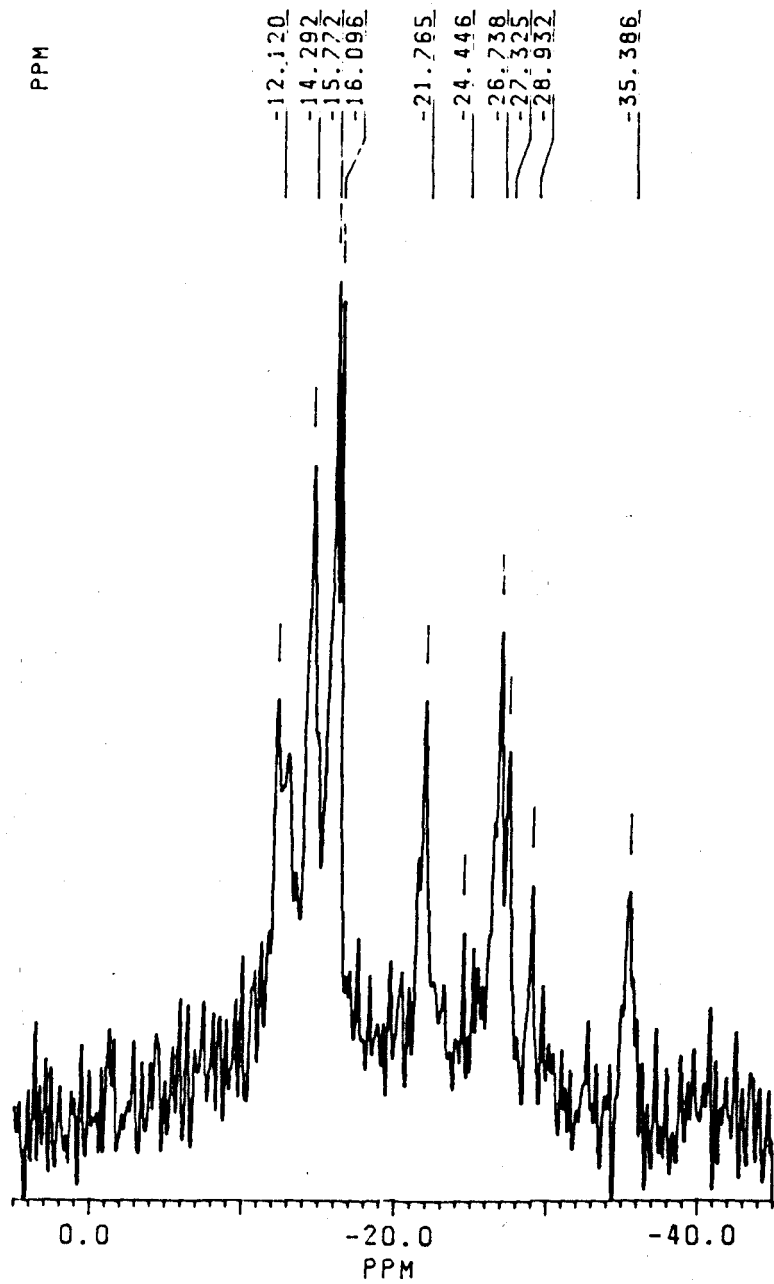
Figure 5.12

31

P-NMR spectra for _Acer_ _pseudoplatanus_
cells undergoing a nitrate synchronized
division.

vacuolar Pi also remained relatively stable. The putative phospho diester was again evident, as in the first division synchrony experiment.

The cytoplasmic pH levels observed during this induced synchronous division were slightly higher than those observed in the cells undergoing the first division after subculture. This may be explained by the fact that the percentage synchrony in the induced division was higher; if some of the cells were out of step in their cell cycles their cytoplasmic pH may be different and this would produce an effect on the resonance peak and thus the estimated pH level.

5.3.2.5 Effect_of_extracellular_pH_on_intracellular_pH

In order to determine whether or not the extracellular pH causes a change in the intracellular pH levels cells were placed in the NMR tube and circulated with medium at pH levels 6.5, 4.06 and 2.9. The cytoplasmic and vacuolar pH levels obtained under these conditions are tabulated in Table 5.4. It can be seen that the extracellular pH changes produced no effect on the vacuolar pH levels and very little effect on the cytoplasmic pH levels. The spectra revealed a slight decrease in the cytoplasmic Pi and NTP resonances as the external pH decreased.

Table 5.4

Cytoplasmic and vacuolar pH levels in cells subjected to a range of extracellular pH levels.

Extracellular pH	Cytoplasmic pH	Vacuolar pH
6.5	7.6	6.1
4.06	7.6	6.1
2.9	7.5	6.1

Thus, it appears that the pH of the bathing medium has no direct causal effect on the intracellular pH. This is probably because of the cell's immense buffering capacity which is essential because defined intracellular pH levels are vital for metabolic processes.

5.3.2.6 Efficiency of circulatory system

Throughout the course of the NMR experiments the cell densities and medium pH levels were monitored both in the NMR tube and in representative control cultures. Generally, the data obtained confirm the efficiency of the circulatory system. The cells in the NMR tube did undergo cytokinesis and the pH profile of their bathing medium was as found in the control cultures. Cell viability data also confirmed that the cells under analysis were maintained in a healthy condition. It is believed that the cells can be maintained in the NMR tube for up to 11 hours without any noticeable deviations in their growth patterns from those of the control cultures. This makes the system ideal for studying processes such as cytokinesis which occupy a time period of several hours.

