STUDIES ON THE ACCUMULATION OF CERTAIN COLOURED SECONDARY METABOLITES BY PLANT CELL AND TISSUE CULTURES.

by

ROBERT DAVID HALL, B.Sc.

A THESIS PRESENTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF EDINBURGH

1984
DECLARATION

I hereby declare that this thesis was composed by myself and the work described herein to be my own.

Robert Hall
Edinburgh, 1984
ACKNOWLEDGEMENTS

I would like to express my thanks to my supervisor and Head of Department, Professor M.M. Yeoman for his guidance and encouragement during my work at the Edinburgh University Botany Department and also for making laboratory and library facilities available for my research. I would also like to thank the academic and technical staff in the department for their friendship and assistance. In particular, I must thank Miss Helen Quinn whose unstinting efforts in the library are greatly appreciated. Thanks are also extended to Mrs. Ranken for her patience and skill in typing this thesis.

Financial assistance in the form of a Postgraduate Research grant from the Department of Education for Northern Ireland which enabled me to carry out this work is gratefully acknowledged. I would also like to thank the DENI, the University of Edinburgh and PATScentre International whose considerable donations permitted me to attend and present a poster at the 5th IAPTC meeting in Japan, 1982.

Finally, I wish to thank my parents, to whom I would like to dedicate this thesis, whose unquestioning support for all my decisions concerning my studies both at school and university has been most deeply appreciated.

RH, 1984
ABBREVIATIONS

spp. species
ssp. subspecies
cv. cultivar
psi. pounds per square inch
rpm. revolutions per minute
cpm. counts per minute
dpm. disintegrations per minute
sec. second(s)
min. minute(s)
h. hour(s)
d. day(s)

xg. x gravitational force
g. gram(s)
l. litre(s)
m. metre(s), milli-

mc. centimetre(s)
ml. millilitre(s)

μ. micro-
n. nano-

G. giga-

M. mega-, molar

mol. mole(s)

N. normal

λ. wavelength

Ci. Curie(s)
Bq. Bequerel(s)
%

percent
>/< greater than/less than

v/v volume per volume (as percentage)

w/v weight per volume (as percentage)

Rf. distance moved by compound / distance moved by solvent

TLC thin layer chromatography

[] concentration

(fr.)wt. (fresh) weight

s.e. standard error

pH negative log of the hydrogen ion concentration

°C. degrees centigrade

no. number

ca. approximately

x̄ mean of x

IOD integrated optical density

MeOH methanol

EtOH ethanol

M+S,MS Murashige and Skoog

K Kinetin

IAA indole-3-acetic acid

NAA 1-naphthylacetic acid

2,4-D 2,4-dichlorophenoxyacetic acid

6-BAP 6-benzyl amino purine

a/c anthocyanin
# CONTENTS

| Title page. | (i) |
| Declaration. | (ii) |
| Acknowledgements. | (iii) |
| Abbreviations. | (iv) |
| Contents. | (vi) |
| Abstract. | (xvii) |

## CHAPTER 1. INTRODUCTION.

## CHAPTER 2. MATERIALS AND METHODS.

**Part 2.1.** Plant material.

**Part 2.2.** Tissue and cell culture.

**Section 2.2.1.:** Media preparation

1. *Daucus* and *Catharanthus* media
2. *Crocus* medium
3. Preparation of growth substance stock solutions.

**Section 2.2.2.:** Sterilization techniques.

1. Sterilization by heat.
2. Sterilization by filtration.
3. Sterilization with hypochlorite.
4. Sterilization with ethanol.
5. Sterilization by ultraviolet light.
Section 2.2.3.: Culture routine.

i. Culture conditions.

ii. Callus initiation, *Daucus carota*.

iii. Callus initiation, *Crocus sativus*, *Crocus spp.*


v. Initiation of cell suspension cultures.

Section 2.2.4.: Protoplast isolation and culture.

i. Protoplast isolation.

ii. Protoplast culture media.

Part 2.3. Analytical techniques.

Section 2.3.1.: Analysis of culture growth.

i. Determination of packed cell volumes, PCV.

ii. Determination of fresh weight.

iii. Determination of dry weight.

iv. Determination of cell population density.

v. Estimation of tracheary element number.

vi. Determination of the viability of cells and protoplasts.

Section 2.3.2.: Analysis of some essential nutrients and sucrose.

i. Sucrose.

ii. Ortho-phosphate.

iii. Ammonium.

iv. Nitrate.

v. Statistical analysis.
Section 2.3.3.: Analysis of pigments.

i. Determination of the proportion of pigmented cells in C. roseus cultures.

ii. Anthocyanin extraction from cells of C. roseus.

iii. Anthocyanin extraction - D. carota.

iv. Chlorophyll extraction and quantification.

v. Extraction and purification of the 'saffron' pigments.

vi. Thin-layer and column chromatography.

Section 2.3.4.: Scanning microdensitometry.

i. Equipment.

ii. Microdensitometry.

Section 2.3.5.: Techniques used involving radioactively-labelled phenylalanine.

i. Material.

ii. 'Feeding' and harvesting procedure.

iii. Thin-layer chromatography and autoradiography.

iv. Scintillation counting.
CHAPTER 3. RESULTS

Part 3.1. Anthocyanin accumulation in callus and cell cultures of two cultivars of Daucus carota L.

Section 3.1.1.: Anthocyanin accumulation in callus cultures of D. carota cv. 'Autumn King'.

i. The growth and anthocyanin accumulation of callus cultures of D. carota cv. 'Autumn King' when incubated on media containing varying auxin/cytokinin supplementation.

ii. The influence of differing levels of 2,4-D and Kinetin on the accumulation of anthocyanin in D. carota cv. 'Autumn King' callus cultures.

Section 3.1.2.: Anthocyanin accumulation in cultures of D. carota cv. 'Takii's Winter Scarlet'.

i. The use of reduced 2,4-D levels as a means to enhance anthocyanin accumulation in callus cultures of D. carota cv. 'Takii's Winter Scarlet'.

ii. The effect of reduced cytokinin concentration on the growth and anthocyanin accumulation of D. carota cv. 'Takii's Winter Scarlet' callus cultures.

iii. Anthocyanin accumulation in cell suspen- sion cultures of D. carota cv. 'Takii's Winter Scarlet' grown in a medium containing reduced levels of 2,4-D.
Part 3.2. Anthocyanin accumulation in callus and cell cultures of the Madagascar Periwinkle, *Catharanthus roseus* (L.) G.Don.

Section 3.2.1.: Anthocyanin accumulation in vitro under standard cultural conditions.

i. Studies on the growth and anthocyanin accumulation of dark-grown cell cultures of *C. roseus* when subcultured and placed in the light.

ii. Studies on the growth and anthocyanin accumulation of dark-grown cell cultures of *C. roseus* when the exposure to light was delayed following subculture.

iii. Studies on the growth and anthocyanin accumulation of light-grown *C. roseus* cell cultures following subculture.

iv. Studies on the growth and anthocyanin accumulation in light-grown cell cultures of *C. roseus* when subcultured and incubated in darkness.

Section 3.2.2.: The influence of inoculum density on growth and anthocyanin accumulation in *C. roseus* cell cultures.

i. A time-course study of growth and anthocyanin accumulation in *C. roseus* cell cultures when initiated at a high inoculum density.
ii. The growth and accumulation of anthocyanins in *C. roseus* cell cultures initiated using different volumes of inocula.

iii. The influence of cell washing on culture growth and anthocyanin accumulation when using high density inocula.

iv. The influence of spent medium addition on the subsequent growth of *C. roseus* cell cultures.

Section 3.2.3.: Anthocyanin accumulation in *C. roseus* cultures under modified conditions.

i. The influence of certain selected growth substance combinations on the growth and anthocyanin accumulation of *C. roseus* callus and cell cultures.

ii. The influence of IAA and 2,4-D on the growth and anthocyanin accumulation of *C. roseus* callus cultures.

iii. The influence of the level of irradiance on the yield of anthocyanin by *C. roseus* tissue and cell cultures.

iv. Growth and anthocyanin accumulation in *C. roseus* callus cultures grown under differing levels of irradiance.

v. The influence of the level of irradiance on the growth and anthocyanin accumulation in *C. roseus* suspension cultures.
vi. Studies on the influence of high sugar concentrations on the accumulation of anthocyanin in *C. roseus* cell cultures.

vii. The influence of inorganic nitrogen and phosphate on culture growth and the accumulation of anthocyanin pigments in *C. roseus* cell suspensions.

Section 3.2.4.: The influence of cellophane on the growth and anthocyanin accumulation of *C. roseus* callus cultures.

i. The influence of prewashed cellophane discs on the growth and anthocyanin accumulation of *C. roseus* callus cultures.

ii. An investigation into the possible mode of influence of cellophane on the growth and anthocyanin accumulation in *C. roseus* callus cultures.

iii. The influence of varying quantities of cellophane on the growth and anthocyanin accumulation of *C. roseus* callus cultures.

iv. The effect of combined stimulatory treatments on anthocyanin accumulation in *C. roseus* callus cultures.

Section 3.2.5.: A study of the variation in growth and anthocyanin accumulation of *C. roseus* callus cultures derived from different cell lines.
Section 3.2.6.: The effects of precursor 'feeding' on the accumulation of anthocyanins in callus and suspension cultures of *C. roseus*.

i. The influence of various precursors on anthocyanin accumulation in *C. roseus* callus cultures when included in the nutrient medium.

ii. The influence of certain precursors on the anthocyanin accumulation of light and dark-grown *C. roseus* cell cultures when supplied during the stationary phase.

iii. An investigation into the time-course of anthocyanin accumulation in *C. roseus* cell cultures following the addition of 1mM. phenylalanine.

Section 3.2.7.: The use of $^{14}$C-phenylalanine to investigate anthocyanin metabolism in *C. roseus* cell cultures grown under potentially inductive conditions.

i. The uptake and incorporation of $^{14}$C-phenylalanine into anthocyanins in early stationary-phase cultures of *C. roseus*.

ii. The influence of 0.05mM. cinnamic acid and 0.05mM. diethyl malonic acid, added simultaneously, on the incorporation of $^{14}$C-labelled phenylalanine into anthocyanins in *C. roseus* cell cultures.
iii. The influence of 1mM. dihydroquercetin upon the incorporation of $^{14}$C-phenylalanine into anthocyanins in C. roseus cell cultures.

iv. The influence of phosphate/nitrogen starvation upon the incorporation of $^{14}$C-phenylalanine into anthocyanins in C. roseus cell cultures.

Section 3.2.8.: Investigations at the cellular level.

i. An investigation into the quantitative and qualitative differences within the pigmented cell population of a stationary-phase, light-grown C. roseus cell culture.

ii. A microdensitometric comparison of the cellular anthocyanin content of cell populations derived from in vivo and in vitro grown tissues.

iii. A microdensitometric investigation into the time course of anthocyanin accumulation in C. roseus cell suspension cultures.

iv. The influence of a high sucrose concentration on the anthocyanin content of C. roseus suspension cultures as determined at the cellular level by the use of microdensitometric techniques.

v. The influence of the level of irradiance on the intracellular anthocyanin accumulation in C. roseus callus cultures.
vi. An investigation into the relationship between aggregate size and anthocyanin content in C. roseus cell cultures.

vii. Microculture techniques using single cells of C. roseus.

CHAPTER 4. DISCUSSION

APPENDIX

"A preliminary study of the culture of the saffron crocus Crocus sativus L. tissues and its potential use as an in vitro source of the valuable spice, saffron."

Section A.1.: The initiation of callus cultures of four species of Crocus.

i. Studies on the ability of different organs to produce callus using explants of C. vernus, C. tomassianianus and C. flavus.

ii. The influence of certain growth substance supplements on the growth of C. tomassianianus cv. 'Whitwell's Purple' callus.

iii. The initiation and growth of callus obtained from tissues of the saffron crocus, C. sativus.

Section A.2.: The pigmentation of C. sativus tissue grown in vitro.

i. TLC and spectrophotometric analysis of the pigment components of C. sativus stigma extracts.
ii. The pigmentation of \textit{C. sativus} callus cultures.

iii. Microspectrophotometric and fluorescence microscopic examinations of \textit{C. sativus} cells derived from \textit{in vivo} and \textit{in vitro} grown tissues.

REFERENCES

PUBLICATIONS
The overall aim of this work was to study in detail the accumulation of visible secondary products in individual plant cells and whole cultures in order to investigate the mechanisms of secondary metabolite accumulation in vitro. Cultures of Daucus carota and Catharanthus roseus which both accumulate anthocyanin pigments were chosen and have been used to study and quantify the extent of both inter- and intracultural heterogeneity with regard to product yield at the cellular level and to determine how this heterogeneity was affected when growth conditions were modified. A brief investigation into the accumulation of the coloured components of saffron in Crocus sativus callus cultures was also undertaken.

The majority of the work performed was on the Catharanthus system and it has been determined that the accumulation of anthocyanins by these cultures was distinctly 'phasal' and took place only in those periods subsequent to cell division. This delimitation between cell division and product accumulation remained, even under modified culture conditions which frequently had a considerable quantitative effect on anthocyanin yield. It is suggested that the results obtained are consistent with a hypothesis proposing that an antagonistic relationship exists between primary metabolism (concerned mainly with cell division) and secondary metabolism. The results from the short investigation using D.carota cultures complement this idea.

To carry out an investigation at the cellular level a novel application of a microdensitometric technique has been employed which has enabled the anthocyanin content of individual cells to be measured on a semi-quantitative basis. Studies have shown
that under standard culture conditions only ca.10% of the cell population accumulated visible levels of anthocyanins. Within this fraction of the population a considerable variation in intracellular anthocyanin content existed (ca.40 fold) with the estimated intracellular anthocyanin concentration varying to a lesser extent (>10 fold). Similar studies on cultures subjected to modified culture conditions and on individual cell lines has revealed that the intracellular anthocyanin concentrations varied relatively little in comparison to the proportion of anthocyanin-accumulating cells. It has been argued that a feedback inhibition mechanism exists which governs the anthocyanin content/cell and consequently it was the proportion of accumulating cells which was the major limitation to anthocyanin yield in these cultures. The factors which might influence this proportion of productive cells have been discussed.
Chapter 1

INTRODUCTION
INTRODUCTION

The growth and manipulation of explanted tissues in a defined and controlled environment, under conditions of total asepsis, has been used repeatedly in the study of many aspects of plant physiology, metabolism and regeneration (e.g. see Fujiwara, 1982; Mantell and Smith, 1983; Evans et al., 1983). The chemical composition of cultured plant tissues, the subject upon which this thesis is based, has received particular attention. Cells in callus, batch, fermenter and more recently, immobilized culture systems have been successfully employed to study many features of plant cell metabolism, both generally, concerning the organisation of such processes and specifically, in relation to the potential use of these systems for the commercial production of desirable plant products. This latter, so-called 'biotechnological' aspect of the subject has been the major cause of the intensive interest shown in this area of research to date. The enormous potential value of a plant cell system dedicated to a specific goal (e.g. the production of a particular metabolite) in the absence of the complications and waste of the intact plant has enticed many researchers into this field for both commercial and scientific reasons.

Plant tissues are an extremely important and often irreplaceable source of many chemicals used in a variety of industries and the advantages of an in vitro system of production have been outlined within detailed reports of the progress made in this area in a number of comprehensive reviews dealing with desirable compounds generally (Barz et al., 1977; Alfermann and Reinhard, 1978a; Staba, 1980; Yeoman et al., 1980; Barz and
to a size appropriate for commercialization (Wagner and Vogelmann, 1977).

In essence, the reasons for this lack of success to date are our very poor understanding of the control of plant metabolic processes in general and in particular our limited knowledge of the influence of and interaction between the elements which govern metabolite accumulation in *in vitro* systems (and indeed *in vivo*). It is hoped that the work reported in this thesis will help to bring us a little nearer to a better understanding of the complexity of these processes.

**A WORKING DEFINITION**

Before proceeding to introduce the original work in this thesis in the context of existing published results it is necessary to define the term 'secondary metabolite'. Many compounds of interest produced by plant tissue culture systems (including those studied here) are grouped under the ill-defined term 'secondary metabolites'. The working definition of this term, provided for the purposes of this thesis is that 'secondary' metabolites are a diverse group of compounds, synthesised and often accumulated by plant (and animal) tissues which, although not essential to the life of the cell in which they are formed, may confer upon the organism which contains such cells, a greater degree of 'fitness' for survival.

**WHAT ARE THE POSSIBLE REASONS FOR THE FAILURE TO OBTAIN SATISFACTORY YIELDS OF SECONDARY METABOLITES FROM IN VITRO SYSTEMS?**

Upon explantation of a plant fragment into a suitable *in*
vitro environment. Neoplastic growth is induced and a callus culture is formed. In association with this neoplastic growth habit the expression of most, if not all secondary metabolic pathways are repressed (Kurz and Constabel, 1979; Böhm, 1980). Therefore, only through an induced derepression of these pathways or possibly through the isolation/induction of a mutation with uncontrolled derepression, will extensive secondary metabolite synthesis and accumulation be possible. Metabolite yields in vitro are consequently, primarily dependent upon culture conditions and also, what might be called 'cellular heterogeneity' which incorporates cellular differences both between and within culture cell populations. Let us now consider each of these governing factors in turn.

a. Culture conditions and secondary metabolite yield.

i. Cytological factors.

In this thesis a considerable amount of the work reported was directed towards the possible influence of specific culture conditions upon the growth and accumulation of anthocyanins in plant tissue cultures in an attempt to elucidate the importance and possible mode of influence of such conditions upon in vitro secondary metabolite yields. Culture conditions both physical and chemical have been shown to be of considerable importance to such yields in many in vitro systems and for a general survey of the literature the reader is referred to Dougall, (1978), Kurz and Constabel, (1979), and Mantell and Smith, (1983b).

The culture environment can be envisaged to influence metabolite yields either through altering certain specific
aspects of cell metabolism or by instigating a more general change in the overall pattern and extent of culture growth and organisation. In this latter respect for example, in some early studies, secondary metabolism in vitro was only observed to be associated with cultures which exhibited either organogenesis (Bhandary et al., 1969; Thomas and Street, 1970; Tabata et al., 1972; Freeman et al., 1974; Ikuta et al., 1975) or the differentiation of specialized cellular structures (Reinhard et al., 1968; Corduan and Reinhard, 1972; Neumann and Mueller, 1974). In other words the accumulation of a particular secondary metabolite only occurred when the structures responsible for its production and accumulation in vivo were induced or observed to form in vitro.

Such a degree of tissue differentiation is now known not to be an essential prerequisite for the accumulation of secondary metabolites in many in vitro systems (e.g. see Tabata and Hiraoka, 1976; Kurz and Constabel, 1979). However, an association between some degree of tissue differentiation (organisation/aggregation) and secondary product accumulation has been suggested as being an important causal factor in increasing culture yields in many in vitro systems (Yeoman et al., 1980, 1982a,b; Lindsey and Yeoman, 1983(b), 1984). Both secondary metabolite accumulation and cell differentiation have also been shown to be closely associated with a particular part of the culture growth cycle - specifically, the period during and after the progressive cessation of cell division (Yeoman et al., 1980). This restriction of secondary metabolism to a specific developmental period has been observed on many occasions (detailed in Chapter 4) and would indicate that strict metabolic controls
operate to determine the direction of the metabolism of a cell at any time during its development.

    Many secondary products are of a highly toxic nature, even to the cells which produce them (Luckner, 1972). Consequently, it is critical for such cells to have a tight control over their synthesis. This would thus enable secondary metabolism to be restricted only to those times when the compounds produced could be 'processed' successfully. For example, if a compound which is cytotoxic and is normally sequestered in the vacuole or is excreted via specific secretory organs, were to be synthesised before such structures had been formed (e.g. in the plant meristem) then the cells concerned would seriously risk self destruction. Consequently, the phase-dependent synthesis of secondary metabolites, reflecting intrinsic control mechanisms within the plant cells, would appear to be an extremely important, if not essential feature of this process. It is therefore not surprising to find that the strict in vivo controls of secondary metabolism (Leete, 1969; Hahlbrock and Grisebach, 1979; Luckner, 1980) are, at least in part, carried over into tissue cultures from the source plant. Clearly, only with a knowledge and an understanding of these control mechanisms will we be able to successfully manipulate plant tissue cultures to produce desirable yields. The relationship between the culture growth cycle, cell differentiation and the control of secondary metabolite accumulation will be discussed in greater detail in Chapter 4 in the light of the results obtained in this investigation.
ii. Environmental factors.

With regard to the influence of specific environmental components upon secondary metabolite yield in vitro perhaps the most studied are the growth substances which have been shown to have both qualitative and quantitative effects. For example, increased metabolite yields were observed in NAA-supplemented medium in comparison to medium containing 2,4-D by Fritsch et al., (1971) for anthocyanin production by cultures of Haplopappus and Zenk et al., (1975) for anthraquinone accumulation by cultures of Morinda citrifolia. Ball et al., (1974) found that Dimorphotheca cultures accumulated anthocyanins solely on media supplemented with the auxin dCPAA. After five years of alkaloid-free growth on a medium containing 2,4-D, Furuya et al., (1971) observed substantial alkaloid accumulation in tobacco callus when transferred to an IAA supplemented medium. This effect was wholly reversible by returning the tissue to the original medium.

The level of auxin has also been shown to influence metabolite yields. It has been observed by Blakely and Steward, (1961) that increased levels of NAA suppressed anthocyanin accumulation in Haplopappus cell cultures. Complementary results have been reported for nicotine accumulation by Nicotiana tabacum cv. NC2512 suspension cultures by Mantell and Smith, (1983b). In contrast, growth substance supplementation was found to have little influence upon secondary metabolite yields in other systems (see Butcher and Connolly, 1971; Hiraoka and Tabata, 1974).

Similar results have been reported for cytokinins.
Coconut milk (in part a source of natural cytokinins) was found to be an essential medium supplement in order to obtain anthocyanin accumulation by *Haplopappus* cultures (Blakely and Steward, 1961) and kinetin was observed to significantly enhance anthocyanin accumulation in tissue cultures of flax (Liau and Ibrahim, 1973). Conversely, Tabata *et al.*, (1971) describe an inhibitory effect of kinetin, when used at high concentrations, upon alkaloid accumulation by *Datura* cultures in the absence of any effect on culture growth. Furthermore, Constabel *et al.*, (1971) report that although kinetin failed to enhance the final level of anthocyanin accumulated by cell cultures of *H.gracilis*, this level was achieved in a shorter time period.

Other medium components such as the macronutrients have been shown to have a considerable influence upon secondary product yields *in vitro*. In general, an increase in macro-nutrient supplementation instigates a subsequent increase in culture growth and a decrease in metabolite yield (Mantell and Smith, 1983b). Consequently, this would agree with the proposal of Yeoman *et al.*, (1980) that an inverse relationship exists between secondary metabolite accumulation and culture growth. In *C.roseus* cultures increased phosphate levels have been reported to prolong the growth of the cultures (MacCarthy *et al.*, 1980) whereas in *Peganum* cultures low initial phosphate strongly stimulated secondary metabolism (Nettleship and Slaytor, 1974). Pertinent observations have also been made by Mantell and Smith, (1983b) who determined that the onset of nicotine accumulation in tobacco cell cultures was associated with the exhaustion of phosphate from the bathing medium. Enhanced nitrogen
supplementation to rose cultures was found to suppress the synthesis and accumulation of phenolic compounds (Mehta and Shalaga, (1978) in Kurz and Constabel, 1979) and to delay alkaloid accumulation in C.roseus cell cultures (Kutney et al., 1980).

Conflicting reports exist concerning the influence of sucrose upon in vitro secondary metabolite yields. For example, decreased anthocyanin accumulation has been observed in cultures subjected to sucrose levels >3% (w/v) by Carew and Krueger (1976) for C.roseus cultures and Colijn et al., (1981) for cultures of Petunia. In contrast, in any early report, Slabecka-Szweykowska, (1955) found abundant anthocyanin in Vitis cultures when grown on medium containing 9% sucrose. Davies, (1972) similarly observed amplified polyphenol synthesis in rose cultures when the sucrose level was increased from 2% to 4%. A modification which induced only a negligible change in biomass production. An enhanced sucrose level (5% w/v) was employed by Zenk in his 'Alkaloid Production' medium, Zenk et al., (1977).

Of the components of the physical culture environment light is perhaps the most studied with respect to secondary metabolite accumulation in vitro. Light is well known to be involved in stimulating the synthesis of many enzymes involved in secondary metabolite pathways, especially those leading to phenolic compounds (see Hahlbrock and Grisebach, (1979) and refs. therein) and it is therefore not surprising that light enhancement of phenolics accumulation in vitro has frequently been reported (Forrest, 1969; Lackmann, 1971; Davies, 1972; Stickland and Sunderland, 1972a, 1972b; Brunet and Ibrahim, 1973).Interestingly, Alfermann and Reinhard, (1971) were able to use auxin to replace
the light requirement by carrot cultures for anthocyanin accumulation although such an effect has never been reported elsewhere. Light has also been found to stimulate alkaloid accumulation in *Solanum* (Seibert and Kadkade, 1980) and *C. roseus* (Röller, 1978) cultures. Conversely, light has proved to be inhibitory to alkaloid accumulation in *Scopolia* cultures (Tabata et al., 1972) and also in *Nicotiana* cultures (Mantell and Smith, 1983b) or to have no observable effect as, for example, on anthraquinone accumulation by *Morinda* cell cultures (Zenk et al., 1975).

b. **Cellular heterogeneity and secondary metabolite yield.**

Due to the difficulties inherent in identifying and quantifying possible intercellular heterogeneity with respect to secondary metabolite content relatively little is known about this phenomenon in plant tissue cultures. Such heterogeneity does exist however, as will become clear from the following text and from the work reported herein and is therefore a factor of some considerable importance in determining metabolite yields *in vitro.*

i. **Intercultural heterogeneity**

Cellular heterogeneity can occur both between and within cultures of the same species even when grown under identical conditions. The former may arise for example, as a result of the differing biosynthetic potential of the explanted tissue. A number of reports exist concerning the relationship between whole plant yield and *in vitro* yield and between the latter and the type of tissue used for explantation. In general it would appear that the "organic" origin of the explant bears no
relation to the subsequent in vitro metabolite yield, unlike the overall biosynthetic capacity of the source plant which would appear to be of considerable importance (Fowler, 1983; Mantell and Smith, 1983b). Yeoman et al., (1980) found that the subsequent capacity of Capsicum cultures to accumulate capsaicin was not correlated with the type of tissue used for culture initiation. Similar observations have been forwarded by Dhoot and Henshaw, (1977) for alkaloid accumulation by Hyoscyamus niger cultures and by Ibrahim et al., (1971) for the accumulation of anthocyanin by cultures of a number of species. One notable exception is the result of Nagel and Reinhard, (1975) who observed that the volatile oil composition of Ruta graveolens cultures was correlated with the varied spectra of such compounds in the explanted tissues.

It has been convincingly argued by Zenk et al., (1977) and Kinnersley and Dougall, (1980b) on the basis of experiments performed on Catharanthus and Nicotiana cultures respectively that a relationship exists between the biosynthetic capacity of a cell culture and that of the source plant. Further evidence for this hypothesis is reported here for cultures of Daucus carota (Chapter 4). Nevertheless, at least one exception to this hypothesis exists in that Roller, (1978) was unable to demonstrate such a relationship concerning the alkaloid yield of C.roseus cultures.

ii. Intracultural heterogeneity.

The existence of intracultural,cellular heterogeneity is much more difficult to demonstrate but has clearly been done so,
both directly and indirectly on several occasions. Direct observations essentially require that the metabolite under examination is visible (i.e. coloured) or can be made so, e.g. by intracellular staining and/or UV microscopy. For example, the accumulation of anthocyanins by *C. roseus* cells in culture was directly observed to be distinctly heterogeneous with only ca. 5% of the cells containing 'high' levels (Knobloch *et al.*, 1982). These cultures also accumulated the alkaloid serpentine which was observed, using fluorescence microscopy to have a very similar highly heterogeneous distribution within the culture cell population. Fluoromicroscopy has also been employed by Sato and Yamada, (1984) to demonstrate the extensive heterogeneity in berberine accumulation by cultured cells of *Coptis japonica*.

Light microscopy enabled Yamakawa *et al.*, (1982) and Colijn *et al.*, (1981) to demonstrate that only limited numbers of cells in *Vitis* and *Petunia* cultures respectively accumulated anthocyanin pigments to visible levels. Furthermore, the heterogeneous distribution of a yellow pigment in cultures of *Macleaya microcarpa* has successfully been exploited in the isolation of high alkaloid yielding cell lines by Koblitz *et al.*, (1975). From some preliminary work in this investigation it was observed that the small amount of the yellow saffron component crocin, accumulated by *Crocus sativus* callus cultures was the result of accumulation by <2% of the cell population (see Appendix). Finally, using a most elaborate short wave microdensitometric technique Ellis was able to determine on a semi-quantitative basis the distribution of certain phenolic compounds within the culture cell populations of five different plant species (Barz and Ellis, 1981; Ellis, 1982). In each of these investigations it was determined that
the cellular frequency distribution on the basis of intracellular 'concentration' was strongly asymmetric with the majority of cells falling in the lower region of the total range of values.

Indirect demonstrations of intracellular secondary metabolite heterogeneity are essentially extrapolations from data obtained from experiments using isolated cell lines. It must be borne in mind however, that in these instances the heterogeneity observed could have been induced by the techniques used for clonal production and that intraclonal heterogeneity may have arisen during the bulking-up procedure. Notwithstanding these provisos, using this method cell populations have been isolated which have exhibited considerable heterogeneity in secondary metabolite yield. Mok et al., (1976) were able to isolate cell lines which contained different pigment compositions from a single carrot culture. The existence of ρ-fluorophenylalanine-resistant cells in Nicotiana cultures permitted the isolation of cell lines with a 2-5 fold increase in cinnamoyl putrescine content (Berlin et al., 1981; see also Widholm, 1980; Maliga et al., 1982). Cell lines with varying capacities for anthocyanin accumulation were isolated by Dougall et al., (1980) from wild carrot cultures. These capacities were found to vary, indicating a 'drift in biosynthetic stability' which could be modified by culture conditions or by reselection. Finally, using rapid screening techniques for alkaloids Zenk et al., (1977) (a radioimmunoassay) and Ogino et al., (1978) (a cell squash technique) were able to isolate cell lines with enhanced secondary metabolite yields in C.roseus and N.tabacum cultures respectively.
AIMS AND OBJECTIVES OF THIS INVESTIGATION

It is clear from the preceding text that both culture conditions and cellular heterogeneity are of considerable importance in determining secondary metabolite yields in plant tissue cultures. For the work reported in this thesis culture systems producing visible secondary products were chosen for study. Due to product visibility in these cultures it has been possible not only to identify the actively accumulating cells within the population but also to assess the intercellular heterogeneity in a semi-quantitative manner using a novel application of a standard microdensitometric technique. This non-destructive means of determining the variation in the chemical production capacity of cell populations has been exploited to enable us to determine directly both the extent of intracultural heterogeneity and how this heterogeneity was influenced by culture conditions such as those detailed above. It was envisaged that the results of these investigations would provide us with a deeper insight into the possible factors which may limit secondary metabolite yields in vitro and how these factors might operate.

The metabolites chosen for study were the anthocyanins. These compounds are extremely common secondary plant products (Harborne, 1967; Timberlake and Bridle, 1975; Markakis, 1982b) and although they have little potential use commercially they are of considerable importance in the food and wine industries (Markakis, 1982a). These compounds were therefore chosen for their scientific rather than commercial value. Anthocyanins have a suitable in vivo absorption spectrum for microdensitometry, are easily detectable in very small quantities, have
characteristics typical of many other, more valuable secondary metabolites (cytoplasmic synthesis, vacuolar storage etc.) and were known to be accumulated in plant cultures (Table 1.1.1.).

The cultures used were of two species, *Daucus carota* and *Catharanthus roseus* although the great majority of the work was carried out on the latter system. The *C. roseus* cultures were almost ideal systems for this type of project in that both the callus and suspension cultures were remarkably friable, with an unusually high degree of cytological homogeneity which resulted not only in very good reproducibility of results but also permitted the cellular investigations to proceed with relative ease, usually without the need for any preliminary tissue digestion.

In essence, the aims and objectives of this work can be summarised by the following five points:

1. To determine the importance of the growth cycle and cell differentiation upon *in vitro* anthocyanin accumulation.
2. To determine how culture yield was modified by growth conditions.
3. To determine the degree of heterogeneity within cell cultures by a direct semi-quantitative method and to investigate how this altered with culture development.
4. To determine how culture conditions influenced anthocyanin accumulation at the cellular level and to identify possible limiting factors.
5. To investigate the nature of possible subcellular control mechanisms for anthocyanin accumulation - does feedback inhibition operate for example?
<table>
<thead>
<tr>
<th>Species</th>
<th>Family</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Catharanthus roseus</em></td>
<td>Apocynaceae</td>
<td>Hall &amp; Yeoman, (1982)</td>
</tr>
<tr>
<td><em>Daucus carota</em></td>
<td>Compositae</td>
<td>Lachele <em>et al.</em>, (1974)</td>
</tr>
<tr>
<td><em>Dimorphotheca sinuata</em></td>
<td>Compositae</td>
<td>Ball &amp; Arditti, (1974)</td>
</tr>
<tr>
<td><em>Euphorbia millii</em></td>
<td>Euphorbiaceae</td>
<td>Yamamoto <em>et al.</em>, (1981)</td>
</tr>
<tr>
<td><em>Haplopappus gracilis</em></td>
<td>Compositae</td>
<td>Lackmann, (1971)</td>
</tr>
<tr>
<td><em>Helianthus tuberosus</em></td>
<td>Compositae</td>
<td>Ibrahim <em>et al.</em>, (1971)</td>
</tr>
<tr>
<td><em>Linum usitatissimum</em></td>
<td>Linaceae</td>
<td>Ibrahim <em>et al.</em>, (1971)</td>
</tr>
<tr>
<td><em>Malus pumila</em></td>
<td>Rosaceae</td>
<td>Ibrahim <em>et al.</em>, (1971)</td>
</tr>
<tr>
<td><em>Petunia hybrida</em></td>
<td>Solanaceae</td>
<td>Colijn <em>et al.</em>, (1981)</td>
</tr>
<tr>
<td><em>Populus spp.</em></td>
<td>Salicaceae</td>
<td>Matsumoto <em>et al.</em>, (1973)</td>
</tr>
<tr>
<td><em>Solanum tuberosum</em></td>
<td>Solanaceae</td>
<td>Gamborg, (1966)</td>
</tr>
<tr>
<td><em>Thea sinensis</em></td>
<td>Theaceae</td>
<td>Forrest, (1969)</td>
</tr>
<tr>
<td><em>Vitis vinifera</em></td>
<td>Vitaceae</td>
<td>Yamakawa <em>et al.</em>, (1983)</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td>Gramineae</td>
<td>Strauss, (1959)</td>
</tr>
</tbody>
</table>

Table 1.1.1. Publications reporting the *in vitro* accumulation of cyanic compounds by tissues of sixteen plant species.
CHAPTER 2

MATERIALS AND METHODS

Part 1: Plant material.

Part 2: Tissue and cell culture.

Part 3: Analytical techniques.
Part 1. PLANT MATERIAL

Daucus carota ssp. sativus (Hoffm.) Arcangeli. (Umbelliferae).
Sources: - D. carota ssp. sativus cv. "Autumn King"
  - Thompson & Morgan Ltd., Ipswich, Suffolk.
  - D. carota ssp. sativus cv. "Takii's Winter Scarlet"
  - Dr. J.F. Horobin, N.V.R.S., Wellesbourne, Warwickshire.

Seed obtained from the above sources were germinated and grown in the Botany Department garden or under greenhouse conditions (16 h. day, 16°C. night temp.), depending on the season. Some plants were grown to anthesis to permit confirmation of their taxonomic identity.

Catharanthus roseus (L.) G.Don. (Apocynaceae)
Source: Thompson & Morgan Ltd., Ipswich, Suffolk.

Seeds were germinated and plants maintained in Levingtons compost (Fisons Ltd.) under greenhouse conditions. Taxonomic identification was confirmed.

Crocus ssp. (Iridaceae)
  - C. tommasinianus Herbert cv. Whitewell Purple - British Horticultural Co. Ltd., King's Lynn, Norfolk.
C. sativus L. - Avon Bulbs, Bathford, Bath and Mrs. E.F. Warburg, British Iris Society, Oxford. (A gift which is gratefully acknowledged).


C. sativus L. (Italian stock) - Prof. F. Tammaro, L'Aquila, Italy. (A gift which is gratefully acknowledged).

Stocks of all the above plants were maintained under greenhouse conditions. All species produced flowers in their first year of growth and this permitted positive confirmation of their taxonomic identity (in accordance with the classification of Mathew, 1980).
Part 2. TISSUE AND CELL CULTURE

Section 2.2.1. Media preparation

i. Daucus and Catharanthus media.

Unless otherwise stated both Daucus and Catharanthus media (henceforth referred to as DC and CR media respectively) contained 4.574 g./litre Murashige and Skoog minimal organic medium as supplied by Gibco Europe Ltd., to which was added 1 ml. of a stock solution of the following:

- 0.5 mg./ml. Nicotinic acid, Sigma Ltd.
- 0.5 mg./ml. Pyridoxine HCl, Sigma Ltd.
- 2.0 mg./ml. Glycine, Sigma Ltd.

made up freshly in distilled water immediately before use. This medium, the complete composition of which is listed in Table 2.2.1., is equivalent to full Murashige and Skoog medium (Murashige and Skoog, 1962).

The following supplements were added:

<table>
<thead>
<tr>
<th>DC medium</th>
<th>CR medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 g./litre Sucrose</td>
<td>30 g./litre Sucrose (BDH)</td>
</tr>
<tr>
<td>2.15 mg./litre Kinetin</td>
<td>-</td>
</tr>
<tr>
<td>2.21 mg./litre 2,4-D.</td>
<td>0.4 mg./litre 2,4-D. Sigma Ltd.</td>
</tr>
<tr>
<td>*(10 g./litre Agar No.3)</td>
<td>8 g./litre Agar No.3 Oxoid Ltd.)</td>
</tr>
</tbody>
</table>

* for solid media only, added after pH adjustment.

All constituents were dissolved in distilled water and made up to the appropriate volume after which the pH was adjusted to 5.8 using 1M.HCl/1M.KOH.
Table 2.2.1.

A complete list of the components of the basal synthetic growth media used in this investigation. Components 1-14 constitute 'Murashige and Skoog Plant Salt Mixture' and 1-19 'Murashige & Skoog Minimal Organics Medium'.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄NO₃</td>
<td>1650.000</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1900.000</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>440.000</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>370.000</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>170.000</td>
</tr>
<tr>
<td>Na₂·EDTA</td>
<td>37.250</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>27.850</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>6.200</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>16.900</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>8.600</td>
</tr>
<tr>
<td>KI</td>
<td>0.830</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>0.250</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>Inositol</td>
<td>100.000</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.100</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.500</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>0.500</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.000</td>
</tr>
</tbody>
</table>

1 mg.L⁻¹
routinely replaced every 3 weeks.

Stock solutions of IAA (10^{-3} M.) were made up in a similar manner, but usually in 10 ml. quantities and always immediately before required. Whereas 2,4-D, NAA, Kinetin and 6-BAP were routinely autoclaved with the other media constituents, IAA was always filter-sterilized after adjusting the pH of the stock solution to 5.8 with 10N.HCl.
Section 2.2.2. Sterilization techniques.

Due to the many problems which arise when plant tissue cultures contain microorganisms it was essential that all in vitro work of this kind was carried out under conditions of total asepsis. This required that all of the tissue used for culture initiation was freed from microbial contaminants and that all equipment, media, glassware etc. used in culture manipulations and for routine subculture were similarly sterilized prior to use. The sterilization procedures used were as follows:

i. Sterilization by heat.

All glassware, instruments, filtration units, distilled water and nutrient media which did not contain heat-labile compounds were sterilized by autoclaving at 121°C. for 20 min. at a steam pressure of 15 psi.

ii. Sterilization by filtration.