5.4 Conclusions

In this section the extracellular pH levels were investigated in parallel with the intracellular pH variations. The cytoplasmic and vacuolar pH levels were

estimated by the technique of ^{31}P NMR spectroscopy. The cytoplasmic and vacuolar compartments contain phosphate pools which are distinguishable on an ^{31}P NMR spectra. The degree of "chemical shift" of these resonance peaks can be related to a pH level with the aid of a calibration curve.

The Acer pseudoplatanus cells used in this present study were routinely grown in a culture medium adjusted to pH 6.4 (Stuart and Street, 1969). The data obtained in Section 4.0 revealed that by Day 21 the medium pH has acidified (approximately 6.0) possibly due to the accumulation of waste and secondary products. On transfer, the acidic nature of the inoculant results in a slight decrease in the medium pH which is then superceded in batch cultures by a marked increase in the alkalinity of the medium as the cells undergo rapid cell division. As asynchrony develops the medium becomes more acidic again until the Day 21 level is established.

In nitrate/phosphate feed induced synchronized cultures the medium pH maintained a near neutral level whilst the cells were dividing and the nutrients were abundant. However, the decline in medium nitrate between feeds was closely paralleled by the onset of the eventual decline in medium pH. This correlation may be due to the efflux of OH^- ions by the cells during nitrate uptake and/or

the accumulation of secondary products in the surrounding medium as nitrate becomes limiting and the cells switch from primary to secondary metabolism.

In order to establish the importance of the near neutral medium pH level associated with cells undergoing rapid synchronous growth, cells were inoculated into medium adjusted to pH 2, 4 and 5. The highly acidic pH 2 culture exhibited no growth and possibly became non-viable due to the severity of the culture conditions. The cells initiated in medium pHs 4 and 5 did exhibit growth although they underwent prolonged lag phases (7+ Days). Their growth rates then increased although the final cell density was higher in the pH 5 culture but not as high as the control culture.

The inhibition of cell culture growth under acidic medium conditions is similar to the affect presumed by Robinson (1982) to be caused by the alleged G2 arrestor, trigonelline (Figure 3.26). As discussed previously in Section 3.3.4 the data from this study indicate that it is possible that if and when trigonelline does cause G2 arrest it may be an indirect affect caused by the acidic nature of medium after trigonelline addition. It is therefore important to check the pH status of culture medium after the addition of any non-routine culture constituent.

The medium pH was monitored in these low pH cultures during the batch culture cycle. The pH 2 culture remained at a relatively stable level of 2.3 throughout. The medium pH in the other two cultures increased such that by Day 10 the pH 5 culture had increased to pH 7.0 and by Day 15 the pH 4 culture had increased to pH 6.8. These levels then declined as in the normal batch cultures. It is unlikely that the neutral to alkaline medium pH associated with the period of rapid cell growth in asynchronous cultures and the synchronous state in synchronised cultures is a specific trigger for cytokinesis. This can be deduced because when the cells undergo their first synchronous division after subculture, the pH in a control culture is approximately 6.6 and in the pH 4 culture considered here, 6.2. It is more likely that the increase in the alkalinity of the medium is a reflection of something else which may be an essential trigger for cytokinesis. This factor may be the availability of nitrate. As the cells take up nitrate there is an efflux of OH^- ions and thus an increase in the alkalinity of the medium. In cultures that become nitrate limited the cells develop into asynchrony, some enter secondary metabolism and produce acidic waste and secondary products, the OH^- efflux ceases due to the cessation of nitrate uptake and the medium eventually becomes more acidic. The extended lag phases in the acidic cultures considered here may be caused by slow nitrate uptake by the cells due to the acidic conditions. When sufficient nitrate had been

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taken in by the cells cytokinesis could ensue regardless of whether the external pH was 6.2; as in the pH 4 culture, or 6.6, as in the control culture.

In cultures where synchrony is maintained by nitrate feeds the external pH remained constantly alkaline whilst the culture was in the synchronous state. This could be explained by the continued OH^- efflux and little acidic secondary product synthesis as the majority of the cells were involved in primary metabolism. As soon as the nitrate began to become depleted between successive feeds the medium pH began to become more acidic, possibly because some of the cells became asynchronous. Thus, it seems likely that nitrate availability is an important trigger for cytokinesis and the extracellular pH merely reflects the nitrate status of the culture. It is likely that other undefined factors associated with cytokinesis also affect the extracellular medium profile.

Although fluctuations were found in the extracellular pH levels during the batch cell cycle, it is likely that the intracellular pH levels are more important to the cell itself, for it is here that the metabolic processes take place. In this study the cellular pH levels were estimated by ^{31}P-NMR which proved to be a reliable non-invasive technique. The cytoplasm and vacuole revealed distinguishable resonance peaks due to their separate Pi

pools and by measuring the degree of the "chemical shift" of these peaks their respective pH levels were estimated.

A preliminary experiment, without the benefit of a circulatory system and with only three time points, revealed mean values for the vacuole and cytoplasm similar to those of 5.5 and 7.0, (respectively), reported by Martin and co-workers (1980). Despite the rapid extracellular pH fluctuations during the exponential phase, little intracellular variation was found. Both compartments became more acidic during the growth cycle.

In order to investigate the intracellular pH variations in more detail in cells maintained for longer time periods in a healthy condition, use was made of the circulatory system (Lee and Ratcliffe, 1983). In a transfer simulation experiment the spectra revealed no distinguishable cytoplasmic peak at first, however, only 17.5 minutes after the transfer simulation the cytoplasmic peak appeared with a corresponding pH level of 6.85. This level then stabilized at 7.0 before becoming more acidic, possibly due to the uncoupling action of compounds such as 2,4-D and dinitrophenol (Hughes et al, 1983; Kime et al, 1982a). The vacuolar pH remained relatively stable throughout the simulation, remaining at a level of approximately 6.1.

The intracellular pH levels were also monitored during the first synchronous division after sub-culture. Again, the vacuolar pH remained stable at approximately 6.1. However, the cytoplasmic pH was found to be 7.25 whilst the cells underwent division. ^{31}P NMR can also reveal information on the presence and relative levels of metabolites. Whilst active cell division took place a peak was apparent which was tentatively assigned to the phosphodiester, glycerol-phospho-glycerol (Ratcliffe, personal communication). Very little is known about such substances although its presence may be indicative of rapid membrane synthesis.

The intracellular pH variations were also estimated during the batch culture cycle. The vacuolar levels were again found to be very stable (approximately 6.1). The cytoplasmic pH was found to be more variable and more alkaline. In dividing cells a level of 7.5-7.6 was achieved and maintained until the end of the growth period (Day 8-9). The cytoplasmic pH then became more acidic until a level of 7.0 was achieved by Day 13. In nitrate synchronized cells the vacuolar pH again remained relatively stable at 6.1 whilst the cytoplasmic pH revealed characteristically high levels of 7.5-7.6 found previously in actively dividing cells. The putative phosphodiester was also evident again.

Thus, the vacuolar pH varied little during the circumstances investigated, whereas the cytoplasmic pH was found to achieve high levels (7.2-7.6) during periods of active semi-synchronous and highly synchronous divisions. In order to determine whether or not the extracellular pH produces a direct effect on the intracellular pH, the cells were studied in medium pH levels of 2.9, 4.06 and 6.5. The extracellular pH was found to produce no effect on the vacuolar pH and very little effect on the cytoplasmic pH. This is probably due to the cell's immense buffering capacity which is essential for its vital metabolic processes.

The results obtained from the cells analysed in the NMR tube are probably representative of the cells in their normal cultural conditions. This is assumed because it is known that cytokinesis occurred in the NMR tube and the pH profile of the circulatory medium was similar to that of the bathing medium in shaken flasks.

The high cytoplasmic pH levels observed during active cell division are probably very significant as this is the pH variation that would closely affect the cells metabolism. It is possible that this level is optimal for the production, distribution, action or uptake of a growth substance essential for cytokinesis. This theory is discussed in more detail in relation to the known endogenous growth substance variations in the following concluding section.

6.0 GENERAL DISCUSSION

In this present study the endogenous cytokinin and IAA levels were investigated during the first synchronous division after sub-culture of <u>Acer</u> <u>pseudoplatanus</u> cells in suspension culture. A synchronization technique involving a nutrient feed system was then developed (Section 4.0) and the IAA levels were further investigated during an induced synchronous division. It is postulated that this "nitrate and phosphate feed" system maintains the cells in primary metabolic pathways rather than the secondary metabolic pathways typical of nutrient depleted asynchronous cells. Doubling the nutrient concentration available produced divisions with shorter cell cycle times implying that nitrate and phosphate availability has a rate-limiting effect on the progress of cells through the cell cycle. A series of highly synchronized divisions (approximately 80% in two hours) were reliably and reproducibly obtained. The synchronization system developed has the potential for complete automization when interfaced to a computer.

The total endogenous cytokinin levels were found to increase to peaks of activity at 20 and between 65-70 hours after sub-culture. Some variations in the actual levels were found but the general trends were consistent. Zeatin-like activity was the main cytokinin detected, being the predominant constituent of the two peaks. Zeatin riboside-, zeatin-O-glucoside- and dihydrozeatin-like activity was also detected. The

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relatively rapid fluctuations between the levels of the different cytokinins suggest some degree of interconversion. The cytokinins have different roles; this may explain why the levels fluctuate. Thus, the cell's cytokinin requirements may be different at different stages of the cell cycle. It is postulated that the 20 hour peak may be associated with protein synthesis and the 65-70 hour peak with mitosis and/or cytokinesis. The cytokinins may act as triggers at these two stages of the cell cycle.

The endogenous IAA levels were found to fluctuate very rapidly with inexplicably higher levels being found immediately after transfer than have previously been reported in stationary phase cells (Robinson, 1982). High levels of IAA are known to be present in the medium and thus it is tentatively assumed that there is movement of IAA between the medium and the cells. This may particularly occur during the shock of subculture when changes in pH and/or permeability could result in transiently high intracellular levels. The IAA levels declined after inoculation but were then seen to increase close to mitosis and/or cytokinesis. This implies that IAA may have a regulatory role at this point of the cell cycle.

The IAA data obtained from the synchronously dividing cells did not reveal a peak coincident with mitosis and/or cytokinesis. Although some slight fluctuations

did occur, generally the levels remained at a relatively stable level of approximately 0.5ng ·10^{-6} cells during the period of rapid synchronous divisions. It is possible that the peak observed after subculture is a reflection of a shock effect of transfer. Conversely, the absence of this peak from the synchronous cultures may be due to a change in the cells usual cultural behaviour caused by the nutrient feed inducement technique. Previous work in these laboratories produced endogenous IAA data from cold-induced semi-synchronous Acer pseudoplatanus cultures (Elliott et al., 1987). Peaks in IAA activity coincident with mitosis were detected but other fluctuations were also observed which it is postulated are a reflection of a change in the biosynthesis : degradation ratio due to the temperature changes.