The sterilization of liquids which contained heat-labile compounds (e.g. certain vitamins, plant growth substances, anthocyanin precursors) was performed using preautoclaved filtration units containing a Millipore cellulose acetate filter (0.22 μm. pore size) (Millipore, Molsheim, France.). For small volumes (<10 ml.) a Millipore Swinnex-13 filtration unit was used and for larger volumes a Sartorius Membranfilter-510 (Sartorius, Göttingen, W. Germany) was employed using vacuum filtration.

iii. Sterilization with hypochlorite.

All plant material used for culture initiation was surface
sterilized before use. Small segments (max. dimension 1 cm.) of plant material were taken from healthy, actively-growing plants, washed thoroughly in tap water and the cut ends sealed with wax (Paraplast-Lancer, Eire.). After a rapid presterilization in 75% (v/v) ethanol in distilled water for 30 sec. the explants were immersed for 20 min. in 15% (v/v) sodium hypochlorite (1.5% available chlorine) in distilled water containing 2 drops of Tween-20 (BDH). The explants were then removed under aseptic conditions and washed in sterile distilled water (x3) before use.

iv. Sterilization with ethanol.

During culture initiation and subculturing all instruments, when not in use, were stored in 96% ethanol. These were then flamed immediately prior to use in order to remove any surface ethanol which could damage the plant tissue. Ethanol was also used to sterilize all work surfaces before and after use.

v. Sterilization by ultraviolet light.

All cultural manipulations were performed either in a laminar flow cabinet (design as Flow Labs., Irvine, Scotland.) or in a 'sterile' culture room reserved for such purposes. This room was irradiated with ultra-violet light for a minimum period of 20 min. before entry and was kept under a continuous positive pressure by an inflow of sterile air to prevent any inward drift of air-borne microbes. Disposable plastic gloves, worn throughout all sterile manipulations were similarly surface sterilized with ultraviolet light.
Section 2.2.3. Culture Routine.

i. Culture conditions.

Unless otherwise stated in the text in vitro plant material was grown under the following cultural conditions.

Temperature - 24°±1°C.
Photon flux density/Illuminance - 25 μmol.m⁻².sec⁻¹/1050 lux.
Light source - Compton warmwhite fluorescent.
Culture dishes (solid)* - 9 cm. polystyrene Petri-dishes (Sterilin) containing 20 ml. medium.
Culture flasks (liquid) - 250 ml. conical (Erlenmeyer) flasks containing 50 ml. medium.
Liquid culture agitation - continuous rotation in a horizontal plane, 98 rpm., 0.8 cm. amplitude.

* All dishes were sealed after inoculation with a double layer of Parafilm to prevent contamination and desiccation.

ii. Callus initiation, *Daucus carota*.

Callus from both cultivars used in this investigation was obtained using explants of root tissue. Transverse segments (1 cm. x 1 cm. x 1 mm.) were removed from presterilized (2.2.2 iii.) pieces of tap root, plated onto full DC medium (2.2.1i.) and placed in the light (15-20 μmol.m⁻².sec⁻¹). As much of the callus was derived from phloem and cambial cells care was taken to include areas of these tissues in each segment. Callus proliferation was rapid and after 2-3 weeks
sufficient (*ca.* 1 g./explant) had grown to permit its isolation from the original explant tissue and subculture.

Subculture was thereafter routinely performed every two weeks when 4 x *ca.* 0.5 g. pieces of friable green callus were used to inoculate each Petri-dish.

**iii. Callus initiation, *Crocus sativus*, *Crocus* spp.**

All organs of *Crocus* spp. were tested for their ability to produce callus. Where possible corms which had not fully "germinated" were chosen as these plants still retained at least one of their outer sheathing cataphylls (Fig. 2.2.1.) intact. As a result entire sterile shoots could be obtained simply by excising them immediately above the corm and sterilizing the complete structure. After removing the waxed end portion and peeling off the outermost cataphyll each of the organs of the shoot and flower-bud(s) were isolated and cultured separately. All organs were cultured entire with the exception of the leaves which were cut into 0.5 cm. segments and the ovaries which were transversely bisected.

To obtain callus from corm tissue all damaged surface tissue, axillary buds and the root crown had to be removed before waxing. After sterilization both longitudinal and transverse sections (1 cm. x 1 cm. x 1 mm.) were excised for culturing.

All tissue types were placed on CS medium (2.2.1ii.) with the addition of growth substances as reported in the text. When sufficient callus had been produced (>2 months) this was separated from the original explant and subcultured onto fresh medium (*ca.* 2-3 g. tissue/plate).

Subculture of all callus was routinely performed every 3 weeks when 50% of the callus mass was transferred onto fresh
Figure 2.2.1.

T.S. *Crocus sativus* plant immediately post anthesis x2.
medium. All cultures were incubated in darkness.


Callus of *C. roseus* was obtained from Prof. J. Berlin, Gesellschaft für Biotechnologische Forschung mbH., Braunschweig, W. Germany. This had originally been isolated in 1977 from hypocotyl segments taken from sterile-grown seedlings. Cultures were initiated and maintained on the same CR medium (2.2.1i.).

Routine subculture was performed on a very strict fortnightly basis and stocks of both light-grown and dark-grown callus were maintained.

v. Initiation of cell suspension cultures.

Liquid cultures of *D. carota* were initiated by the addition of 1-2 g. of friable callus to 50 ml. DC medium. After three weeks agitation ca. 5 ml. of the resultant fine cell suspension was poured into a flask containing fresh medium. Thereafter routine subculture was performed in this manner at three weekly intervals and all cultures were grown in the light.

To initiate liquid cultures of *C. roseus* ca. 0.5 g. of callus was added to 50 ml. CR medium and the mixture agitated for 2 weeks. By this time an exceptionally finely dispersed cell suspension had been produced. Aliquots (3 ml.) of this were then used to inoculate new flasks and routine subculture proceeded in this manner after each interval of 2 weeks. Both light and dark-grown stocks were maintained.
Section 2.2.4. Protoplast isolation and culture.

i. Protoplast isolation.

During the course of this investigation protoplasts were prepared from both in vivo and in vitro-grown tissues using a single method.

The following solutions were prepared fresh:-

Solution 1. 0.6M. mannitol (Sigma Ltd.) in distilled water containing 0.5% (0.034M.) CaCl₂·2H₂O, pH 5.4.
Solution 2. 0.75M. mannitol in distilled water containing 0.5% (0.034M.) CaCl₂·2H₂O, pH 5.4.
Solution 3. 10% cellulase "Chozuka" R-10¹, 5% Macerozyme R-10¹ and 0.5% CaCl₂·2H₂O in distilled water, pH 5.4.

1 Yakult Honsha Ltd., Nishinomiya Japan.

2 g. (fr.Wt.) tissue was washed with 18 ml. solution 1. in a 25 ml. conical flask. After the cells had settled out the washing solution was replaced with 16 ml. Solution 2. and the mixture agitated on a rotary shaker (98 r.p.m.) for 30 min. in darkness. 4 ml. Solution 3 was then added with continued agitation for a further 5 h. Digestion proceeded in darkness under normal culture conditions. Although low speeds of rotation are normally recommended for protoplast isolation a low degree of agitation was successfully achieved by having a relatively large volume of liquid in a small flask (20/25 ml.) when using a medium-speed shaker (98 r.p.m.).

Following the enzyme incubation period the cell suspension was filtered through nylon mesh (64 µm. H. Simon Ltd.,
Stockport, Cheshire) and then centrifuged at 10xg. for 5 min. The supernatant was removed and the pellet resuspended in 16 ml. Solution 1. and again centrifuged. This procedure was repeated two more times before finally resuspending the pellet in a small volume of culture medium containing 0.3M. mannitol to prevent cell lysis.

ii. Protoplast culture media.

In addition to modifications of the normal culture medium, details of which are reported in the text, the following very rich medium, composed to meet the needs of low density protoplast culture (Kao and Michayluk, 1975), was used. This medium (KM), the full composition of which is given in Table 2.2.2., was made up in distilled water as a 10X. concentrated stock solution and stored, until required, at -40°C. Upon dilution of this stock before use the pH was adjusted to 5.5 (1M HCl/KOH) and the complete medium filter sterilized. When solid medium was required double-strength KM medium was prepared and sterilized and to this was added an equal volume of autoclaved distilled water (pH 5.5) containing 10 g./litre Oxoid No.3 Agar before pouring the plates.
Table 2.2.2.

Composition of KM medium as Kao & Michayluk, 1975. All components were obtained from BDH. or Sigma Ltd. with the exception of 1CIBA-Geigy Ltd., Cambridge (the kind gift of Dr. M. Davey, University of Nottingham), 2decanted from mature, fresh fruits heated to 60°C. for 30 min. and filtered, 3Difco Labs., Detroit, USA.

<table>
<thead>
<tr>
<th>Mineral salts (mg./litre)</th>
<th>Organic acids, sugars, sugar alcohols (mg./litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄NO₃ 600.000</td>
<td>Sodium pyruvate 20</td>
</tr>
<tr>
<td>KNO₃ 1900.000</td>
<td>Citric acid 40</td>
</tr>
<tr>
<td>CaCl₂·2H₂O 600.000</td>
<td>Malic acid 40</td>
</tr>
<tr>
<td>MgSO₄·7H₂O 300.000</td>
<td>Fumaric acid 40</td>
</tr>
<tr>
<td>KH₂PO₄ 170.000</td>
<td>Sucrose 250</td>
</tr>
<tr>
<td>KCl 300.000</td>
<td>Glucose 68400</td>
</tr>
<tr>
<td>KI 0.750</td>
<td>Fructose 250</td>
</tr>
<tr>
<td>H₃BO₃ 3.000</td>
<td>Ribose 250</td>
</tr>
<tr>
<td>MnSO₄·H₂O 10.000</td>
<td>Xylose 250</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O 2.000</td>
<td>Mannose 250</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O 0.250</td>
<td>Rhamnose 250</td>
</tr>
<tr>
<td>CuSO₄·5H₂O 0.025</td>
<td>Cellobiose 250</td>
</tr>
<tr>
<td>CoCl₂·6H₂O 0.025</td>
<td>Sorbitol 250</td>
</tr>
<tr>
<td>Sequestrene 330Fe¹ 28.000</td>
<td>Mannitol 250</td>
</tr>
</tbody>
</table>

| Vitamins mg./litre | | |
|-------------------| | |
| Inositol 100      | Folic acid 0.40 |
| Nicotinamide 1    | p-Aminobenzoic acid 0.02 |
| Pyridoxine HCl 1  | Biotin 0.01 |
| Thiamine HCl 1    | Riboflavin 0.02 |
| D-Calcium pantothenate 1 | Vitamin A 0.01 |
| Choline chloride 1 | Vitamin D₃ 0.01 |
| Ascorbic acid 2   | Vitamin B₁₂ 0.02 |

| Organic supplements | |
|---------------------| |
| Coconut milk² 20 ml./litre |
| Casamino acids³(Vit.free) 250 mg./litre |
Part 3. ANALYTICAL TECHNIQUES.

Section 2.3.1. Analysis of culture growth.

i. Determination of Packed Cell Volume, PCV.

9-10 ml. samples, taken from well-mixed suspension cultures, were poured into 10 ml. graduated, conical centrifuge tubes (Volax) and centrifuged for 5 min. at a force of 1000xg. The PCV was calculated as the percentage cell volume of the total culture volume. That is,

\[
PCV = \frac{\text{Volume of cell mass}}{\text{Total volume of sample}} \times 100
\]

ii. Determination of Fresh wt.

The fresh weight of callus was determined by placing the entire cell mass from each Petri dish into separate, pre-weighed glass vials. These were then reweighed and the appropriate subtraction made.

The fresh weight of suspended cells was determined after samples (usu. 10 ml.) of 50 ml. suspension cultures or entire 20 ml. cultures had been vacuum filtered through Miracloth (Calbiochem, Calif.) discs inserted into a Millipore Filtration Unit (Millipore, Molsheim, France). This procedure was standardised to ensure a consistent degree of separation of the cells from their bathing medium.

iii. Determination of dry weight.

The dry weights of cell samples were determined after drying overnight (16 h.) in a hot air oven at 75°C.
iv. Determination of the cell population density in liquid cultures.

The cell population density of liquid cultures was estimated with a Hawksley Crystalite Haemocytometer (grid volume 1.8 μl.).

From each thoroughly mixed culture a 200μl. sample of cell suspension was taken and diluted with a known volume of distilled water until a cell density of 150-200 cells grid⁻¹ was achieved. Due to the exceptionally fine nature of the suspension cultures used maceration was not necessary to facilitate accurate counting. Ten grids were counted for each sample, the mean of which was used to estimate the density of the original cell population.

Results have been presented in terms of cell no. per ml. of culture.

v. Estimation of tracheary element number.

In order to estimate the number of tracheary elements in *D.caro*ta callus samples an initial tissue digestion was necessary to obtain a suspension of cells sufficiently dispersed to permit accurate counting.

To a known weight (ca.0.4 g. fr.wt.) of callus was added 5 ml. of 5% Chromic acid solution (w/v chromium trioxide in distilled water). This mixture was then left for 24 h. at room temperature after which it was homogenised by pumping the solution into and out of a Pasteur pipette twenty times. Sufficient distilled water was then added to give a tracheary element* density of 150-200 elements.grid⁻¹. Six grids were counted, the mean of which was inserted into the following formula to determine the number of tracheary elements per
g. fresh weight of callus.

\[
\text{no. of tracheary elements per g. fresh wt.} = \frac{a \times b}{c \times d}
\]

where \(a\) = mean t.e. number. grid\(^{-1}\)

\(b\) = total volume of digest (ml.)

\(c\) = volume of grid (ml.)

\(d\) = weight of sample (g.)

* Tracheary elements were considered to be those cells which showed any signs of visible secondary thickening (see Fig.2.3.1.).

vi. Determination of the viability of cells and protoplasts.

The method of Widholm (1972) was used which is based upon the ability of living cells to cleave fluorescein diacetate molecules to produce free fluorescein which is detectable by its bright yellow/green fluorescence in ultra violet light.

A stock solution of fluorescein diacetate (Sigma Ltd.) was prepared by dissolving 25 mg. in 5 ml. Acetone. Immediately before analysis the stock solution was added dropwise to 5 ml. culture medium (cells) or 0.6M mannitol (protoplasts) until a slight turbidity persisted. One drop of this solution was then mixed with one drop of cell suspension on a microscope slide and covered with a glass coverslip. After 10 min. the cells were observed under visible and ultra violet light using a Vickers Photoplan UV. Microscope. The proportion of cells with fluorescent (yellow/green) protoplasm was determined in a random sample of ca. 500 cells. Results have been presented as percentages of the total cell number.
Figure 2.3.1.

For the purposes of this investigation "tracheary elements" (for typical examples, see above) were taken to be those cells which were observed to exhibit cell wall patterning characteristic of cells with lignified secondary walls.
Section 2.3.2. Analysis of some essential mineral nutrients and sucrose.

In certain liquid culture experiments the disappearance of some essential nutrients was monitored over a single growth cycle. Four "essential" nutrients were examined; orthophosphate, ammonium, nitrate and sucrose. A simple quantitative test for each was chosen on the basis of sensitivity, specificity and the ability to produce a visible colour reaction which could be rapidly measured on a Pye-Unicam SP8-100 spectrophotometer.

Samples of medium for analysis were obtained by gravity filtration of the liquid cultures through Whatman No.1 filter paper. In all cases the reference blank used to "zero" the spectrophotometer consisted of a sample of MS. medium containing all the components used in the experiment but lacking the particular nutrient under test. Standard curves were prepared at each analysis.

i. Sucrose.

The 'Anthrone method' as described by Ashwell (1957) was used. For each analysis fresh anthrone reagent was prepared by dissolving 2 g. anthrone (Sigma) in 1 l. conc. H₂SO₄ (BDH). Samples of medium (1 ml., after dilution with distilled water if necessary) were thoroughly mixed, on ice, with 2 ml. of the anthrone reagent and then incubated in a 90°C. water bath for 10 min. The resulting coloured solutions were rapidly cooled in an ice bath and the optical density of each determined at 620 nm. A typical calibration curve obtained using standard sucrose solutions is presented (Fig.2.3.2.).
Calibration curves for sucrose (Fig. 2.3.2.) and ortho-phosphate (Fig. 2.3.3.)
ii. Orthophosphate.

The method of Allen, (1940) was used.

For each analysis fresh amidol reagent was prepared as follows:

0.2 g. amidol (2,4-diaminophenol hydrochloride, BDH) plus 4.0 g. sodium metabisulphite (BDH) were dissolved in distilled water to give a final volume of 20 ml.

Each medium sample (1 ml., after dilution with distilled water) was initially decolourised with 440 μl. of 60% (w/v) perchloric acid (BDH) after which they were diluted with 4 ml. distilled water. To each was added 400 μl. amidol reagent and 200 μl. 8.3% (w/v in distilled water) ammonium molybdate (BDH) and after thorough mixing the solutions were left to stand for 30 min. at room temperature. The optical densities were then determined at 620 nm. A typical calibration curve using KH₂PO₄ is given in Fig.2.3.3.

iii. Ammonium.

The method of Havilah et al., (1977) was used.

The following reagents were required:

Salicylate reagent (Reagent I)

34 g. sodium salicylate (BDH) and 0.24 g. sodium nitroprusside (BDH) were dissolved in distilled water to give a final volume of 1 l.

Cyanurate reagent (Reagent II)

0.25 g. sodium dichloroisocyanurate was dissolved in 200 ml. distilled water to which was added 15 ml. of 40% (w/v) NaOH. The resulting solution was then made up to 1 l. with distilled
water before use.

5 ml. of Reagent I was mixed with 5 ml. of Reagent II and to this was added a 50 μl. sample of the medium to be tested. After thorough mixing the solution was allowed to stand for 30 min. before the optical density was determined at 660 nm. A typical calibration curve using NH₄NO₃ is given in Fig.2.3.4.

iv. Nitrate

The method of Woolley et al. (1960) was used. The following reagents were prepared.

Reagent I.
20% (v/v) glacial acetic acid in distilled water containing 0.2 ppm. Cu⁺⁺ as CuSO₄.

Reagent II.
An intimate powder mixture of:

- 100 g. BaSO₄
- 75 g. Citric acid
- 12 g. MnSO₄.4H₂O (BDH)
- 4 g. Sulfanilic acid
- 2 g. Powdered zinc
- 2 g 1-naphthylamine (Sigma)

prepared as a solid >15 days before use and having been thoroughly mixed on a Coulter rotary mixer for a minimum of 3 days.

To a 1 ml. sample of medium (diluted with distilled water if necessary) was added 9 ml. of reagent I. 0.8 g. reagent II was then added and the mixture immediately shaken for 15 sec.
Calibration curves for Ammonium (Fig.2.3.4.) and nitrate (Fig.2.3.5.).
Section 2.3.3. Analysis of Pigments

i. Determination of the proportion of pigmented cells in C.roseus cultures

The proportion of cells which contained visible levels of anthocyanins was estimated by counting the number of cells with red/blue pigmentation in a random sample of 1500-2000 cells taken from a well-mixed culture. Where possible this was carried out concurrently with estimations of cell population density (2.3.1iv.). The results have been presented as percentages of the total cell population.

ii. Anthocyanin extraction from cells of C.roseus

The method of Knobloch et al., (1982) was used with minor modifications.

To a known weight (ca. 0.5 g. fr.wt.) of tissue was added 5 ml. of cold (4°C.) methanol containing 1% (v/v) conc. HCl (MeOH. 1% HCl). The mixture was left overnight in the dark at 4°C. on a Coulter rotary mixer (Coulter Elect., Harpenden) and was then centrifuged at 1250xg. for 5 min. The supernatant was decanted and the pellet resuspended in 2 ml. of fresh cold MeOH.1% HCl. and again centrifuged. The supernatants were pooled and the procedure repeated with a final 2 ml. of solvent.

Sufficient cold solvent was then added to the combined supernatants to make the volume up to 10 ml. after which the optical density of the solution was determined at $\lambda_{\text{max}}$ 535 nm. using a Pye-Unicam SP8-100 spectrophotometer. A non-pigmented cell extract was used as the reference blank.

Results are presented in OD units/g.fr.wt./10 ml. solvent.
iii. Anthocyanin extraction - D.carota.

D.carota callus, in comparison to that of C.roseus was considerably aggregated, more heterogeneous as regards anthocyanin production and contained chlorophyll. As a result, a different method of anthocyanin extraction was required for this tissue.

Initially, in order to obtain a more homogeneous tissue mass, the entire callus from each Petri-dish was separately ground in a pestle (4°C.) and mortar to form a slurry. From this slurry a 2 g. sample was taken for analysis.

To this sample 4 ml. of cold (4°C.) 1% aqueous HCl (v/v) and 0.5 g. washed sand were added. The mixture was then reground for 2 min. and after rapid vacuum filtration the solid material was reextracted for 1 min. with another 2 ml. of 1% aq.HCl. After a second filtration the pellet was washed with 2 ml. 1% aq.HCl. before making the volume of the combined filtrates up to 10 ml.

In order to remove contamination from any photosynthetic pigments which may have been extracted by this procedure 3 ml. of chloroform was added to form a Folsch partition (Folsch et al., 1957). The mixture was then vigorously shaken and centrifuged for 1 min. at 1250xg. to rapidly reform the bilayer. The upper aqueous layer was then removed, filtered under gravity through Whatman No.1. filter paper and the optical density of the filtrate measured at \( \lambda_{\text{max}} \) 533 nm. using a chloroform-extracted sample of 1% aq.HCl as the reference blank.

iv. Chlorophyll extraction and quantification.

The method of Harborne (1976) was used. All procedures
were performed at 4°C.

2 g. slurry samples were extracted following the procedure outlined in Sect.2.3.3iii. but substituting Acetone for aq.1%HCl. When the extracts had been made up to a volume of 10 ml., each was filtered under gravity through Whatman No.1 filter paper and the optical density of the filtrate determined at 663 nm. and 645 nm. using 80% acetone (v/v in distilled water) as the reference blank. This procedure took less than 4 min. to complete. To estimate the total concentration of chlorophyll in the extracts the following formula was used:

\[
\text{Total chlorophyll (a+b) mg.l}^{-1} = 20.2 \ A_{645} + 8.02 A_{663}
\]

where \( A_{645} \) and \( A_{663} \) are the optical densities of the extract at 645 nm. and 663 nm. respectively.

Results are presented in μg. chlorophyll g.\(^{-1}\) (fr.wt.).

Note. Anthocyanin interference in this analysis would be negligible. Only a small proportion of the relatively small amount of anthocyanin present in the original tissue would be extracted by this method and at the wavelengths chosen anthocyanin absorption is at a minimum.

v. Extraction and purification of the 'saffron' pigments.

One complete stigma (including the style) (Sigma Ltd. or Culpepers, London) or 0.5 g.fr.wt. \textit{Crocus sativus} callus was ground in a glass/glass homogeniser with 5 ml. 80% acetone (v/v in distilled water) for 3 min. Following a 5 min centrifugation at 1250xg. the supernatant was removed and the pellet reextracted as above until the supernatant became clear. To the combined supernatants (usually \textit{ca.20 ml.}) 10 ml. diethyl
ether was added along with sufficient distilled water to produce a bilayer. The mixture was then shaken vigorously and when the bilayer had reformed the upper phase was removed. The remaining aqueous phase was then continuously reextracted with diethyl ether until the upper layer ceased to be coloured. The resulting aqueous extract (containing all of the main saffron pigments) was then concentrated (if necessary) in vacuo at <30°C. If callus extracts were found to contain polyphenols (brown pigments) these could be separated from the desired yellow pigments by reextracting the latter into an equal volume of n-Butanol (BDH). This last step was essential if the extracts were to be chromatographed.

vi. Thin-layer and column chromatography.

Plastic-backed TLC plates using Kieselgel-60 (Merck, saffron pigments and anthocyanins) or Cellulose MN-300 (Camlab, anthocyanins) as the adsorbant were routinely used. The solvent systems tested are reported in the results section.

Large-scale separation of anthocyanins was performed using 0.8 cm.X10 cm. columns of Polyclar AT (BDH, Polyvinyl-polypyrrolidone) prepared as Van Teeling et al. (1971). Aqueous 0.25M.HCl was used as the eluting solvent. Separation proceeded under gravity and a LKB Ultratrac fraction collector was used to collect the eluted pigments.
Section 2.3.4. Scanning microdensitometry

It is not feasible to present here a detailed description of the principles of scanning microdensitometry (see Meek & Elder, 1977) but it is hoped that the following, very brief account will serve to explain the basics behind the technique in order that those unfamiliar with it may understand the results obtained.

Scanning microdensitometry concerns the precise measurement of light absorbing components (which are coloured) within small particles (usually cells) using an instrument specifically designed for this purpose. These light-absorbing components (chromophores) may be natural (e.g. haemoglobin, anthocyanin) or artefactual (biological stains for specific cellular components e.g. Feulgen/DNA.). In order to accurately quantify the chromophore content of a specimen, the instrument scans the object using a very narrow light beam of defined wavelength and measures its absorbance (10,000-50,000 times) at regular intervals over the entire specimen area. All of these measurements are accumulated, summed and then integrated to yield a single number (the IOD) the value of which is directly proportional to the total amount of chromophore present within the specimen. The wavelength of the light beam used is chosen in accordance with the spectral maximum of the chromophore to be measured.

It is a requirement of this instrument that only well dispersed microscopic preparations are used for analysis as each cell must be totally isolated and surrounded by freely illuminated background, necessary for both accurate measurement and machine calibration. This essentially required that
a preparation of single cells had to be obtained and therefore a protoplast preparation of each culture sample was made before analysis. Although this was an unfortunate extra complication in an already very time-consuming technique it does offer one advantage in that the cell population thus produced for analysis consisted of regularly spherical cells. This entailed that as the microdensitometer used provided a value for the cross sectional area of each cell in association with the IOD value, an accurate estimate of cell volume could be made using the formula \( V = \sqrt[3]{0.5659A^3} \) (\( V = \text{volume}, \ A = \text{cross-sectional area} \)). Consequently a relative value for the anthocyanin concentration could also be estimated for each cell. It must be pointed out however, that if we are to compare relative concentration values of different cells, it has to be assumed that they were all initially plasmolysed to the same degree during the protoplast preparation procedure. Although the extent of plasmolysis, which is dependent upon the osmotic potential of the cell, is likely to vary from cell to cell the differences are likely to be relatively small in a cell population of this type. This point should nevertheless, be borne in mind when considering the results.

i. Equipment.

*In vivo* microdensitometric and microspectrophotometric measurements on individual plant cells were made using a Vickers M85 scanning integrated microdensitometer (Vickers Instruments Ltd., London)

The following machine settings were routinely used:

- objective, X40;
- scan size selector, 1/1;
- gating mask selector B.3.;
- band width, 25;
- spot aperture, 2;
- wavelength, variable (400 nm.-700 nm.).
ii. Microdensitometry.

In all of the experiments in which microdensitometric measurements were made the analyses were carried out on a random sample of visibly pigmented protoplasts prepared as detailed in Chapter 2.2.4. Unfortunately due to the very tedious and time-consuming procedure required to make each individual measurement, this random sample had to be restricted to 100 cells/treatment.

It was found that the \( \lambda_{\text{max}} \) of anthocyanin-accumulating C. roseus cells varied over a small wavelength range. Therefore, in order to obtain a suitably accurate IOD determination three readings were taken for each cell at wavelengths 580 nm. (machine setting 60), 554 nm. (55) and 530 nm. (50). Of these the maximum value was selected as the IOD value for the cell.

* In all experiments the proportion of pigmented cells was determined before and after protoplast isolation in order to determine if this was affected by the procedures employed. IN ALL CASES THE PROPORTION OF VISIBLY PIGMENTED CELLS WAS NOT ALTERED BY MORE THAN 0.5% BY THIS TREATMENT.

iii. Microspectrophotometry.

A crude absorption spectrum could be obtained for individual plant cells or protoplasts by measuring the IOD of a selected cell over a range of wavelengths and presenting the results graphically. The wavelength range used for both saffron and anthocyanin-containing cells was 400-700 nm.
Section 3.2.5. Techniques used involving radioactively-labelled phenylalanine

i. Material.

In all experiments where a radioactive label was used the compound chosen was phenylalanine which had the $^{14}$C isotope for each of its carbon atoms. This L-[U-$^{14}$C] phenylalanine was obtained from Amersham International plc., Amersham, UK. The compound was obtained in a sterile aqueous solution containing 2% (v/v) ethanol and was stored at -4°C. The specific activity was ca. 500 mCi./mmol. (18.5 G.Bq./mmol.) and the radioactive concentration 50 µCi./ml. (1.85 MBq./ml.).

ii. 'Feeding' and harvesting procedure.

In labelling experiments, unless otherwise stated in the text 40 µl. (ca. 2 µCi.) of the stock solution of $^{14}$C-phenylalanine was added to each 10 ml. experimental culture using a Hamilton microsyringe. After the appropriate incubation period (usually 24 h.) the cultures were harvested by vacuum filtration, and the cell mass washed with 4x10 ml. distilled water. The filtrate was collected and made up to 50 ml. with distilled water. Aliquots (0.5 ml.) were then taken for scintillation counting to determine the loss of radioactivity from the medium, which has been taken as an indication of cellular uptake.

Anthocyanin extracts of the washed cell mass were made as described previously. Soluble protein extracts were obtained by the following method. To a known weight (ca. 1 g. fresh wt.) of cells was added 5 ml. of 0.1N NaOH. The mixture was shaken and left overnight at 4°C. Following centrifugation (2500 rpm.,
10 min.) the supernatant was removed and the pellet resuspended three times in 3x5 ml. 0.1N NaOH with intervening centrifugations. To the pooled supernatants was then added an equal volume of cold 20% (w/v) trichloroacetic acid (TCA) in distilled water. The mixture was then stored at 0°C for 2.5 h. after which the precipitate was collected by centrifugation (3250 rpm., 30 min.) at 4°C. The pellet thus obtained was washed in 2x5 ml. of 10% aq. TCA before being redissolved in 5 ml. 0.1N NaOH. Small aliquots (100 µl.) were then taken for scintillation counting and if the protein content was to be quantified the standard method of Lowry et al., (1951) was used.

iii. Thin Layer Chromatography (TLC) and autoradiography.

In order to isolate the anthocyanin components from the methanolic cell extracts two dimensional thin layer chromatography (2DTLC) was employed. Extract samples were initially concentrated ca. 20 fold by placing known volumes (2 ml.) under a stream of slightly warm (35°C) air. After ca. 2-3 min. the volume of each was redetermined using a Gilson micropipette. Aliquots of known volume (usually ca. 5 µl.) were then spotted onto 9x9 cm. silica gel TLC plates (Merck) 1 cm. from the bottom and 1 cm. from the left-hand side. The plates were developed firstly in n-Butanol:Acetic acid:water (4:1:5) and then after thorough drying in warm (ca. 35°C) air for 5 min. in n-Butanol:Formic acid:water (4:1:5).

The three anthocyanin spots were then clearly visible and separate from all other visible (and UV-visible) compounds. Spraying with Ninhydrin-Acetic acid (0.3 g. ninhydrin was dissolved in 100 ml. n-butanol to which was then added 3 ml.
acetic acid) revealed the positions of all ninhydrin-positive compounds (including free amino acids) which were also all distinctly separate from the anthocyanin spots. This technique also identified the position of phenylalanine which was identified by its distinctive colour (Randerath, 1968) and by its co-chromatography with a standard.

Autoradiography was performed using Dupont Cronex X-ray film with an exposure time of 5 weeks.

iv. Scintillation counting.

Scintillation counting was performed using an Intertechnique SL3000 scintillation counter. The scintillation fluid consisted of toluene (BDH) with the scintillant butyl PBD [2-(4'-t-butylyphenyl)-5-(4"-biphenyl)-1,3,4-oxadiazole, Sigma] and with Triton-X-100 (Koch Light) as an emulsifier (1000 ml.:6.1 g.: 500 ml.). For the scintillation of protein extracts 100 μl. of extract was mixed with 4.9 ml. of the scintillation fluid. For the scintillation of chromatographed anthocyanins and phenylalanine the spots were carefully scraped off the TLC plates and added directly to 5 ml. of scintillation fluid. The results, after 10 minutes counting (in cpm.) were corrected for quenching and consequently are presented in dpm.
Chapter 3

RESULTS

Part 1. "Anthocyanin accumulation in callus and cell cultures of two cultivars of Daucus carota L."

Part 2. "Anthocyanin accumulation in callus and cell cultures of the Madagascar Periwinkle Catharanthus roseus (L.) G.Don."
Part 3.1.

"ANTHOCYANIN ACCUMULATION IN CALLUS AND CELL CULTURES OF TWO CULTIVARS OF DAUCUS CAROTA L."
As outlined in Chapter 1 cultures of two plant species, *Daucus carota* and *Catharanthus roseus* were used in this investigation to study the accumulation of the group of secondary compounds, the anthocyanins, *in vitro*. The results of the work using cultures of the former species are presented in this first part of the Results. In this study root sections of two distinctly different cultivars of the cultivated carrot were used to obtain cultures for experimentation (for details see Section 2.2.3ii.). These were, an orange cultivar - *D. carota* cv. "Autumn King" and a red cultivar *D. carota* cv. "Takii's Winter Scarlet" (Fig.3.1.1.). The much darker and redder appearance of the tap root of the latter cultivar is due primarily to extremely high levels of carotenoids present in the outer cell layers of these organs and also to the accumulation of anthocyanins, particularly in those upper portions of the roots which become exposed to light (Widholm, (1980); Mok *et al.*, (1976)).

Cultures of *D. carota* were chosen for investigation for two main reasons. Firstly, cultures of this species have previously been observed to accumulate relatively high levels of anthocyanins *in vitro* (Schmitz & Seitz (1972); Alfermann *et al.*, (1975); Dougall *et al.*, (1980)). Secondly, cultures initiated from root segments of the cultivar "Autumn King" in this laboratory were observed to contain small numbers of deeply red cells on the callus surface when grown under standard cultural conditions (Fig.3.1.2.). Attempts were made to increase the proportion of these pigmented cells by chemical manipulation and by initiating cultures from a cultivar (Takii's Winter Scarlet) which would have, theoretically, a
The root phenotype of the two carrot varieties used in this investigation. Left: *D. carota* cv. 'Takii's Winter Scarlet'; Right: *D. carota* cv. 'Autumn King', (50% of life size).
Figure 3.1.2.

The phenotype of *D. carota* callus after 3 weeks growth on standard DC medium supplemented with $10^{-5}$ M. 2,4-D and $10^{-5}$ M. kinetin under low irradiance levels. (a) *D. carota* cv. 'Autumn King', (b) *D. carota* cv. 'Takii's Winter Scarlet'.

Bar = 500µm.
higher potential for anthocyanin accumulation (see Zenk et al., 1977; Kinnersley & Dougall, 1980b). It was hoped that such modifications would bring about enhanced anthocyanin accumulation to a level which would permit a detailed investigation into the factors influencing the occurrence of these compounds in vitro as examined at both the cultural level (by extraction) and also the cellular level (by microscopy/microdensitometry).
Light-grown callus cultures of the carrot variety "Autumn King" were observed to contain small numbers of red/purple cells when grown on the standard initiation/sub-culture medium which contained $10^{-5}$ M. 2,4-D and $10^{-5}$ M. kinetin (see Fig.3.1.2a). These cells occurred at very low frequency (>0.1%, total cell population) and were only observed in the outermost cell layers of the callus. The amount of pigment produced was insufficient to permit extraction and identification of the causal compounds. However, in vivo microspectrophotometry revealed that the pigment had a $\lambda_{max}$ in the range 542-552 nm. (Fig.3.1.3.) and this spectral pattern, in association with the vacuolar location of the pigment would suggest that it was anthocyanidin-based. (The other likely possibility, that the pigment was betacyanic in nature, can be eliminated as such compounds are solely produced by representatives of the Centrospermae of which Daucus is not a member (Smith (1976); Harborne (1973)).

As these cultures appeared capable of anthocyanin accumulation under standard conditions of culture it was considered that the extent of this pigmentation and the proportion of accumulating cells could be increased by quantitative/qualitative, modifications of the culture conditions. It has been shown that both auxins and cytokinins can have considerable influence on the in vitro accumulation of anthocyanins (Constabel et al., (1971); Liau & Ibrahim, (1973); Kinnersley & Dougall, (1980b); Blakeley & Steward, (1961)) and consequently experiments were carried out to determine the effect of qualitative (i) and quantitative (ii) variation of the growth substance supplementation of the nutrient medium upon which these cultures were grown.
Figure 3.1.3.
Microspectrophotometric absorption spectra of 3 visibly red/purple cells from a *D. carota* cv. Autumn King callus culture.
i. The growth and anthocyanin accumulation of callus cultures of *D. carota* cv. "Autumn King" when incubated on media containing varying auxin/cytokinin supplementation.

Three experiments were performed using IAA and 6-BAP as alternative growth substances to those used in the standard growth medium (2,4-D and Kinetin). Three combinations of these four supplements were tested over a wide range of concentrations to determine the effect of such modifications on culture growth and anthocyanin accumulation. The concentrations and combinations of growth substances tested are given in Table 3.1.1. Fourteen day old, light-grown callus was taken from several Petri-dishes, pooled and well mixed. From this ca. 2.5 g. samples (exact weight determined) were taken and used to inoculate each 9 cm. Petri-dish containing 20 ml. of the medium to be tested. Two replicates of each treatment were used. After sealing, the cultures were incubated in standard lighting conditions (25-30 μmol.m⁻²sec⁻¹) for 3 weeks before harvesting. The fresh weight and anthocyanin content of the cultures were determined, the results of which are presented in Table 3.1.1.

In Tables 3.1.1a. and 3.1.1b. which include the data for those treatments in which IAA had been used it is clear that all of the growth substance combinations tested generally promoted relatively little growth of the cultures. The culture fresh weight was doubled in only two of the twenty four treatments tested over the three week experimental period. In the 2,4-D/6-BAP treatments (Table 3.1.1c.), although culture growth was generally improved the best levels attained were still
<table>
<thead>
<tr>
<th>Treatment</th>
<th>% increase in fr.wt.</th>
<th>Red areas(^1)</th>
<th>Anthocyanin cont. /g.(fr.wt.)(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K/1AA(^3)</td>
<td>10 0.1 0</td>
<td>10 0.1 0</td>
<td>10 0.1 0</td>
</tr>
<tr>
<td>100</td>
<td>49 58 68</td>
<td>17 46 5</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>56 34 77</td>
<td>16 2 5</td>
<td>all treatments</td>
</tr>
<tr>
<td>0.1</td>
<td>69 38 58</td>
<td>8 7 0</td>
<td>n.d.</td>
</tr>
<tr>
<td>0</td>
<td>70 67 48</td>
<td>12 6 0</td>
<td></td>
</tr>
<tr>
<td>6-BAP/1AA(^3)</td>
<td>10 0.1 0</td>
<td>10 0.1 0</td>
<td>10 0.1 0</td>
</tr>
<tr>
<td>100</td>
<td>51 53 82</td>
<td>9 13 25</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>47 61 168</td>
<td>12 12 34</td>
<td>all treatments</td>
</tr>
<tr>
<td>0.1</td>
<td>63 49 148</td>
<td>3 0 10</td>
<td>n.d.</td>
</tr>
<tr>
<td>0</td>
<td>67 32 48</td>
<td>0 0 0</td>
<td></td>
</tr>
<tr>
<td>6-BAP/2,4-D(^3)</td>
<td>10 0.1 0</td>
<td>10 0.1 0</td>
<td>10 0.1 0</td>
</tr>
<tr>
<td>100</td>
<td>77 85 100</td>
<td>62 41 49</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>121 206 211</td>
<td>66 45 53</td>
<td>all treatments</td>
</tr>
<tr>
<td>0.1</td>
<td>137 174 189</td>
<td>48 5 18</td>
<td>n.d.</td>
</tr>
<tr>
<td>0</td>
<td>42 91 63</td>
<td>0 8 0</td>
<td></td>
</tr>
</tbody>
</table>

\(^{1}\) Red areas consisted of 1-4 pigmented cells.

\(^{2}\) OD./g./10ml.

\(^{3}\) μM.

Table 3.1.1.

Fresh weight increase and anthocyanin content of *D. carota* cv. 'Autumn King' callus cultures after incubation for 3 weeks on DC medium supplemented with the differing growth substance combinations tabulated.

n.d. not detectable.
considerably less than the 'control' cultures grown on the standard medium.