It is proposed that the nutrient feed technique employed here is likely to be less disruptive than the cold-induced synchronization technique or subculture itself. Thus, the data presented in this study are likely to be more akin to the situation in the whole plant. That is, during active synchronous cell division a base level, rather than a peak in IAA activity is observed.

Unfortunately, the cytokinins were not analyzed during nutrient feed induced synchrony and this is an area for important future work. However, the cytokinin peaks detected during first division synchrony were coincident with the points in the cycle where protein synthesis and

cytokinesis are known to occur, both processes being closely linked with the cytokinins. Nevertheless, in the light of the previously mentioned IAA data, these cytokinin results must be considered tentatively.

The medium pH profiles were determined in both asynchronous and synchronous cultures. In asynchronous cultures the medium pH was found to increase after subculture until the exponential phase when a rapid decline was observed. This decline was found to be coincident with the point at which nitrate was depleted from the medium. The acidification of the medium is believed to be at least partially caused by the cessation of OH^- efflux and the production of acidic secondary products by cells in secondary metabolic pathways. In nutrient feed induced synchronized cells the pH increased as before but then remained relatively stable at a level of approximately 7.5-7.6 as the cells underwent division. This may be explained by the continuing OH^- efflux and the fact that the majority of the cells remained in primary metabolism.

The technique of ^{31}P-NMR spectroscopy was employed to estimate the intracellular pH levels. Alteration of the extracellular pH was found to have little or no effect on the vacuolar or cytoplasmic pHs. This is probably due to the buffering capacity of the cells. The vacuolar pH was found to remain relatively stable at a level of approximately 6.1 during both the batch culture

cycle and in nitrate/phosphate synchronized cells. The cytoplasmic pH was found to be more variable, except in the nitrate induced synchronized cultures where a stable level of 7.5-7.6 was observed. This stable level is probably a reflection of a metabolic process associated with cytokinesis.

During both the first synchronous division and the induced division a resonance peak was found which was tentatively assigned to a phosphodiester. The presence of this substance is indicative of rapid membrane synthesis which would be taking place during cytokinesis.

Regardless of the exact mechanisms determining the extra and intracellular pH levels, an important factor to consider is the effect that these pH levels will have on the distribution of auxin.

Auxin transport is greatly affected by the lipid-soluble weakly acidic nature of both the naturally occurring IAA and of most synthetic auxins such as 2,4-D. Auxin distribution is believed to be achieved by polar transport which is a pH dependent process (Raven, 1975). In cell suspension cultures with higher intracellular than extracellular pH levels, the net uptake rate of IAA is reported to be larger the greater the pH difference

between the cytoplasm and the bathing medium (Rubery and Sheldrake, 1973). However, in this present study during periods of rapid asynchronous or synchronous divisions the cytoplasmic pH and the extracellular pH levels were found to be similarly high and so little diffusion of IAA would be expected on this basis.

However, the pH difference between the cytoplasm (pH 7.5) and vacuole (pH 6.1) during periods of cytokinesis would affect the IAA distribution. In this case the IAA will be almost exclusively in the cytoplasm. The cytoplasmic/vacuolar volume ratio for suspension culture sycamore cells is approximately 1:9 (Bligny and Douce, 1976). Thus, effectively, the intracellular IAA concentration of the cytoplasm would be ten times greater than the previously calculated per cell levels.

Much of the previous data available from these laboratories were in accord with the simple hypothesis that cells had to achieve a critical peak ("trigger") concentration of endogenous IAA before each division. However, the results obtained from the nitrate synchronized cultures implied that this hypothesis may require modification. It is apparent that the variations in the endogenous IAA levels may be less significant to the cells than the absolute concentration of IAA within them. It seems likely that a specific IAA

concentration is a pre-requisite for cytokinesis and provided the level is maintained above a defined base level, cytokinesis will ensue regardless of any fluctuations.

The exact determinant of this hypothetical base level may be one of many factors. However, it is proposed that this level may be associated with an IAA binding protein required for the utilization of IAA by the cell. A specific high affinity IAA binding protein has been detected in these cells (Bailey et al., 1985) which has characteristics similar to those of the "soluble receptor" described by Libbenga and co-workers (Bogers et al, 1980; Oostram et al, 1980; Van der Linde et al, 1984). The apparent K_a for IAA was found to be approximately $1 \times 10^8 M^{-1}$. The data obtained by Robinson (1982) for cold-induced synchrony (Figure 6.1) have been recalculated on the basis of actual cell concentration by taking into account the MCV. Figure 6.2 illustrates these data with the endogenous IAA expressed as $\log_{10}$ [IAA]. The horizontal lines define the limits of the K_a values determined for the auxin binding protein ($1.6 \times 10^8 M^{-1}$ to $1 \times 10^8 M^{-1}$). The endogenous IAA concentration is below the K_a value of the binding protein on transfer of the cells to fresh medium but it rapidly rises above the 50% saturation level prior to cytokinesis and remains above this level despite the fluctuations. Figure 6.3 illustrates the data obtained in Section 4.0 for the endogenous IAA levels during
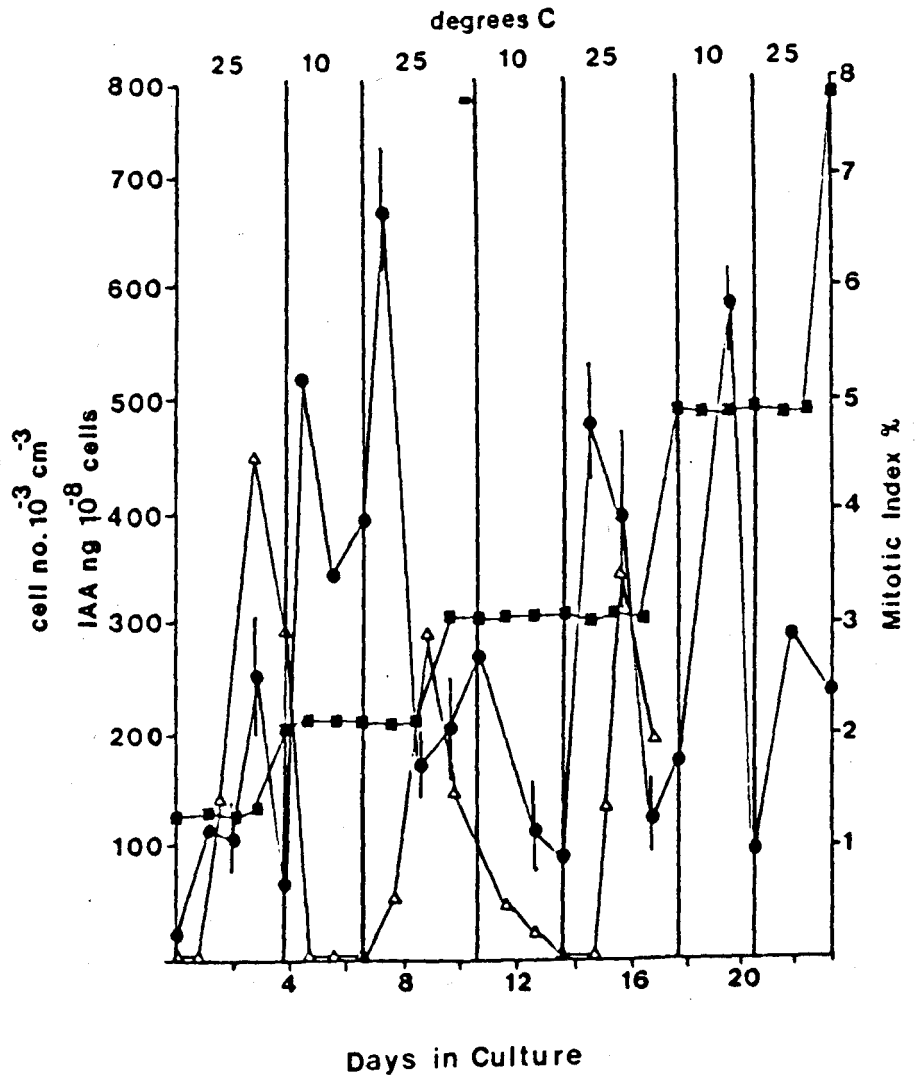
Figure 6.1

Acer pseudoplatanus endogenous IAA changes
during cold-induced synchrony
(Robinson, 1982).

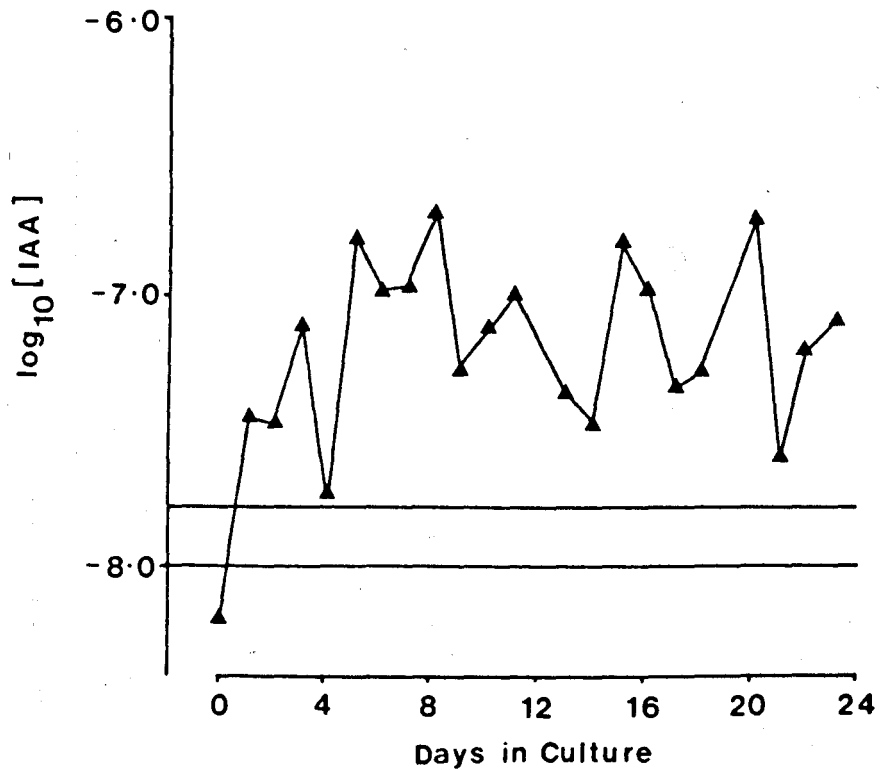Figure 6.2

Acer pseudoplatanus intracellular IAA
concentrations during cold-induced synchrony.
(The horizontal lines define the limits of
the Ka values determined for the auxin

binding protein, $1.6 \times 10^8 M^{-1}$ to $1 \times 10^8 M^{-1}$).

nitrate/phosphate induced synchrony expressed in terms

of log $_{10}$ [IAA]. As the vacuolar and cytoplasmic pH data

for these cells (obtained in Section 5.0), indicated

that the IAA would be almost exclusively within the

cytoplasm the concentrations have been increased tenfold

accordingly. It can be seen that the endogenous IAA

concentration is far above that necessary to achieve 50%

saturation of the auxin binding protein. It is likely

that the same tenfold factor should be applied to

Robinson's data.