In each of these experiments, despite the use of different auxin/cytokinin combinations over a wide range of concentrations, none were able to increase the anthocyanins accumulated to a detectable level after extraction. In the control cultures 100-200 'red cell areas'/culture generally appeared at the end of each subculture period (each 'red cell area' consisted of 1-4 cells, as observed using a binocular microscope). In all of the treatments tested the number of 'red cell areas' was considerably reduced rather than increased.

Consequently, as none of the growth substance modifications tested in these experiments promoted increased levels of anthocyanin in these cultures a further experiment was undertaken in order to investigate the effect of quantitative variation of the growth substances which were included in the standard (most productive) control medium, on the accumulation of these compounds.

ii. The influence of differing levels of 2,4-D and Kinetin on the accumulation of anthocyanin in D.carota cv. "Autumn King" callus cultures.

In this experiment 12 different treatments based upon variations to the 2,4-D/Kinetin balance were tested for their ability to promote anthocyanin accumulation in D.carota cv. "Autumn King" callus cultures grown on agar media. The procedure used involved the use of a nurse culture technique which enabled small areas of callus already containing some
red cells to be successfully subcultured onto modified media. By using only very small callus isolates the risk of chemical carryover was minimised and also the ratio of green/red cells was considerably reduced thus decreasing any possible competition for nutrients which may have existed between the cells.

Three, 9-cm. Petri-dishes, each containing 20 ml. of medium were prepared for each treatment (see Table 3.1.2.). In the centre of each was placed a small piece of *D. carota* callus (1 cm. diameter, ca. 0.5-1 g.). Around these were then placed small pieces of similar callus (1 mm. diameter) which included some (10-15) red cells on their upper surface. These callus "islands" were placed in a ring around the nurse callus, 5 mm. from its outer edge. Six "islands"/nurse callus were used. Each "island" was coded and the number of red cells determined using a binocular microscope. As it was not possible to accurately determine the weight of these small callus pieces the values for the red cell population have been expressed on a per unit surface area basis. The callus pieces chosen were all roughly hemispherical and thus their surface areas were easily determined after measuring the diameter of each microscopically. After 16 days the size and red cell number of each isolate were redetermined and from these values the changes in red cell no./mm.² were estimated. These results are presented in Table 3.1.2.

Once again the numerous modifications of the growth substance supplementation of the medium failed to induce a substantial increase in the proportion of anthocyanin-accumulating cells. Although in a few treatments the red cell no./mm².
<table>
<thead>
<tr>
<th>Medium</th>
<th>Time 0d mean no. red cell areas/mm²</th>
<th>Time 16d mean no. red cell areas/mm²</th>
<th>Δχ</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D K.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 OM. OM.</td>
<td>8.9 ± 1.1</td>
<td>18.2 ± 3.1</td>
<td>+9.3</td>
</tr>
<tr>
<td>2 0 10⁻⁷</td>
<td>8.6 ± 1.2</td>
<td>19.5 ± 2.2</td>
<td>+10.9</td>
</tr>
<tr>
<td>3 0 10⁻⁵</td>
<td>7.3 ± 1.8</td>
<td>9.4 ± 2.0</td>
<td>+2.1</td>
</tr>
<tr>
<td>4 0 10⁻⁴</td>
<td>8.3 ± 1.3</td>
<td>12.9 ± 1.0</td>
<td>+4.6</td>
</tr>
<tr>
<td>5 10⁻⁷ 0</td>
<td>11.6 ± 1.7</td>
<td>11.7 ± 2.0</td>
<td>+0.1</td>
</tr>
<tr>
<td>6 10⁻⁷ 10⁻⁷</td>
<td>8.7 ± 1.0</td>
<td>10.8 ± 2.9</td>
<td>+2.1</td>
</tr>
<tr>
<td>7 10⁻⁷ 10⁻⁵</td>
<td>11.8 ± 2.2</td>
<td>7.8 ± 2.4</td>
<td>-4.0</td>
</tr>
<tr>
<td>8 10⁻⁷ 10⁻⁴</td>
<td>7.7 ± 1.4</td>
<td>6.2 ± 0.8</td>
<td>-1.5</td>
</tr>
<tr>
<td>9 10⁻⁵ 0</td>
<td>8.0 ± 1.6</td>
<td>5.2 ± 0.8</td>
<td>-2.8</td>
</tr>
<tr>
<td>10 10⁻⁵ 10⁻⁷</td>
<td>9.7 ± 1.1</td>
<td>9.4 ± 0.9</td>
<td>-0.3</td>
</tr>
<tr>
<td>11 10⁻⁵ 10⁻⁵</td>
<td>10.9 ± 1.2</td>
<td>10.1 ± 1.1</td>
<td>-0.8</td>
</tr>
<tr>
<td>12 10⁻⁵ 10⁻⁴</td>
<td>10.8 ± 1.2</td>
<td>5.7 ± 1.7</td>
<td>-5.1</td>
</tr>
</tbody>
</table>

**Table 3.1.2.**

The change in the frequency of 'red cell areas' per mm.² surface area of callus fragments of *D. carota* cv. 'Autumn King' cultured on media supplemented with a variety of different growth substance levels. Each value is the mean of 18 replicates ± s.e.
was approximately doubled the levels attained were still extremely low and the anthocyanin yield would still not have been sufficient to be easily measured by extraction techniques. It is interesting to note that the greatest increases in red cell density occurred in those treatments lacking any 2,4-D supplementation. This might indicate that in this situation auxin, or perhaps more specifically 2,4-D was inhibitory to the appearance of anthocyanin accumulating cells. Unfortunately however, continued exposure of the cultures to 2,4-D-free media resulted in tissue death. After the measurements detailed above had been taken some of the material from the "most productive" 2,4-D-free treatments were selectively sub-cultured onto fresh medium of the same type and were observed to undergo very little further growth, turn dark green and then become necrotic. Very few extra red cells appeared during this time.

The final point to raise concerning these results is that anthocyanin accumulation, even in the treated cultures appeared to occur as rather isolated events. Rarely were more than 3-4 red cells observed adjacent to each other. Although occasionally the red cells appeared to be concentrated into certain areas of the callus even in these instances they were generally well separated by non-red (usually green) cells.
The following points have emerged from the experiments reported in this section.

1. Callus cultures of *D. carota* cv. 'Autumn King' produced small numbers of deeply red/purple cells under standard culture conditions.

2. Microscopic examination and *in vivo* microspectrophotometry indicated that the pigments concerned were almost certainly anthocyanins.

3. Attempts to enhance anthocyanin accumulation through modification of the growth substance supplementation of the nutrient medium were unsuccessful.

In conjunction with the above experiments using the 'Autumn King' variety another series of experiments using cultures of the red variety 'Takii's Winter Scarlet' was carried out. The results of these experiments are reported in the following section.
Section 3.1.2.

"Anthocyanin accumulation in cultures of D. carota cv. 'Takii's Winter Scarlet'."
There still remains some controversy as to the relationship between the level of in vitro secondary metabolite accumulation and the levels generally accumulated by the source plant. However, it is becoming more widely accepted, as a result of some very convincing reports such as those by Kinnersley & Dougall, (1980a) and Zenk et al., (1977) that high yielding plants generally give rise to the higher yielding cultures. Accordingly, cultures of a cultivar of D.carota with a greater propensity to accumulate anthocyanins (cv. 'Takii's Winter Scarlet') were initiated in an attempt to improve upon the in vitro levels previously observed in order to obtain a culture system which was more amenable for further research. The cultures produced did appear to contain a higher proportion of red/purple cells when grown on the standard induction/growth medium (Fig.3.1.2b.) and the following three experiments were performed to determine means to enhance these levels yet further.

i. The use of reduced 2,4-D levels as a means to enhance anthocyanin accumulation in callus cultures of D.carota cv. 'Takii's Winter Scarlet'.

The standard medium used to initiate and maintain the callus cultures of D.carota cv. 'Takii's Winter Scarlet' was identical to that used for the cultivar 'Autumn King' and consequently contained a high concentration of 2,4-D. For the reasons stated in the previous section the following experiment was performed in order to determine if a reduction of this level of 2,4-D could induce increased levels of anthocyanin accumulation in these cultures.
Cultures of *D. carota* cv. 'Takii's Winter Scarlet' which had been grown in the light for >6 months (3 week subculture period) were used for experimentation. Two, 14 day old cultures were pooled and thoroughly mixed and from this were taken ca. 2.5 g. samples of the very friable callus which were used to inoculate three 9 cm. Petri dishes containing 20 ml. of standard DC medium and three similar plates containing medium with a reduced 2,4-D supplementation ($10^{-7}$M.). The cultures were maintained on these media (with 1 subculture) for 5 weeks. At this time the callus from the control (DC) treatment was pooled, mixed and 2.5 g. samples were taken to inoculate 3 fresh Petri dishes containing the same control medium. Callus from the $10^{-7}$M. 2,4-D treatment was similarly used to inoculate 6 Petri dishes, 3 containing the reduced 2,4-D level and 3 the control level. After three weeks incubation in the light the plates were harvested and measurements of fresh weight, dry weight, chlorophyll content, anthocyanin content and tracheary element no. were determined by the methods detailed in Cpt. 2.

The results are presented in Fig. 3.1.4. and Table 3.1.3. Clearly the reduction of the 2,4-D concentration has had a substantial morphological and biochemical effect on these cultures. The fresh and dry weights were both significantly reduced and the cultures became exceedingly hard and brittle. The very compact nodular appearance of the treated callus was very much in contrast to the very friable and spongy appearance of the controls. It had been considered that these changes may have arisen as a result of, or in parallel with, an increase in cell lignification. However, measurements of the number of tracheary elements present revealed that no significant
<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>2,4-D/K.(^1)</th>
<th>10(^{-5})/10(^{-5})</th>
<th>10(^{-7})/10(^{-5})</th>
<th>10(^{-5})/10(^{-5})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicates</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh wt.</td>
<td>9.160 ±1.007</td>
<td>6.762**</td>
<td>8.912 ±0.938</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry wt.</td>
<td>0.611 ±0.032</td>
<td>0.531*</td>
<td>0.589 ±0.009</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorophyll cont.</td>
<td>10.9</td>
<td>63.7***</td>
<td>17.7 ±2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>µg./g. (fr.wt.)</td>
<td>±0.3</td>
<td>±6.7</td>
<td>±2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthocyanin cont.</td>
<td>0.025 ±0.003</td>
<td>0.141***</td>
<td>0.037 ±0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OD/g. (fr.wt.)/10ml.</td>
<td></td>
<td>±0.014</td>
<td>±0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tracheid no.</td>
<td>8.7 ±1.1</td>
<td>8.0 ±0.4</td>
<td>9.6 ±1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(x10(^{-5}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Appearance</td>
<td>pale green;</td>
<td>dark green;</td>
<td>pale green;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>v.friable;</td>
<td>v.compact/</td>
<td>v.friable;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>nodular;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>few, isolated</td>
<td>extensive dark</td>
<td>few, isolated</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>red cells;</td>
<td>red areas;</td>
<td>red cells;</td>
<td>roots absent.</td>
<td>roots present. roots absent.</td>
</tr>
</tbody>
</table>

Table 3.1.3.

Measurements of the growth and pigmentation of callus cultures of *D.carota* cv. 'Takii's Winter Scarlet' after a 3 week incubation period on media supplemented with a standard (10\(^{-5}\)M.) or reduced (10\(^{-7}\)M.) level of 2,4-D. The cultures had been preincubated for 5 weeks on 10\(^{-5}\)M. 2,4-D (CONTROL) or 10\(^{-7}\)M. 2,4-D (both remaining treatments). \(^1\)M.

Significantly different from controls at: * P=0.05, ** P=0.01, *** P=0.001.
(a+b) The appearance of *D. carota* cv. 'Takii's Winter Scarlet' callus after growth on a low auxin medium (see text for precise details). In comparison to cultures grown on the standard ($10^{-5}$ M. 2,4-D) medium (c) these cultures became much less friable, darker green and exhibited considerable red pigmentation. Roots were also frequently observed (b). Bar = 1 cm.
difference in the proportion of lignified cells occurred between the treatments.

Reduced 2,4-D supplementation also resulted in two very distinct changes to the pattern of pigmentation in these cultures (Fig. 3.1.4a.). Firstly, the callus became very dark green in colour in contrast to the much paler green of the controls. Chlorophyll extraction revealed that a six fold increase in the chlorophyll content had occurred in the treated cultures. Secondly, the anthocyanin content of the treated cultures was much more pronounced. In contrast to the controls which contained small numbers of red cells, distributed more or less evenly over the callus surface, the cultures grown on low 2,4-D levels contained many large areas, often nodular in form, which were dark red in colour. Callus extraction once again revealed that the level of these pigments were also increased ca. 6 fold. (The levels produced permitted spectrophotometric confirmation of the anthocyanic identity of the pigments (Fig. 3.1.5.).)

The remaining very obvious feature of the cultures grown on the low 2,4-D medium was the presence of roots, emerging at random from the callus mass (Fig. 3.1.4b.). As many as 8-10, 1 cm.-2 cm. roots per dish were observed. As is clearly indicated by the results in the third column of Table 3.1.3. this effect and all of the others mentioned above, which resulted from the reduction in 2,4-D medium supplementation, were all very rapidly reversed when the 2,4-D level was returned to its original high level. Those cultures which had been grown on \(10^{-5}\) M. 2,4-D after a 5 week period on the \(10^{-7}\) M. 2,4-D medium (by which time they begun to take on all the modified charac-
Figure 3.1.5.

Absorption spectrum of an acidified aqueous extract of *D. carota* cv. 'Takii's Winter Scarlet' callus after growth on a low-auxin medium. The extract was pre-extracted with chloroform prior to spectral analysis. Solvent: 1% conc. HCl in distilled water.
teristics detailed above) were insignificantly different from those which had been exposed to $10^{-5}$M. 2,4-D throughout.

It would appear therefore that reducing the 2,4-D supplementation does indeed enhance the levels of anthocyanin accumulated by these cultures and that this comes about in association with several indications of increased internal organisation of the callus (compact, nodular growth; root production; greening). These results thus appeared very promising and consequently a second experiment was performed in which the cytokinin concentration was modified in order to determine if this also could influence the level of anthocyanin accumulation in this in vitro system.

ii. The effect of reduced cytokinin concentration on the growth and anthocyanin accumulation of *D. carota* cv. 'Takii's Winter Scarlet' callus cultures.

Investigations involving other in vitro systems have shown that cytokinins can prove inhibitory to anthocyanin accumulation in plant tissue cultures (Kinnersley & Dougall, (1980a), Part 3.2. this volume). The following experiment was thus performed to determine the effect of reducing the normally high kinetin concentration ($10^{-5}$M.) on pigment accumulation in these cultures.

The procedure followed was identical to that used in the previous experiment with the exception that it was the kinetin concentration, not the 2,4-D concentration, which was reduced to $10^{-7}$M. The results are presented in Table 3.1.4.

The results would indicate that in this culture system the influence of kinetin on the growth and pigmentation was
<table>
<thead>
<tr>
<th>2,4-D/K (M.)</th>
<th>CONTROL</th>
<th>10^{-5}/10^{-5}</th>
<th>10^{-5}/10^{-7}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate no.</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Fresh wt. (g.)</td>
<td>11.293</td>
<td>9.269</td>
<td></td>
</tr>
<tr>
<td>±1.357</td>
<td>±1.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry wt. (g.)</td>
<td>0.469</td>
<td>0.461</td>
<td></td>
</tr>
<tr>
<td>±0.051</td>
<td>±0.032</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorophyll content (µg./g.(fr.wt.))</td>
<td>22.64</td>
<td>20.00</td>
<td>±2.57</td>
</tr>
<tr>
<td>±2.57</td>
<td>±1.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthocyanin content OD/g.(fr.wt.)/10ml.</td>
<td>0.015</td>
<td>0.010</td>
<td>±0.003</td>
</tr>
</tbody>
</table>

Table 3.1.4.

Biomass production and pigmentation of *D. carota* cv. 'Takii's Winter Scarlet' callus cultures after a 3 week culture period on DC medium supplemented with a standard (10^{-5} M.) or reduced (10^{-7} M.) level of kinetin.
much less pronounced than that for 2,4-D, at least at the
levels tested. Apart from a small (20%) reduction in culture
fresh weight all of the measurements made were closely com-
parable between the treatments. However, one difference was
observed in that although no difference in the level of
chlorophyll was detected, the treated cultures were obviously
more yellow in appearance. It would seem likely therefore that
the reduction in cytokinin concentration had affected carotenoid
levels although no measurements were made to confirm this.

Accordingly, although it cannot be excluded that exceedingly
high or low kinetin concentrations may have some effect, it
would seem likely that this cytokinin has little influence on
anthocyanin accumulation in these cultures.

Before continuing with more detailed experiments it was
necessary to repeat the auxin effect using liquid cultures as
it was this system which would be required to perform those
types of experiment suggested in Chapter 1. The results of
this experiment are now reported.

iii. Anthocyanin accumulation in cell suspension cultures
of D.carota cv. 'Takii's Winter Scarlet' grown in a
medium containing reduced levels of 2,4-D.

To fulfil the aims of this project it was essential that
easy, accurate examination of the culture cell population was
possible. It was hoped that individual cells could be
observed in order to permit, for example, accurate counts of
specific cell types to be made. This essentially required a
liquid culture system where the extreme friability of the
culture would meet the experimental requirements. Accordingly the experiment reported in the first part of this section was repeated using liquid media.

The experimental procedure followed was similar to that for the previous experiment but using liquid rather than callus cultures. The inoculum size was 4 ml. and measurements were made at the end of the 3rd subculture period from transfer to the modified conditions. Three treatments (5 replicates) were tested, a control using the standard DC medium and a further two using reduced levels of 2,4-D (10\(^{-7}\) M., 10\(^{-8}\) M.). The results are presented in Table 3.1.5.

In these cultures, when 10\(^{-7}\) M. 2,4-D (the level used in the previous, callus experiment) had been used little difference in either culture growth or pigment content had occurred. The cultures remained bright green (although the chlorophyll content was actually slightly reduced in comparison to the controls) and contained only negligible numbers of red cells. Reducing the 2,4-D level to 10\(^{-8}\) M. resulted in significantly reduced growth and chlorophyll content but still had no inductive influence on anthocyanin accumulation. Maintenance of these cultures in either media for a further two subculture periods (6 weeks) still failed to induce visible levels of anthocyanin accumulation (the 10\(^{-8}\) M. 2,4-D treatment failed to maintain full culture viability for this period, although the cultures given 10\(^{-7}\) M. 2,4-D appeared to grow normally).
Table 3.1.5.

Biomass production and pigmentation of \textit{D. carota} cv. 'Takii's Winter Scarlet' suspension cultures after a 3 week culture period using media with varying levels of 2,4-D.

Significantly different from controls at ** P=0.01, *** P=0.001.

<table>
<thead>
<tr>
<th>2,4-D/K. (M.)</th>
<th>CONTROL</th>
<th>10^{-5}/10^{-5}</th>
<th>10^{-7}/10^{-5}</th>
<th>10^{-8}/10^{-5}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicates</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Fresh wt. g.</td>
<td>11.430</td>
<td>12.185</td>
<td>6.370**</td>
<td></td>
</tr>
<tr>
<td>±1.478</td>
<td>±1.432</td>
<td>±0.581</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry wt. g.</td>
<td>0.588</td>
<td>0.698</td>
<td>0.259**</td>
<td></td>
</tr>
<tr>
<td>±0.052</td>
<td>±0.031</td>
<td>±0.012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorophyll content</td>
<td>47.11</td>
<td>43.51</td>
<td>28.70***</td>
<td></td>
</tr>
<tr>
<td>µg./g.(fr.wt.)</td>
<td>±2.98</td>
<td>±2.17</td>
<td>±1.67</td>
<td></td>
</tr>
<tr>
<td>Anthocyanin content</td>
<td>0.005</td>
<td>0.007</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>OD/g.(fr.wt.)/10ml.</td>
<td>±0.005</td>
<td>±0.004</td>
<td>±0.004</td>
<td></td>
</tr>
</tbody>
</table>
The following salient points arose from the work reported in this section.

1. Callus cultures of *D. carota* cv. Takii's Winter Scarlet were observed to contain deeply red/purple cells at low frequency when grown under standard culture conditions.

2. Spectrophotometric examination confirmed the pigment identity as being anthocyaninic.

3. In callus cultures the kinetin concentration was found to have little influence upon anthocyanin accumulation.

4. In callus cultures the reduction of the 2,4-D concentration to $10^{-7}$M decreased culture growth and enhanced both chlorophyll and anthocyanin levels. Evidence of increased internal organisation were also apparent.

5. The auxin-induced effects upon callus cultures could not be reproduced when cell suspension cultures were used.

Although these results are intrinsically very interesting, and include features commonly found in other systems (which will be discussed further in Chapter 4) this system was likely to prove of little value for the type of project originally planned, as detailed in Chapter 1. Consequently, as cultures of another species, *C. roseus* which were ideal for this type of work had been obtained, it was decided to terminate this line of investigation and to concentrate upon the second system for the major part of this project.
PART 3.2.

"ANTHOCYANIN ACCUMULATION IN CALLUS AND
CELL CULTURES OF THE MADAGASCAR PERIWINKLE,
CATHARANTHUS ROSEUS (L.) G.DON."
Cultures of *C. roseus* (syn. *Vinca rosea* L.) were obtained from Professor J. Berlin, W.Germany (Cpt.2) in November, 1981. These cultures had the capacity to accumulate anthocyanins (Fig.3.2.1.) and subsequently became the subject of an intensive investigation into the factors which influence this accumulation in vitro. The results of this work constitute this second and main part of the results chapter.

These cultures had two important features which made them ideal for use in an investigation of this type:

1. **Both solid-phase and liquid cultures consisted of very small cell aggregates (in the latter few were >200μm. in size).** This exceptional friability permitted an accurate quantitative and qualitative examination of the cell population, without the need for any preliminary tissue digestion with chromic acid or pectolytic/cellulolytic enzymes.

2. **The cell populations in these cultures were remarkably homogeneous and apparently lacked any structural organisation or vascular differentiation.** (During the course of this study in excess of 1,000,000 cells were counted by hand and observations were made on a great many more and of all these cells only one was seen to have secondary thickening characteristic of tracheary elements.)

Both of these features contributed greatly to the relative ease of experimentation using this system and to the consistency in the results obtained.

Before giving the experimental results in detail it is important to forward evidence to confirm that the pigmented
(a) The appearance of *Catharanthus roseus* callus after 14d. incubation in darkness (right) or under low irradiance levels (left) on the standard CR medium. Please note the 'creamy' appearance of the callus. (9 cm. Petri dishes).

(b+c) Cells from a 14d. old *C. roseus* suspension culture grown in standard CR medium in the light. A considerable quantitative and qualitative variation in intracellular pigmentation was apparent within the cell population. Bar = 20 μm.
compounds examined were actually anthocyanins. The following combination of characteristics of the pigmentation observed clearly identifies the causal compounds as being anthocyanic in nature:

1. The pigmentation was red/purple in colour.
2. Microscopic observations clearly indicated the intra-cellular site of accumulation as being the vacuole.
3. The pigments were intensely UV-absorbant and the absorption spectrum (1% HCl in methanol) also had a major peak in the visible region at ca.530-540nm. (Fig.3.2.2.).
4. The intensity and colour of the pigment was strongly pH dependent. Fig.3.2.3. indicates the qualitative and quantitative changes in pigment expression in a methanolic solution of varying pH.
5. Pigmentation was stable in acidic solutions but was rapidly lost in alkaline solutions.
6. The chromatographic properties of the pigments coincided with those indicated by Harborne, (1973) for anthocyanidin glycosides.

It was thus confirmed that the compounds responsible for the coloured appearance of these cultures were anthocyanins although no attempt was made to structurally identify them. This has already been carried out using both in vivo (Forsyth and Simmonds, 1957) and in vitro Knobloch et al., 1982) grown tissues and it has been determined that three compounds are involved, petunidin, malvidin and hirsutidin, all of which are derivatives of the more common aglycone delphinidin (Fig.3.2.4.). All three of these anthocyanidins have been shown to exist in vivo
Figure 3.2.2.

The absorption spectrum (400-650nm.) of an acidified methanol (1% HCl in methanol, v/v) extract of a light-grown *C. roseus* cell culture. Acidified methanol was used as the reference blank.
Figure 3.2.3.

Quantitative and qualitative changes in the pigmentation of an acidified methanol extract of a light-grown C. roseus cell culture. A large volume of extract was initially obtained using 1% (v/v) HCl in methanol which was subsequently divided up into 25 ml. aliquots. Taking these solutions singly the pH was increased (0.01 or 0.1N KOH) to one of the values shown and the spectral properties determined 'immediately' (within 1 min.). A 'fresh' aliquot was used for each pH value.

p = plateau
Figure 3.2.4.

Molecular structures of delphinidin and three of its derivatives which are found in *C. roseus* tissues. Me = methyl (CH₃) group.
as glucosides but it has yet to be determined as to number and position of attachment of the glucose moieties (Knobloch et al., 1982).

Thus, having determined the suitability of this culture system for study and having confirmed the identity of the secondary compounds to be investigated the following series of experiments was undertaken to research into the factors influential in anthocyanin accumulation in vitro.
Section 3.2.1.

"Anthocyanin accumulation in vitro under standard cultural conditions."
The callus and cell suspension cultures used in this investigation were known to accumulate considerable levels of anthocyanins when grown in the light. However, when actively-growing or freshly subcultured cell suspensions were exposed to light a delay of several days was always observed prior to pigment appearance. Similarly, when light-grown (and thus already pigmented) cultures were subcultured an initial visible decrease in the level of anthocyanin invariably occurred, with pigmentation often reaching undetectable levels before re-appearing once again during the latter part of the subculture period. From this it was apparent that the light dependent accumulation of anthocyanin in these cultures was related to culture age and that the onset of this accumulation may be correlated with certain events in the growth cycle and/or with the nutrient status of the cells.

Accordingly, the following four experiments were performed to investigate fully the nature of the relationship between anthocyanin accumulation and growth in these cultures as related to both the onset of accumulation in recently illuminated cultures and also to the continuation of accumulation in cultures which were already pigmented as a consequence of their prior exposure to light during preceding subculture periods.

i. Studies on the growth and anthocyanin accumulation of dark-grown cell cultures of *C. roseus* when subcultured and placed in the light.

The aim of this experiment was to determine the relationship between the onset of anthocyanin accumulation and the pattern of cell division and growth in cultures of *C. roseus*
when exposed to light following a prolonged period (>3 months) of culture in darkness.

At time 0 forty-two, 100 ml. conical flasks each containing 20 ml. CR medium were inoculated with 1 ml. of a stock cell suspension to give an initial cell population density of $5 \times 10^5$ cells/ml. This stock suspension had been obtained by the pooling of two eleven-day old dark-grown stock cell cultures. The flasks, with the exception of 3 which were harvested "immediately" (1 h. post inoculation), were then placed on an orbital shaker in continuous light (see Section 2.2.3i.).

Three flasks taken at random were harvested daily for the first eight days and then on every second day for a further eight days. The remaining 3 flasks were harvested on day 23. Fresh wt., dry wt., PCV, cell population density, cell viability, anthocyanin content and the proportion of pigmented cells in these cultures were determined as detailed earlier (Section 2.2.3.). Samples of medium (5 ml.) were also taken, stored at -40°C and later analysed for nutrient content at a time when all samples could be tested together. The results are presented in Figures 3.2.5. and 3.2.6.

**Cell population density. (Fig.3.2.5(b).)**

After an initial brief lag-phase of ca. 2 days the cell population density of these cultures increased linearly (mean increase, $6.5 \times 10^5$ cells/ml./day) until day 10. Beyond this time the number of cells/ml. remained relatively constant.

**Culture growth: Fresh wt., PCV. (Fig.3.2.5(a).)**

Packed cell volume and filtered-cell fresh weight, both used as estimates of true culture fresh weight followed very similar patterns of development. Increases in both were
Figure 3.2.5.

The changes which take place subsequent to the subculture of a dark-grown \textit{C. roseus} cell culture (1 ml. inoculum : 20 ml. fresh medium). The cultures were incubated in low light levels (25\mu mol. m\textsuperscript{-2}sec\textsuperscript{-1}). Each value is a mean of 3 replicates \pm s.e.

\begin{itemize}
  \item [\(\nabla\)] Cell viability
  \item [\(\Box\)] Dry weight
  \item [\(\bigcirc\)] 'Fresh' weight
  \item [\(\nabla\)] PCV
  \item [\(\bullet\)] Cell number
  \item [\(\bigcirc\)] Proportion of visibly pigmented cells
  \item [\(\Delta\)] Anthocyanin content per culture
  \item [\(\text{OD/cult.}/10\text{ml.}\)]
\end{itemize}
Figure 3.2.5.
Figure 3.2.6.

Nutrient uptake by *C. roseus* cell cultures over a 23d. period following subculture. Values are means of 3 replicates ± s.e.

(▲) ortho-phosphate  
(■) ammonium  
(●) nitrate  
(▼) sucrose
found to precede the increase in cell population density and continued until day 16 after which a slight decline was observed. At the time when maximum cell number was reached the estimated culture fresh weight was approximately 50% of its subsequent maximum value.

Culture growth: Dry weight. (Fig.3.2.5(a).)

The pattern of dry wt. increase in these cultures was similar to that for fresh weight. Again, little or no lag phase was observed and maximum levels were reached by day 16. A more substantial decrease was however observed towards the end of the experiment.

Cell viability. (Fig.3.2.5(a).)

With the exception of a temporary decline immediately following subculture cell viability was exceptionally high in these cultures. A maximum of 97% was found at the time when the maximum cell population density was achieved and subsequent to this a slow but steady decline was observed.

Anthocyanin accumulation. (Fig.3.2.5(b).)

Anthocyanins were not detected in these cultures until the tenth day of culture. At this time less than 1% of the cells had become visibly pigmented but during the following six days this increased to ca. 10%. Although no further increase in the size of the pigmented cell population was observed the level of extractable anthocyanin continued to increase substantially over the seven day period up to day 23.

Nutrient status. (Fig.3.2.6.)

Monitoring the nutrient disappearance from the bathing medium (Fig.3.2.6.) indicated that both NH$_4^+$ and sucrose,
after an initial increase in concentration concomitant with cell death, decreased relatively steadily over the experimental period and by day 23 had reached negligible amounts. The NO$_3^-$ content of the medium was removed somewhat more rapidly after a shorter and less pronounced phase of increase and maximum uptake of this nutrient (ca. 90%) was reached on day 12-14. ϕ-Phosphate, in contrast disappeared from the medium remarkably rapidly. As much as 25% uptake was observed 1h. after inoculation and 23 h. later only negligible levels remained.

As illustrated in Fig. 3.2.5, it would appear that an inverse relationship existed between the increase in cell number and anthocyanin accumulation in these cultures. Anthocyanin pigmentation first began to appear only when the period of cell division was nearing completion. However, in this experiment it was possible that such a correlation was purely coincidental and that the observed delay in anthocyanin accumulation was simply the result of a nett illumination effect whereby a necessary period of exposure to light was required before anthocyanin accumulation was possible. In order to test this possibility the following experiment was performed.

**ii. Studies on the growth and anthocyanin accumulation of dark-grown cell cultures of *C. roseus* when the exposure to light was delayed following subculture.**

The objective of this experiment was to determine the relationship between the duration of the observed delay in anthocyanin accumulation in these cultures and the period of pre-exposure to light. To do this freshly subcultured cell suspensions were preincubated in darkness for varying lengths
of time prior to their exposure to the usual lighting conditions.

At time 0, sixty nine, 100 ml. conical flasks, each containing 20 ml. CR medium were inoculated as detailed in Section 3.2.1. The initial cell population density was $6.5 \times 10^5$ cells/ml. Three flasks were harvested immediately and the remainder were incubated under the usual conditions of illumination after 0, 5 or 10 days in darkness. As a control three flasks were retained in the dark for the full duration of the experiment. Three replicate flasks from each treatment were harvested on every second day for the first 12 days of culture and then on days 16 and 20. Measurements were made as detailed in the previous experiment. The results are presented in Fig.3.2.7.

Cell population density. (Fig.3.2.7(a).)

With the exception of a slightly shorter lag-phase the pattern of change in cell population density was similar to that found previously. Maximum cell number was reached between days 10-12 and although no significant difference between treatments was observed the cell population density of those cultures subjected to a period of darkness were generally slightly greater than those which had been grown in the light for the full duration of the experiment.

Culture growth-Fresh weight/PCV. (Fig.3.2.7(b).)

Again no significant difference in the pattern of growth was observed between the treatments and in all cases maximum levels were reached by ca. day 16.
Figure 3.2.7.

The growth and anthocyanin content of \textit{C. roseus} cell cultures following subculture. The cultures were placed in the light after 0 (circles), 5 (squares) or 10d. (triangles) in darkness. Each value is a mean of 3 replicates ± s.e.

(a) closed symbols - cell no.
open symbols - anthocyanin content/culture
(OD/cult./10ml.)

(b) closed symbols - PCV
open symbols - proportion of pigmented cells
Figure 3.2.7.
Anthocyanin accumulation. (Fig.3.2.7(a).)

Anthocyanin accumulation was detected in those cultures subjected to either 0 or 5 days of darkness from day 10 onwards. However, initial levels remained very low and were only found to increase dramatically after day 12, the time when maximum cell number was reached. Although no qualitative difference in the pattern of anthocyanin accumulation was observed between these treatments throughout the experiment, in the latter case the response was slightly reduced.

In those cultures grown in darkness for 10 days little or no delay in anthocyanin accumulation was observed with pigmentation appearing from day 12 onwards. In these cultures the levels of anthocyanin subsequently produced were closely similar to those of the cultures which had received only 5d. darkness. Finally, cultures grown in continuous darkness throughout the experiment were not found to accumulate anthocyanin at any time.

These results thus indicate that the observed delay in anthocyanin accumulation in these cultures was not the consequence of a requirement for a period of pre-exposure to light. The accumulation of anthocyanin began in illuminated cultures when the cell division phase was completed, or nearly so, irrespective of the period of preillumination.

Both the above experiment and the preceding one have been concerned with the relationship between culture growth and the onset of anthocyanin accumulation in cultures previously lacking such pigments as a consequence of the absence of the required stimulus (light). The following experiment was
thus performed using cultures which were already accumulating anthocyanins to determine if the continuation of this accumulation could also be correlated with the cessation of cell division as was found for its initiation.

iii. Studies on the growth and anthocyanin accumulation of light-grown *C.roseus* cell cultures following subculture.

The aim of this experiment was to determine the relationship between the changes in the pattern of anthocyanin accumulation and the changes in the growth and cell division in cultures already 'dedicated' to anthocyanin synthesis by their previous exposure to light.

At time 0, thirty, 100 ml. conical flasks, each containing 20 ml. CR medium were inoculated with 1 ml. of a stock cell suspension, producing an initial cell population density of 9×10^5 cells/ml. This stock suspension was obtained by pooling two, 11 day-old cell cultures which had been grown in the light for three months prior to experimentation and had regularly produced 6-8% pigmented cells at the end of each subculture period. Three flasks were harvested on day 0 and then on every second day until day 16. A final harvest was made on day 23. Measurements were made as detailed previously. The results are presented in Fig.3.2.8.

Cell population density. (Fig.3.2.8(a).)

Due to the slightly higher inoculum density used a much shorter lag-phase was observed in this experiment and the cell division phase was completed within eight days from initiation.

Culture growth "Fresh" wt./PCV. (Fig.3.2.8(a).)

Both the PCV and the filtered-cell fresh weight increased
Figure 3.2.8.

The growth and anthocyanin content of a light-grown *C. roseus* cell culture following subculture and continued exposure to light. Inoculum 1 ml.: 20 ml. fresh medium. Each value is the mean of 3 replicates ± s.e.

- (●) Cell no. ml.\(^{-1}\) x10\(^6\)
- (■) Culture fresh weight
- (◊) PCV
- (△) Total anthocyanin/culture (OD/cult./10ml.)
- (○) % pigmented cells
- (▲) Total pigmented cell no./culture
Figure 3.2.8.
at a relatively steady rate for the first 12-14 days following subculture after which levels underwent little change.

*Anthocyanin accumulation.* *(Fig.3.2.8(b).)*

During the first 8 days of culture both the percentage and the total number of visibly pigmented cells decreased, the former from 5.9% to 0.4% and the latter from $5.5 \times 10^4$ cells/ml. to $1.7 \times 10^4$ cells/ml. By day 8 the cultures had become almost pure white in colour. After this time anthocyanins accumulated rapidly, the cultures once again became visibly pigmented and by day 16 maximum levels of pigmentation had been reached. However, in terms of the total amount of extractable anthocyanin/culture no statistically significant change was observed until day 8, by which time a slight enhancement was found. Levels then continued to increase rapidly for a further 6-8 days.

Two important points arise from these results concerning the accumulation of anthocyanin by these cultures. Firstly, the visible changes observed in the pigmented cell population can once again be correlated with the pattern of cell division in these cultures. The time of the cessation of cell division again appears as a turning point in the observed pattern of accumulation. Secondly, it is clear from the results that upon subculture of a cell suspension already accumulating anthocyanins the nett accumulation is initially halted, with levels remaining unaltered for a time coincident with the period of cell division. Therefore the observed decrease in visible levels of pigment during this time cannot be due to anthocyanin degradation and consequently must result from pigment dilution within the cells to visibly undetectable levels brought about by divisions within the pigmented cell
population. (The absence of an initial increase in pigmented cell number, as might have been expected following early cycles of cell division, would have resulted from the large number of very weakly-coloured cells present in the original cell population (see Section 3.2.8.) which after one cycle of division would have ceased to be visibly coloured and would subsequently not have been counted.)

The preceding three experiments have all been concerned with the accumulation of anthocyanin by \textit{C.roseus} cultures when exposed to light. As stated in Section 3.2.1(ii) it became clear that the presence of light was essential for the onset of anthocyanin accumulation in this system. The remaining experiment in this section was designed to investigate whether the presence of light remained as a necessity for the continuation of this accumulation.

\textit{iv. Studies on the growth and anthocyanin accumulation in light-grown cell cultures of \textit{C.roseus} when subcultured and incubated in darkness.}

This experiment was carried out to determine if the accumulation of anthocyanin by these cultures remained light-dependent after the initial stimulus had been given during a previous subculture period.

At time 0 thirty three, 100 ml. conical flasks each containing 20 ml. CR medium were each inoculated with 1 ml. of a light-grown stock cell suspension, giving an initial cell population density of \(6.7 \times 10^5\) cells/ml. Fifteen flasks were placed in the light and 15 in darkness. Harvesting took
place on day 0 and subsequently on every fourth day until day 20. Measurements were made as described previously. The results are presented in Fig.3.2.9.

**Cell population density.** *(Fig.3.2.9(a)).*

In this experiment no significant difference was observed between the cell population densities of the two treatments. In both instances maximum cell number was reached between days 8 and 12.

**Culture growth.** "Fresh" wt./PCV. *(Fig. 3.2.9(a)).*

As with cell number both treatments showed very similar patterns of growth over the experimental period. However, in this experiment growth was atypical in that maximum levels were not reached within the 20 day experimental period.

**Anthocyanin content.** *(Fig.3.2.9(b)).*

In those cultures grown in the light the pattern of anthocyanin accumulation followed closely to that found previously *(Fig.3.2.9(b)).* with the exception that maximum levels were not achieved within the period of the experiment. In the dark-grown cultures the proportion of visibly pigmented cells decreased during the cell division phase in a similar manner to that of cultures grown in the light. However, in this treatment no subsequent increase was observed once the minimum level had been reached on day 8 (0.28%). Irrespective of these visible changes, no significant alteration in the total amount of extractable anthocyanin/culture was observed throughout the 20 days of the experiment.

The results of this experiment thus demonstrate the continued requirement for light by these cultures for the
Figure 3.2.9.

Changes in the growth and anthocyanin content of a light-grown *C. roseus* cell culture following subculture (1 ml. inoculum : 20 ml. fresh medium). The cultures were either grown in the light (open symbols) or were placed in darkness immediately following subculture (closed symbols). Each value is the mean of 3 replicates ± s.e.