Related work in these laboratories with _Nicotiana_

tabacum cell suspension cultures (Bailey _et_ _al_., 1985)

has demonstrated an auxin receptor that appears to cycle

between the cytoplasm and the nucleus during the culture

cycle. It is postulated that such a system might also

be involved in cell division regulation in _Acer_

pseudoplatanus cells. Therefore, it seems that cell

cycle regulation is unlikely to be achieved by plant

growth regulator concentrations alone but by a complex

interaction of growth regulator and receptor

concentration and distribution.

The distribution of extracellular and intracellular IAA

is probably very important in this regulatory process

and may explain rapid fluctuations. Providing that no

active transport system operates, it is possible to

predict the distribution of IAA between two compartments
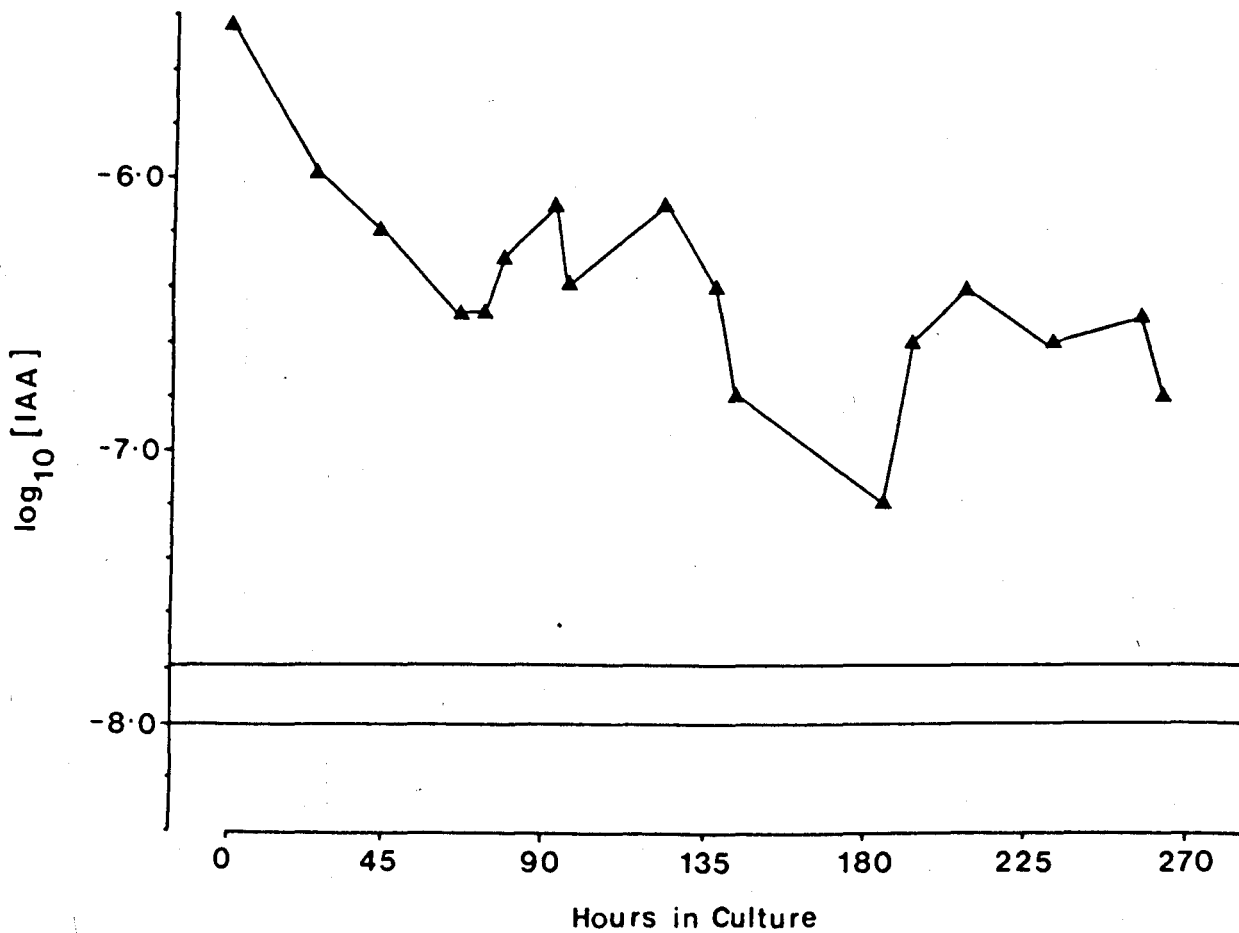
if the relative pH's are known (Rubery and Sheldrake,

Figure 6.3

Acer pseudoplatanus intracellular IAA
concentrations, corrected for
cytoplasmic:vacuolar ratios, during nitrate/
phosphate feed induced synchrony. (The
horizontal lines define the limits of the Ka
values determined for the auxin binding
protein, $1.6 \times 10^8 M^{-1}$ to $1 \times 10^8 M^{-1}$).

1973). This approach has been utilized in these laboratories to predict, from experimentally determined intracellular values, extracellular IAA concentrations, which may then be compared with experimentally measured values (Figure 6.4). The observed extracellular IAA concentrations show clear discrepancies from the predicted values. Thus differences in pH between the two compartments are not solely responsible for the distribution; these data suggest that active transport may be involved in achieving the observed distribution of IAA (Elliott et al., in preparation).

Further work in these laboratories has been focused on the role of NPA (N-1-naphthylphthalamic acid) binding sites (Elliott et al., in preparation). NPA has been shown to reduce IAA transport (Morgan and Soding, 1958; Morgan, 1964) and NPA binding sites have been described by Lembi et al., (1971). NPA appears to specifically block any auxin efflux via these membrane-bound binding sites, (Rubery and Sheldrake, 1974; Rubery, 1979; Sussman and Goldsmith, 1981), and thus stimulates the net uptake of IAA by cultured cells and other tissues, (Hertel, 1983). Jacobs and Gilbert (1983) have shown that the NPA binding site interacts with IAA.

A high affinity NPA binding site has been detected in membrane preparations from the Acer pseudoplatanus cells used in this present study (Elliott et al., in preparation). NPA stimulated cell division when the
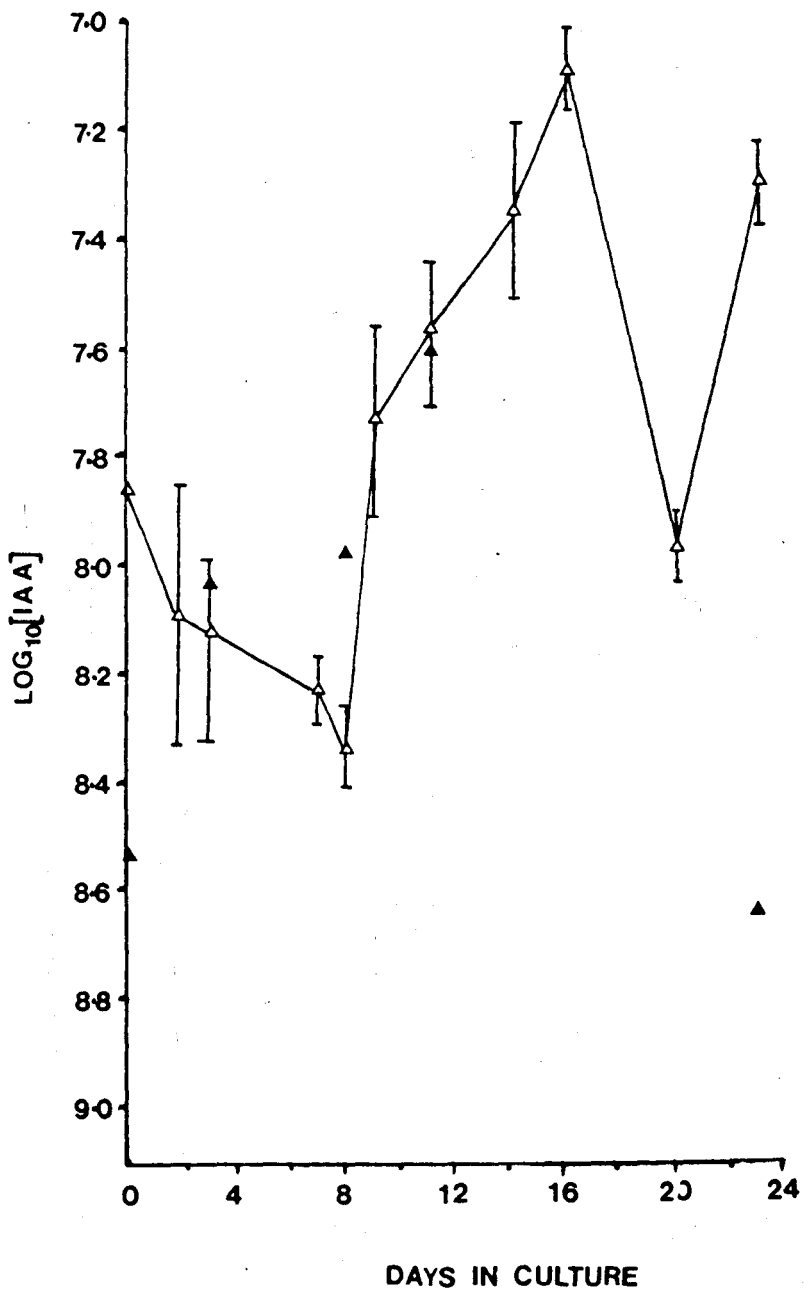
Figure 6.4

A comparison of the measured extracellular
IAA concentration ($\log_{10}$ mol dm^{-3}) with
those predicted from the intracellular
IAA concentration assuming that IAA partitions
according to pH only (Elliott et al.,
in preparation).

cells were transferred to fresh standard medium or to medium lacking 2,4-D. The NPA treatment caused an increase in the intracellular IAA concentration of cells cultured in the presence or absence of 2,4-D. These data support the hypothesis that intracellular IAA concentrations influence the growth rate of the cells used in this study. In cells routinely cultured without 2,4-D lower numbers of NPA binding sites were detected. Thus, it seems likely that habituation to growth without 2,4-D eventually results in a reduction in the capacity of cells to export IAA via the NPA binding protein.

Cells habituated to growth in the absence of 2,4-D have been found to maintain intracellular IAA levels approximately six times higher than that of cells maintained in the standard culture medium (Elliott et al., in preparation). It is possible that the reduction in NPA binding sites may be largely responsible for the elevated intracellular levels and the ensuing culture growth in the absence of 2,4-D.