- ○ and • Cell no. ml\(^{-1}\) x 10\(^6\)
- ▼ and ▼ Total anthocyanin content/culture (OD/cult./10ml.)
- □ and ■ PCV
- △ and ▲ Proportion of pigmented cells
Figure 3.2.9.
process of anthocyanin accumulation. When cultures already accumulating anthocyanins were subsequently deprived of light no further enhancement in pigment level occurred.

It is interesting to note that when conditions did not favour the accumulation of anthocyanin (e.g. in darkness, or during the period immediately following subculture) the existing level of pigment remained unaltered. This would suggest either that no turnover of anthocyanins occurred in this system, or that on these occasions anthocyanin synthesis was always balanced by its degradation.
The following notable points have arisen from the results reported in this section.

(1) Anthocyanin accumulation in these cultures was light-dependent.

(2) When grown in the light, cultures initiated from dark-grown or light-grown stocks had very similar patterns of development.

(3) Of the essential nutrients examined 6'-phosphate was taken up most rapidly (max. uptake 24h.). Maximum uptake of \( \text{NO}_3^- \) was first observed on day 14, sucrose on day 23 and \( \text{NH}_4^+ \) on day 20.

(4) The delay in anthocyanin accumulation observed following subculture could not be correlated with the time of preexposure to light although a correlation may exist between the former and the duration of the cell division phase.

(5) Cells which accumulated pigments in the latter stages of the culture growth cycle were capable of undergoing further division when the growth conditions were altered accordingly.

(6) The apparent loss of pigmentation at certain times (eg. immediately following subculture, or when cultures were returned to darkness) was the result of anthocyanin 'dilution' within the cell population and not its degradation.
Having determined the pattern of anthocyanin accumulation under standard cultural conditions the following groups of experiments were performed to determine how this accumulation was altered when cultural conditions were modified. It was hoped that these experiments would provide us both with a means to enhance anthocyanin yields and also with some understanding as to how this increase comes about.
Section 3.2.2.

"The influence of inoculum density on growth and anthocyanin accumulation in *C. roseus* cell cultures."
In the first section of this 2nd part of the Results chapter, accounts of four experiments were given concerning the pattern of anthocyanin accumulation in *C. roseus* liquid cultures. As indicated in the first experiment (3.2.1i.) it was found that a delay in anthocyanin accumulation was observed immediately following subculture which may be the result of a requirement by these cultures for a period of pre-exposure to light. To determine if this was the case two experiments were performed one of which, involving preincubation of the cultures in darkness prior to illumination has already been reported (3.2.1ii.). The other experiment however, (actually performed first) gave rather unexpected results which, it was thought, were worthy of further investigation.

In this experiment it was aimed to shorten the period of cell division and reduce the overall time taken to reach maximum cell number by increasing the initial inoculum density of the cultures. However, in association with achieving this desired aim a very different pattern of growth was observed and anthocyanins were not accumulated at any time. Consequently, as considerable emphasis has been placed in this investigation upon studying the possible relationship between culture growth/cell division and secondary metabolite (anthocyanin) accumulation in this experimental system three further experiments were designed to investigate the basis of this effect. The results of these experiments and of the preliminary one are presented in this section.
A time-course study of growth and anthocyanin accumulation in *C. roseus* cell cultures when initiated at a high inoculum density.

In this experiment it was aimed to alter the growth pattern of *C. roseus* cell cultures by increasing the original inoculum density in order to determine if the observed delay in anthocyanin accumulation was the consequence of a light effect.

Conical flasks (100 ml.) containing 20 ml. CR medium were inoculated with 3 ml. of a 12d. old dark-grown stock suspension, which resulted in an initial cell population density of ca. $2 \times 10^6$ cells/ml. The cultures were then incubated in the light and measurements (3 replicates) of culture growth were made at suitable intervals as described previously (Section 3.2.1i). The experiment was terminated on day 12 for reasons to be given later. The results are presented graphically in Fig.3.2.10.

Cell population density.

As has been found for other systems the use of a relatively high inoculum density resulted in a very short (or possibly non-existent) lag phase and the subsequent maximum cell number was reached within the much shorter time of 4 days. Typically, the peak value (ca.$3.5 \times 10^6$ cells/ml.) was also somewhat less than was generally found in cultures initiated with a small inoculum (>4.5$ \times 10^6$ cells/ml.).

Culture growth. (Fresh wt., PCV.).

Estimates of culture fresh weight indicated that during the period of cell division, growth (fr.wt. increase) proceeded in a similar manner to that which had been observed when using a small inoculum. (see Section 3.2.1i.). Consequently, on
Figure 3.2.10.

A time-course study of the growth and anthocyanin content of a *C. roseus* cell culture initiated with 3 x standard inoculum size. Each value represented the mean of 3 replicates ± s.e.

(φ) Cell viability  (•) Cell number
(□) Culture fresh st.  (✓) PCV
(■) Culture dry wt.  (○) Proportion pigmented cells
day 4 culture fresh weight was very similar to that found for the equivalent cell number in this previous experiment. However, in contrast to the standard inoculum experiment, no subsequent enhancement of culture fresh weight was observed after the cell division phase was complete. Indeed, by day 12 levels had actually undergone a considerable decline.

*Culture growth, (dry weight.)*

Although slightly greater variation in culture dry weight was found it is apparent from the results that values continued to increase until day 6. However, as with culture fresh weight, levels then proceeded to decline.

*Cell viability.*

The fluorescein diacetate test once again demonstrated the normally high level of cell viability in these cultures. The initial decline in cell viability, as observed when using a small inoculum (Section 3.2.1i.) was absent from these cultures and the percentage of viable cells in the total cell population, as measured up to day 7 was always ca. 90%. However, subsequent to day 7 a rapid decrease was observed, indicative of the onset of culture senescence and as a result, the experiment was terminated on day 12.

*Anthocyanin content.*

Accumulation of anthocyanin by these cultures was not observed at any time during this experiment.

In this thesis considerable emphasis has been placed upon studying the possible associations between the accumulation of secondary metabolites (anthocyanins) and the culture growth
cycle. In the above experiment the use of a larger than standard volume of inoculum has resulted in a very different pattern of culture growth in association with a total absence of the usual anthocyanin accumulation response. Before investigating this effect further this interesting and potentially very important result was tested for reproducibility in a second, more elaborate experiment carried out as follows.

ii. The growth and accumulation of anthocyanins in *C. roseus* cell cultures initiated using different volumes of inocula.

In this experiment 3 sets of 3 conical flasks (100 ml./20 ml. CR medium) were each inoculated with different volumes of a stock suspension to further test the effect of inoculum size on the subsequent culture growth and anthocyanin accumulation.

20 ml. cultures were initiated using either 1.5 ml., 3 ml. or 4.5 ml. of an 11 day old dark-grown stock suspension culture to give initial cell population densities of ca.6x10^5, 1.2x10^6 and 1.8x10^6 cells/ml. In order to introduce a constant volume into each flask 3, 1.5 or 0 ml. of filter-sterilized distilled water (pH 5.8) was added as appropriate. All flasks were incubated in the light for the duration of the experiment and harvesting took place 14 days post inoculation. This time was chosen as a compromise between the risk of considerable culture senescence taking place in the high inoculum treatments and the usual time required for the control flasks to produce maximum levels of anthocyanins (16-20 days).
The results are presented in Table 3.2.1. As found in the previous experiment cell viability tests indicated that senescence had again begun earlier in the higher inoculum cultures. Nevertheless, cell viability was still reasonably high at the time of harvesting.

As can be seen from the results, in both cases where a larger than standard inoculum had been used the final cell population density was reduced. In the control flasks the levels attained were ca. 20-25% greater than those for the other two treatments. Similarly, culture dry weight was also significantly reduced in these cultures, by approximately the same amount (25-30%). However, in contrast culture fresh weight was more drastically affected and levels in both high inoculum treatments were reduced by some 50-60% over the control cultures.

Anthocyanin accumulation, in accordance with the relatively early termination of the experiment was less than is generally found in control (standard inoculum) cultures. Nevertheless with levels at ca. 50% of the usual maximum level accumulation was clearly well under way. In contrast, the amounts of extractable anthocyanin in both of the high inoculum treatments remained negligible.

From both of the preceding experiments it has become clear that increasing the volume of inoculum by as little as two times the standard value had a considerable physiological effect on these cultures. Growth was significantly reduced particularly with respect to culture fresh weight, apparently as a result of the absence of the cell expansion phase of the
## Table 3.2.1.

The influence of inoculum size upon the subsequent growth and anthocyanin accumulation in illuminated *C. roseus* cell cultures. Harvesting: day 14. Each value is the mean of 3 replicates ± s.e.

<table>
<thead>
<tr>
<th>Inoculum size (ml.)</th>
<th>CONTROL</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial cell density</td>
<td>(1.5ml H₂O)</td>
<td>(1.5ml H₂O)</td>
</tr>
<tr>
<td>(cells/ml.)</td>
<td>1.5</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>6.1x10⁵</td>
<td>1.23x10⁶</td>
</tr>
<tr>
<td>Cell no. (cells/ml.x10⁻⁶)</td>
<td>6.3</td>
<td>5.02***</td>
</tr>
<tr>
<td></td>
<td>±0.10</td>
<td>±0</td>
</tr>
<tr>
<td>% PCV</td>
<td>46.3</td>
<td>19.0***</td>
</tr>
<tr>
<td>Fresh wt. (g.)</td>
<td>4.11</td>
<td>1.63**</td>
</tr>
<tr>
<td></td>
<td>±0.62</td>
<td>±0.15</td>
</tr>
<tr>
<td>Dry wt. (g.)</td>
<td>0.180</td>
<td>0.134*</td>
</tr>
<tr>
<td></td>
<td>±0.008</td>
<td>±0.007</td>
</tr>
<tr>
<td>Cell viability (%)</td>
<td>90.0</td>
<td>75.5*</td>
</tr>
<tr>
<td>Pigmented cell population (%)</td>
<td>4.1***</td>
<td>0.1***</td>
</tr>
<tr>
<td>Anthocyanin content</td>
<td>0.30</td>
<td>0.01***</td>
</tr>
<tr>
<td>OD/g⁻¹(fr.wt.)/10ml.</td>
<td>±0.05</td>
<td>±0.01</td>
</tr>
</tbody>
</table>
culture growth cycle. Anthocyanin accumulation was also absent under these conditions. It was not possible however, to determine from the experiments performed whether these effects were the result of simply having a larger initial cell population or whether they arose due to the larger transfer of spent medium. Consequently, the two remaining experiments in this section were performed to investigate this.

iii. The influence of cell washing on culture growth and anthocyanin accumulation when using high density inocula.

In this experiment C.roseus cell cultures were initiated by inoculation with either washed or unwashed cells. The former were transferred in water and the latter in spent medium in order to investigate the influence of spent medium and increased cell inocula on the subsequent growth of the cultures.

Two 12 day old dark-grown stock cell suspensions provided the source material for initiating the cultures used in this experiment. These were pooled, thoroughly mixed and then divided to give two uniform cell stocks. One half was subsequently gently centrifuged (500xg, 3 min.) after which the supernatant was removed and the pellet thoroughly resuspended in 30 ml. sterile distilled water (pH 5.8). This procedure was repeated a further two times before finally resuspending the pellet in sufficient of the sterile water to return the cell population density to its original level. The second half of the stock suspension was used directly.

Two sets of 3 flasks (100 ml./20 ml. CR medium) were inoculated with either 1.5 ml. or 3 ml. of the washed cell
suspension and a further two sets with 1.5 ml. or 3 ml. of the unwashed cell suspension. Sterile distilled water (1.5 ml., pH 5.8) was added, where appropriate, to maintain equal volumes in all flasks. The flasks were then incubated in the light and after 16 days the experiment was terminated and measurements taken.

The results are presented in Table 3.2.2. The effects of increasing the inoculum size were again clear from this experiment. In the cultures initiated with twice the standard inoculum both cell number and culture dry weight were again reduced by ca. 20%, fresh weight by ca. 50% and with anthocyanin levels remaining negligible in comparison to the control (6x10^5 cells/ml. inoculum). These results are however very much in contrast to those for the cultures inoculated with double the standard number of cells alone, in the absence of any medium transfer.

From the results of the washed cell control (6.8x10^5 cells/ml. inoculum) it is evident that the washing procedure had no detrimental effect on the subsequent growth of the cultures. Values obtained in all cases were very similar to those for the unwashed control (6.2x10^5 cells/ml. inoculum) with the exception of the cell population density which was actually slightly increased. When double the inoculum of these cells was used this was found not to alter the culture development in any way. No significant difference was detected between any of the estimates of culture growth, cell viability or anthocyanin content in comparison to either controls (again with the exception of cell number in the unwashed control).

These results would thus suggest that the observed changes
<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial cell density (cells ml⁻¹)</td>
<td>6.2×10⁵ 1.45×10⁶ 6.8×10⁵ 1.4×10⁶</td>
</tr>
<tr>
<td>Treatment</td>
<td>UNWASHED UNWASHED WASHED WASHED</td>
</tr>
<tr>
<td>Cell no. (cells/ml×10⁻⁶)</td>
<td>6.18 4.85** 7.7 7.7</td>
</tr>
<tr>
<td></td>
<td>±0.04 ±0.27 ±0.43 ±0.30</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>38.8 20.5*** 43.7 36.6</td>
</tr>
<tr>
<td>Fresh wt. (g.)</td>
<td>3.812 1.845** 3.569 3.568</td>
</tr>
<tr>
<td></td>
<td>±0.051 ±0.250 ±0.186 ±0.301</td>
</tr>
<tr>
<td>Dry wt. (g.)</td>
<td>0.200 0.163* 0.225 0.217</td>
</tr>
<tr>
<td></td>
<td>±0.011 ±0.011 ±0.010 ±0.001</td>
</tr>
<tr>
<td>Cell viability (%)</td>
<td>92.0 80.0* 94.5 89.8</td>
</tr>
<tr>
<td>Proportion pig.cells (%)</td>
<td>8.1 0.4*** 8.33 8.13</td>
</tr>
<tr>
<td>Anthocyanin content (OD/g.(fr.wt.)/10ml.)</td>
<td>0.50 0.02*** 0.59 0.62</td>
</tr>
<tr>
<td></td>
<td>±0.02 ±0.02 ±0.06 ±0.06</td>
</tr>
</tbody>
</table>

Table 3.2.2.

The growth and anthocyanin content of 16d. old illuminated *C.roseus* cell cultures initiated with either washed or unwashed cell stocks at two different cell population densities. Significance levels as Table 3.2.1.
in culture growth following the use of double (or greater) the standard inoculum were not due to the transfer of more than the standard number of cells but were the consequence of transferring extra spent medium. The experiment needed to confirm this is now reported.

iv. The influence of spent medium addition on the subsequent growth of *C. roseus* cell cultures.

The aim of this experiment was to confirm that the previously described effects on culture growth resulting from increasing the inoculum size was a medium rather than a cellular effect.

Two 12 day old dark-grown stock suspensions were pooled and from this was taken a 20 ml. sample. That which remained was then 'washed' as described previously before again resuspending the cell pellet in sufficient sterile distilled water (pH 5.8) to return the cell population density to its original level. Three conical flasks (100 ml./20 ml.CR medium) were then inoculated with 1.5 ml. of the unwashed stock and six with 1.5 ml. of the washed stock. It had been determined by filtration that in the unwashed stock 1 ml. of cell suspension contained 0.5 ml. spent medium and consequently to three of the 'washed cell' cultures was added 0.75 ml. spent medium (obtained from the initial centrifugation). In the remaining treatment 3x1.5 ml. samples of washed stock suspension were gently centrifuged (500xg, 3 min.) and having removed the supernatant the cells were resuspended in 1.5 ml. (2x standard) spent medium. These samples were then used to inoculate a further three flasks. In all cases where necessary, 0.75 ml.
sterile distilled water (pH 5.8) was added to make up the total volume of liquid added to each flask to 1.5 ml. All 12 cultures were then incubated in the light for 16 days before harvesting.

The results can be found in Table 3.2.3.

Cell population density.

Although a lower cell population density was observed in those flasks inoculated with washed cells in addition to either 0 or 0.75 ml. of spent medium as compared with the unwashed control, this was not found to be significant. However, a statistically significant decrease was found between the cell population densities of these cultures and those given double the standard volume of spent medium.

Culture growth, 'fresh' weight/PCV.

Both estimates of culture fresh weight indicated that in those cultures given double quantities of medium a significant decrease (>40%) was present over all other treatments, the values for which were closely similar.

Culture growth, dry weight.

Similarly, for culture dry weight the only significant difference found was between the result for the 'double medium' treatment and all the others. In this instance however, values were reduced by only ca.20%.

Anthocyanin content.

The accumulation of anthocyanin by the control cultures in this experiment had proceeded remarkably well. Circa 13% of the cells had become visibly pigmented by the end of the experiment and once again, of all the treatments tested levels
<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>UNWASHED</th>
<th>WASHED</th>
<th>WASHED</th>
<th>WASHED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial cell density (cells/ml.)</td>
<td>$6.1 \times 10^5$</td>
<td>$6.1 \times 10^5$</td>
<td>$6.1 \times 10^5$</td>
<td>$6.1 \times 10^5$</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vol. medium added (ml.)</td>
<td>0.75</td>
<td>0</td>
<td>0.75</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Vol. water added (ml.)</td>
<td>0.75</td>
<td>1.5</td>
<td>0.75</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cell no. (cells/ml. $\times 10^{-6}$)</td>
<td>6.4</td>
<td>5.7</td>
<td>5.5</td>
<td>3.9**</td>
<td></td>
</tr>
<tr>
<td>PCV (%)</td>
<td>42.9</td>
<td>45.1</td>
<td>43.9</td>
<td>26.2**</td>
<td></td>
</tr>
<tr>
<td>Fresh wt. (g.)</td>
<td>4.454</td>
<td>4.531</td>
<td>4.496</td>
<td>2.560*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\pm 0.108$</td>
<td>$\pm 0.219$</td>
<td>$\pm 0.252$</td>
<td>$\pm 0.507$</td>
<td></td>
</tr>
<tr>
<td>Dry wt. (g.)</td>
<td>0.209</td>
<td>0.199</td>
<td>0.205</td>
<td>0.168*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\pm 0.008$</td>
<td>$\pm 0.018$</td>
<td>$\pm 0.011$</td>
<td>$\pm 0.015$</td>
<td></td>
</tr>
<tr>
<td>Proportion pig. cells (%)</td>
<td>13.4</td>
<td>13.3</td>
<td>12.4</td>
<td>3.8***</td>
<td></td>
</tr>
<tr>
<td>Anthocyanin content (OD/g.(fr.wt.)/10ml.)</td>
<td>1.096</td>
<td>1.126</td>
<td>1.076</td>
<td>0.179***</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\pm 0.062$</td>
<td>$\pm 0.086$</td>
<td>$\pm 0.112$</td>
<td>$\pm 0.027$</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2.3.

The influence of spent medium upon the subsequent growth and anthocyanin content of illuminated *C.roseus* cell cultures. Significance levels as Table 3.2.1.
were only significantly altered in the 'double medium' cultures. In this treatment the anthocyanin content was substantially reduced with levels reaching less than 20% of the control values.

It is clear from these results that the changes brought about by the increase in the volume of transferred medium alone, in the absence of any alteration in the initial cell population density resembled closely those observed when both had been increased together. It follows therefore that it was the extra transfer of spent medium, rather than the extra transfer of cells which was responsible, at least in the large part, for the effects observed.
The following points have emerged from the experiments described in this section.

(1) Increasing the inoculum size very significantly affected the subsequent development of these cultures.

(2) Although growth, in terms of either cell no. or total culture dry weight were both significantly reduced by this treatment the greatest effect found was on culture fresh weight which fell to ca. 50% of the control level apparently as a result of the almost total absence of the cell expansion phase of the culture growth cycle.

(3) Anthocyanin accumulation was not observed in cultures initiated with double (or greater) the standard inoculum size.

(4) It has been determined that all of these effects can be induced by transferring extra spent medium at the time of inoculation, but not by increasing the initial cell number/culture.

In the next section the experiments reported were carried out to determine the effect of altering the cultural conditions, both physical and chemical, on anthocyanin accumulation in this culture system.
Section 3.2.3.

"Anthocyanin accumulation in \textit{C. roseus} cultures under modified conditions".

The experiments described in the previous sections concerned the possible correlative associations between the events in the culture growth cycle, in particular with respect to anthocyanin accumulation. One feature of all these experiments, the importance of which has not yet been touched upon, was that the anthocyanin response involved, as far as was discernable from microscopic observations, only a very small proportion (ca. 10%) of the total cell population. As the basic theme of this work was to investigate the factors influential in the accumulation of secondary metabolites *in vitro* it is clearly critical that an understanding as to why this was so was realised. Should this phenomenon occur in other systems, and there is evidence to suggest that it does [see Appendix, Yamakawa *et al.* (1982), Colijn *et al.* (1981)] then clearly there is enormous potential for enhancing productivity "simply" by increasing the proportion of cells within the total population which contribute to the overall metabolite yield.

Accordingly, a number of experiments were undertaken to investigate the influence of certain cultural conditions (growth substance complement, light) on anthocyanin yield and also two 'inductive' media, shown to be effective in other systems, were tested for their ability to enhance productivity in these cultures. In these experiments it was hoped that, by exploiting the visible properties of anthocyanins, it would be possible to determine how the above factors were actually influencing the population of productive cells within the culture.
i. The influence of certain selected growth substance combinations on the growth and anthocyanin accumulation of C.roseus callus and cell cultures.

There are many reports in the literature concerning the influence of plant growth substances on anthocyanin accumulation in *in vitro* systems (see Ch.1.). Although the results are far from consistent between the different species tested, it is clear that these compounds exert considerable influence on this process. Consequently, both this experiment and the one following were performed to determine if the anthocyanin yield (particularly with respect to the proportion of productive cells) could be improved in the culture system used in this investigation by altering the balance of growth substances supplied in the nutrient medium.

Six different growth substance combinations, involving 2,4-D, NAA, Kinetin and coconut milk were tested in this experiment (see Table 3.2.4. for details). Three, 5 cm. Petri dishes containing 10 ml. medium were prepared for each treatment and all were inoculated with 0.25 g. of a well-mixed 11 day old light-grown callus stock, taking care to spread the callus evenly over the agar surface. All cultures were then incubated in the light for 18 days before harvesting.

The results may be found in Table 3.2.4. It is apparent that despite the considerable qualitative variation in the growth substance combinations tested, no significant difference in culture growth (fresh or dry weight) was found between treatments. Indeed surprisingly, most growth was actually observed in those cultures from which all exogenously supplied growth substances were withheld.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fresh wt.</th>
<th>Dry wt.</th>
<th>Anthocyanin content/g. fr.wt.</th>
<th>Anthocyanin content/culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>2μM. 2,4-D (CONTROL)</td>
<td>2.809</td>
<td>0.099</td>
<td>0.53</td>
<td>1.49</td>
</tr>
<tr>
<td>+8% C.milk</td>
<td>±0.196</td>
<td>±0.003</td>
<td>±0.01</td>
<td>±0.08</td>
</tr>
<tr>
<td>2μM. 2,4-D</td>
<td>2.387</td>
<td>0.113</td>
<td>0.21***</td>
<td>0.56**</td>
</tr>
<tr>
<td>+1μM. Kinetin</td>
<td>±0.225</td>
<td>±0.004</td>
<td>±0.05</td>
<td>±0.04</td>
</tr>
<tr>
<td>1μM. NAA</td>
<td>2.879</td>
<td>0.123</td>
<td>0.16***</td>
<td>0.51***</td>
</tr>
<tr>
<td></td>
<td>±0.502</td>
<td>±0.019</td>
<td>±0.04</td>
<td>±0.18</td>
</tr>
<tr>
<td>1μM. Kinetin</td>
<td>2.616</td>
<td>0.110</td>
<td>0.23***</td>
<td>0.62**</td>
</tr>
<tr>
<td></td>
<td>±0.295</td>
<td>±0.004</td>
<td>±0.07</td>
<td>±0.25</td>
</tr>
<tr>
<td>0</td>
<td>3.157</td>
<td>0.126</td>
<td>0.23***</td>
<td>0.74**</td>
</tr>
<tr>
<td></td>
<td>±0.328</td>
<td>±0.008</td>
<td>±0.03</td>
<td>±0.16</td>
</tr>
</tbody>
</table>

Table 3.2.4.

The growth and anthocyanin accumulation of C. roseus callus cultures after incubation in the light for 18d. on CR medium supplemented with different growth substance combinations.

1 expressed in OD units/g./10ml.  2 OD units/cult./10ml.

Significantly different from the controls at * P=0.05, ** P=0.01, *** P=0.001.
Considerable differences were however found in the anthocyanin content of these cultures. For example, it is clear that the inclusion of cytokinins (kinetin, coconut milk) in the medium was detrimental to anthocyanin accumulation by these cultures. Similarly, poor levels of anthocyanin were found in cultures grown on media in which 2,4-D was omitted or substituted by NAA. Consequently, it was found that the best yield was actually effectuated by the control medium itself, which contained 2 μM 2,4-D alone.

As it had been anticipated that these treatments would alter culture growth as well as anthocyanin content it was decided to repeat part of this experiment, this time using liquid cultures in order to determine if similar results were obtained and also to permit easy examination of the cell population density which is somewhat more difficult in callus cultures. The results of this experiment are presented in Table 3.2.5., the legend of which contains the experimental details.

In these cultures it was found that, in the absence of 2,4-D, growth (fresh wt. or dry wt.) was only slightly reduced. However, from the cell number data it was evident that this must have resulted from considerable extra cell expansion as the cell population densities of these cultures were very much less than that for the 2,4-D control. This was indeed evident from microscopical observations which revealed that cultures grown in 2,4-D-free media, either in the presence or absence of kinetin, contained visibly larger cells, some of which reached >1 mm. in length (Fig.3.2.11.). Reduced viability was also observed in these cultures.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>2μM. 2,4-D</th>
<th>O</th>
<th>1μM. Kinetin</th>
<th>2μM. 2,4-D +1μM. Kinetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell no. ml⁻¹</td>
<td>6.26 ±0.57</td>
<td>2.40***</td>
<td>1.89***</td>
<td>8.47*</td>
</tr>
<tr>
<td>x10⁻⁶</td>
<td></td>
<td>±0.25</td>
<td>±0.36</td>
<td>±1.22</td>
</tr>
<tr>
<td>Cell viability (%)</td>
<td>82 ±0.297</td>
<td>54**</td>
<td>72*</td>
<td>92*</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>38.0 ±0.09</td>
<td>36.5</td>
<td>37.2</td>
<td>40.2</td>
</tr>
<tr>
<td>Fresh wt. (g)</td>
<td>3.034 ±0.297</td>
<td>2.547</td>
<td>2.774</td>
<td>3.837*</td>
</tr>
<tr>
<td></td>
<td>±0.230</td>
<td></td>
<td>±0.076</td>
<td>±0.085</td>
</tr>
<tr>
<td>Dry wt. (g)</td>
<td>0.110 ±0.009</td>
<td>0.093</td>
<td>0.099</td>
<td>0.139*</td>
</tr>
<tr>
<td></td>
<td>±0.004</td>
<td></td>
<td>±0.009</td>
<td>±0.004</td>
</tr>
<tr>
<td>% Pig. cells</td>
<td>5.2 ±0.005</td>
<td>0.1***</td>
<td>0.7***</td>
<td>2.6*</td>
</tr>
<tr>
<td>Anthocyanin cont.</td>
<td>0.424 ±0.005</td>
<td>0.010***</td>
<td>0.010***</td>
<td>0.151**</td>
</tr>
<tr>
<td>OD/g.(fr.wt.)/10 ml.</td>
<td>±0.005</td>
<td>±0.002</td>
<td>±0.071</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2.5.

The growth, anthocyanin content and cell viability of C. roseus cell cultures grown for 18d. in the light. The 20ml. cultures (3 per treatment) were initiated with 1ml. of a 12d old dark-grown stock and then incubated under standard conditions. The media (modified CR) were supplemented with the above growth substance combinations which were added prior to autoclaving.

Significant from the control at * P=0.05, ** P=0.01, *** P=0.001.
Figure 3.2.11.

Three typical cells from an 18d. old *C. roseus* cell culture which had been incubated in the light in a medium lacking 2,4-D supplementation. A very large increase in the volume of many cells was observed. Bar = 100 μm.
1 μM. kinetin, when added in association with 2 μM. 2,4-D was found to significantly increase the final cell population density although increases in culture fresh and dry weights were only slight. The anthocyanin content of these cultures was however significantly reduced to <50% of the control level.

The levels of anthocyanins accumulated in the 2,4-D-free treatments were negligible and consequently the standard CR medium containing 2 μM. 2,4-D, used as the control, once again proved to be the most productive in terms of metabolite yield.

The following experiment was performed to determine if the yield of anthocyanins obtained on the standard CR medium could be further improved by altering the level of exogenously applied 2,4-D or by using IAA as an alternative auxin supplement.

ii. The influence of IAA and 2,4-D on the growth and anthocyanin accumulation of *C. roseus* callus cultures.

Of the treatments tested in the previous two experiments none were able to improve upon the levels of anthocyanin accumulated by cultures grown on the standard 2 μM. 2,4-D-supplemented medium. As well as testing IAA as a possible substitute for 2,4-D, this experiment was also carried out to determine if anthocyanin yield could be improved by modifying the level of 2,4-D applied.

Culture dishes were set up as detailed in the previous subsection using media containing either 2,4-D at levels 0.2,
2.0 or 20 μM., or 1 μM. IAA (the latter was filter sterilized and added immediately before pouring the plates). All plates were inoculated with 0.25 g. callus taken from a well mixed, 11 day old, light-grown stock culture, and which was spread evenly over the agar surface. After incubation in the light for 18 days the cultures were harvested and measurements made.

From the results listed in Table 3.2.6., it is apparent that once again the standard 2 μM. 2,4-D medium yielded the greatest amounts of anthocyanin. Increasing the level of 2,4-D to 20 μM. was found to inhibit both growth and anthocyanin accumulation and although reducing the level to 0.2 μM. resulted in slightly enhanced growth the anthocyanin yield was once again substantially reduced. In the final treatment where IAA was used, growth (fresh wt., dry wt.) was again slightly increased but the anthocyanin content was even more severely affected than before, falling to <25% of the maximum level obtained.

Therefore of all the growth substance modifications tested none were successful in improving the anthocyanin yield. The standard medium, containing 2 μM. 2,4-D, routinely gave the best results and was therefore retained as the standard (control) medium for all subsequent experiments.

iii. The influence of the level of irradiance on the yield of anthocyanin by *C. roseus* tissue and cell cultures.

It has clearly been demonstrated (Section 3.2.1.) that the accumulation of anthocyanin by these cultures is under the influence of an essential stimulus, light. It may therefore be possible to improve anthocyanin yield by increasing
### Table 3.2.6.

The growth and anthocyanin content of *C. roseus* callus cultures grown in the light for 18d. on media containing differing amounts of 2,4-D or 1AA.

Significantly different from the controls at * P=0.05, ** P=0.01, *** P=0.001.
the intensity of the stimulus normally used. Consequently, two preliminary experiments were carried out using cultures initiated with either dark-grown or light-grown cell inocula and incubated in two different light levels to determine if an enhancement of metabolite yield was possible using this method. Two further experiments were then performed to examine more closely the influence of the level of incident light on anthocyanin accumulation in this system.

Petri dishes (5 cm.), each containing 10 ml. CR medium were inoculated with 0.25 g. cell samples taken from a well-mixed 11 day old light or dark grown callus stock culture. After sealing, the plates were then placed either in standard lighting conditions (30 μmol./m²/sec.) or in enhanced conditions (130 μmol./m²/sec.) for 18 days before harvesting. To achieve the different light levels the plates were placed at different distances away from the same light source. This did not result in any change in the incubation temperature.

The results for the experiment set up using light-grown callus are presented in Table 3.2.7. and those using dark-grown callus in Table 3.2.8. From the results presented in Table 3.2.7. it is apparent that increasing the irradiance level did not affect culture growth (fresh, dry wts.). The anthocyanin content was however substantially increased by this treatment, with the amount of extractable anthocyanin from the 'high light' cultures reaching over 3 times that for the control. The proportion of visibly pigmented cells was also significantly increased, by approximately 2 times. In those cultures where dark-grown cells had been used for the inocula (Table 3.2.8.) the results were very similar. No alteration
<table>
<thead>
<tr>
<th>Irradiance level</th>
<th>30$\mu$mol.m$^{-2}$sec$^{-1}$</th>
<th>130$\mu$mol.m$^{-2}$sec$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh weight</td>
<td>2.058</td>
<td>1.646</td>
</tr>
<tr>
<td>(g)</td>
<td>±0.184</td>
<td>±0.210</td>
</tr>
<tr>
<td>Dry weight</td>
<td>0.079</td>
<td>0.057</td>
</tr>
<tr>
<td>(g)</td>
<td>±0.009</td>
<td>±0.009</td>
</tr>
<tr>
<td>Anthocyanin content</td>
<td>0.465</td>
<td>1.586***</td>
</tr>
<tr>
<td>OD/g.(fr.wt.)/10 ml.</td>
<td>±0.049</td>
<td>±0.22</td>
</tr>
<tr>
<td>Total anthocyanin content</td>
<td>0.970</td>
<td>2.596**</td>
</tr>
<tr>
<td>OD/culture/10 ml.</td>
<td>±0.123</td>
<td>±0.361</td>
</tr>
<tr>
<td>% Pig. cells</td>
<td>7.7</td>
<td>17.4***</td>
</tr>
</tbody>
</table>

Table 3.2.7.

The influence of irradiance level upon the growth and anthocyanin accumulation of *C.roseus* callus cultures initiated with material from a light-grown stock culture. Harvest time - 18d. post Inoculation.

Significant from low light treatment at ** $P=0.01$, *** $P=0.001$. 
<table>
<thead>
<tr>
<th></th>
<th>30 μmol.m⁻²sec⁻¹</th>
<th>130 μmol.m⁻²sec⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irradiance level</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh weight</td>
<td>3.192 ±0.161</td>
<td>3.060 ±0.171</td>
</tr>
<tr>
<td>(g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry weight</td>
<td>0.113 ±0.005</td>
<td>0.105 ±0.007</td>
</tr>
<tr>
<td>(g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthocyanin content</td>
<td>0.352 ±0.049</td>
<td>1.252 ±0.123</td>
</tr>
<tr>
<td>OD/g.(fr.wt.)/10 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total anthocyanin content</td>
<td>1.097 ±0.100</td>
<td>3.832 ±0.434</td>
</tr>
<tr>
<td>OD/culture/10 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Pig. cells</td>
<td>7.6</td>
<td>17.3</td>
</tr>
</tbody>
</table>

Table 3.2.8.

The influence of irradiance level upon the growth and anthocyanin accumulation of *C. roseus* callus cultures initiated with material from a dark-grown stock culture. Harvest time - 18d. *post* inoculation.

Significantly different from low light treatment at *** p=0.001.
in culture growth was evident between the two treatments and the anthocyanin content was enhanced in the 'high light' cultures as detailed above.

It is clear therefore that increasing the intensity of the stimulus (light) does indeed increase anthocyanin yield in these cultures. Consequently a further experiment was performed to determine more precisely the relationship between irradiance and pigment accumulation.

iv. Growth and anthocyanin accumulation in C. roseus callus cultures grown under differing levels of irradiance.

This experiment was set up using the method described in the previous subsection. Light-grown callus (11 days old) was used for the inocula and the cultures were placed at varying distances away from the same light source to give irradiance levels of 130, 60, 30 and 15 μmol./m²/sec. Three plates were set up for each of these treatments and a further three were placed in darkness for the duration of the experiment. Harvesting took place on day 18. The results are presented in Fig.3.2.12.

As can be seen from the figure, little difference in growth (fresh wt., dry wt.) was found between any of the irradiated treatments although those cultures totally deprived of light did actually show slightly reduced levels of fresh and dry weights in comparison. Measurements of the anthocyanin content of these cultures revealed that on a per g. (fr.wt.) basis or on a total culture basis the level increased linearly with increased irradiance up to 60 μmol./m²/sec. but above this irradiance level little further enhancement was observed.
Figure 3.2.12.

The influence of the level of irradiance upon the growth and anthocyanin content of *C. roseus* callus cultures incubated on CR medium for 18d. Each value is the mean of 3 replicates ±s.e.

(■) Culture fresh wt.
(□) Culture dry wt.
(▲) Anthocyanin content (OD/g./10ml.)
(○) Proportion pig.cells
(△) Anthocyanin content (OD/cult./10ml.)

![Graph showing the influence of irradiance on growth and anthocyanin content of *C. roseus* callus cultures.](image)
In contrast, the proportion of visibly pigmented cells increased linearly only up to the 30 μmol./m²/sec. level above which it proceeded to level off at a maximum level of ca.20%. This was only ca.65% greater than that for the standard treatment (30 μmol./m²/sec.) whereas the maximum amount of extractable anthocyanin obtained was ca.150% greater.

From these results it is clear that increasing the level of irradiance did indeed increase the anthocyanin accumulation response in these cultures, but only up to a level of ca.60 μmol./m²/sec. The maximum yield obtained at this level (or above) still however, involved only a relatively small proportion of the total cell population with ca.80% of cells remaining visibly unpigmented. Although it is possible that this poor response might have been due to the heterogeneity of conditions present within the cultures (with certain cells receiving more incident light than others, for example) this could not be the full explanation for the paucity of the response. Many of the cells on the callus surface in the 'high light' treatment, despite receiving maximum levels of the stimulus, still did not accumulate anthocyanins and the cultures remained densely speckled in appearance. Nevertheless, the next experiment to be described was performed to determine to what degree (if any) the use of suspension cultures in which conditions are more homogeneous, could improve the overall anthocyanin yield and increase the proportion of pigmented cells.
v. The influence of the level of irradiance on the growth and anthocyanin accumulation in *C. roseus* suspension cultures.

As indicated above, it was suspected that the failure of the majority of cells to accumulate visible levels of anthocyanins in callus cultures might (partly) have been the consequence of the cells receiving poor illumination due to their shading by the surrounding cells. In this experiment, by using cell suspensions in which, theoretically, all cells are grown under the same conditions as a result of constant culture agitation, it was hoped to resolve this problem.

Twelve, 100 ml. conical flasks, each containing 20 ml. CR medium were inoculated with 1 ml. of an 11 day old, light-grown stock suspension. After sealing, 3 flasks were enclosed in tin foil to completely exclude light and were placed on the orbital shaker. Those flasks remaining were randomly grouped into threes and placed on the shaker at varying distances from the light source so that each group received 30, 60 or 120 μmol./m²/sec. incident light. After 18 days all flasks were harvested and measurements of culture growth and anthocyanin content were made.

The results are presented in Fig.3.2.13. It is clear from the graph that the pattern of growth and anthocyanin accumulation was very similar in these cultures as compared to those for callus cultures used previously. The production of cell biomass, measured in terms of PCV/fresh weight or dry weight was not found to vary significantly under the different light regimes.

However, the irradiance level did considerably influence
Figure 3.2.13.

The influence of the level of irradiance upon the growth and anthocyanin content of *C. roseus* cell cultures incubated for 18d. in CR medium. Each value represents the mean of three replicates ±s.e.

- (o) Culture dry wt.
- (■) Culture fresh wt.
- (●) PCV
- (▲) Anthocyanin content (OD/g./10ml.)
- (△) Anthocyanin content (OD/cult./10ml.)
- (○) Propr. pig. cells

<table>
<thead>
<tr>
<th>Irradiance (μmol.m⁻².sec⁻¹)</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>dry wt. g.</td>
<td>0.30</td>
<td>50</td>
<td>70</td>
<td>60</td>
<td>50</td>
</tr>
<tr>
<td>fresh wt. g.</td>
<td>0.28</td>
<td>50</td>
<td>70</td>
<td>60</td>
<td>50</td>
</tr>
<tr>
<td>PCV</td>
<td>5</td>
<td>70</td>
<td>60</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>a/c g. fr. wt.</td>
<td>0.5</td>
<td>1.0</td>
<td>1.5</td>
<td>2.0</td>
<td>5.0</td>
</tr>
<tr>
<td>a/c pig. cells</td>
<td>0</td>
<td>5.0</td>
<td>10</td>
<td>20</td>
<td>50</td>
</tr>
</tbody>
</table>
the anthocyanin content of these cultures. Similar to what had been found previously an approximately linear response was observed between the irradiance level and the anthocyanin content (extracted/g. fresh wt. and pigmented cell proportion) up to ca. 60 μmol./m²/sec. Once again, little further enhancement occurred when the irradiance level was increased beyond this value. In this experiment the maximum proportion of pigmented cells detected was ca. 18% of the total cell population.

These results would therefore indicate that the poor response previously observed in callus cultures concerning the proportion of cells visibly accumulating anthocyanins under inductive conditions was unlikely to be due to the heterogeneity of conditions within the culture. In the above experiment, in which all cells theoretically were grown under the same conditions, a similar low level of response was observed.

The two experiments remaining in this section were carried out to investigate the possible influence of two specific chemical treatments, previously shown to enhance secondary metabolite accumulation in other in vitro systems, on the accumulation of anthocyanins in C.roseus cultures.

vi. Studies on the influence of high sugar concentrations on the accumulation of anthocyanin in C.roseus cell cultures.

Knobloch et al., (1981) have reported that enhanced yields of plant phenolics and alkaloids were obtained when C.roseus cell cultures were subjected to high sucrose concentrations
(8% w/v). The following experiment was carried out to determine if such a response occurred in the cultures used in this investigation and if so what was its cellular basis.

In this experiment four sugar solutions were tested for their ability to enhance anthocyanin yield. These were: 8% aq. sucrose, 8% aq. mannitol, 3% aq. sucrose + 5% aq. mannitol and 5% sucrose-enriched CR medium (giving 8% sucrose in total). Conditions were repeated in accordance with the above paper; 3x250 ml. conical flasks each containing 50 ml. of the solution to be tested were inoculated with 5 ml. (10-fold dilution) of a thoroughly mixed 12 day old light-grown suspension. Three control flasks containing CR medium were also initiated using the standard 3 ml. inoculum of the same stock suspension. All cultures were then incubated in the light for 14 days before harvesting. (50 ml. cultures were observed to grow faster than the previously used 20 ml. experimental cultures and by day 14 would have reached their maximum level of anthocyanin accumulation).

The results are presented in Table 3.2.9. In the control cultures typical levels of biomass and anthocyanin had accumulated by the end of the 14 day experimental period. However, in all of the 8% sugar treatments the pattern of culture development had been considerably modified. Firstly, it is clear that subjecting these cultures to 8% mannitol was detrimental to culture viability. Decreases in fresh and dry weight of these cultures were found and substantial losses of cell viability also occurred in this treatment. Substituting 3% of the mannitol added with sucrose resulted in unchanged culture biomass over the 14 day period. However, the
<table>
<thead>
<tr>
<th>Treatment</th>
<th>CONTROL</th>
<th>8% aq. Sucrose</th>
<th>8% Mannitol</th>
<th>3% aq. Sucrose +5% aq. Mannitol</th>
<th>8% Sucrose (in CR medium)</th>
<th>Time 0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell no. ml^{-1} x10^{-6}</td>
<td>7.53</td>
<td>0.6</td>
<td>0.62</td>
<td>0.59</td>
<td>7.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>±0.92</td>
<td>±0.01</td>
<td>±0.05</td>
<td>±0.03</td>
<td>±0.95</td>
<td>±0.02</td>
</tr>
<tr>
<td>PCV %</td>
<td>61.4</td>
<td>7.4</td>
<td>4.6</td>
<td>5.34</td>
<td>21.4</td>
<td>7.1</td>
</tr>
<tr>
<td>Fr.wt. (g.)</td>
<td>16.210</td>
<td>1.941</td>
<td>0.981</td>
<td>1.587</td>
<td>8.330</td>
<td>1.664</td>
</tr>
<tr>
<td></td>
<td>±1.09</td>
<td>±0.069</td>
<td>±0.059</td>
<td>±0.161</td>
<td>±0.510</td>
<td>±0.142</td>
</tr>
<tr>
<td>Dry wt. (g.)</td>
<td>0.570</td>
<td>0.121</td>
<td>0.021</td>
<td>0.098</td>
<td>0.484&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.066</td>
</tr>
<tr>
<td></td>
<td>±0.041</td>
<td>±0.009</td>
<td>±0.005</td>
<td>±0.002</td>
<td>±0.021</td>
<td>±0.004</td>
</tr>
<tr>
<td>% Pig Cells</td>
<td>11.0</td>
<td>13.0</td>
<td>3.5</td>
<td>6.4</td>
<td>2.6</td>
<td>10.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Anthocyanin cont. OD/g./10ml.</td>
<td>0.611</td>
<td>0.803</td>
<td>0.371</td>
<td>0.492</td>
<td>0.111</td>
<td>0.571&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>±0.141</td>
<td>±0.042</td>
<td>±0.027</td>
<td>±0.030</td>
<td>±0.010</td>
<td>±0.051</td>
</tr>
<tr>
<td>Total anthocyanin OD/culture/10ml.</td>
<td>9.46</td>
<td>1.55</td>
<td>0.36</td>
<td>0.77</td>
<td>0.93</td>
<td>0.948</td>
</tr>
<tr>
<td></td>
<td>±1.43</td>
<td>±0.10</td>
<td>±0.03</td>
<td>±0.03</td>
<td>±0.08</td>
<td>±0.08</td>
</tr>
<tr>
<td>Cell viability %</td>
<td>92</td>
<td>89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48</td>
<td>85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>91&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 3.2.9. The influence of 8% aq. sugar solutions upon the growth and anthocyanin content of *C. roseus* liquid cultures. See text for details. All those values excluding those denoted with <sup>a</sup> are significantly different from the equivalent control value at least at P=0.05. <sup>1</sup>=5ml. inoculum.
anthocyanin content of these cultures was, as had been found for the 8% mannitol treatment, significantly reduced. In those cultures subjected to 8% aq. sucrose, no significant change in the cell number or culture fresh weight was found although the culture dry weight was double the original value. Cell viability remained high and the anthocyanin yield was actually enhanced by ca. 50%. In contrast, however, those cultures given 5% sucrose in association with the normal nutrient medium (8% sucrose in total) accumulated very much less anthocyanin/g. fresh weight of tissue. Although the cell population density of these cultures was equivalent to that found in the controls the culture fresh weight was considerably lower and the dry weight slightly higher in comparison.

It would appear therefore that using 8% sucrose is not a very successful way to enhance anthocyanin yield in this culture system. However, it is very interesting to note that when cells are subcultured into a medium containing both a high sucrose concentration and the usual CR medium constituents, although cell number increases normally the culture fresh weight (but not dry weight) is substantially reduced. This would suggest that the cells were very much smaller under these conditions, presumably as a result of poor cell expansion. This has very interesting parallels with certain other findings and will be discussed further in Chapter 4.

The remaining experiment in this section concerns the potential use of nutrient starvation as a means to enhance secondary metabolite yield.
The influence of inorganic nitrogen and phosphate on culture growth and the accumulation of anthocyanin pigments in \textit{C.roseus} cell suspensions.

There are several reports in the literature describing the success of nutrient starvation (especially i-N and i-P) in enhancing the yield of secondary metabolites in plant tissue cultures (see Mantell & Smith, 1983). The following experiment, the last in this section, was performed to investigate the effect of i-N and i-P-free media on \textit{C.roseus} cell cultures.

Beginning with the basic constituents (BDH./Sigma) of CR medium (see Chapter 2.) four media types were made up: Full medium, containing all of the normal constituents; i-Phosphate-free medium, lacking KH$_2$PO$_4$; i-Nitrogen-free medium, lacking both KNO$_3$ and NH$_4$NO$_3$; i-P/i-N-free medium lacking KH$_2$PO$_4$, NH$_4$NO$_3$ and KNO$_3$. Four sets of 3x100 ml. conical flasks containing 20 ml. of the medium to be tested were inoculated with 1.5 ml. of a well-mixed, 12 day old, light-grown stock culture. Then, after 18 days incubation in the light the cultures were harvested and measurements made.

The results, presented in Table 3.2.10. clearly illustrate how these nutrients are essential for culture growth. In cultures deprived of i-phosphate no increase in biomass occurred over the 18 days of the experiment, cell viability was reduced and a substantial loss of anthocyanin content was observed. Although those cultures deprived of i-N had grown slightly (a doubling of fresh wt., PCV and cell no. was observed) by the end of the 18 day incubation period both
<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>-i-P</th>
<th>-i-N</th>
<th>-i-P/i-N</th>
<th>Time 0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh wt.</td>
<td>3.699</td>
<td>0.428</td>
<td>1.038</td>
<td>0.820</td>
<td>0.358</td>
</tr>
<tr>
<td>(g.)</td>
<td>±0.419</td>
<td>±0.008</td>
<td>±0.089</td>
<td>±0.040</td>
<td>±0.001</td>
</tr>
<tr>
<td>PCV. (%)</td>
<td>60.3</td>
<td>6.2</td>
<td>13.5</td>
<td>11.1</td>
<td>5.9</td>
</tr>
<tr>
<td>Dry wt.</td>
<td>0.253</td>
<td>0.022</td>
<td>0.047</td>
<td>0.045</td>
<td>0.025</td>
</tr>
<tr>
<td>(g.)</td>
<td>±0.021</td>
<td>±0.004</td>
<td>±0.008</td>
<td>±0.002</td>
<td>±0.001</td>
</tr>
<tr>
<td>Cell viability</td>
<td>96</td>
<td>73</td>
<td>59</td>
<td>91(^a)</td>
<td>93(^a)</td>
</tr>
<tr>
<td>(%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell no.</td>
<td>6.33</td>
<td>0.52</td>
<td>0.94</td>
<td>0.57</td>
<td>0.58</td>
</tr>
<tr>
<td>ml(^{-1}) x (10(^{-6})</td>
<td>±0.29</td>
<td>±0.04</td>
<td>±0.02</td>
<td>±0.05</td>
<td>±0.05</td>
</tr>
<tr>
<td>% Pig.cells</td>
<td>9.0</td>
<td>2.3</td>
<td>0.4</td>
<td>19.7</td>
<td>10.1</td>
</tr>
<tr>
<td>Anthocyanin</td>
<td>0.915</td>
<td>0.376</td>
<td>0.075</td>
<td>1.643</td>
<td>0.818(^a)</td>
</tr>
<tr>
<td>content OD/ g./10ml.</td>
<td>±0.073</td>
<td>±0.004</td>
<td>±0.014</td>
<td>±0.164</td>
<td>±0.073</td>
</tr>
<tr>
<td>Total a/c</td>
<td>3.443</td>
<td>0.161</td>
<td>0.080</td>
<td>1.354</td>
<td>0.292</td>
</tr>
<tr>
<td>content OD/ culture/10ml.</td>
<td>±0.301</td>
<td>±0.020</td>
<td>±0.004</td>
<td>±0.091</td>
<td>±0.031</td>
</tr>
</tbody>
</table>

**Table 3.2.10.**

The growth and anthocyanin content of *C.roseus* cell cultures incubated in media lacking the major i-Phosphate and/or i-Nitrogen sources. See text for details. All those values, excluding those denoted \(^a\) are significantly different from the corresponding control value at least at \(P=0.05\).

\(^1\) =full CR medium.
the cell viability and the anthocyanin content were again substantially reduced. In those cultures deprived of both nutrient types the results for culture growth are somewhat of a 'compromise' between those for the separate treatments although interestingly cell viability in this 'double starvation' medium was not affected and remained >90%. However, in these cultures a significant increase in anthocyanin accumulation was observed with the proportion of visibly pigmented cells doubling during the experimental period with a slightly smaller (80%) increase in the amount of extractable anthocyanin/g. fresh wt. present.

It is apparent therefore that in this culture system the sole deprivation of either i-nitrogen or i-phosphate sources was ineffective in improving metabolite yields and indeed was detrimental to culture viability. This however is in contrast to the combined removal of i-P and i-N sources from the medium which was found to have no such undesired effect on cell viability and resulted in almost doubling the anthocyanin yield.
The following points have arisen from the experiments reported in this section.

1. The growth substance complement of the nutrient medium was found to greatly influence anthocyanin yield. The standard supplement, 2μM 2,4-D, proved the most suitable to give maximum yields.
2. Increasing the irradiance level increased the anthocyanin yield by a maximum of 3-4 times.
3. 8% (w/v) sucrose slightly increased the yield of anthocyanins in aqueous solutions but in the presence of the full CR medium constituents, proved inhibitory to anthocyanin accumulation.
4. In the combined absence of i-N and i-P sources anthocyanin productivity was approximately doubled.
5. Of the numerous treatments tested, none increased the proportion of pigmented cells above 20% of the total cell population.

Concerning other modifications to the culture environment which influenced secondary metabolite yield, the following section reports on a very unusual and surprising stimulatory effect induced by a substance which was initially expected to be inert.
Section 3.2.4.

"The influence of cellophane on the growth and anthocyanin accumulation of *C. roseus* callus cultures".
As will be mentioned in more detail in a later section, several attempts were made to culture single C. roseus cells in isolation, in order to carry out detailed microdensitometric analyses on individual cells over chosen time intervals. One of the techniques tested was to place the selected cells between layers of visking tubing which were then placed on top of an actively-growing callus, used as a 'nurse' culture. However, this method and several modifications of it, failed to sustain the life of the cells for more than a few days.

Consequently, due to the potential value of this technique a further method was planned in which cellophane (Cellophane PT., British Cellophane Ltd.) was to be used instead of visking tubing. It was known that this substance was gas permeable and provided no detectable barrier to nutrient movement (D. Park, Q.U.B., pers.comm.) but it was not known if it contained components which were toxic to plant cells. Accordingly a simple preliminary experiment was set up to determine if this was so (Expt. 3.2.4i.). Surprisingly, the results of this experiment indicated that not only did cellophane appear non-toxic to the cells but also it proved promotory to both culture growth and anthocyanin yield. This rather unusual but interesting result instigated the following short series of experiments performed to further investigate this effect.
The influence of prewashed cellophane discs on the growth and anthocyanin accumulation of *C. roseus* callus cultures.

As a preliminary to attempting certain proposed nurse culture techniques using cellophane 'chambers', the following experiment was performed using *C. roseus* callus cultures to determine if the cellophane to be used (Cellophane PT., British Cellophane Ltd.) was in any way detrimental to the growth and viability of the cultures.

**Cellophane pretreatment:** Cellophane sheets (ca. 20 µm. thick) were first cut into 5 cm. discs before boiling in a large (500 ml.) volume of distilled water for 5 min. After partial cooling this water was decanted and the discs washed in 500 ml. tap water. This procedure was then repeated a further two times before placing the discs in 200 ml. distilled water (pH 5.8) for autoclaving. Finally, immediately prior to use each disc was individually washed in 3×10 ml. sterile distilled water (pH 5.8) before its placement on the nutrient medium.

**Callus culture.** CR medium (10 ml.) was placed in each of 18 5 cm. Petri dishes, allowed to gel and cool completely. 6 plates were then inoculated directly using 0.25 g. samples of a well-mixed callus taken from two 12 day old light-grown stock plates, spreading the cells evenly over the agar surface. A single layer of cellophane was placed on the agar surface of all remaining plates before their inoculation in an identical manner. Finally, to six of these plates was added a second layer of cellophane, placed on top of the callus mass after which all plates were sealed and incubated in the light for 18 days before harvesting.
The results are presented in Table 3.2.11. Clearly the placement of *C. roseus* callus between layers of cellophane was detrimental to the growth of the cultures. Very little growth was observed under these conditions and areas of cell necrosis were clearly present. Final values of fresh weight, dry weight and cell viability were all significantly reduced and the anthocyanin content was equivalent only to that which had been present in the original inoculum (0.25 g. contained 0.114 OD units/10 ml. solvent at time 0).

In contrast however, a very different picture emerged when only a single (under) layer of cellophane had been used. In these cultures cell viability remained at its normally high level and culture fresh weight was actually significantly increased. No significant difference was however observed in culture dry weight. The anthocyanin content of these cultures was also significantly increased - by ca. 50% on a g\textsuperscript{−1} fresh weight basis and almost doubled on a total culture basis.

The proposed use of cellophane 'sandwiches' to culture single cells in a way which allows easy manipulation and observation is not feasible. From the results given above it would appear that, as *C. roseus* callus could grow on top of one, but not between two layers of cellophane, the gas permeability of this material may not have been sufficient to meet the requirements of the cells. However, the very unexpected result that growing this callus above a single layer of cellophane actually enhances culture growth and almost doubles the yield of anthocyanins was rather curious and the following three experiments were performed to further investigate this phenomenon.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>CONTROL (no cellophane)</th>
<th>1 Layer</th>
<th>2 Layers &quot;Sandwich&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh wt. (g)</td>
<td>2.400 ±0.091</td>
<td>3.279***±0.154</td>
<td>1.117***±0.072</td>
</tr>
<tr>
<td>Dry wt. (g)</td>
<td>0.098 ±0.003</td>
<td>0.112 ±0.020</td>
<td>0.051*±0.003</td>
</tr>
<tr>
<td>Cell viability (%)</td>
<td>88</td>
<td>87</td>
<td>67*</td>
</tr>
<tr>
<td>Anthocyanin content OD/g./10 ml.</td>
<td>0.571 ±0.05</td>
<td>0.752*±0.090</td>
<td>0.100***±0.01</td>
</tr>
<tr>
<td>Total a/c content OD/culture/10 ml.</td>
<td>1.360 ±0.114</td>
<td>2.431***±0.234</td>
<td>0.110***±0.01</td>
</tr>
</tbody>
</table>

Table 3.2.11.

The growth and anthocyanin content of *C. roseus* callus cultures grown on top of one, or between two layers of prewashed cellophane. See text for details. Measurements made 18d. after inoculation.

Significantly different from control at * P = 0.05, *** P = 0.001.
ii. An investigation into the possible mode of influence of cellophane on the growth and anthocyanin accumulation in C.roseus callus cultures.

Several possible explanations were envisaged for the observed effect of cellophane on the growth of these cultures:

1. The cellophane may act as a semipermeable membrane by preventing either the outward diffusion of stimulatory compounds from the cell mass or the inward diffusion of inhibitory compounds from the agar medium.

2. Boiling/autoclaving may bring about partial degradation of the cellophane, instigating the release of certain 'active' compounds (sugars? cell wall fragments?).

3. The cellophane may possess adsorptive properties which could influence the pattern of nutrient availability or may bind to inhibitory compounds present in the medium or released by the cells thus preventing their action.

The following experiment was performed to investigate these proposals.

Six treatments (3 replicates) were set up as listed below using 5 cm. Petri dishes each containing 10 ml. CR medium. Each plate was inoculated using 0.25 g. cell samples taken from two pooled and well mixed 12 day old light-grown callus stocks, spreading each inoculum evenly over the entire agar surface. All of the cellophane used in this experiment was pretreated as detailed previously.

CON - no cellophane added.
DISC - 5 cm. cellophane discs (1/plate) placed on the agar surface prior to inoculation.
MASC - 3x5 cm. cellophane discs were cut into ca. 2 mm. squares and autoclaved in the agar medium (30 ml.). This mixture was mixed well before pouring. (The cellophane fragments were always observed to sink before gelling occurred).

D/MASC - as MASC but plates ultimately incubated in darkness.

X/MASC - as MASC but removing the cellophane fragments by filtering the medium prior to pouring the plates.

FP - as DISC but substituting Whatman No.1. filter paper discs for cellophane. (Paper prewashed as cellophane)

All plates (excl. D/MASC) were then incubated under normal conditions of illumination for 18 days before harvesting. The results are tabulated in Table 3.2.12. Of the five treatments tested, four produced results which were significantly different from those of the control. In the exceptional treatment (X/MASC), as none of the results obtained were found to deviate significantly from those of the control this would indicate that autoclaving the cellophane in situ does not produce a nutrient medium modified in such a way as to promote culture growth and anthocyanin accumulation above normal control levels.

In the D/MASC treatment where cultures were grown on a medium containing cellophane in darkness, anthocyanin accumulation was very poor and the level detected was only slightly greater than the total amount present initially (0.25 g. contained 0.26±0.01 OD units/10 ml. solvent at time 0). It is clear therefore, that light remains essential for anthocyanin accumulation even under these modified conditions.
## Table 3.2.12.

The growth and anthocyanin content of *C. roseus* callus cultures when cultured in the presence or absence of cellophane. Harvesting took place 18d. after inoculation.

1 For explanation of treatments see text.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fresh wt. (g)</th>
<th>Dry wt. (g)</th>
<th>Anthocyanin content OD/g.(fr.wt.)</th>
<th>Total anthocyanin OD/culture/10 ml./10 ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>3.23 ±0.167</td>
<td>0.106 ±0.013</td>
<td>0.712 ±0.082</td>
<td>2.557 ±0.437</td>
</tr>
<tr>
<td>DISC</td>
<td>4.118 ±0.243</td>
<td>0.126</td>
<td>1.062 ±0.079</td>
<td>4.401 ±0.559</td>
</tr>
<tr>
<td>MASC</td>
<td>4.059 ±0.062</td>
<td>0.120</td>
<td>1.128 ±0.054</td>
<td>4.569 ±0.148</td>
</tr>
<tr>
<td>XMASC</td>
<td>3.552 ±0.254</td>
<td>0.107</td>
<td>0.780 ±0.080</td>
<td>2.714 ±0.574</td>
</tr>
<tr>
<td>DMASC</td>
<td>3.950 ±0.140</td>
<td>0.126</td>
<td>0.080 ±0.015</td>
<td>0.299 ±0.052</td>
</tr>
<tr>
<td>FP</td>
<td>3.405 ±0.375</td>
<td>0.102</td>
<td>1.071 ±0.012</td>
<td>3.432 ±0.373</td>
</tr>
</tbody>
</table>

Significantly different from controls (CON) at * P = 0.05, ** P = 0.01, *** P = 0.001.
In the DISC and MASC treatments results were very closely similar. This demonstrates not only the reproducibility of the observed effects (significant enhancement of culture fresh wt. and anthocyanin content) but also indicates that these effects can be induced when the cellophane is not in a position to act as any sort of barrier/membrane.

In the final treatment where washed filter paper discs were used as a substitute for cellophane, growth (fresh wt., dry wt.) was quantitatively similar to that of the control although it was apparent that many areas of cell necrosis were present throughout the callus mass. Nevertheless, the anthocyanin content of this callus was of a level equivalent to that of the DISC/MASC cellophane treatments on a / g. (fr. wt.) basis although due to the absence of any growth enhancement the increase was not significant on a total culture basis.

These results would therefore suggest that simply the physical presence of cellophane in or on the nutrient medium was sufficient to bring about the observed enhancement of secondary metabolite yield by increasing both culture fresh weight and the anthocyanin content / g. (fr.wt.). The following experiment was thus performed to determine if this effect could be further intensified by increasing the amount of cellophane included in the nutrient medium.
iii. The influence of varying quantities of cellophane on the growth and anthocyanin accumulation of *C. roseus* callus cultures.

This experiment was carried out to determine if a dose response existed between the amount of cellophane included in the nutrient medium and the anthocyanin yield of the cultures grown thereon.

The culture plates for this experiment were prepared in an identical manner to that described for the MASC treatment in the previous experiment with the modification that the amount of cellophane/dish was varied between 0.5-4 discs (35-280 mg.). All plates were inoculated with 0.25 g. of a well mixed 12 day old light-grown stock and incubated in the light for 18 days before harvesting.

The results are tabulated in Table 3.2.13. and presented graphically in Figure 3.2.14. From these results it would appear that as little as 35 mg. (50% of a 5 cm. disc) was sufficient to bring about a significant response in terms of both fresh weight and anthocyanin content. Increasing this amount (by up to 8x) increased the response only slightly and the results for the 280 mg. (4 discs) treatment were not significantly greater than those for the 35 mg. (¼ disc) treatment. In dry weight terms, although increases were observed from the 35 mg. treatment upwards, this only became statistically significant when the maximum level of 280 mg. had been used.

It would appear therefore that only relatively small amounts of cellophane were required to induce the maximum response. As little as 35-70 mg. of cellophane/plate was
<table>
<thead>
<tr>
<th>Cellophane supplement</th>
<th><strong>0</strong> (CONTROL)</th>
<th><strong>35 mg.</strong></th>
<th><strong>70 mg.</strong></th>
<th><strong>140 mg.</strong></th>
<th><strong>280 mg.</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh wt. (g.)</td>
<td>3.379 ±0.064</td>
<td>4.157* ±0.100</td>
<td>4.115* ±0.182</td>
<td>4.345* ±0.125</td>
<td>4.209* ±0.086</td>
</tr>
<tr>
<td>Dry wt. (g.)</td>
<td>0.100 ±0.004</td>
<td>0.100 ±0.011</td>
<td>0.110 ±0.002</td>
<td>0.120 ±0.018</td>
<td>0.125* ±0.003</td>
</tr>
<tr>
<td>% Pig. cells</td>
<td>6.5</td>
<td>10.4*</td>
<td>12.6**</td>
<td>11.0*</td>
<td>12.1*</td>
</tr>
<tr>
<td>Anthocyanin content</td>
<td>0.308 ±0.020</td>
<td>0.464* ±0.034</td>
<td>0.510* ±0.021</td>
<td>0.479* ±0.083</td>
<td>0.561** ±0.037</td>
</tr>
<tr>
<td>10 ml.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total a/c OD/culture</td>
<td>1.04 ±0.08</td>
<td>1.91** ±0.11</td>
<td>2.11*** ±0.04</td>
<td>2.09** ±0.21</td>
<td>2.37*** ±0.28</td>
</tr>
<tr>
<td>10 ml.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2.13.

The influence of varied amounts of prewashed cellophane, included in the nutrient medium, upon the growth and anthocyanin content of *C. roseus* callus cultures. Culture period: 18d.

1 Dry weight.

Significantly different from controls at * P = 0.05, ** P = 0.01, *** P = 0.001.
Figure 3.2.14.
Graphic presentation of some of the data reported in Table 3.2.13. Each value is the mean of 3 replicates ± s.e.
sufficient to more than double the yield of anthocyanins. Consequently, if a dose response does exist in this system then this must therefore occur below the lowest amount of cellophane tested.

In addition to this cellophane effect, the two other treatments which have been found amongst all those tested which repeatedly increased the anthocyanin yield of these cultures by $\geq 2$ times were i-phosphate/i-nitrogen starvation and increased irradiance. In the final experiment in this section, combinations of these treatments have been tested for their influence on anthocyanin accumulation in vitro in a further attempt to increase the proportion of pigmented cells to $>20\%$.

iv. The effect of combined stimulatory treatments on anthocyanin accumulation in *C. roseus* callus cultures.

In this experiment three treatments which had previously been shown to enhance anthocyanin yield in *C. roseus* callus and cell cultures were tested in combination to determine whether the effects were cumulative or perhaps synergistic.

The treatments tested were i-P/i-N stress, cellophane addition and enhanced irradiance. Each was tested singly as a control and also in combination with either one or both of the other treatments (see Table 3.2.14.). The i-P/i-N-free medium was made up as described in Expt.3.2.3vi. and the cellophane medium was equivalent to the "MASC" treatment in Expt.3.2.4ii. The enhanced irradiance level was 130 $\mu$mol/
m^2/sec. as compared to the 30 μmol./m^2/sec. control level.

Three 5 cm Petri dishes were set up for each treatment using 0.25 g. inocula (CR medium treatments) or 0.5 g. inocula (P/N-free medium treatments). All inocula were taken from two pooled and well mixed 11 day old callus cultures. The cultures were harvested 18 days after initiation. The results are presented in Table 3.2.14.

Under standard lighting conditions both of the single treatments tested produced similar results to those found in previous experiments. In those cultures which had been grown on a cellophane supplemented medium significant increases in culture fresh weight and anthocyanin content were observed. The total anthocyanin yield/culture was enhanced by 80-90%.

In the P/N stress treatment although the culture growth (fresh wt., dry wt.) was considerably reduced in comparison to the control value, this was nevertheless somewhat higher than had previously been observed when using liquid cultures (Section 3.2.3iv.). This difference may have arisen from a larger nutrient carryover in this experiment or perhaps may have been due to the presence of some i-P or i-N sources in the agar. The approximate doubling of both the proportion of pigmented cells and the anthocyanin content/g. fresh weight in this treatment was closely similar to previous observations.

When both these treatments were combined under these lighting conditions the enhancement in the culture anthocyanin content closely paralleled that found in the cellophane treatment. No cellophane-induced increase in culture fresh weight was however observed in this treatment.

Under enhanced lighting conditions control cultures contained two times the proportion of pigmented cells and
<table>
<thead>
<tr>
<th>Treatment/medium</th>
<th>Fr.wt. (g.)</th>
<th>Dry wt. (g.)</th>
<th>% Pig. cells</th>
<th>Anthocyanin OD/g. x 10ml.</th>
<th>Total a/c OD/culture/10 ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>30μmol.m⁻².sec⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>3.093 ±0.483</td>
<td>0.138 ±0.003</td>
<td>8.1</td>
<td>0.274 ±0.046</td>
<td>0.85 ±0.06</td>
</tr>
<tr>
<td>+cellophane</td>
<td>3.868 ±0.088</td>
<td>0.134 ±0.005</td>
<td>12.0*</td>
<td>0.395 ±0.031</td>
<td>1.55 ±0.34</td>
</tr>
<tr>
<td>-P/-N</td>
<td>1.940 ±0.163</td>
<td>0.099 ±0.010</td>
<td>15.0**</td>
<td>0.553 ±0.088</td>
<td>1.07 ±0.20</td>
</tr>
<tr>
<td>+cellophane</td>
<td>1.532 ±0.015</td>
<td>0.085 ±0.002</td>
<td>13.0*</td>
<td>0.372 ±0.043</td>
<td>0.57 ±0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>130μmol.m⁻².sec⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>3.230 ±0.359</td>
<td>0.130 ±0.006</td>
<td>16.3</td>
<td>0.802 ±0.051</td>
<td>2.58 ±0.30</td>
</tr>
<tr>
<td>+cellophane</td>
<td>3.830 ±0.262</td>
<td>0.130 ±0.009</td>
<td>16.5</td>
<td>1.132 ±0.149</td>
<td>4.13 ±0.65</td>
</tr>
<tr>
<td>-P/-N</td>
<td>1.684 ±0.114</td>
<td>0.099 ±0.004</td>
<td>26.5*</td>
<td>2.021 ±0.152</td>
<td>3.40 ±0.05</td>
</tr>
<tr>
<td>+cellophane</td>
<td>1.639 ±0.112</td>
<td>0.084 ±0.003</td>
<td>24.3*</td>
<td>1.545 ±0.338</td>
<td>2.53 ±0.38</td>
</tr>
</tbody>
</table>

Table 3.2.14.

The growth and anthocyanin content of *C. roseus* callus cultures when incubated in the presence or absence of prewashed cellophane and/or i-P and i-N sources. Two irradiance levels were used. See text for details. Culture period: 18d.

Significantly different from controls at *P = 0.05, **P = 0.01.
three times the level of extractable anthocyanin found in the low light controls. No significant alteration in culture growth resulted from this treatment.

In the presence of cellophane, cultures exposed to high irradiance levels produced slightly more biomass in terms of fresh weight but not dry weight. This had also been observed in the low irradiance treatments but to a slightly greater extent. Interestingly, under these conditions the presence of cellophane did not increase the proportion of pigmented cells in these cultures although an increase in the level of extractable anthocyanin was found. In comparison to the 'high light' control an increase of 60% in the total anthocyanin yield/culture was observed (40% on a g\textsuperscript{-1} fresh weight basis) in this treatment producing a final level almost five times that of the standard irradiance level control.

The combined effect of a P/N deficient medium and high irradiance levels resulted in cultures with the highest proportion of pigmented cells ever observed in this investigation. In these cultures 26% of the cells were observed to contain visible levels of anthocyanin which is more than three times the value for the low light control. A considerably larger effect however, was observed on the actual level of anthocyanin accumulated by these cultures. Although, in comparison to the high light control the pigmented cell proportion was only increased by ca. 60% in this treatment, the anthocyanin content (g\textsuperscript{-1} fr.wt.) was increased by >150%. The actual level present was ca. 4 times that for the equivalent treatment in low light and ca. 7 times that for the low light control.
In the final treatment tested, which combined all three of the individual treatments used although the proportion of pigmented cells was roughly equivalent to the level found in the previous (high light/nutrient stress) treatment the actual level of extractable anthocyanin present was significantly reduced. This level was however still considerably greater than either of the controls.
The following points have emerged from the experiments detailed in this section.

(1) The inclusion of small amounts of washed cellophane in the nutrient medium approximately doubled the yield of anthocyanin in these cultures.

(2) This effect was light dependent.

(3) The stimulatory factor was not released from the cellophane on autoclaving.

(4) The combined effects of cellophane, nutrient stress and enhanced irradiance have been investigated. In those cultures subjected to the best combination of treatments the proportion of pigmented cells was still only \( \text{ca.} 25\% \) of the total population.

In all of the experiments so far reported, despite extensive modifications to the culture conditions the proportion of cells visibly accumulating the desired products has remained relatively low with a maximal level of 26\% observed. One possible explanation for this continued low level of response is that the basis of the difference between accumulating and non-accumulating cells was genetic rather than environmental. Consequently the work reported in the following section was initiated to investigate this possibility.
Section 3.2.5.

"A study of the variation in growth and anthocyanin accumulation of *C. roseus* callus cultures derived from different cell lines."
From the results so far reported it is evident that the red-purple pigmentation taken on by the cultures used in this study in the latter part of the culture growth cycle was the result of anthocyanin accumulation by (as far as was discernable from microscopical observations) a very small but relatively consistent proportion (ca. 10%) of the total cell population. Attempts to increase this proportion through modifications of the cultural conditions (Section 3.2.3.) were not very successful and only rarely were levels of >20% achieved.

One possible explanation for this low level of response is that in these cultures, two distinct cell types existed, only one of which (possibly a mutant?), constituting ca. 10% of the total cell population was capable of accumulating anthocyanins under the conditions used. If this was the case then it might be expected that quite distinct distributions of pigmented and nonpigmented cells would appear, particularly in callus cultures where there would be minimal dissociation of cell progeny. However, although there was occasionally some indication of clumping of pigmented cells (Fig. 3.2.1.), in general coloured and colourless cells occurred in close association with one another and only rarely existed in distinctly separate groups. This being rather tentative evidence against a theory which has very important implications concerning the main theme of this thesis it was thus decided to investigate this 'mutation' theory more thoroughly and consequently the following work was carried out.

In this experiment a number of individual cell lines were isolated using established procedures and after reaching a suitable size each was analysed for its ability to accumulate
anthocyanins under standard inductive conditions. To obtain these cell lines both protoplast isolation and suspension filtration techniques were tested. Details of these techniques are now presented in the preliminary part of this section and this is then followed by a full description of the results of the analysis of the cell lines thus obtained.

Protoplast culture

Protoplasts were isolated in accordance with the method detailed in Section 2.2.4. In a preliminary 'trial run' protoplasts were obtained from cells removed from a 4 day old callus culture and plated at a density of ca. 25,000 cells.ml⁻¹ in either full CR medium or in CR medium containing 50% (v/v) conditioned medium obtained from a 5 day old cell suspension. Mannitol (0.3M) was added to both media to prevent cell lysis and each was solidified using 0.4% (w/v) agar. Approximately 2-2.5 ml. of the cell suspension was placed in each 5 cm. Petri dish.

No growth was observed in any plate incubated in the light and of those kept in darkness callus was obtained only where conditioned medium had been included. In this treatment the agar surface was completely covered by the callus produced within 7 weeks of plating.

However, despite this initial success all subsequent attempts to reproduce this result failed. In addition to repeating the above procedure exactly several modifications were tested using 95% conditioned CR medium, KM medium, 50% conditioned KM medium and finally 50% KM medium/50% conditioned
CR medium. Using cultures of different ages (2d., 4d., 7d. or 14d. post inoculum) or using suspended cells in place of callus cells similarly had no effect. In all of these treatments the protoplasts, when plated were observed to regenerate a cell wall within 1-2 days after which they proceeded to expand in volume. After 7 days a few cell divisions (<1%) had occurred but by this time many cells had already undergone senescence. By day 14 all cells had died. (see Fig.3.2.15.).

Although several other procedures had been planned, by this time success had been achieved using filtered-cell plating and it was thus decided to curtail this particular line of investigation and to use the cell lines derived from filtered cells for analysis.

The culture of filtered cells

Using nylon mesh with a very uniform and defined pore size (H. Simon Ltd., Stockport, Cheshire) it is possible to obtain from a heterogeneous cell suspension a population of cells and cell aggregates of a specified, limited size range. In this study, to obtain a suspension of cells suitable for plating a double layer of 64 μm. nylon mesh was placed over the end of a glass tube into which was then poured a 4 day old light-grown cell suspension. Without applying any external force the liquid was allowed to drain through the mesh and was collected in a sterile flask. The population of cells obtained in this way consisted of groups of 1-4 cells the majority of which were likely the progeny of single cells (Fig.3.2.16.). The cell population density of the filtrate was then determined and adjusted by removing some of the bathing medium to give
Figure 3.2.15.

The appearance of cells plated into 50% conditioned CR medium 7d. after their isolation as protoplasts from a 4d. old C.roseus callus culture. Although a few cell divisions were visible (b) many of the cells had already undergone senescence and after a further 7d. all cells were dead.
Figure 3.2.16.

The cell aggregates present in a filtrate from a 4d. old *C. roseus* suspension culture having passed through a double layer of 64 μm. mesh nylon filter. Most cells occurred either singly or in groups of <4 which had very likely been derived from single cells. (eg. arrowed). Bar = 30 μm.
a final value of 50,000 cells/ml.

To this prepared suspension was added an equal volume of autoclaved CR medium containing 1% (w/v) agar and which had been allowed to cool to precisely 40°C. The solution was then thoroughly mixed and one half was poured into 5 cm. Petri dishes (2 ml./plate) and allowed gel. To the remainder was quickly added a further equal volume of cooled agar medium (thus giving a cell population density of 12,500 cells/ml. and 25% 'conditioning') and plated as above. Half of each set of plates were incubated in low intensity (15 μmol./m²/sec.) fluorescent light and half in total darkness.

Due to the variation in cell grouping present initially it was not possible to determine exactly the plating efficiency in these treatments. However, by counting the number of colonies consisting of >4 cells on day 7 some indication as to the plating efficiency was obtained. These data are presented in Table 3.2.15.

It is apparent from the table that very poor plating efficiencies were found in those plates inoculated at a cell density of 12,500/ml. with 25% conditioning. However, at the higher inoculation density (25,000 cells/ml.) the plating efficiencies were somewhat better, both in light and dark incubated cultures with values for the latter giving the better result of ca.50%.

When many colonies had reached a size of 30-50 cells (estimated) the cultures were individually examined using an Olympus IMT inverted microscope and the positions of those colonies well isolated from all neighbouring ones were marked by a cross on the underside of the dish. After a further 2-3
Table 3.2.15.

The proportion of cell aggregates consisting of >4 cells after 7d. incubation in 1 50% or 2 25% 'conditioned' CR medium in the light or in darkness (3 replicate plates/treatment).
weeks and thereafter, when any of the marked colonies which remained isolated had burst through the agar surface (ca. 1 mm. diameter) they were removed using a sterile dissecting needle and plated onto individual 5 cm. Petri dishes containing 5 ml. of 50% conditioned CR medium. 6-8 weeks later (including one subculture after 4 weeks onto similar medium) the callus produced had reached approximately 0.3-0.5 g. (fr. wt.) which was sufficient to inoculate a 5 cm. Petri dish containing 10 ml. of non-conditioned CR medium. Thereafter, each cell line was subcultured every 14 days.

As some of the colonies were isolated slightly later than others and some took longer to reach a size suitable for transfer onto standard medium all cell lines were cultured for at least 2 months on the standard medium prior to analysis.

All of the cell lines used for analysis were selected from the 25,000 cells/ml. plates which had been incubated in darkness. To ensure maximal survival all were kept in darkness at all times until their transfer into the light to determine their ability to accumulate anthocyanins.