The concentrations of IAA in the culture medium supporting the growth of the non-2,4-D requiring cells were similar to the levels predicted by physico-chemical calculations. Similar extracellular IAA levels were found for cells cultured in standard medium but in this case they were not in accord with the physico-chemical predictions but require the involvement of a metabolic (carrier) component. Clearly the regulation of the cell

cycle is likely to be closely linked to the availability and distribution of such receptors (Elliott, et al., 1987).

The cell cycle and various proposed regulatory theories have been detailed in Section 1.0. The data obtained from this present study confirm that stationary phase cells are nutrient limited and accumulate in the G1 state. Thus nutrients (nitrate and/or phosphate) appear to regulate progress through the cell cycle at a point between G1 and S. Although the endogenous cytokinin data must be considered tentatively, the two peaks at 20 and 66-70 hours imply a role in the initiation of protein synthesis and mitosis and/or cytokinesis. Therefore the cytokinins may have a regulatory role in both the G1 and G2. The IAA data has been discussed in depth, essentially it seems that some fluctuations in levels do occur which may be related to cell cycle stages, notably mitosis and cytokinesis. However, the relatively stable baselevel may be optimal for an IAA receptor and/or a reflection of IAA having many regulatory roles at various points in the cell cycle.

The levels of intracellular growth regulators are likely to be affected by the pH of the external medium. Thus, pH may have an indirect regulatory affect on the cell cycle. In this study the alleged G2 arrestor, trigonelline, was found to cause cell arrest or impeded growth probably simply as a result of medium

acidification. Medium pH may also affect the uptake of nitrate, thus indirectly having a regulatory role in the G1.

It seems likely that all of the putative regulatory factors considered in this study can interact or cause indirect affects upon one another. In many cases it is unclear whether an effect is causal or indirect. For example, the high cytoplasmic pH associated with cytokinesis may be a trigger level essential for cytokinesis or a reflection of intracellular levels of some other regulatory substance.

The data outlined here do seem to essentially support the "two principal control points" hypothesis but this model does not really explain why some cells never progress through the mitotic cycle but begin to differentiate or produce secondary products. Quastler and Sherman (1959) proposed a "decision" point shortly after mitosis where cells could be directed towards division or differentiation. This period of the cell cycle was later defined as the G0 period, a period of indefinite length for cells not committed to nuclear division.

Smith and Martin (1973) expanded this theory further by defining the S, G2, M and part of the G1 period as the "B-phase". They proposed that after mitosis the cells enter an "A-phase" in which they do not progress towards

cytokinesis. Cells may remain in this "A-phase" for any
length of time but always have a constant probability of
entering the "B-phase". However, it was assumed that
all of the cells entered the "A-phase", unlike the
earlier mentioned GO-phase.

From the evidence available, particularly from
asynchronous batch cultures, there does seem to be a
point in the cell cycle where cells can "rest" (as in
lag phase) or switch to secondary metabolism. However,
even in an old asynchronous batch culture some cells
still exhibit cytokinesis and thus it seems unlikely
that all of the cells enter this proposed "A-phase", and
then some leave to rejoin the main cycle. It seems more
logical that a system might exist involving an
"optional" GO phase. Cells could enter this stage if
they could no longer progress through the main cycle
because of a blockage, eg nitrate or growth regulator
limitations. If these requirements are not met within a
defined time period the cells might divert to secondary
metabolism or senesce and die. If they overcome their
metabolic blockage they could re-enter the main cycle at
the G1 stage. The progression to the S-phase may then
be triggered by a substance such as cytokinin or IAA.
After DNA synthesis the cells would enter mitosis
provided the necessary postulated trigger (possibly
cytokinin and IAA) was present. In the absence of this
trigger or in the presence of a mitotic inhibitor the
cells would accumulate in the G2 state. It has been

tentatively suggested that in such cells DNA turnover may occur (Firby, 1985) and the cells may return to the G1 state.

It is proposed that the nitrate feed induced synchronization technique employed here is effective because a large number of cells are in the GO phase predominantly due to nitrate limitation. When nitrate is supplied the blockage is overcome and the cells re-enter the G1 phase at a similar time and proceed (if unimpeded) through the remainder of the cycle, provided that the essential growth regulators are also present in the required quantities. The fact that synchrony can be quickly lost if the nitrate feed is added more than 5 hours after the first synchronous division implies that the period of time spent in GO before commitment to non-mitotic cycles is relatively short. However, if the GO is a "short stay" stage this would not explain why old stationary phase cells can be induced to divide synchronously on transfer to fresh medium. One possibility is that such cells may be able to revert back from secondary cycles to mitotic cycles if conditions become optimal. Another explanation may be that many stationary phase cells may be held in a state unsuitable for either primary or secondary metabolism due to the shortage of metabolites and accumulation of toxic substances. On transfer to fresh medium these cells may be able to rejoin the main cell cycle.

Thus, it seems that cell cycle regulation is a very

complex process which may not take place in a clear step-wise manner. Undoubtedly there is a great deal of interaction between the growth regulators which explains why many data obtained from this and other studies are difficult to interpret and often apparently contradictory.

The synchronized cell suspension cultures developed and investigated in this study proved to be a suitable system for investigating cell cycle regulation. Future work involving estimations of the levels of the other growth regulators in these synchronized cultures is likely to reveal much useful information about the complex process of cell cycle regulation.

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