**Cell line analysis. i. Methods.**

Of the 30 cell lines originally isolated, 26 survived to reach the analysis stage (2 were lost through contamination and 2 failed to survive when transferred to nonconditioned medium). To determine the ability of these lines to accumulate anthocyanins groups of 5 or 6 were randomly selected at the time of each subculture and callus from each was used to inoculate (0.25 g./plate) 3 x 5 cm. Petri dishes containing 10 ml.
CR medium. The plates were then placed in the standard uniform conditions of illumination and after 18 days, were harvested and estimates of culture fresh wt., dry wt., the proportion of pigmented cells and the amount of extractable anthocyanin were made. This procedure was repeated until all cell lines had been analysed. The data obtained from these analyses are collectively presented in Table 3.2.16.

It had been hoped that this analysis could be repeated at least once for each cell line but this proved not to be possible. However, a randomly selected group of eight different cell lines were analysed a second time (ca. 3 months later) to test both the reproducibility of the results and also the stability of the cell lines and the data from both these tests are presented together in Table 3.2.17.

Cell line analysis: ii. Quantitative differences.

As will be described in detail shortly, considerable differences were observed between the cell lines analysed and it is perhaps appropriate to indicate at this point that from the results of those cell lines which were tested a second time (Table 3.2.17.) it would appear that these differences were not only reproducible but were also quite stable, at least for 3-5 months.

In Table 3.2.16., which contains all the data for the 26 cell lines isolated, the results have been listed in decreasing order of mean total anthocyanin yield/culture. The first important feature of these results to draw attention to is that no cell line failed to accumulate anthocyanin when exposed to light. Although the response varied considerably (a differenc
The growth and anthocyanin content of *C. roseus* cell lines which had been isolated from a single stock culture after incubation for 18d. in the light. Each value is the mean of three replicates. For the purposes of clarity standard errors have not been presented. Nevertheless statistical analyses were carried out on the majority of the data (see Cpt.2) and least significant differences (LSD) are reported. Anthocyanin (a/c) contents were determined in OD units/culture/10 ml. solvent (total a/c) or OD units/g./10 ml. solvent (a/c/g.fr.or dry wt.).
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Total a/c</th>
<th>A/c.g(^{-1}) (fr. wt.)</th>
<th>A/c.g(^{-1}) (dry wt.)</th>
<th>% Pig. cells</th>
<th>Fresh wt.(g.)</th>
<th>Dry wt.(g.)</th>
<th>Fr./Dry wt./ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>109</td>
<td>1.18</td>
<td>1.01</td>
<td>22.4</td>
<td>20.2</td>
<td>3.17</td>
<td>0.142</td>
<td>22.4</td>
</tr>
<tr>
<td>103</td>
<td>1.46</td>
<td>0.70</td>
<td>16.3</td>
<td>15.3</td>
<td>2.07</td>
<td>0.089</td>
<td>23.2</td>
</tr>
<tr>
<td>013</td>
<td>1.45</td>
<td>0.34</td>
<td>11.6</td>
<td>5.8</td>
<td>4.30</td>
<td>0.123</td>
<td>34.2</td>
</tr>
<tr>
<td>201R</td>
<td>1.40</td>
<td>0.59</td>
<td>15.9</td>
<td>14.9</td>
<td>2.37</td>
<td>0.088</td>
<td>27.0</td>
</tr>
<tr>
<td>002</td>
<td>1.20</td>
<td>0.46</td>
<td>11.1</td>
<td>11.9</td>
<td>2.65</td>
<td>0.109</td>
<td>24.4</td>
</tr>
<tr>
<td>006</td>
<td>1.19</td>
<td>0.49</td>
<td>10.0</td>
<td>10.2</td>
<td>2.39</td>
<td>0.119</td>
<td>21.7</td>
</tr>
<tr>
<td>008</td>
<td>1.16</td>
<td>0.37</td>
<td>11.7</td>
<td>10.5</td>
<td>3.11</td>
<td>0.100</td>
<td>31.1</td>
</tr>
<tr>
<td>019</td>
<td>1.04</td>
<td>0.28</td>
<td>8.9</td>
<td>11.2</td>
<td>3.74</td>
<td>0.117</td>
<td>32.1</td>
</tr>
<tr>
<td>104</td>
<td>1.00</td>
<td>0.36</td>
<td>9.4</td>
<td>11.0</td>
<td>2.73</td>
<td>0.106</td>
<td>25.7</td>
</tr>
<tr>
<td>108</td>
<td>0.85</td>
<td>0.27</td>
<td>7.3</td>
<td>5.9</td>
<td>3.18</td>
<td>0.117</td>
<td>27.3</td>
</tr>
<tr>
<td>012</td>
<td>0.84</td>
<td>0.35</td>
<td>8.9</td>
<td>10.8</td>
<td>2.36</td>
<td>0.095</td>
<td>25.0</td>
</tr>
<tr>
<td>001</td>
<td>0.80</td>
<td>0.30</td>
<td>6.5</td>
<td>9.7</td>
<td>2.71</td>
<td>0.125</td>
<td>21.7</td>
</tr>
<tr>
<td>007</td>
<td>0.79</td>
<td>0.31</td>
<td>6.5</td>
<td>6.8</td>
<td>2.58</td>
<td>0.121</td>
<td>21.3</td>
</tr>
<tr>
<td>010</td>
<td>0.79</td>
<td>0.31</td>
<td>7.2</td>
<td>8.3</td>
<td>2.57</td>
<td>0.110</td>
<td>23.4</td>
</tr>
<tr>
<td>101</td>
<td>0.61</td>
<td>0.30</td>
<td>6.7</td>
<td>9.2</td>
<td>2.04</td>
<td>0.092</td>
<td>22.3</td>
</tr>
<tr>
<td>011</td>
<td>0.56</td>
<td>0.11</td>
<td>4.7</td>
<td>3.0</td>
<td>3.87</td>
<td>0.118</td>
<td>32.8</td>
</tr>
<tr>
<td>009</td>
<td>0.53</td>
<td>0.15</td>
<td>3.9</td>
<td>2.9</td>
<td>3.57</td>
<td>0.137</td>
<td>26.1</td>
</tr>
<tr>
<td>021</td>
<td>0.46</td>
<td>0.17</td>
<td>4.2</td>
<td>4.7</td>
<td>2.74</td>
<td>0.111</td>
<td>24.8</td>
</tr>
<tr>
<td>015</td>
<td>0.37</td>
<td>0.14</td>
<td>3.8</td>
<td>3.8</td>
<td>2.54</td>
<td>0.097</td>
<td>26.0</td>
</tr>
<tr>
<td>107</td>
<td>0.26</td>
<td>0.08</td>
<td>2.3</td>
<td>1.9</td>
<td>3.26</td>
<td>0.113</td>
<td>28.9</td>
</tr>
<tr>
<td>003</td>
<td>0.23</td>
<td>0.08</td>
<td>1.9</td>
<td>2.7</td>
<td>2.75</td>
<td>0.122</td>
<td>22.6</td>
</tr>
<tr>
<td>018</td>
<td>0.23</td>
<td>0.09</td>
<td>2.2</td>
<td>2.7</td>
<td>2.62</td>
<td>0.102</td>
<td>25.8</td>
</tr>
<tr>
<td>105</td>
<td>0.17</td>
<td>0.07</td>
<td>2.1</td>
<td>2.1</td>
<td>2.58</td>
<td>0.082</td>
<td>31.6</td>
</tr>
<tr>
<td>106</td>
<td>0.15</td>
<td>0.04</td>
<td>1.3</td>
<td>0.8</td>
<td>3.55</td>
<td>0.119</td>
<td>29.9</td>
</tr>
<tr>
<td>017</td>
<td>0.14</td>
<td>0.04</td>
<td>1.3</td>
<td>1.2</td>
<td>3.49</td>
<td>0.106</td>
<td>32.8</td>
</tr>
<tr>
<td>014</td>
<td>0.10</td>
<td>0.03</td>
<td>1.1</td>
<td>0.6</td>
<td>3.40</td>
<td>0.094</td>
<td>36.2</td>
</tr>
<tr>
<td>LSD</td>
<td>0.26</td>
<td>0.07</td>
<td>2.6</td>
<td>-</td>
<td>0.46</td>
<td>0.018</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.2.16.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Fresh wt. (g)</th>
<th>Dry wt. (g)</th>
<th>% Pig.cells</th>
<th>Anthocyanin OD/g./10 ml.</th>
<th>Total a/c OD/cult./10 ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>201R</td>
<td>2.365±0.066</td>
<td>0.088±0.004</td>
<td>14.9</td>
<td>0.587±0.060</td>
<td>1.395±0.173</td>
</tr>
<tr>
<td>(2)</td>
<td>2.565±0.109</td>
<td>0.088±0.008</td>
<td>16.2</td>
<td>0.639±0.058</td>
<td>1.541±0.139</td>
</tr>
<tr>
<td>006</td>
<td>2.592±0.242</td>
<td>0.119±0.004</td>
<td>10.2</td>
<td>0.489±0.037</td>
<td>1.191±0.162</td>
</tr>
<tr>
<td>(2)</td>
<td>3.070±0.129</td>
<td>0.123±0.010</td>
<td>9.5</td>
<td>0.518±0.049</td>
<td>1.403±0.109</td>
</tr>
<tr>
<td>008</td>
<td>3.109±0.249</td>
<td>0.100±0</td>
<td>10.5</td>
<td>0.373±0.028</td>
<td>1.164±0.162</td>
</tr>
<tr>
<td>(2)</td>
<td>2.541±0.251</td>
<td>0.100±0.003</td>
<td>9.0</td>
<td>0.341±0.028</td>
<td>0.871±0.078</td>
</tr>
<tr>
<td>001</td>
<td>2.709±0.073</td>
<td>0.125±0.006</td>
<td>9.7</td>
<td>0.298±0.022</td>
<td>0.804±0.045</td>
</tr>
<tr>
<td>(2)</td>
<td>2.460±0.210</td>
<td>0.095±0.009</td>
<td>9.8</td>
<td>0.325±0.030</td>
<td>0.795±0.090</td>
</tr>
<tr>
<td>018</td>
<td>2.621±0.097</td>
<td>0.102±0.003</td>
<td>2.7</td>
<td>0.087±0.010</td>
<td>0.226±0.019</td>
</tr>
<tr>
<td>(2)</td>
<td>2.519±0.088</td>
<td>0.098±0.010</td>
<td>3.1</td>
<td>0.080±0.007</td>
<td>0.193±0.024</td>
</tr>
<tr>
<td>105</td>
<td>2.577±0.061</td>
<td>0.082±0.004</td>
<td>2.1</td>
<td>0.067±0.012</td>
<td>0.172±0.027</td>
</tr>
<tr>
<td>(2)</td>
<td>2.863±0.210</td>
<td>0.090±0.009</td>
<td>2.5</td>
<td>0.078±0.008</td>
<td>0.217±0.018</td>
</tr>
<tr>
<td>106</td>
<td>3.552±0.151</td>
<td>0.119±0.007</td>
<td>0.8</td>
<td>0.043±0.008</td>
<td>0.151±0.024</td>
</tr>
<tr>
<td>(2)</td>
<td>3.314±0.300</td>
<td>0.112±0.008</td>
<td>1.3</td>
<td>0.059±0.001</td>
<td>0.186±0.004</td>
</tr>
</tbody>
</table>

Table 3.2.17.

A repeat analysis of 7 cell lines to test for cell line stability. Analysis (2) was performed 3-5 months after analysis (1).
of >30 fold was observed) all cell lines had accumulated at least some anthocyanin by the end of the eighteen day experimental period. It was also observed that, with the exception of the most productive cell line which produced over two times more anthocyanin/culture than the next 'best' line, the variation in total culture yield between the cell lines was more or less continuous over its range.

The data for the proportion of pigmented cells found in these cultures also reveals a similar 30 fold difference in response to that mentioned above and in general, the ordering of these data (determined of course by the order of the total anthocyanin yield data) is, with only a few exceptions (eg. Nos. 013, 108, 007 and 021) in the order of highest to lowest. This would suggest that a good correlation is likely to exist between the total product yield and the proportion of productive cells within the culture and this is indeed the case as indicated by Fig.3.2.17.

When the anthocyanin content of these cell lines was expressed on a per g. (fr.wt.) basis once again a wide range of values were found, the extremes of which show a ca. 30 fold difference. In this case also the ordering of the data would suggest that there is a close correlation with total anthocyanin yield and this is borne out by Fig.3.2.18(a). although again there are a few notable exceptions (eg. Nos. 013, 019, 008 and 021). There is, as might be expected a very good correlation between the anthocyanin content/g. (fr.wt.) and the proportion of pigmented cells (see Fig.3.2.19.). When these results are expressed on a per g. (dry wt.) basis a slightly reduced range of values is observed but once again there is a good correlation with total product yield (Fig.3.2.18(b).).
Figure 3.2.17 (above). The relationship between mean anthocyanin yield/culture and the proportion of visibly pigmented cells for 26 isolated *C. roseus* callus cell lines (see Table 3.2.16.).

Figure 3.2.18(a). The relationship between the anthocyanin content/g.(fr.wt.) and the total anthocyanin yield/culture for the isolated cell lines.

(b) The relationship between the anthocyanin content/g.(dry wt.) and the total anthocyanin yield/culture for the isolated cell lines.

Figure 3.2.19. The relationship between the anthocyanin content /g.(fr.wt.) and the proportion of pigmented cells for the isolated cell lines.

Figure 3.2.20. The relationship between the anthocyanin yield/culture and culture fresh wt. for the isolated cell lines.
Figure 3.2.18.
Figure 3.2.19.

Figure 3.2.20.

r = 0.95

r = -0.03
The growth of these cultures, as measured either on a fresh weight or a dry weight basis after 18 days incubation showed relatively little variation with a maximum difference of ca. 2 times. However, in this case it is clear from the results that there is no correlation between the anthocyanin content and the final biomass attained by the cultures. (see Fig.3.2.20.). Similarly, no correlation was apparent between the total anthocyanin yield and the fresh weight/dry weight ratio which might, albeit naively be suggested to crudely indicate the degree of cell expansion which had occurred within the cultures.

Microdensitometric analysis.

When a microdensitometric analysis was carried out on the callus from which these cell lines had been derived (Section 3.2.8.) it became clear that a considerable variation in anthocyanin content occurred within the visibly pigmented cell population. It was thus considered that such an investigation carried out on the various cell lines might yield some very valuable information concerning both the nature of the overall yield differences between these lines as described above and also the basis of the variation observed within the original callus stock.

As mentioned previously microdensitometry is an extremely tedious and very time-consuming procedure and thus, unfortunately it was not practical to study each cell line individually by this method. Consequently four cell lines were analysed which were 'randomly' selected, two from the 6 highest yielding lines and two from the 6 lowest. Protoplasts were obtained as
detailed previously (using 18 day old callus) and micro-
densitometric measurements were made on a random sample of
100 pigmented cells from each cell line. Using the data
obtained mean values for both the anthocyanin content per cell
and also for the estimated anthocyanin concentration of each
protoplast were determined. These results are presented in
Table 3.2.18. Histograms of cell frequency vs. anthocyanin
content were also prepared for each cell line and these are
presented in Fig.3.2.21. The data obtained for the original
stock callus are included with these results for easy comparison.

In terms of the anthocyanin content/pigmented cell slightly
modified ranges were observed in the low yielding cell lines
in comparison to the source callus (Fig.3.2.21.). The values
obtained showed a slightly more restricted distribution with
a greater proportion of the cells occurring at the lower end
of the scale. In the two higher yielding lines both the over-
all range and the modal range were increased in comparison to
the low yielding lines but differed only slightly from the
original stock. In all cases the difference between highest
and lowest values was always 35-45 fold in comparison to 40 fold
in the source callus.

In terms of the estimated anthocyanin concentration of
the protoplasts in the lowest yielding cell line (014) a
definite reduction in the overall range was observed with
almost 70% of the cells falling into a single range category.
However, in the remaining 3 cell lines the differences both
between each other and between the original callus were very
much reduced. For example, the mean values for cell lines
105 and 006 were insignificantly different despite a 7 fold
<table>
<thead>
<tr>
<th>Cell line</th>
<th>IOD&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Range</th>
<th>IOD/vol&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>014</td>
<td>76.7 ± 6.8</td>
<td>8-337 (42x)</td>
<td>0.176 ± 0.007</td>
<td>0.07-0.53 (7x)</td>
</tr>
<tr>
<td>105</td>
<td>100.4 ± 8.9</td>
<td>8-377 (45x)</td>
<td>0.239 ± 0.014</td>
<td>0.08-0.75 (9x)</td>
</tr>
<tr>
<td>006</td>
<td>149.9 ± 9.4</td>
<td>12-465 (38x)</td>
<td>0.229 ± 0.011</td>
<td>0.09-0.69 (8x)</td>
</tr>
<tr>
<td>103</td>
<td>144.6 ± 10.7</td>
<td>18-614 (34x)</td>
<td>0.297 ± 0.017</td>
<td>0.08-0.87 (11x)</td>
</tr>
<tr>
<td>Stock callus</td>
<td>161.0 ± 14.1</td>
<td>14-645 (46x)</td>
<td>0.247 ± 0.015</td>
<td>0.10-0.79 (8x)</td>
</tr>
</tbody>
</table>

Table 3.2.18.

Microdensitometric analysis of the visibly pigmented cell populations within 18d. old callus cultures of 4 of the cell lines isolated from a *C.roseus* stock culture.

1 IOD is equivalent to the mean anthocyanin content/cell.

2 IOD/vol. is equivalent to the mean intracellular anthocyanin concentration.
Figure 3.2.21.

Intercellular variation in anthocyanin content of 4 C. roseus cell lines (see also Table 3.2.18.).
difference in the overall anthocyanin yield per culture. In all the cell lines examined a 7-11 fold range difference was observed between the highest and lowest estimated anthocyanin concentrations as compared to an 8 fold difference in the original callus.

From these results it would appear therefore that the most important difference between these cell lines as concerns the overall culture yield of anthocyanins was the proportion of visibly productive cells as the anthocyanin content, when measured on a cellular basis varied relatively little. This has indeed been reflected by the close relationship observed when the values for total extractable anthocyanin were plotted against the pigmented cell percentage of the total cell population (Fig.3.2.17.).

The remaining observation to which attention must be drawn is that although considerable variation in the total anthocyanin yield of these cell lines was evident, in comparison to the original source callus the great majority of the cell lines isolated accumulated less, rather than more anthocyanin. Although the stock callus showed some variation in anthocyanin productivity, in general levels of anthocyanin of ca. 0.5 OD units/g.(fr.wt.)/10 ml. with callus fresh weight/culture of ca. 3 g. would put it on a par with the second and third highest yielding cell lines. The implications of these findings will be discussed in detail in Chapter 4.

Cell line analysis: iii. Qualitative differences.

Surprisingly, very few qualitative differences were observed between the 26 cell lines isolated.
**Culture friability:** In all cases the friability of the cultures was very similar to that of the original stock callus in that their extreme friability resulted in an apparently homogeneous "creamy" callus which grew in a flat layer over the agar surface.

**Culture pigmentation:** When grown in the dark all of the cell lines produced uniformly creamy-white callus. In one cell line however, no. 201R, it was observed that frequently (but not always) a few visibly pigmented red cells (<0.1%) appeared towards the end of the subculture period.

When grown in the light none of these lines failed to accumulate anthocyanin to some degree and in all cases the coloured cells which appeared were distributed relatively uniformly over the callus surface.

Microscopic observations on the original stock culture revealed (see Fig.3.2.1.) that the pigmented cell population consisted of three basic cell types, one of which was magenta in colour, one purple and the third an intermediate colour. As the expression of anthocyanin colour *in vivo* is determined by two main factors - the actual anthocyanin(s) present (cyanidin glycosides, delphinidin glycosides etc.) and the intracellular conditions (pH, copigments present) and it is known that *C. roseus* plants and tissue cultures produce three different anthocyanins (Forsyth & Simmonds, 1957, Knobloch *et al.*, 1982, and this volume) it is therefore possible that these colour differences may indicate that different cells were producing different anthocyanins in these cultures. However, microscopic observations revealed that even after cell line selection all of the lines isolated continued to produce cells of each colour.
type and TLC analysis of cell extracts (Fig.3.2.22.) indicated that each cell line accumulated all three of the anthocyanins originally present. It would appear therefore, that the colour differences observed within the pigmented cell population either did not reflect qualitative intracellular differences in anthocyanin content or if they did then they were not based upon stable heritable differences between the cells.
Figure 3.2.22. TLC analysis of methanolic extracts of the C. roseus cell lines isolated as detailed in the text. The spots drawn were all magenta in colour. Solvent n-Butanol : Acetic acid : H₂O 5:1:4.
The following points have arisen from the work reported in this section.

1. Cell plating techniques have been used successfully to isolate a number of individual cell lines.

2. Analysis of these cell lines has revealed differences in both growth and anthocyanin accumulation after an 18 day period of incubation of the cultures in the light.

3. The anthocyanin content of these lines, in terms of total yield/culture, yield/g.(fr.wt.) and the proportion of visibly pigmented cells was found to vary by >30x.

4. Microdensitometric analyses have revealed that within the pigmented cell population of four of these lines the mean values of either the anthocyanin content/cell or the intracellular anthocyanin concentration varied only slightly despite considerable variation in the overall culture yield.

5. Few of the cell lines isolated accumulated anthocyanins to an extent similar to, or greater than that of the original source callus.

6. Very few qualitative differences in either culture appearance or pigment accumulation were observed between the various cell lines isolated.

In the following section the use of precursor feeding techniques are investigated as a means to increase the proportion of pigmented cells in C.roseus cultures and thus increase the overall metabolite yield.
Section 3.2.6.

"The effects of precursor 'feeding' on the accumulation of anthocyanins in callus and suspension cultures of *C. roseus."

There are now many reports in the literature which demonstrate the value of exogenously supplying plant tissue cultures with specific metabolic precursors as a means to enhance the accumulation of certain desired secondary metabolites in vitro (see Yeoman et al. (1980), Fowler, (1983) and Mantell&Smith, (1983b) for refs.). The following five experiments were performed to investigate whether the exogenous application of suitable precursors could enhance the yield of anthocyanins in this culture system and to determine if such a treatment could alter the level of predominance of the nonpigmented (non producing?) cells within the cultures.

In these experiments a wide range of precursors were used and the very simplified biosynthetic pathway charted in Fig.3.2.23. has been included to demonstrate the relative positions of these compounds in the currently proposed anthocyanin metabolic pathway. However, it must be pointed out that anthocyanin biosynthesis has not specifically been studied in Catharanthus and that in those species which have been studied a less than complete picture has yet been obtained and in certain instances slight variations in the pathway have been found. Consequently, Fig.3.2.23. represents a compendium of the possible pathways which may occur and it should be understood that not all of the arrowed links may be present in Catharanthus tissues, especially in those instances where alternative pathways to the same compound are shown.
Figure 3.2.23

A compendium of the possible metabolic steps of the anthocyanin biosynthetic pathway. This figure has been drawn up from findings reported in the literature some of the most important of which may be found in the following: Wiering, 1974; Kho and Bennink, 1975; Steiner, 1975; Forkmann, 1977; Kho et al., 1977; Tabak et al., 1978; Forkmann, 1980; Forkmann et al., 1980; Heinsbroek and Brederode, 1980; Kamsteeg et al., 1980; Schram et al., 1981; Tabak et al., 1981; Dooderman et al., 1982; Gerats et al., 1982; Jonsson et al., 1982; Spribille and Forkmann, 1982.
Shikimate pathway

Phenylalanine → Cinnamate → p-Coumarate*

Caffeic acid → p-Coumaroyl CoA

Ferulic acid* → Acetyl CoA

Caffeoyl CoA

Sinapic acid*

Naringenin chalcone = Naringenin

Naringin

Apigenin

Eriodictiol chalcone

Dihydrokaempferol → Kaempferol

Flavonols

Quercetin → Dihydroquercetin

Dihydromyrecetin

Myrecetin

Cyanidin

Delphinidin

Pelargonidin

Petunidin

Malvidin

* involved in acylation

Figure 3.2.23
The influence of various precursors on anthocyanin accumulation in *C. roseus* callus cultures when included in the nutrient medium.

Two preliminary experiments involving 6 different anthocyanin precursors were performed using *C. roseus* callus cultures. Both experiments were set up in a similar manner (on successive subculture periods) using 11 day old dark-grown stock callus. Three Petri dishes (5 cm.)/treatment were used into which was placed 10 ml. CR medium supplemented with the desired concentration of precursor. Each plate, when cool was inoculated with 0.25 g. callus and all were harvested 18 days later. The plates were incubated in either standard lighting conditions or in darkness.

The precursors were generally added to give final concentrations of 5, 1 or 0.1 mM. (Apigenin 0.5 and 0.1 mM.) and with one exception were dissolved in distilled water. Each solution was adjusted to pH 5.8 before filter-sterilization. In the 5 mM. cinnamate treatment the precursor was heated to 50°C for 5 min. and filtered immediately in order to obtain a sterile solution of the necessary concentration. In the naringin treatment it was not possible to obtain a 5 mM. concentration in vitro and so in this case the highest concentration used was ca. 2 mM., produced by mixing equal volumes of 2xconc. CR medium and a saturated solution of naringin. Apigenin is only slightly soluble in nonalkaline solutions and consequently this compound was added to the medium prior to autoclaving.

The results are presented in Tables 3.2.19, 3.2.20. and 3.2.21. From Table 3.2.19, which includes
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fresh wt. (g)</th>
<th>Dry wt. (g)</th>
<th>Anthocyanin content</th>
<th>Total a/c. OD/g.(fr.wt.)</th>
<th>10 ml. OD/cult./10 ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5mM. CIN</td>
<td>0.201 ±0.044³</td>
<td>nd</td>
<td>0.005 ±0³</td>
<td>0.001 ±0³</td>
<td></td>
</tr>
<tr>
<td>1mM. CIN</td>
<td>0.696 ±0.329³</td>
<td>nd</td>
<td>0.027 ±0.016³</td>
<td>0.019 ±0.022³</td>
<td></td>
</tr>
<tr>
<td>0.1mM. CIN</td>
<td>1.597 ±0.343</td>
<td>0.044</td>
<td>0.289 ±0.096²</td>
<td>0.507 ±0.221²</td>
<td></td>
</tr>
<tr>
<td>5mM. MAL</td>
<td>0.979 ±0.131²</td>
<td>0.042</td>
<td>0.191 ±0.054³</td>
<td>0.200 ±0.083³</td>
<td></td>
</tr>
<tr>
<td>1mM. MAL</td>
<td>1.811 ±0.306</td>
<td>0.053</td>
<td>0.356 ±0.017¹</td>
<td>0.650 ±0.124¹</td>
<td></td>
</tr>
<tr>
<td>0.1mM. MAL</td>
<td>1.784 ±0.236</td>
<td>0.045</td>
<td>0.231 ±0.019²</td>
<td>0.418 ±0.079²</td>
<td></td>
</tr>
<tr>
<td>5mM. PHE</td>
<td>2.100 ±0.170²</td>
<td>0.057</td>
<td>0.204 ±0.015²</td>
<td>0.423 ±0.002²</td>
<td></td>
</tr>
<tr>
<td>1mM. PHE</td>
<td>2.059 ±0.194</td>
<td>0.054</td>
<td>0.379 ±0.116</td>
<td>0.798 ±0.269</td>
<td></td>
</tr>
<tr>
<td>0.1mM. PHE</td>
<td>1.836 ±0.380²</td>
<td>0.044</td>
<td>0.234 ±0.077²</td>
<td>0.489 ±0.222²</td>
<td></td>
</tr>
<tr>
<td>CONTROL</td>
<td>2.274 ±0.090</td>
<td>0.063</td>
<td>0.565 ±0.111</td>
<td>1.279 ±0.262</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2.19.

The influence of precursor-supplemented media upon the growth and anthocyanin content of illuminated *C. roseus* callus cultures. For details see text.

CIN = Cinnamate, MAL = Malonate, PHE = Phenylalanine.

Significantly different from control (+H₂O) at

¹ P=0.05, ² P=0.01, ³ P=0.001.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fresh wt. (g)</th>
<th>Dry wt. (g)</th>
<th>Anthocyanin content</th>
<th>Total a/c OD/g./10ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5mM.DEM</td>
<td>0.385±0.019³</td>
<td>nd</td>
<td>0.010±0³</td>
<td>0.004±0³</td>
</tr>
<tr>
<td>1mM.DEM</td>
<td>0.825±0.218²</td>
<td>nd</td>
<td>0.020±0.010³</td>
<td>0.017±0.010³</td>
</tr>
<tr>
<td>0.1mM.DEM</td>
<td>2.473±0.107</td>
<td>0.119±0.002</td>
<td>0.071±0.010</td>
<td>1.730±0.111</td>
</tr>
<tr>
<td>50%Sat.NAR</td>
<td>2.348±0.233</td>
<td>0.125±0.010</td>
<td>0.362±0.120²</td>
<td>0.845±0.049³</td>
</tr>
<tr>
<td>1mM.NAR</td>
<td>2.198±0.059</td>
<td>0.120±0.001</td>
<td>0.920±0.101</td>
<td>2.020±0.195</td>
</tr>
<tr>
<td>0.1mM.NAR</td>
<td>2.416±0.063</td>
<td>0.114±0.005</td>
<td>0.671±0.071</td>
<td>1.619±0.113</td>
</tr>
<tr>
<td>0.5mM.API</td>
<td>1.480±0.584¹</td>
<td>nd</td>
<td>0.191±0.142³</td>
<td>0.281±0.050³</td>
</tr>
<tr>
<td>0.1mM.API</td>
<td>2.565±0.092</td>
<td>0.119±0.011</td>
<td>0.773±0.121</td>
<td>1.975±0.203</td>
</tr>
<tr>
<td>CONTROL</td>
<td>2.609±0.085</td>
<td>0.119±0.001</td>
<td>0.862±0.050</td>
<td>2.241±0.154</td>
</tr>
</tbody>
</table>

Table 3.2.20.

The influence of precursor-supplemented media upon the growth and anthocyanin content of illuminated *C. roseus* callus cultures. For details see text. DEM = Diethyl malonate, NAR = Naringenin 7-Rhamnosidoglucoside, API = Apigenin. Significantly different from control (+H₂O) at ¹ p=0.05, ² p=0.01, ³ p=0.001.
Table 3.2.21.

The influence of precursor-supplemented media upon the growth and anthocyanin content of *C. roseus* callus cultures incubated in darkness. For full experimental details see text.

1 NAR = Naringenin 7-rhamnosidoglucoside

2 DEM = Diethyl malonate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fr. wt. (g)</th>
<th>Dry wt. (g)</th>
<th>Anthocyanin content OD/g./10 ml.</th>
<th>Total a/c OD/cult./10 ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>2.338 ±0.518</td>
<td>0.113 ±0.021</td>
<td>0.016 ±0.016</td>
<td>0.037 ±0.021</td>
</tr>
<tr>
<td>1mM.NAR ¹</td>
<td>2.353 ±0.132</td>
<td>0.200 ±0.042</td>
<td>0.050 * ±0.006</td>
<td>0.118 ** ±0.011</td>
</tr>
<tr>
<td>1mM.DEM ²</td>
<td>1.172 * ±0.435</td>
<td>0.073 ±0.015</td>
<td>0.010 ±0</td>
<td>0.012 ±0.003</td>
</tr>
</tbody>
</table>

Significantly different from the control (+H₂O) at * P=0.05, ** P=0.01.
the results of the first experiment using cinnamic acid, malonic acid and phenylalanine it is apparent that these compounds had considerable effects on both culture growth and anthocyanin accumulation. Cinnamic acid proved toxic at the two higher concentrations tested, producing total necrosis in the cultures. Some necrosis was also visible in the 5 mM. malonate treatment resulting in a significant reduction in culture biomass. Growth was also reduced in all of the other treatments in comparison to the controls but not to the level of statistical significance. The anthocyanin levels accumulated in the treated cultures were also reduced, significantly so with the exception of only one treatment (1 mM. Phenylalanine).

In the second experiment (Tables 3.2.20 and 3.2.21) diethyl malonate (an ester of malonic acid) was tested as were Naringin (Naringenin-7-rhamnosidogluicoside) and Apigenin, two compounds which are separated by a single biochemical step from Naringenin, the first component of the anthocyanin biosynthetic pathway to have the basic C\textsubscript{15} flavonyl skeleton and which plays a key role in anthocyanin biosynthesis (Fig.3.2.23). Some cultures were grown in darkness (Table 3.2.21.) to determine if precursor feeding could overcome the requirement for light by these cultures. The remainder (Table 3.2.20.) were grown under standard lighting conditions to determine if the use of precursors could increase anthocyanin accumulation beyond normal control levels.

In the light experiment the final biomass of the cultures was found to be reduced significantly in only three treatments, 0.5 mM. apigenin, 5 and 1 mM. diethyl malonate. (In the 5 mM. diethyl malonate treatment total necrosis of the cultures
was evident). All of the other treated cultures produced fresh weight values comparable to those of the controls.

With the exception of the 1 mM. naringin treatment, the levels of accumulated anthocyanins in the treated cultures were reduced in comparison to that of the control, significantly so in at least those treatments involving the highest precursor concentrations.

In the dark-grown cultures (Table 3.2.21.) 1 mM. diethyl malonate again showed indications of toxicity. However, in those cultures exposed to 1 mM. naringin a significant increase in anthocyanin yield was observed over the control value. The final levels attained were however pitifully low (6%) in comparison to the light-grown controls.

Callus cultures are not very suitable in vitro systems to use when studying the effect of precursors on cell metabolism. As the precursors must be added to the medium at the time of subculture it is possible that they may become degraded (by physical or chemical means) or utilised by the cells in primary metabolism before the phase of secondary metabolism is reached. Also, if the precursors have even slightly toxic properties then the prolonged exposure of the cells to the compounds may result in detrimental effects on culture viability.

Most, if not all of these problems can be overcome by using liquid cultures to which the addition of precursors may be delayed until such a time when they are likely to have most effect. Consequently, when space became available the following two experiments were carried out using C.roseus suspension cultures in order to determine the effect of
precursors when specifically applied during the stationary phase of the culture growth cycle.

\textit{ii. The influence of certain precursors on the anthocyanin accumulation of light and dark-grown \textit{C.roseus} cell cultures when supplied during the stationary phase.}

Both of the experiments, the results of which are reported in this subsection, were performed using 20 ml. liquid cultures which had been inoculated with 1 ml. of a 12d old dark-grown cell suspension. The cultures were incubated in the light or in darkness for 11 days after which sterile solutions of certain precursors were added to give the final concentrations listed in Tables 3.2.22-3.2.24. As the cells in these cultures are held in more intimate contact with the nutrient medium, in general the precursor concentrations tested were lower than those previously used in the callus experiments. On day 18 all flasks were harvested and the usual measurements made.

The precursors were added either in aqueous solutions (pH 5.8) after filter-sterilization or in the case of the poorly soluble compounds, in an aqueous suspension (pH 5.8) after ethanol sterilization. The latter was carried out by placing a known amount of precursor (e.g. Dihydroquercetin) in a sterile vial to which was then added 1 ml. of 96% ethanol. The vials were sealed and after 16 h. the seals were removed aseptically in a laminar flow cabinet and when all the liquid had evaporated sterile water (pH 5.8) was added and the solid resuspended. Aliquots of the suspension were then added as necessary to the culture flasks.
The results are presented in Tables 3.2.22, 3.2.23 and 3.2.24. In the first experiment (Table 3.2.22) in which all cultures were grown in darkness the addition of either naringin or phenylalanine at 0.05 or 0.5 mM. was found to have no significant effect on culture growth or anthocyanin accumulation. The levels of the latter were however in all cases increased (20-60%) in the treated cultures.

In the second experiment, in those cultures grown in the light (Table 3.2.23) one treatment gave significantly enhanced anthocyanin yields. Naringin (0.5 mM.) was found to increase the accumulation of anthocyanin in these cultures by ca. 50% in comparison to control levels. In both of the remaining treatments the culture anthocyanin content was also increased but to a lesser extent. In none of these treatments was the growth of the cultures significantly affected by the compounds added.

Of the 8 treatments which were tested in the second experiment in the absence of light (Table 3.2.24) only 2 were found to have significantly affected the culture fresh weight. In the 0.5 mM. Malonate/0.05 mM. cinnamate combined treatment the culture growth was inhibited whereas surprisingly, in the 1 mM. dihydroquercetin treatment culture growth had been significantly increased.

The levels of anthocyanin accumulated by these cultures were generally very low although in three treatments a significant increase above the control value was found. Two of these involved dihydroquercetin, a dihydroflavonol which occurs very near the end of the anthocyanin biosynthetic
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fresh wt. (g)</th>
<th>Dry wt. (g)</th>
<th>Anthocyanin content</th>
<th>Total a/c OD/g./10ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>4.056 ± 0.28</td>
<td>0.108 ± 0.004</td>
<td>0.024 ± 0.009</td>
<td>0.102 ± 0.044</td>
</tr>
<tr>
<td>0.05mM.NAR</td>
<td>3.328 ± 0.582</td>
<td>0.098 ± 0.009</td>
<td>0.030 ± 0.008</td>
<td>0.105 ± 0.042</td>
</tr>
<tr>
<td>0.5mM.NAR</td>
<td>4.248 ± 0.136</td>
<td>0.112 ± 0.001</td>
<td>0.029 ± 0.008</td>
<td>0.123 ± 0.033</td>
</tr>
<tr>
<td>0.05mM.PHE</td>
<td>3.581 ± 0.599</td>
<td>0.107 ± 0.003</td>
<td>0.032 ± 0.010</td>
<td>0.124 ± 0.052</td>
</tr>
<tr>
<td>0.5mM.PHE</td>
<td>3.689 ± 0.567</td>
<td>0.114 ± 0.004</td>
<td>0.038 ± 0.014</td>
<td>0.124 ± 0.062</td>
</tr>
</tbody>
</table>

Table 3.2.22.

The influence of exogenously supplied precursors upon the growth and anthocyanin content of non-illuminated *C. roseus* cell cultures when added 10d. after initiation. For full experimental details see text.

NAR = Naringenin 7-rhamnosidoglucoside   PHE = Phenylalanine
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fresh wt. (g)</th>
<th>Dry wt. (g)</th>
<th>Anthocyanin content</th>
<th>Total a/c. OD/g./10ml.</th>
<th>% Pig cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>4.088 ±0.224</td>
<td>0.155 ±0.006</td>
<td>0.842 ±0.063</td>
<td>3.424 ±0.174</td>
<td>7.8</td>
</tr>
<tr>
<td>0.5mM.PHE</td>
<td>3.684 ±0.261</td>
<td>0.176 ±0.012</td>
<td>0.992 ±0.085</td>
<td>3.614 ±0.133</td>
<td>8.5</td>
</tr>
<tr>
<td>0.5mM.NAR</td>
<td>3.953 ±0.309</td>
<td>0.165 ±0.012</td>
<td>1.288 * ±0.038</td>
<td>5.101 ** ±0.482</td>
<td>11.6</td>
</tr>
<tr>
<td>1.0mM.DHQ</td>
<td>3.967 ±0.300</td>
<td>0.160 ±0.007</td>
<td>0.943 ±0.100</td>
<td>3.723 ±0.413</td>
<td>9.1</td>
</tr>
</tbody>
</table>

Table 3.2.23.

The influence of exogenously supplied precursors upon the growth and anthocyanin content of illuminated *C. roseus* cell cultures when added 10d. after initiation. For full details see text. PHE=Phenylalanine, NAR=Naringenin 7-rhamnosido-glucoside, DHQ=Dihydroquercetin. Significantly different from controls at *P=0.05, **P=0.01.

Table 3.2.24. (overleaf)

The influence of exogenously supplied precursors upon the growth and anthocyanin content of non-illuminated *C. roseus* cell cultures when added 10d. after initiation. For full details see text. (Precursor abbreviations as previously plus FER=Ferulic acid, QUE=Quercetin, NAR=Naringenin). Significance levels as above. n.d. = not detectable.
<table>
<thead>
<tr>
<th>Type</th>
<th>OD</th>
<th>OD/9.5M</th>
<th>OD/25M</th>
<th>OD/25M CIN</th>
<th>OD/25M MAT</th>
<th>OD/25M CIN</th>
<th>OD/25M MAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pu</td>
<td>9</td>
<td>0.010</td>
<td>0.016</td>
<td>0.016</td>
<td>0.015</td>
<td>0.016</td>
<td>0.015</td>
</tr>
<tr>
<td>Pu</td>
<td>1</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
</tr>
<tr>
<td>Pu</td>
<td>1.5</td>
<td>0.088</td>
<td>0.098</td>
<td>0.098</td>
<td>0.098</td>
<td>0.098</td>
<td>0.098</td>
</tr>
<tr>
<td>Pu</td>
<td>2</td>
<td>0.032</td>
<td>0.032</td>
<td>0.032</td>
<td>0.032</td>
<td>0.032</td>
<td>0.032</td>
</tr>
<tr>
<td>Pu</td>
<td>3</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td>Pu</td>
<td>4</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
</tr>
<tr>
<td>Pu</td>
<td>5</td>
<td>0.032</td>
<td>0.032</td>
<td>0.032</td>
<td>0.032</td>
<td>0.032</td>
<td>0.032</td>
</tr>
<tr>
<td>Pu</td>
<td>6</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td>Pu</td>
<td>7</td>
<td>0.007</td>
<td>0.007</td>
<td>0.007</td>
<td>0.007</td>
<td>0.007</td>
<td>0.007</td>
</tr>
</tbody>
</table>

**Table 3.2.2.4.**
pathway. However, despite its close proximity to the final product, culture yields of anthocyanin were still only at best ca.2% of the usual levels achieved in standard light-grown cultures. Similarly, 5 mM phenylalanine induced a significant increase in anthocyanin accumulation but in this instance levels of only ca.1% of typical control values were detected.

The clear, general conclusion to draw from all of these experiments is that despite using logical precursors, applied theoretically at the most suitable time in the culture growth cycle very little/no enhancement of anthocyanin accumulation occurred. One possible explanation of this failure is that the precursors were not taken up by the cells. This point will partly be investigated in the next section. One other possibility is that in the light-grown cultures the precursors may influence the rate of accumulation of anthocyanins but not the final level attained. This is an important point which has considerable implications with regard to the control of in vitro anthocyanin accumulation. Accordingly, the following experiment was performed to investigate this.

iii. An investigation into the time course of anthocyanin accumulation in C. roseus cell cultures following the addition of 1 mM phenylalanine.

In previous precursor feeding experiments end-point sampling was used to investigate the effect of specific precursors on anthocyanin accumulation. Such a procedure
cannot give us any indication as to the rate of accumulation, a factor which may also be influenced by precursor presence. This experiment was thus performed to investigate this possibility. The precursor used, phenylalanine, was chosen because it was simple to apply, known to be taken up by the cells (see following section) and had consistently given slightly enhanced anthocyanin yields over the controls in previous experiments.

Thirty, 100 ml. conical flasks each containing 20 ml. CR medium were inoculated with 1 ml. of a 12d. old dark-grown cell suspension. After 9 days of incubation in the light, by which time the first signs of anthocyanin accumulation were apparent, half of the cultures were given 1 ml. filter-sterilized water (pH 5.8) and half, 1 ml. of a filter-sterilized aqueous phenylalanine stock solution (pH 5.8) to give a final culture concentration of 1 mM. The cultures were then returned to the previous incubation conditions. Three flasks from each treatment were harvested on 0, 2, 4, 6 and 9 days after feeding. Estimates of culture growth and anthocyanin accumulation were made for each sample day and the results have been presented graphically in Fig. 3.2.24.

The results indicate that this experiment was initiated at a time when the cell division phase had been completed (Fig.3.2.24e.) and the cultures were in (or entering) a phase of rapid cell expansion (Fig.3.2.24a/b.). By the end of the experimental period this latter phase was nearing completion as the culture fresh wt./PCV had begun to tail off and the culture dry weights (Fig.3.2.24c.) had reached their maximum values. No significant differences were found between the
Figure 3.2.24

The growth and anthocyanin content of illuminated *C. roseus* cell cultures (20 ml.) between the 9th and 18th days from subculture. The cultures were given 1 ml. H₂O (●) or 1 ml. 21mM. phenylalanine (O) on day 9. For full details see text.
pattern of growth in the control cultures and that of the cultures given 1 mM phenylalanine.

The anthocyanin accumulation of these cultures followed the standard pattern with an initial rapid increase up to ca. day 15-16 after which values quickly levelled off (Fig. 3.2.24d/f.). Very little difference was observed between the treated and untreated cultures although the values for the former were generally slightly higher than those of the controls. At one sample time however, 4 days after precursor application a 2-3 fold increase in the anthocyanin content (g.\textsuperscript{-1} fr. wt.) of the treated cultures was observed in association with a 1.5 fold increase in the proportion of pigmented cells. Although this difference was not statistically significant and was only observed at a single sample time, these values may indicate a slight enhancement in the rate of anthocyanin accumulation in the phenylalanine-treated cultures.
The following points have arisen from the precursor feeding experiments reported in this section.

(1) Numerous attempts have been made to enhance the *in vitro* accumulation of anthocyanins by the exogenous supply of suitable precursors to callus and cell cultures of *C. roseus*.

(2) Precursor supplementation did not overcome the light requirement of these cultures for anthocyanin accumulation to any considerable degree.

(3) In the light, precursor addition on only one occasion significantly increased anthocyanin levels above those for the untreated controls.

(4) 1mM. phenylalanine was found to only slightly increase the rate of anthocyanin accumulation in illuminated cell suspensions.

In the following section are reported the results of a small number of experiments which were carried out using $^{14}$C-labelled phenylalanine in order to investigate further aspects of anthocyanin metabolism in this culture system.
Section 3.2.7.

"The use of $^{14}$C-phenylalanine to investigate anthocyanin metabolism in *C. roseus* cell cultures grown under potentially inductive conditions."
The use of radioactively labelled precursors has, in the past, proved to be an enormously valuable tool with which to study various aspects of plant cell metabolism in vivo, using either whole plants or tissue culture systems (see for example, Phillips and Henshaw, 1977; Yeoman et al., 1980; Lindsey and Yeoman, 1983b; Turnbull et al., 1980). In this investigation a small number of experiments were performed using $^{14}$C-phenylalanine as a means to study possible alterations to cell metabolism as induced by modified culture conditions, namely exogenous precursor feeding and nutrient starvation.

In previous precursor feeding experiments (preceding section) it was observed that exogenously applied precursors failed, on all but one occasion, to significantly enhance anthocyanin accumulation in light grown *C. roseus* cell cultures. There are many possible explanations for these negative results, including uptake failure, incorporation failure, increased product/precursor turnover etc., and it was hoped that many of these possibilities could be eliminated by using $^{14}$C labelling experiments, thus yielding a more precise and detailed picture of the control of anthocyanin accumulation in this system. Similarly, it was aimed to use $^{14}$C-phenylalanine as a probe with which to study the possible metabolic effects of nutrient starvation upon *C. roseus* cell cultures, a treatment which has previously been demonstrated (Section 3.2.3(vii).) to yield increased levels of anthocyanin/g. fresh weight of cells.

In the following section a slight alteration to the style of presentation has been employed. Due to the intrinsic difficulties and complications involved in analysing the results
of labelling experiments it was considered advisable to discuss briefly each experiment immediately following the experimental results. This has enabled a brief outline of their implications to be presented and also avoids undesirable overconsideration of a particular set of results in the Discussion which would otherwise have occurred.

i. The uptake and incorporation of $^{14}$C-phenylalanine into anthocyanins in early stationary-phase cultures of C. roseus

Prior to carrying out some of the experiments proposed in the introduction to this section, the following two preliminary experiments were undertaken to determine both the applicability of the proposed practical techniques to be used and also the kinetics of $^{14}$C-phenylalanine uptake and incorporation into anthocyanins in this system. From these results the most appropriate practical procedures to choose for future experiments could then be determined.

In the first experiment the major aim was to determine if $^{14}$C-phenylalanine uptake and incorporation into anthocyanins over a 48h period proceeded to a measurable level using the techniques available. The aim of the second experiment was to determine the kinetics of $^{14}$C-phenylalanine uptake and incorporation over a 48h period by using an intermittent sampling regime.

Both of these experiments were set up in the identical manner now described. From a well mixed cell suspension, obtained by pooling two, 9 day old, light-grown stock cultures
which were showing the first indications of anthocyanin accumulation, were removed 3x10ml. samples of suspension culture. These were placed in 3 sterile, 100 ml. conical flasks to which was then added 2μCi. of L-U-1\(^4\)C-phenylalanine (40 μl. of the stock solution) and the cultures gently but thoroughly mixed. In the first experiment the cultures were harvested after a 48h period of incubation in the light and the extractions/measurements made as detailed in Chapter 2.

In the second experiment 200 μl. cell suspension samples were removed from the well-mixed cultures at regular intervals using a Gilson micropipette fitted with a wide bore disposable tip. These samples were placed in 1 ml. Eppendorf tubes and immediately centrifuged at ca.10,000xg for 3 min. Aliquots (50 μl.) of the supernatant (nutrient medium) were then taken for scintillation counting. Having initially determined the total volume of medium/culture, the % uptake of radioactivity could be estimated from the values obtained. (As a small but steady decline in the total volume of medium/culture would have occurred over the 48h period as a result of cell expansion, the uptake values calculated towards the end of the experiment would have been slight underestimations.)

To determine the level of \(^1\)C-phenylalanine incorporation into anthocyanins in this experiment, the cell pellets obtained above were freed from as much of the remaining bathing medium as possible before the addition of 200 μl. cold (4°C) MeOH. 1% HCl. The pellets were then resuspended and the mixtures allowed to stand for 1h in the dark at 4°C. After a second centrifugation the supernatant in each tube was removed and the pellet
washed twice using 2x100 μl. MeOH. 1% HCl. The supernatants were pooled and to this were added the supernatants from the other two samples from the same harvest time in order to yield sufficient material to permit an accurate estimation of radioactively-labelled anthocyanin. The total volume of these combined extracts was accurately determined using a Gilson micropipette after which a sample of known volume was concentrated as detailed in Chapter 2 for TLC. Routine TLC and scintillation counting were carried out again following the procedures outlined in Chapter 2.

The results of experiment 1 are presented in Table 3.2.25. and those for experiment 2 in Fig.3.2.25a. and 3.2.25b.

In the first experiment 95% of the $^{14}$C-phenylalanine which had been applied to the cultures was found to have been taken up by the cells in the 48h experimental period. After analysis of the various extracts it was determined that substantial amounts of radioactivity were present in the protein and methanol-soluble fractions, the latter of course, containing the remaining free phenylalanine. *Circa* 25% of the total label applied was found in the NaOH/Methanol-insoluble fraction which would have contained membrane, insoluble protein and cell wall material.

Two dimensional TLC of the concentrated methanol extracts followed by scintillation counting of the anthocyanin and phenylalanine components revealed that of the $^{14}$C-phenylalanine taken up, *ca.* 3.6% had been incorporated into anthocyanin over the 48h experimental period with *ca.* 1.4% remaining as the free amino acid.

In the second experiment it was found that the uptake of
Fresh wt. (g) 2.990 ± 0.358

Dry wt. (g) 0.186 ± 0.020

Anthocyanin content

(OD/g.(fr.wt.)/10ml.) 1.05 ± 0.130
(OD/culture/10ml.) 3.14 ± 0.321

Protein (mg./g. fr.wt.) 6.56 ± 0.070

Label uptake (%) 95

Total radioactivity

<table>
<thead>
<tr>
<th>Fraction</th>
<th>(dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH extract</td>
<td>2.41 x 10⁵</td>
</tr>
<tr>
<td>Methanol extract</td>
<td>1.75 x 10⁶</td>
</tr>
<tr>
<td>Insoluble fraction</td>
<td>9.50 x 10⁵</td>
</tr>
<tr>
<td>(Medium)</td>
<td>(1.09 x 10⁶)</td>
</tr>
<tr>
<td>Total</td>
<td>4.03 x 10⁶</td>
</tr>
<tr>
<td>% Recovery¹</td>
<td>99.8</td>
</tr>
</tbody>
</table>

dpm. Protein

<table>
<thead>
<tr>
<th>Fraction</th>
<th>(dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(dpm/g.(fr.wt.))</td>
<td>3.65 ± 0.41 x 10⁵</td>
</tr>
<tr>
<td>(dpm/mg.(protein))</td>
<td>0.56 ± 0.06 x 10⁵</td>
</tr>
</tbody>
</table>

dpm. Anthocyanin

<table>
<thead>
<tr>
<th>Fraction</th>
<th>(dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(dpm/g.(fr.wt.))</td>
<td>4.65 ± 0.63 x 10⁴</td>
</tr>
<tr>
<td>(dpm/culture)</td>
<td>1.39 ± 0.15 x 10⁵</td>
</tr>
</tbody>
</table>

dpm. free phenylalanine

<table>
<thead>
<tr>
<th>Fraction</th>
<th>(dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(dpm/culture)</td>
<td>5.29 ± 0.99 x 10⁴</td>
</tr>
</tbody>
</table>

Table 3.2.25.
The distribution of radioactivity in different fractions of a C.roseus cell culture after incubation in the presence of 2µCi. ¹⁴C-phenylalanine for 48 h. See text for details

¹ 40 µl. of the L-U-¹⁴C-phenylalanine stock solution (≈ 2µCi.) was determined to yield 4.04 x 10⁶ dpm.
Figure 3.2.25.
The uptake (a) of $^{14}$C-phenylalanine and its incorporation into anthocyanins (b) in C.roseus cell cultures over a 48 h period.
$^{14}$C-phenylalanine (as measured by loss from the medium) by the cultures was exceedingly rapid (Fig. 3.2.25a.). The maximum level of uptake (90%) had occurred within the first hour after $^{14}$C-phenylalanine application. (Circa 20% was taken up in the 5-10 min. period between label application and the analysis of the first sample).

Time course measurements of $^{14}$C-phenylalanine incorporation into anthocyanins (Fig. 3.2.25b.) have revealed that the appearance of $^{14}$C-anthocyanin also occurred remarkably rapidly. Labelled anthocyanins were detectable in the first samples taken ca. 5 minutes after $^{14}$C-phenylalanine addition to the cultures. From the time of maximum $^{14}$C-phenylalanine uptake (<1h.) the rate of $^{14}$C incorporation into anthocyanin was approximately linear although this may have begun to decline slightly towards the end of the experiment (48h.). The overall rate of label incorporation into anthocyanin was ca. $10^3$ dpm./h./culture during this period.

From the results of these experiments it was concluded that the rate of uptake and incorporation of $^{14}$C-phenylalanine into anthocyanins by this system was sufficient to yield measurable levels of $^{14}$C-anthocyanin using the techniques available, within a matter of hours. As it appeared that the linear phase of incorporation did not extend as far as 48h. post application of the labelled precursor it was decided to use a 24h. labelling period in all future experiments.

The labelling experiments carried out under modified culture conditions are now reported.
ii. The influence of 0.05mM. cinnamic acid and 0.05mM. diethyl malonic acid, added simultaneously, on the incorporation of 14C-labelled phenylalanine into anthocyanins in C. roseus cell cultures.

In previous experiments (Section 3.2.6.) it was found that the addition of cinnamate or malonate/diethyl malonate at concentrations of >0.5mM., was detrimental to both the growth and anthocyanin accumulation in C. roseus callus and cell cultures. The addition of 0.05mM. cinnamate and/or 0.05mM. diethyl malonate had no apparent toxic effect although neither did it enhance anthocyanin accumulation.

The failure of these treatments to enhance anthocyanin accumulation in vitro may simply have been due to a failure in the uptake or incorporation of the exogenously-applied precursors. Alternatively it may have been that cinnamate/malonate availability was not a limiting factor in anthocyanin accumulation in this system. As cinnamate and malonate are components of the separate branches of the early part of the anthocyanin biosynthetic pathway (Fig.3.2.23.) it was decided to determine the effect of these compounds in combination, upon the incorporation of 14C-labelled phenylalanine into anthocyanins in the cultures used. It was considered that the results of this experiment would give some indication as to the reasons for the previously observed failure to enhance anthocyanin accumulation by this treatment.

Samples (10 ml.) of two pooled and well mixed, 9 day old, light-grown cell cultures (in the early stages of anthocyanin accumulation) were placed in 6, 100 ml. conical flasks. To 3 of these was added 1 ml. of sterile distilled water and to the
remaining three, 1 ml. of a filter sterilized stock solution of 0.55mM. cinnamic acid and 0.55mM. diethyl malonic acid, thus giving a final concentration of 0.05mM. for both compounds. It had been determined that the pH of the medium in these cultures was 5.1 and consequently the pH of both of the solutions added were adjusted to 5.1 also. L-U-\(^{14}\)C-phenylalanine (2μCi. in 40μl of a stock solution) was immediately added to each flask and the cultures gently agitated. After a 24h period of incubation in the light the cultures were harvested and measurements made as detailed in Chapter 2. The results are presented in Table 3.2.26.

**Culture development**

The application of the precursors used in this experiment was found to have had no significant effect upon either culture biomass (fresh, dry weights) or cell viability. As had been observed previously, the precursor concentrations used failed to induce any increase in anthocyanin accumulation in the treated cultures.

**Label uptake**

The uptake of L-U-\(^{14}\)C-phenylalanine, as estimated by the loss of radioactivity from the bathing medium was unaffected by the presence of the other precursors added to the treated cultures. In the 24h. experimental period 90-95% uptake was observed in all flasks.
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.05mM. Cinnamate + 0.05mM. Diethyl malonate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh wt.</td>
<td>2.459 ± 0.003</td>
<td>2.510 ± 0.018</td>
</tr>
<tr>
<td>(g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry wt.</td>
<td>0.092 ± 0.001</td>
<td>0.109 ± 0.005</td>
</tr>
<tr>
<td>(g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell viability</td>
<td>93.0</td>
<td>92.0</td>
</tr>
<tr>
<td>(%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthocyanin content</td>
<td>0.572 ± 0.005</td>
<td>0.564 ± 0.004</td>
</tr>
<tr>
<td>(OD/g. (fr. wt.)/10 ml.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Label uptake 1</td>
<td>94.5</td>
<td>93.4</td>
</tr>
<tr>
<td>(%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Label incorp. into anthocyanin</td>
<td>3.71 x 10^4 ± 0.49</td>
<td>2.96 x 10^4 ± 0.39</td>
</tr>
<tr>
<td>(dpm/culture)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific activity (anthocyanin)</td>
<td>2.64 x 10^4 ± 0.32</td>
<td>2.10 x 10^4 ± 0.27</td>
</tr>
<tr>
<td>(dpm/OD/cult./10 ml.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14C-phenylalanine 2</td>
<td>1.37 x 10^5 ± 0.05</td>
<td>1.34 x 10^5 ± 0.07</td>
</tr>
<tr>
<td>(dpm/culture)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2.26.

The influence of 0.05mM. cinnamic acid + 0.05mM. diethyl malonic acid on the uptake of 14C-phenylalanine into C. roseus cultures and its incorporation into anthocyanins. Labelling period 24 h.

1 loss from medium
2 in cell extracts
Precursor uptake

Prior to considering the radioactivity data in detail it is important to give some indication as to the likelihood of the applied precursors having been taken up by the cells. In this context, spectrophotometric techniques have previously been used successfully to monitor the uptake of a phenolic precursor (dihydroquercetin) into plant tissues (eg. Kho et al., 1977). By measuring the OD of the bathing medium at the wavelength corresponding to the $\lambda_{\text{max}}$ of the compound concerned, these authors were able to monitor the disappearance of the precursor from the medium. This disappearance was then, upon comparison with a cell-free medium + precursor sample, equated with precursor uptake. (The main proviso being that the compound of interest was not extracellularly degraded by the cells). It must be borne in mind however, that precursor 'uptake' as measured in this way is not a definite indication that the compound enters the cells and becomes involved in their metabolism.

In this experiment the above technique was used to estimate cinnamate uptake (diethyl malonate unfortunately exhibits negligible absorption between 250-700nm.). Spectrophotometric measurements indicated that 0.05mM. cinnamic acid in CR medium was intensely UV.absorbant and had an OD of 11.30 (measured after the necessary dilution) at $\lambda_{\text{max}}$, 274nm. In the 24 hours of the experiment this absorbance was found to have fallen by 89% whereas the OD of the control medium and also the OD of a cell-free medium + precursor sample had altered only slightly (<5%).
It is apparent therefore, that in this experiment a considerable proportion of, at least, cinnamic acid was lost from the nutrient medium during the 24 hour period. This loss was dependent upon the presence of cells and it is thus presumed to have been the result of precursor uptake into these cells.

$L-U-^{14}C$-phenylalanine incorporation into anthocyanins

Although the total levels of free $^{14}C$-phenylalanine which remained in the cells at the end of the experiment were closely similar in the two treatments, the level of radioactivity incorporated into anthocyanins in the cultures given cinnamate/diethyl malonate (total amount or specific activity) was ca. 20% less than that of the controls. Although this decrease was relatively small (and less than had been hoped for) if one is to argue in terms of a direct influence of the exogenously applied precursors upon the anthocyanin metabolic pathway then a larger effect may not have been possible under the conditions used.

Concerning cinnamic acid for example, Billett et al., (1981) in the first direct analysis of the free cinnamate contents of plant tissue (gherkin hypocotyls), report that the endogenous levels of this compound were of the order of 0.2mM. In the experiment reported here, due to the very high sensitivity of the cells to the precursors used, only very low concentrations (0.05mM.) could be exogenously applied to the cultures in order to avoid the risk of considerable disruption of cell metabolism or even, cell death. Consequently, assuming a ca. 90% uptake of cinnamate into the cells, it has been estimated that the maximum possible increase in intracellular cinnamic acid
concentration, as would have resulted from a more or less instantaneous uptake (e.g. as observed by Fry, (1984) for $^{14}$C-cinnamate using spinach cultures), would therefore have been 0.2mM. Accordingly, if the internal cinnamate pool size in this tissue was of a size similar to that reported above (and if not the likelihood would be that it would be larger, rather than smaller due to the type of cells involved and their metabolic state at the time of labelling) the increase in intracellular cinnamate concentration, arising as a direct result of exogenous supplementation, may not even have been as much as 100%. Therefore, on the basis of this calculation, in conjunction with the likely rapid turnover of the endogenous cinnamate pool (see Fry, 1984) it is perhaps not surprising that the observed decrease in $^{14}$C-phenylalanine incorporation was only ca. 20%.

Autoradiography.

Autoradiography of the chromatographed extracts of each of the cultures revealed no qualitative and no major quantitative differences in labelling pattern between the two treatments.

There are a number of possible interpretations of this result. Firstly, it may be proposed that the observed decrease in label incorporation into anthocyanins resulted from an (indirect) effect of the exogenously-supplied cinnamate and/or malonate which decreased anthocyanin turnover and thus decreased the overall flow of metabolites down the biosynthetic pathway.
However, such an effect would seem unlikely not only because it is difficult to envisage a reason for the existence of such a mechanism and how it might operate in vivo, but also because evidence has already been presented [Section 3.2.1(iv), 3.2.3(iv).] which would suggest that, at least in certain instances, anthocyanin turnover was absent or negligible in the cultures used.

Secondly, the observed decrease in $^{14}$C-labelled anthocyanin may have arisen through a direct effect of the exogenously-supplied cinnamic acid increasing the intracellular concentration beyond the normal level. This may simply have resulted in a dilution of the $^{14}$C-cinnamate synthesised from $^{14}$C-phenylalanine, or it may have induced a reduction in phenylalanine deamination by the enzyme PAL (phenylalanine ammonia lyase) through end-product feedback inhibition. Both of these effects would have resulted in a decreased flow of $^{14}$C from phenylalanine through the complete pathway, thus decreasing the level of $^{14}$C-labelled anthocyanin, as was observed.

It would appear likely therefore, that exogenously-applied cinnamate (and possibly malonate) became actively involved in the anthocyanin biosynthetic pathway. Consequently, this would indicate that, as all of the attempts to enhance anthocyanin accumulation in vitro by the extracellular addition of these precursors to the cultures had failed, the availability of these precursors cannot have been a limiting factor in anthocyanin accumulation in this system.

In the following subsection the experiment described was of a similar type to that detailed above with the modification
that the cold (non-radioactive) precursor used was more closely related, biosynthetically, to the final products (anthocyanins).

iii. The influence of 1mM. Dihydroquercetin upon the incorporation of \(^{14}C\)-phenylalanine into anthocyanins in *C. roseus* cell cultures.

2,3-Dihydroquercetin (DHQ) is a complex colourless flavonoid intermediate of the anthocyanin biosynthetic pathway (Fig.3.2.23.). It is situated only a single metabolic step away from the most common anthocyanin aglycone, cyanidin and is considered, although this has yet to be positively confirmed, to be just two metabolic steps away from the anthocyanidin delphinidin, which is the first coloured (purple/blue) precursor of all three of the anthocyanidins (petunidin, malvidin, hirsutidin) present in *Catharanthus roseus* tissues. DHQ is the most closely related (biosynthetically) anthocyanin precursor which is commercially available (from a single British source: Apin Chemicals, Abingdon, U.K.).

In previous experiments (Section 3.2.6.) it was found that exogenous DHQ application only slightly enhanced anthocyanin accumulation in light grown *C. roseus* cell cultures, when used at a concentration of 1mM. Considering the relatively close proximity of this compound to the final product and also the concentration which could be applied without any apparent detrimental effect to the cultures, the effects observed were considerably less than might have been anticipated. As similarly stated in the previous experiment, it was not known
if the paucity of this response was perhaps the result of these compounds failing to enter the cells or possibly the inability of the cells to incorporate the exogenously-applied precursor into anthocyanins.

However, the success of Kho et al., (1977) in an experiment carried out in an attempt to induce anthocyanin accumulation in albino mutant petals of *Petunia hybrida*, through exogenous application of DHQ would indicate the potentiality of the proposed technique. This might suggest that the precursor used could indeed have been taken up by the *C. roseus* cells but failed to enhance anthocyanin accumulation significantly as the availability of this compound was not a limiting factor in anthocyanin accumulation in this system, at least at the time of application.

In order to investigate the validity of these various hypotheses, in the absence of any available directly labelled DHQ, the effect of the exogenous application of DHQ upon the movement of radioactivity from 14C-phenylalanine into anthocyanins was examined.

To carry out this experiment 2x50 ml., 9 day old, light-grown cell suspensions were pooled and well-mixed. Samples (10 ml.) of this stock (which was showing the first indications of anthocyanin accumulation) were placed into 6, 100 ml. conical flasks. To 3 of these was added 1 ml. sterile distilled water (pH 5.8) and to the remaining 3, 11 μmol. dihydroquercetin (suspended in 1 ml of sterile distilled water, pH 5.8). The precursor had been ethanol-sterilized and was added as detailed in Section 3.2.6(ii). Previous results (Kho et al., 1977) had indicated that DHQ was taken up very slowly by the plant tissues
used and accordingly, a 24h. preincubation period was included prior to label application. L-U-\(^{14}\)C-phenylalanine (2\(\mu\)Ci.) was then added to each flask and after a further 24h. all flasks were harvested and measurements made as detailed in chapter 2.

The results are presented in Table 3.2.27.

**Culture biomass, cell viability, anthocyanin content.**

The treatment used was found to have had no significant effect upon culture biomass (fresh or dry weight), cell viability or anthocyanin content.

**DHQ uptake.**

DHQ is intensely UV-absorbent and a 1mM. solution in CR medium was found to have an absorbance of ca. 20 OD units at the \(\lambda_{\text{max}}\) 291nm. It was therefore possible to use the method of Kho et al., (1977), as described in the previous experiment, to accurately determine the loss of this precursor from the nutrient medium over the experimental period. In the 48h. experiment the OD\(\lambda_{\text{max}}\) of the medium was found to have fallen to ca. 10% of the original level whereas the OD of a medium + precursor sample, from which the cells had been removed, fell by only a negligible amount (<2%).

**\(^{14}\)C-phenylalanine uptake.**

The exogenously-applied DHQ was found to have had no significant effect upon the overall uptake of \(^{14}\)C-phenylalanine by the cells. In all flasks the total uptake, as estimated by the loss of radioactivity from the bathing medium, was between 83-90%.
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DHQ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fresh wt.</strong> (g)</td>
<td>2.749 ± 0.058</td>
<td>2.823 ± 0.024</td>
</tr>
<tr>
<td><strong>Dry wt.</strong> (g)</td>
<td>0.109 ± 0.009</td>
<td>0.125 ± 0.006</td>
</tr>
<tr>
<td><strong>Cell viability (%)</strong></td>
<td>91</td>
<td>89</td>
</tr>
<tr>
<td><strong>Anthocyanin content</strong></td>
<td>2.831 ± 0.220</td>
<td>2.460 ± 0.226</td>
</tr>
<tr>
<td>(OD/culture/10ml.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>14C-phenylalanine</strong></td>
<td>89</td>
<td>84.5</td>
</tr>
<tr>
<td><em>uptake (%)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Label incorp. into</strong></td>
<td>4.51 x 10^4</td>
<td>5.62 x 10^4</td>
</tr>
<tr>
<td>anthocyanin (dpm/cult.)</td>
<td>±0.41</td>
<td>±0.54</td>
</tr>
<tr>
<td><strong>Anthocyanin sp.activity</strong></td>
<td>1.64 x 10^4</td>
<td>2.28 x 10^4</td>
</tr>
<tr>
<td>(dpm/OD/cult./10ml.)</td>
<td>±0.18</td>
<td>±0.21</td>
</tr>
<tr>
<td><strong>Free 14C-phenylalanine</strong></td>
<td>7.95 x 10^5</td>
<td>6.95 x 10^5</td>
</tr>
<tr>
<td>(dpm/cult.extract)</td>
<td>±0.83</td>
<td>±0.75</td>
</tr>
</tbody>
</table>

Table 3.2.27.
The influence of exogenously applied dihydroquercetin (1mM.) upon the incorporation of 14C-phenylalanine into anthocyanins in *C. roseus* cell cultures. Labelling period: 24 h. See text for precise details.
$^{14}$C-phenylalanine incorporation: Autoradiography.

Figure 3.2.26. illustrates the combined TLC/Autoradiograph tracings of chromatographed cell extracts from both control and treated cultures. No qualitative or major quantitative differences were observed between the patterns of radioactivity distribution of the different cell extracts. However, it was observed that a quite distinct increase in pigment intensity of some of the visible yellow components, particularly those which had $R_f$ values similar to the anthocyanins, had occurred in the DHQ-treated cultures, relative to the controls.

$^{14}$C-phenylalanine incorporation: Scintillation counting.

In those cultures exposed to 1mM. DHQ, although the anthocyanin content was closely similar to that of the controls, the level of label incorporation (either in terms of total dpm, or specific activity) was increased by ca. 40%. However, this difference did not prove statistically significant. Three major reasons were envisaged for this apparent failure of a considerable concentration of DHQ to significantly influence the passage of $^{14}$C from phenylalanine into anthocyanins in this system.

(a) The DHQ did not enter the cells.

or (b) The DHQ did not enter the active intracellular 'precursor' pool.

or (c) The DHQ had been metabolised prior to $^{14}$C-phenylalanine application.

However, as DHQ has been shown to have been taken up intracellularly in other systems (Kho et al., 1977) and as DHQ was found to have increased the accumulation of some of
Figure 3.2.26.

Tracings of TLC plates (and their corresponding autoradiographs) on which radioactively-labelled *C. roseus* cell extracts had been chromatographed in (1) n-Butanol: Acetic acid:water (4:1:5) and then (2) n-Butanol:Formic acid:water (4:1:5). The stippled areas represent regions of radioactivity, spots with solid lines were visible in daylight (A = anthocyanin, Y = yellow pigments) and spots with broken lines were ninhydrin positive compounds (P = Phenylalanine).
the other flavonoid compounds produced by this system both (a) and (b) would appear improbable explanations of the result observed. (It could however be argued that two separate active precursor pools of DHQ existed in these cells but this would seem a rather unlikely complication). It would thus seem probable that the exogenously-applied DHQ had indeed entered the cells and must therefore have been metabolised prior to the addition of $^{14}$C-phenylalanine. The increase in accumulation of some of the yellow flavonoid components, in the absence of any apparent change in their total radioactivity, would suggest that these effects had been induced in the preincubation period and had not been carried over into the labelling period, which is consistent with the above hypothesis.

Consequently, if the exogenously-applied DHQ had entered the active precursor pool within the cells the failure to observe any increase in anthocyanin accumulation under these conditions would strongly suggest that DHQ availability can not have been a factor which was limiting anthocyanin synthesis in the cultures at the time of experimentation. The alternative hypothesis, that the total level of anthocyanin accumulated in these cultures at any one time was strictly controlled and that any 'excess' anthocyanin would have been degraded, would seem unlikely as anthocyanin turnover did not appear to occur in this system (Section 3.2.1(iv), 3.2.3(iv)).

In the final experiment in this section $^{14}$C-phenylalanine was used to study the possible effects of nutrient starvation upon cell metabolism, particularly in relation to anthocyanin synthesis and accumulation.
iv. The influence of phosphate/nitrogen starvation upon the incorporation of $^{14}$C-phenylalanine into anthocyanins in C. roseus cell cultures.

In Section 3.2.3(vii) an experiment was reported, the results of which indicated that the incubation of C. roseus cells in nutrient medium lacking sources of i-phosphate and i-nitrogen, in the light, resulted in enhanced levels of anthocyanin/g. fresh wt. In the following experiment this effect was studied in greater detail in order to determine how this treatment influenced both the rate of anthocyanin accumulation and also the incorporation of $^{14}$C-phenylalanine, applied exogenously, into anthocyanins in this system.

Three, 100 ml. conical flasks containing 20 ml. CR medium were each inoculated with 1 ml. of two pooled, 12 day old, well-mixed, light-grown stock suspension cultures. At the same time 3 flasks containing 20 ml of i-P/i-N free medium (see Section 3.2.3(vii).) were similarly inoculated with 1 ml. samples taken from the same stock suspension. These cultures were then incubated under standard culture conditions in the light.

It had previously been observed that the level of anthocyanin accumulated by phosphate/nitrogen starved cultures increased most prominently between the 3rd and 7th days after initiation. Similar observations (see Section 3.2.1. for example) had indicated that the main anthocyanin accumulation phase in 20 ml. control cultures occurred between days 8 and 15. Consequently, the fate of $^{14}$C-phenylalanine was monitored in these cultures between days 4 and 5 in the nutrient starvation treatment and between days 10 and 11 in the control.
The experimental procedure was as follows. On the appropriate day for label application the cultures were thoroughly, but carefully mixed and using a wide-bore pipette, half of each was transferred to three empty, sterile, 100 ml. flasks. Then, after the removal of a 1 ml. sample for anthocyanin determination these transferred cultures were incubated for the full, standard experimental period (18 days), after which they were harvested and the final level of anthocyanin accumulation determined.

To the 10 ml. of culture which remained in each original flask, was added 4μCi. of L-U-14C-phenylalanine as detailed in Chapter 2. The flasks were harvested 24h. later and measurements made as detailed in the previous experiments. The amount of 14C-phenylalanine added to each flask in this experiment was double that which had been used previously as it was considered that the much smaller amount of tissue present in the nutrient starved cultures and the apparently lower rate of anthocyanin accumulation observed under these conditions might not have yielded sufficient labelled product in the 24h. period to permit accurate analysis.

The results are presented in Table 3.2.28.

**Anthocyanin accumulation.**

During the 24h. experiment >15 times more anthocyanin was accumulated by the control cultures in comparison to those starved of certain nutrients. However, on a per g. (fr.wt.) basis this difference was ca. 4-fold. In both treatments the amount of anthocyanin present in the cultures at the time of 14C-phenylalanine application was ca. 50% of the final (18d.) level.
<table>
<thead>
<tr>
<th></th>
<th>Control (full medium)</th>
<th>i-P/i-N depleted medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell no. $^1$ (10^6/culture)</td>
<td>83 ± 1.97</td>
<td>8.7 ± 0.28</td>
</tr>
<tr>
<td>Fresh wt. $^1$ (g)</td>
<td>2.539 ± 0.72</td>
<td>0.645 ± 0.029</td>
</tr>
<tr>
<td>% Pig.cells Day 0 $^2$</td>
<td>7.4</td>
<td>7.4</td>
</tr>
<tr>
<td>&quot; Day 18</td>
<td>12.5</td>
<td>17.5</td>
</tr>
<tr>
<td>&quot; 24h after label appln.</td>
<td>7.4</td>
<td>13.2</td>
</tr>
<tr>
<td>Anthocyanin cont. Day 0 $^2$</td>
<td>1.103 ± 0.103</td>
<td>1.103 ± 0.103</td>
</tr>
<tr>
<td>&quot; Day 18</td>
<td>1.068 ± 0.128</td>
<td>1.693 ± 0.099</td>
</tr>
<tr>
<td>&quot; at time of label appln.</td>
<td>0.576 ± 0.018</td>
<td>0.850 ± 0.028</td>
</tr>
<tr>
<td>&quot; 24h after label appln.</td>
<td>0.979 ± 0.095</td>
<td>0.977 ± 0.056</td>
</tr>
<tr>
<td>(OD/g. (fr.wt.)/10ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthocyanin accumulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>in 24h. labelling period</td>
<td>1.195 ± 0.195</td>
<td>0.079 ± 0.006</td>
</tr>
<tr>
<td>(OD/culture/10ml.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Label uptake (%)</td>
<td>94</td>
<td>63</td>
</tr>
<tr>
<td>&quot; (10^5 dpm/cult.)</td>
<td>7.60 ± 0.03</td>
<td>5.07 ± 0.40</td>
</tr>
<tr>
<td>$^{14}$C-anthocyanin (10^5 dpm/cult.)</td>
<td>2.67 ± 0.29</td>
<td>1.76 ± 0.31</td>
</tr>
<tr>
<td>$^{14}$C-phenylalanine $^3$ (10^3 dpm/cult.)</td>
<td>2.79 ± 0.25</td>
<td>7.71 ± 0.79</td>
</tr>
<tr>
<td>$^{14}$C-protein $^4$ (10^5 dpm/cult.)</td>
<td>13.80 ± 0.12</td>
<td>5.40 ± 0.27</td>
</tr>
</tbody>
</table>

Table 3.2.28.

The influence of i-P/i-N starvation upon the incorporation of $^{14}$C-phenylalanine into proteins and anthocyanins in *C. roseus* cell cultures over a 24 h. period during the active anthocyanin accumulation phase. See text for full details.

$^1$ After 24 h. labelling period.

$^2$ Culture initiation.

$^3$ In cell extracts.

$^4$ Soluble in 0.1N.NaOH.
**14C-phenylalanine uptake.**

The uptake of radioactively labelled phenylalanine by the control cultures was of a level comparable to that found in previous experiments. However, in the nutrient starved cultures the uptake level was somewhat less. In these cultures only an estimated 63% of the 14C-phenylalanine was found to have been taken up by the cells in the 24h. experimental period. This is presumed to have been the result of the much smaller amount of biomass present in these cultures. This decreased uptake must therefore be taken into account when considering the results.

**14C-phenylalanine incorporation: Protein.**

Upon measuring the level of radioactivity in the extracted protein from these cultures it was found that the total amount of label present in the 'soluble' protein was considerably reduced in the i-P/i-N deprived cultures, by a factor of 2.6X. If the assumption is made that the size of the free phenylalanine pool in these cultures was directly proportional to culture fresh weight, it can be estimated that if both the amount of protein synthesised/g. (fr.wt.) and the size and rate of turn-over of the endogenous phenylalanine pool had remained unaltered by the nutrient starvation treatment, the expected decrease in the level of radioactively-labelled protein in the treated cultures would have been 1.5 fold. (In the treated cultures the fresh weight was reduced by a factor of 4 whereas the intracellular specific activity of the precursor was increased by a factor of 2.7, thus the overall change would have been a decrease by a factor of 4/2.7=1.5).
Therefore, as the actual level of $^{14}$C-phenylalanine incorporation into 'soluble' protein was considerably less than the 'expected' level this would indicate either that much less protein had been synthesised per g. (fr.wt.) of cells in the treated cultures over the 24h. labelling period or that the specific activity of the endogenous phenylalanine pool was decreased relative to that of the control. The latter could have come about as a result of either a relative increase in size of the endogenous phenylalanine pool or an increase in its turnover into non protein products. Considering that the treated cells were deprived of both of their major nitrogen sources the former possibility of an increased intracellular phenylalanine level would appear unlikely.

$^{14}$C-phenylalanine incorporation: Anthocyanins

i. Autoradiography. The pattern of radioactivity distribution in chromatographed cell extracts was found to have been altered only slightly as a result of the nutrient depletion treatment (Fig.3.2.27.). In the extracts from the cultures deprived of phosphate/nitrogen a single, extra, unidentified spot was visible on the autoradiographs close to the position of phenylalanine, but clearly distinct from it. However, outwith this difference no other variation (either qualitative or quantitative (relative)) was observed.

ii. Scintillation counting: Although the total amount of labelled phenylalanine which became incorporated into anthocyanin in the treated cultures was slightly less than the total amount detected in the controls the anthocyanin specific activity
Figure 3.2.27.
The pattern of radioactivity distribution (stippling), visible components (solid lined spots) and ninhydrin positive components (broken lined spots) on TLC plates of chromatographed C. roseus culture extracts (MeOH.1%HCl.). The cultures had been grown under standard (upper) or nutrient limiting (lower) conditions. Solvents and symbols as Fig.3.2.26.
(dpm/unit of anthocyanin) was ca. 2.6X greater as a result of
the much smaller amount of pigment present in the nutrient-
depleted cultures. Comparing this value with the 'expected'
value, calculated as detailed below, has revealed some interesting
information concerning the possible effects of this nutrient
starvation treatment upon these cultures.

After a period of exposure of cells to a labelled precursor
(phenylalanine) the specific activity of the product (antho-
cyanin) would be determined by the following 5 parameters:

a. The specific activity of the intracellular precursor
pool.

b. The amount of new product synthesised in the labelling
period.

c. The amount of product originally present prior to
label application.

d. Product turnover.

e. Precursor pool turnover.

If d=0 then the specific activity of product \( \frac{axb}{c} \), assuming
that the availability of label does not become limiting. (The
results given in Section 3.2.7(i). and the measured levels of
free \(^{14}\)C-phenylalanine remaining at the end of this experiment
(Table 3.2.28.) would suggest that it does not).

In the treated cultures (a) was 2.6X the control value
(making the previously-stated assumption that the phenylalanine
pool size was directly proportional to culture fresh weight),
(b) was 0.07X the control value and (c)0.43X the control value.
Consequently, if (d) and (e) were unchanged by the treatment
the 'expected' anthocyanin specific activity in the nutrient
starved cultures would have been 0.41X that of the control.
The actual value was therefore considerably (ca. 6X) greater than the expected value upon the basis of the above calculations.

It must be concluded therefore, that the treatment used in this experiment either brought about an increase in anthocyanin turnover in the cultures or an increase in the specific activity of the intracellular phenylalanine pool relative to the controls, both of which could have brought about the observed disproportionate increase in the transfer of $^{14}$C from phenylalanine into anthocyanins. As evidence has already been presented which suggests that anthocyanin turnover was unlikely to have occurred in this system, the latter possibility would appear the most plausible.

A relative increase in the specific activity of the endogenous pool could have come about either as a result of a decrease in pool size or a decrease in its overall rate of turnover. Both of these possibilities are the direct antitheses of those proposed upon the basis of the protein results and this would hence strongly intimate that the original hypothesis, that reduced protein synthesis in the treated cultures was the cause of the reduced level of protein-associated radioactivity, was correct.

Under the modified conditions employed in this experiment it is quite understandable that a decrease in the endogenous phenylalanine level and/or a decrease in the turnover of the phenylalanine pool could have occurred. Severe nitrogen limitation is likely not only to restrict amino acid synthesis but also to curtail considerably the overall cell metabolism thus decreasing both the size and turnover of the intracellular phenylalanine pool.
One final, very interesting point which has emerged from these results concerns the degree of conversion of labelled precursor into product. In the control cultures the percentage conversion of $^{14}$C-phenylalanine (which was taken up by the cells) into anthocyanin over the 24h. labelling period was 2.63% and in the treated cultures, 3.47% on a total culture basis. However, as it has been possible to identify the active anthocyanin-accumulating cells in this culture system it has therefore also been possible to determine the percentage conversion of $^{14}$C-precursor into product, specifically within the productive cell population. If it is assumed that all cells had an equal ability to take up the labelled precursor and that those cells which were not visibly pigmented were accumulating negligible amounts of anthocyanin it can be estimated that in both sets of cultures, of the total amount of label taken up by the pigmented cell population, 26.2% was incorporated into anthocyanin over the 24h. period of the experiment. Considering the extremely general nature of the precursor used, $^{14}$C-phenylalanine, which is not only involved in many secondary metabolic pathways but also of course is an extremely important metabolite in primary metabolism, this level of conversion would appear unexpectedly high and clearly emphasises the considerable degree of 'dedication' of the pigmented cells towards anthocyanin synthesis and accumulation in these cultures at the time of experimentation.
The use of $^{14}$C-phenylalanine labelling experiments has enabled the following points to be reported concerning anthocyanin metabolism in *C. roseus* cell cultures.

1. $^{14}$C-anthocyanin can be detected remarkably rapidly (5min.) after label application to *C. roseus* cell suspensions.

2. Of the estimated amount of $^{14}$C-phenylalanine taken up by the anthocyanin-accumulating cells a remarkable 26% was incorporated into anthocyanin in a 24h. labelling period.

3. Exogenously applied cinnamic acid and dihydroquercetin appear to enter *C. roseus* cells and become actively involved in flavonoid biosynthesis.

4. In the light of the failure of the above (3) treatments to enhance anthocyanin levels in these cultures the results would indicate that the availability of neither of these compounds was rate limiting to this process.

5. Deprivation of these cultures of i-P and i-N sources permitted continued anthocyanin synthesis but at a reduced rate. Protein synthesis was also decreased.

6. From the results obtained it has been proposed that the nutrient starvation treatment applied instigates a decrease in the intracellular phenylalanine precursor pool size. This may partly explain the reduced rate of anthocyanin accumulation observed under these conditions.

The remaining section of results to be described contains reports of a number of investigations which were carried out, essentially, at the cellular level. These experiments were designed to exploit the visible properties of the metabolites
under investigation in order to obtain further information concerning the control of accumulation of these compounds in cultured cells.
Section 3.2.8.

"Investigations at the cellular level"
It has been emphasised previously that the basic aim of this work was to exploit the visible properties of anthocyanins in order to study in greater detail than is normally possible the accumulation of secondary metabolites in *in vitro* systems. In the preceding sections many experiments have been reported which were essentially concerned with the variation in anthocyanin accumulation between *C. roseus* cultures when grown under different conditions or subjected to certain specific treatments. In the following set of experiments brought together under the heading of 'Investigations at the cellular level' the emphasis has been placed upon studying the variation in anthocyanin accumulation within cultures rather than between them. As a result of anthocyanin visibility it was possible to reduce the level of the investigation to that of the cell rather than the whole culture and it was possible to identify within the total cell population those cells which were responsible for the majority of the culture pigmentation. Consequently, the basic aim of these experiments was to assess in a semiquantitative way the degree of heterogeneity with regard to anthocyanin distribution within this pigmented cell population in cultures grown under standard conditions and to determine how (if at all) this alters when the culture conditions are modified.

Many of the experiments reported in this section involved the use of a Vickers M85 scanning integrated microdensitometer in a novel technique to study both quantitatively and qualitatively the anthocyanin content of individual plant cells. A brief introduction to this instrument and its application has been
given in Chapter 2. The other experiments in this section were performed to investigate the relationship between aggregate size and anthocyanin productivity in *C. roseus* liquid cultures and also the use of microculture techniques to study the expression of secondary metabolism *in vivo*.

**NOTE.**

Upon making the conclusions which are to follow concerning the IOD values obtained using the microdensitometer the basic assumption has been made that the IOD of a cell was directly correlated with its anthocyanin content. This assumption must be justified as the *in vivo* expression of anthocyanin colour (both quantitative and qualitative) is dependent not only upon the actual amount of such compound(s) present but also upon the amount of copigment present. The importance of copigmentation upon the *in vivo* visibility of anthocyanins is well documented (Saito, 1967; Asen *et al.*, 1971, 1972; Ishikura, 1975, 1978) and it is consequently of considerable importance to indicate that any differences observed between cultures (e.g. cell lines, different culture treatments etc.) was not merely an effect via the altered presence of the copigments.

In Figure 3.2.28a. the estimated, mean intracellular anthocyanin concentration x the proportion of pigmented cells has been plotted against the anthocyanin yield/g. fresh weight. The points are from experiments many of which are reported herein and represent values for cultures grown under standard or modified conditions and for the cell lines which were analysed. As can be seen, despite the wide range in the overall culture
Figure 3.2.28(a).

Anthocyanin content per gram fresh weight of callus vs. the mean intracellular anthocyanin concentration x the proportion of visibly pigmented cells in C. roseus tissue cultures grown in the light. See text for details.
yield this was always found to correlate closely with the values calculated from microscopic determinations. Although this is not irrefutable evidence, it is proposed that this would indicate that the values obtained by microdensitometric techniques were indeed a true reflection of the intracellular anthocyanin content.

i. An investigation into the quantitative and qualitative differences within the pigmented cell population of a stationary-phase, light-grown *C. roseus* cell culture.

It became apparent from microscopic observations that within the cell population of light-grown *C. roseus* callus or suspension cultures considerable variation in both the intensity and colour of the pigmentation occurred (see Fig.3.2.1.). In the following experiment a Vickers M85 scanning microdensitometer was used in an attempt to quantify these differences and also to ascertain if this instrument could be used to detect cells in the total cell population which were accumulating anthocyanins at subliminal levels.

From two pooled and well mixed, 14 day old, light-grown *C. roseus* cell cultures were taken 4 x ca.0.5 g. samples. A protoplast preparation was made using one of these samples and the remaining three were analysed for the level of extractable anthocyanin, the proportion of pigmented cells and the cell no./g. fresh weight.

When the protoplasts had been prepared and washed they were resuspended in a known volume of 0.6M. mannitol. Samples were then taken and estimates of the cell population density, viability and the proportion of pigmented cells were made. From these values were then estimated the percentage yield of protoplasts and also it was determined if the isolation
procedure had affected the proportion of pigmented cells within the total cell population.

Three investigations were carried out on this prepared material. Firstly, determinations of the \textit{in vivo} absorption spectrum of individual cells were made. This was made possible by the ability to vary the wavelength of the scanning light beam. By altering the wavelength and measuring the IOD of the same cell at intervals over the complete instrument range (400-700 nm.) and plotting the results graphically a crude absorption spectrum of individual cells could be produced. This was carried out for a number of cells and the results not only demonstrated the spectral differences between cells but also indicated the most appropriate wavelength to select in future investigations when making single measurements on a large number of cells in order to quantify intrapopulation differences.

Secondly, IOD measurements were made on a random sample of 100 apparently non-pigmented cells. Four measurements were taken for each cell at the following wavelengths: 580 nm. (machine setting 60), 554 nm (55), 530 nm. (50) and 511 nm. (45) and from these data spectral patterns were determined. Any cell which exhibited an increased absorbance within the two extreme values was assumed to have a spectral peak in this region and consequently contained anthocyanin. From these results it was determined if this instrument could identify cells which were accumulating anthocyanins at a level which was visibly undetectable.

The third set of measurements was made on a random sample of 100 visibly pigmented cells in order to assess the
range of variation in both the total anthocyanin content per cell and also the intracellular anthocyanin concentration. As slight differences in the spectral maxima were observed for different cells three readings were taken for each cell at 580 nm., 554 nm. and 530 nm. the greatest of which was used for analysis.

**Qualitative variation**

From the crude spectral maps presented in Fig.3.2.28b. quite a range of $\lambda_{\text{max}}$ values are apparent, varying from ca.530 nm. to ca.570 nm. As might be expected, these spectral differences were reflected by visible colour differences between the cells. Those cells with spectral maxima of ca. 540 nm. were (deep) magenta in colour whereas those with maxima of ca.560-570 nm. were deep purple. Those with intermediate spectra exhibited intermediate colour types. The magenta cells were by far the most abundant with the purple cells occurring at an estimated frequency of <10% of the total pigmented cell population. As is clear from the IOD plots for the non-pigmented cells, values were consistently very low over the entire visible wavelength range measured.

**Instrument sensitivity**

Table 3.2.29. contains the results of the IOD analysis of the random sample of visibly non-pigmented cells. Of the 100 cells examined only 2 gave any indication of having an absorbance peak within the wavelength range used. Both of these cells had very low IOD values (<10) with a difference
Figure 3.2.28b.

Microspectrophotometric analysis of *C. roseus* cells which had been grown in liquid medium for 14d. in the light. The cells were visibly: (●) unpigmented, (○) deep purple, (•) magenta.
Table 3.2.29. Spectral properties of random sample of visibly non-pigmented *C. roseus* suspension culture cells (14d. old). Measurements made using a Vickers M85 scanning microdensitometer.

<table>
<thead>
<tr>
<th>Property</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell no. examined</td>
<td>100</td>
</tr>
<tr>
<td>Number visibly pigmented</td>
<td>0</td>
</tr>
<tr>
<td>No. with $\lambda_{\text{max}}$ 511–580 nm.</td>
<td>2</td>
</tr>
<tr>
<td>No. with $\lambda_{\text{max}} \leq 511$ or $\geq 580$ nm.</td>
<td>98</td>
</tr>
</tbody>
</table>

Quantitative variation

From the results presented in Fig. 3.2.29. and Table 3.2.30. it is clear that a considerable variation in the anthocyanin content was present within the pigmented cell population of these cultures. In terms of the total cellular anthocyanin content (IOD) a difference of >43 fold was observed between the highest (IOD, 615) and lowest (14.3) values. Also, the distribution of cells within this range (Fig. 3.2.29.) was by no means uniform and was very much weighted towards the lower end of the scale. Approximately 70% of the cells were found to occur in the lower one third of the total range covered.

The variation in cell size is clearly one factor which may be influential in the variation in anthocyanin content noted above and naively it might be suggested that larger cells
Figure 3.2.29.
Frequency distribution of the visibly pigmented cell population of a *C. roseus* suspension culture on the basis of cellular anthocyanin content (left) or intracellular concentration (right).

**Wt. callus used** 
0.5051 g.

**Anthocyanin content (callus)** 
0.66 OD/g.(fr.wt.)/10 ml.

**Cell viability - callus** 
94%

**- protoplasts** 
93%

**% Pigmented cells** 
13.8%

**% " proplasts** 
14.5%

**Cell no. used** 
8.4x10^6

**Protoplast no. obtained** 
3.9x10^6

**% Yield** 
47%

**Microdensitometry:**

<table>
<thead>
<tr>
<th></th>
<th>x</th>
<th>Range</th>
<th>Multiplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>IOD (mach units)</td>
<td>182.6±12.72</td>
<td>615-14.3</td>
<td>43 x</td>
</tr>
<tr>
<td>IOD/vol.</td>
<td>0.261±0.018</td>
<td>0.899-0.112</td>
<td>8x</td>
</tr>
<tr>
<td>Volume (mach units)</td>
<td>798±53</td>
<td>2184-103</td>
<td>21x</td>
</tr>
</tbody>
</table>

Table 3.2.30.
Protoplast isolation efficiency and the results of a microdensitometric analysis of the pigmented cell population thus obtained from a 14d. old *C. roseus* cell culture.
are likely to contain larger amounts of anthocyanin. However, in Fig.3.2.30, where IOD values have been plotted against estimated cell volumes it is clear that only a very poor relationship existed between the two and cells with similar sizes had considerably different anthocyanin contents and **vice versa**. This observation is borne out by the intracellular concentration data (Fig.3.2.29) in which, although somewhat less than the previous data, an 8-9 fold difference was found between the extreme values. The distribution histogram again shows a non-uniform distribution of cells over the range of values found with 70% of the cells falling, as before in the lower one third of the total range.

The use of the scanning microdensitometer in a novel way has thus enabled us to express in a quantitative manner the differences observed within these cultures. As anticipated, considerable quantitative and qualitative variations within the cell population were apparent from the results. The question which now arises is how do the values obtained and the differences observed compare with those normally found **in vivo** in a mature plant? The following investigation was thus performed to answer this.

**ii. A microdensitometric comparison of the cellular anthocyanin content of cell populations derived from in vivo and in vitro grown tissues.**

In this experiment an assessment of the variation in intracellular anthocyanin content of pigmented cells isolated
Figure 3.2.30

IOD vs. estimated cell volume of a visibly pigmented *C. roseus* protoplast preparation (light-grown, 14d.).
from various parts of intact *C. roseus* plants was carried out in order that a comparison could be made between the values for these tissues and those found previously in *in vitro* grown cells.

Three distinct tissues of *C. roseus* plants contain red/purple cells as a result of anthocyanin accumulation. These are the upper cell layer of the petals, the uppermost part of the perianth tube (both see Fig. 3.2.31) and the epidermal and sub-epidermal tissue of young stems. Taking three healthy, greenhouse grown plants in their flowering period, areas of each of these tissues were excised and from which protoplast preparations were raised by the standard method. (Gentle teasing of the tissues with dissecting needles was found to greatly ease the release of protoplasts). Microdensitometric measurements were then made on 100 randomly selected pigmented cells from each of these preparations as described previously. A small number of spectra were also obtained for typical cells from each tissue type.

The results have been expressed as before, in terms of both the total amount of anthocyanin/cell and also the intracellular anthocyanin concentration and may be found in Fig. 3.2.32. and Table 3.2.31. The data obtained from the previous *in vitro* investigation have been included for ease of reference.

Interestingly, the crude absorption spectra obtained for these cells (Fig. 3.2.33) showed very similar $\lambda_{\text{max}}$ values irrespective of their tissue of origin. The colour of these cells was also very similar although varying considerably in intensity (Fig. 3.2.31) and with peak values of 535-540 nm.
Figure 3.2.31.

(a) A *C. roseus* plant at anthesis (x1.5). Please note the variation in floral pigmentation. The pale outer lamina (p) and the central fused section (t) denoted as the 'perianth tube' in the text were used as sources of floral tissue protoplasts.

(b) A partial enzyme digest of the outer petal lamina. Please note the very regular pale pigmentation. Bar = 40 μm.

(c) Perianth tube tissue as (b). Pigmentation in these cells was consistently very intense. Bar = 75 μm.

(d) A partial enzyme digest of the peeled epidermis and subepidermal layers of *C. roseus* stems. Bar = 100 μm.
Figure 3.2.32.

Variation in cellular anthocyanin content (left) and intracellular anthocyanin concentration (right) of *C. roseus* cells grown *in vitro* and *in vivo*.
<table>
<thead>
<tr>
<th>Tissue source</th>
<th>Somatic tissue</th>
<th>Petal lamina</th>
<th>Perianth tube</th>
<th>Cell culture (14d.old)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IOD</td>
<td>508.1±45.7</td>
<td>66.6±3.7</td>
<td>265.4±32.3</td>
<td>182.6±12.72</td>
</tr>
<tr>
<td>Range</td>
<td>272-1107 (4x)</td>
<td>47.6-110 (2x)</td>
<td>164.4-576 (3.5x)</td>
<td>14.3-615 (43x)</td>
</tr>
<tr>
<td>Volume</td>
<td>1718±152</td>
<td>304.2±28.4</td>
<td>599.1±60.5</td>
<td>798.0±53</td>
</tr>
<tr>
<td>Range</td>
<td>342-2582 (8x)</td>
<td>148-463 (3x)</td>
<td>227-1130 (5x)</td>
<td>2184-103 (21x)</td>
</tr>
<tr>
<td>IOD/volume</td>
<td>0.333±0.021</td>
<td>0.227±0.015</td>
<td>0.440±0.035</td>
<td>0.261±0.018</td>
</tr>
<tr>
<td>Range</td>
<td>0.191-0.686 (4x)</td>
<td>0.157-0.322 (2x)</td>
<td>0.235-0.677 (3x)</td>
<td>0.112-0.899 (8x)</td>
</tr>
</tbody>
</table>

Table 3.2.31.

Microdensitometric analysis of *C. roseus* cells derived from tissues grown *in vivo* and *in vitro*.
Figure 3.2.33.
Microspectrophotometric analysis of *C. roseus* cells isolated from whole plants. (•) Somatic cells, (○) perianth tube cells, and (●) perianth lamina cells.
they would appear equivalent to the magenta cells observed in quantity \textit{in vitro}. Although rarely some of the outer petal cells had a slight purple appearance no cells from any of these preparations were observed to have the intensity of purple pigmentation observed in the \textit{in vitro} cell populations.

Quantitative differences as indicated in the table of mean values were found to be considerable between the different tissue types. The total cellular anthocyanin content was very much greatest in the somatic cells which contained on average >7 times the mean amount present in the petal (lamina) cells. The cells from the top of the perianth tube contained an amount intermediate between these two. The mean cell volume of these tissue types was however considerably different with the somatic cells being generally much larger than the others. When this is taken into account (concentration data, Table 3.2.31.) maximum values were found to occur in the perianth tube cells, but again the lowest levels were present in the petal lamina cells which contained, on average, approximately half the concentration of the perianth tube cells. In comparison, the \textit{in vitro} cell population value falls between the two lowest \textit{in vivo} estimates.

One very striking feature of these results was that, very much in contrast to the \textit{in vitro} data, the variation in anthocyanin content between cells within the \textit{in vivo} cell populations was considerably reduced. In these populations the differences observed between maximum and minimum values both for the total cellular anthocyanin content and also the intracellular anthocyanin concentration were \textit{ca}.2–4 fold as compared to \textit{ca}.40 fold
and 8 fold respectively for the in vitro data. These differences are reflected in the histograms, where the plots for the whole plant protoplast preparations are generally restricted (in some cases considerably so) in comparison to those for the in vitro derived cells (Fig. 3.2.32.).

One final point of interest arising from these data is that on comparison of the overall range of values in terms of the cellular anthocyanin concentration for the plant cell populations with that found for the culture cell population the values for the latter tissue type were found to have an expanded range not only on the lower side but on the upper side also.

Having compared the stationary-phase in vitro cell population data with those for in vivo derived cells the following experiment was performed to investigate how the variation in the cellular anthocyanin content in the former system altered over the culture growth cycle.

iii. A microdensitometric investigation into the time course of anthocyanin accumulation in C. roseus cell suspension cultures.

The following experiment was performed in order to observe and quantify the changes which take place within the pigmented cell population of C. roseus liquid cultures during a single subculture period of 14 days.

Two 50 ml. light-grown C. roseus stock suspension cultures were pooled and thoroughly mixed after 11 days of culture. From this suspension was then taken 4 x 3 ml. samples which
were used to inoculate 4 x 250 ml. flasks containing 50 ml. of fresh CR medium. These flasks were then incubated on an orbital shaker under standard light conditions and were harvested as follows.

To study the development of these cultures with time samples were taken on days 0, 4, 7, 10 and 14. At time 0 the samples were taken from the unused stock but on all other days sampling was carried out by removing aseptically 5 ml. of each of the four cultures after their thorough but careful mixing. As time did not permit four individual analyses, all samples were pooled before measurements were made. Filtered cell fresh weight was determined as described in Chapter 2 after which estimates of the proportion of pigmented cells were made. The cell mass was then divided into two parts, one of which was extracted to determine the anthocyanin content and the other was used to raise protoplasts for microdensitometry. The microdensitometric analysis was carried out as detailed in previous experiments.

The results are presented in Table 3.2.32. and Fig.3.2.34. and for a more convenient comparison of the tabulated results these have also been presented graphically in Fig.3.2.35.

**General culture development.**

Although there were only a relatively small number of sample days the results available strongly suggest that the growth of these cultures (Fig.3.2.35(a).) was of the typical sigmoidal pattern found previously. After an initial period of little increase (which would have included the lag phase) the culture fresh weight increased linearly up to ca. day 10
<table>
<thead>
<tr>
<th>Time (days)</th>
<th>0</th>
<th>4</th>
<th>7</th>
<th>10</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fr.wt. g./ml.</td>
<td>0.025</td>
<td>0.061</td>
<td>0.172</td>
<td>0.285</td>
<td>0.324</td>
</tr>
<tr>
<td>% Pig.cells</td>
<td>10.0</td>
<td>1.1</td>
<td>1.5</td>
<td>8.2</td>
<td>10.0</td>
</tr>
<tr>
<td>Anthocyanin</td>
<td>0.449</td>
<td>0.184</td>
<td>0.201</td>
<td>0.452</td>
<td>0.571</td>
</tr>
<tr>
<td>OD/g./10 ml.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthocyanin OD/ml.cult./10 ml.</td>
<td>0.011</td>
<td>0.011</td>
<td>0.035</td>
<td>0.129</td>
<td>0.185</td>
</tr>
<tr>
<td>IOD(^1)</td>
<td>133</td>
<td>57</td>
<td>65</td>
<td>139</td>
<td>175</td>
</tr>
<tr>
<td>±10.0</td>
<td>±5.1</td>
<td>±5.9</td>
<td>±11.0</td>
<td>±12.8</td>
<td></td>
</tr>
<tr>
<td>Cell volume(^1)</td>
<td>435</td>
<td>291</td>
<td>300</td>
<td>501</td>
<td>612</td>
</tr>
<tr>
<td>±41</td>
<td>±21</td>
<td>±20</td>
<td>±32</td>
<td>±58</td>
<td></td>
</tr>
<tr>
<td>IOD/volume(^1)</td>
<td>0.310</td>
<td>0.195</td>
<td>0.211</td>
<td>0.290</td>
<td>0.30</td>
</tr>
<tr>
<td>±0.018</td>
<td>±0.012</td>
<td>±0.011</td>
<td>±0.018</td>
<td>±0.018</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2.32.

Time course analysis of the growth and anthocyanin content of a *C.roseus* cell culture over a single subculture period (for details see text).

\(^1\) in machine units.
Figure 3.2.34.
Variation in cellular anthocyanin content (left) and intracellular anthocyanin concentration (right) of the visibly pigmented cell population of a C.roseus cell culture over a 14d. subculture period.
Figure 3.2.35.
Graphic presentation of data from Table 3.2.32.
after which it proceeded to level off. Although it was not possible to measure the cell population density in this experiment, on the basis of previous experiments and observations (Section 3.2.1.), the results available would indicate that cell division would have ceased by day 6-7. At this time the culture fresh weight was ca. 50% of its final value and the pattern of anthocyanin accumulation was reversing.

The variation in the anthocyanin content of these cultures over the 14 day period was also closely similar to the pattern observed in previous experiments (Fig.3.2.35b+c). In the early part of the subculture period the anthocyanin content/g. fresh weight and the proportion of pigmented cells decreased while the total culture anthocyanin content remained unchanged. Then from day 7 all values were observed to increase up to and beyond initial (11 day) levels and by day 14 the total culture anthocyanin content had increased by >16 fold.

**Microdensitometric analysis**

The estimates of mean protoplast volume (Fig.3.2.35(a).) indicated that considerable variation in cell size occurred over the 14 day subculture period. Initially, a decrease was observed, associated with the period of cell division which was then followed by an increase up to and beyond that of the original value. The difference between minimum and maximum values was ca. 2 fold which was similar to values estimated from fresh weight/cell number data from previous experiments (Section 3.2.1.). These observations, in association with the further observation that the changeover first occurred on day 7 provide support for the proposal that the transition between
the cell division and cell expansion phases occurred on 
ca.days 6-7.

Substantial changes in the IOD values were also observed 
over the experimental period (Fig.3.2.35(e).). In association 
with the considerable decline in the proportion of pigmented 
cells noted previously, a similar decrease was also observed 
in both the mean cellular anthocyanin content and total range 
of IOD values (Fig.3.2.34.) following subculture. On days 4 
and 7 more than 80% of the cells occurred in a single (the 
lowest) range category. However, this trend was later 
reversed beginning on ca.day 7 and by day 14 the cell distribu-
tion was more uniform and the range of observed values had 
increased beyond that found in the original 11 day old (day 0) 
population.

It is possible that these observed changes in the cellular 
anthocyanin content might largely have been due to the changes 
in cell size. However, on converting the IOD values into 
units of concentration it can be seen (Figs.3.2.34. and 
3.2.35(f).) that a very similar overall trend was observed. 
In the first half of the subculture period the great majority 
(70%) of the concentration values fell within a single range 
category. Only between days 7-14 did the values become more 
evenly distributed over the total range. However, in contrast 
to the IOD data very little reduction in the overall range 
of concentration values occurred within the cultures over the 
entire experimental period.

Some very interesting relationships appear in these 
results between the 'cellular' data and those for the total
culture. Upon plotting the mean intracellular anthocyanin concentration data against the cultural anthocyanin content/g. (fr.wt.) (Fig.3.2.36 (a).) it became clear that although the mean cellular concentration increased during the early phase of anthocyanin accumulation by day 10 a maximum mean value had been reached. Consequently, during a considerable proportion of the anthocyanin accumulation phase the increase in the cultural anthocyanin content resulted from an increase in the size of the pigmented cell population and also an increase in mean cell volume but not from any increase in intracellular pigment concentration.

From Figs.3.2.36 (b). and 3.2.36 (c). it also becomes clear that during the period of active anthocyanin accumulation, which is known to coincide with the period of cell expansion, the level of extractable anthocyanin expressed in a per g. (fr.wt.) or total culture basis, bears a direct relationship (r=0.99) to the culture fresh weight, at least up to day 14. A similar direct correlation (r=0.99) is apparent between culture fresh weight and the mean anthocyanin content (IOD) per pigmented cell over the same period (Fig.3.2.36 (b).).

Finally, a direct relationship (r=1) was also found between the mean IOD values and the level of anthocyanin/g. (fr.wt.) not only during the anthocyanin accumulation phase as might be expected but during the complete growth cycle (Fig.3.2.36 (d).).

The next two experiments were performed to investigate how the variation in the intracellular anthocyanin content was influenced when the cultures were grown under modified conditions.
Figure 3.2.36.
Graphic presentation of data presented in Table 3.2.32.
See text for details.
iv. The influence of a high sucrose concentration on the anthocyanin content of *C. roseus* suspension cultures as determined at the cellular level by the use of microdensitometric techniques.

Increased sucrose concentration has previously been shown to enhance anthocyanin yield in *C. roseus* cell cultures (Section 3.2.3.). This experiment was performed to repeat this result and to investigate the basis of these changes at the cellular level.

The experiment was set up as described previously (Section 3.2.3.). Two pooled, 12 day old, light-grown suspensions were used as the source of inocula and five replicates of each treatment were initiated.

Measurements were taken at zero time (using 5 extra inoculum samples) and after 14 days of subculture. For the microdensitometric analysis 5 x 5 ml. samples were pooled for each treatment and following protoplast isolation measurements were made on a random sample of 100 pigmented cells. The results are presented in Table 3.2.33. and Fig.3.2.37.

From the tabulated values it is apparent that the previously observed effects of 8% aq. sucrose on culture growth and anthocyanin accumulation, although relatively small are quite reproducible. In this treatment a significant increase in total culture dry weight was observed in the absence of any such change in fresh weight. In comparison to the control flasks (CR medium) both the proportion of visible anthocyanin-accumulating cells and the level of extractable anthocyanin/unit fresh weight were increased by the treatment,
Figure 3.2.37
Variation in intracellular anthocyanin content of visibly pigmented *C. roseus* cells incubated in 8% aqueous sucrose (right) or CR medium (left) for 14d.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time 0 (5ml inoculum)</th>
<th>8%aq.Sucrose Day 14</th>
<th>Control Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fr.wt.(g.)</td>
<td>1.620</td>
<td>1.588 ±0.061</td>
<td>16.643 ±0.634</td>
</tr>
<tr>
<td>Dry wt.(g.)</td>
<td>0.058</td>
<td>0.084 ±0.002</td>
<td>0.526 ±0.018</td>
</tr>
<tr>
<td>% Pig.cells</td>
<td>10.0</td>
<td>12.0</td>
<td>9.5</td>
</tr>
<tr>
<td>Anthocyanin content OD/g./10ml.</td>
<td>0.570 ±0.009</td>
<td>0.800* ±0.037</td>
<td>0.533 ±0.016</td>
</tr>
<tr>
<td><strong>IOD</strong></td>
<td>175 ±12.8</td>
<td>227** ±16.8</td>
<td>183 ±12.7</td>
</tr>
<tr>
<td><strong>Cell volume</strong></td>
<td>612 ±53</td>
<td>1001** ±91</td>
<td>644 ±49</td>
</tr>
<tr>
<td><strong>IOD/volume</strong></td>
<td>0.286 ±0.020</td>
<td>0.227* ±0.019</td>
<td>0.283 ±0.020</td>
</tr>
</tbody>
</table>

Table 3.2.33.
The influence of 8% aq. sucrose upon the growth and accumulation of anthocyanin in *C. roseus* cell cultures. Significantly different from either Day 0 or control at * P=0.05, ** P=0.01.
the former by 26% and the latter by 51%. From these values it can be estimated that, assuming cell number and cell size remain unchanged (and the fresh weight and cell population density data from both experiments would indicate that they do) then a mean increase in the anthocyanin content/pigmented cell of ca. 20% can be proposed. From the microdensitometric analysis of the random cell sample an estimated increase of ca. 24%/cell was found and was thus in quite close agreement with the expected value.

The distribution of cells within the pigmented cell population on the basis of their anthocyanin content (Fig. 3.2.37.) was, in the control cultures closely similar to that found previously. This pattern was however, somewhat modified in the sucrose-treated cell population. In these cultures the level of predominance of the lowest range categories was reduced and a slight extension in the overall range was observed.

One slight peculiarity in these results requires explanation. Despite the indication given by the fresh weight and cell number data that only very minor changes in cell size and population density had occurred in the treated cultures from the direct measurements made on individual cells it would appear that the cell size, at least of the pigmented cells had indeed increased by almost 100%. Consequently the estimated mean intracellular anthocyanin concentration appears to have been decreased by this treatment. This is almost certainly artefactual and the following proposal is offered in explanation. All of the microdensitometric measurements are, through necessity made on cells which have been plasmolysed. The degree of
plasmolysis primarily depends upon the initial mean osmotic potential of the cell population and it would seem likely that such a value would increase when the cell population was incubated in a considerable excess of sucrose (4 g./1.5 g. cells). Consequently, upon exposure to a high mannitol concentration such a cell population would become plasmolyzed to a lesser degree than those incubated in standard CR medium and this appears to be what has occurred.

The following experiment was carried out to investigate in a similar way the effect of light on anthocyanin accumulation.

v. The influence of the level of irradiance on the intracellular anthocyanin accumulation in C.roseus callus cultures.

Light considerably influenced the accumulation of anthocyanin in these cultures (Section 3.2.3.) and it has been found that the total culture yield can be increased 3-4 times over the levels obtained under standard conditions by increasing the level of irradiance. The following experiment was thus performed to determine how this treatment influences anthocyanin accumulation as examined at the cellular level.

Two, 12 day old light grown callus cultures were harvested and the cell masses pooled and well mixed in a sterile Petri dish. Samples (0.25 g.) were taken and used to inoculate six 5 cm. Petri dishes containing 10 ml. CR medium by the standard method. Three of these plates were then incubated in low light levels (15-20 μmol./m²/sec.) and three in enhanced
lighting (130 μmol./m²/sec.). These different levels were again simply attained by placing the two sets of cultures at different distances from the same light source. After 18 days the plates were harvested and measurements of the culture fresh and dry weights, the proportion of pigmented cells and the amount of extractable anthocyanins were made. 0.5 g. samples of each of the three (well mixed) callusses from each treatment were also taken, pooled and used to make a protoplast preparation for each treatment. These were then used for microdensitometric analysis.

The results are presented in Table 3.2.34. and Fig.3.2.38. and from the table it is clear that the results obtained reflect closely those found previously (Section 3.2.3(iv).). Total culture biomass, in terms of either fresh or dry weight remained unaffected by increased irradiance. However, anthocyanin accumulation was altered significantly with a ca.4 (3.94) fold increase in the yield/g.(fr.wt.) in association with a ca.3 (2.87) fold increase in the proportion of pigmented cells.

On the basis of the above results it would be expected that the mean increase in the anthocyanin concentration of the pigmented cell population would be ca.1.37 times in the high light treatment. Microdensitometric measurements revealed an estimated actual increase of 1.34 times. The increase in the mean cellular anthocyanin content (IOD) was however considerably more than this (1.85 times) as a consequence of the apparent increase (1.37 times) in the mean cell volume in this treatment.

The high light treatment also resulted in a considerable alteration in the pigment distribution within the pigmented
<table>
<thead>
<tr>
<th></th>
<th>Irradiance level 15μmol.m^−2 sec^−1</th>
<th>Irradiance level 130μmol.m^−2 sec^−1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh wt. (g.)</td>
<td>2.201 ±0.195</td>
<td>2.198 ±0.262</td>
</tr>
<tr>
<td>Dry wt. (g.)</td>
<td>0.095 ±0.013</td>
<td>0.103 ±0.015</td>
</tr>
<tr>
<td>Anthocyanin content</td>
<td>0.372 ±0.044</td>
<td>1.467** ±0.301</td>
</tr>
<tr>
<td>OD/g.(fr.wt.)/10ml.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total anthocyanin</td>
<td>0.819 ±0.062</td>
<td>3.224** ±0.412</td>
</tr>
<tr>
<td>OD/culture/10ml.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Pig.cells</td>
<td>8.3</td>
<td>15.5**</td>
</tr>
<tr>
<td>IOD</td>
<td>111.1 ±9.8</td>
<td>204.7*** ±14.6</td>
</tr>
<tr>
<td>Cell volume</td>
<td>454 ±31</td>
<td>623*</td>
</tr>
<tr>
<td>IOD/volume</td>
<td>0.245 ±.018</td>
<td>0.329*</td>
</tr>
</tbody>
</table>

Table 3.2.34.

The influence of irradiance level on the anthocyanin accumulation of *C. roseus* callus cultures as measured using extraction and microdensitometric techniques. 18d. Culture period. Significantly different from low light treatment at * P=0.05, ** P=0.01, *** P=0.001.
Figure 3.2.38.

Variation in intracellular anthocyanin content (upper) and concentration (lower) of visibly pigmented *C. roseus* callus cells grown for 18d. under low (15μmol.m⁻².sec⁻¹) or high (130μmol.m⁻².sec⁻¹) irradiance levels.
cell population as indicated by the histograms in Fig. 3.2.38. Despite the considerable increase in total anthocyanin yield only a relatively small increase in the maximum range of values (14-615 (low light), 15-768 (high light)) was observed and consequently the main increase in yield was the result of an increase in the proportion of cells in the upper (3rd and 4th) IOD range categories at the 'expense' of the lowest (0-100 units) category which was by far the most predominant in the low irradiance treatment.

To continue the theme of the intracultural variation as concerns anthocyanin content the following experiment was carried out in order to determine if a relationship existed between aggregate size in *C. roseus* cell cultures and their anthocyanin content.

vi. An investigation into the relationship between aggregate size and anthocyanin content in *C. roseus* cell cultures.

Using liquid cultures of *D. carota*, Kinnersley and Dougall (1980a) report the existence of a link between aggregate size and anthocyanin accumulation and indicate that enhanced yields could be achieved by continually reselecting for a particular size range of cell aggregate for subculture. The following experiment was carried out to determine whether such a relationship existed in the culture system used in this investigation and if so to investigate its cellular basis.

As it was not possible to take individual measurements for replicate cell suspensions, three flasks were pooled,
thoroughly mixed and from this one third (45 ml.) was taken for analysis. The cultures used had been grown in the light under standard conditions for 14 days before harvesting.

To separate the different cell aggregate sizes from the total culture mass a series of nylon meshes of very defined pore size were used (H. Simon Ltd., Stockport). Five different meshes with pore sizes 500, 200, 140, 100 and 64 µm. were employed to give the six aggregate size groupings listed in Table 3.2.35. These meshes were held taut over 5 cm. glass funnels and to begin a small sample (ca. 5 ml.) of the stock suspension was poured onto the largest mesh. The cells were then gently washed through with distilled water taking great care not to exert any extra downward force when doing so. Washing was continued until cells ceased to pass through the mesh. The eluant was collected and using this the process was repeated with each of the other meshes in turn (largest-smallest). When completed the cell aggregates trapped on each of the meshes were washed off into separate containers. The complete procedure was then repeated with further suspension samples until all had been processed.

Due to the nature of this procedure the smallest aggregates (<64 µm.) were suspended in a final volume of ca. 2.5 l. of water and in order to reduce this to a more convenient volume the final eluant was gently vacuum filtered to remove most of the bathing solution.

When the separation process was complete each of the cell aggregate suspensions were taken in turn and measurements of the total PCV (meas. as per % PCV (Section 2.3.1.) but giving the results in absolute units, ml.), fresh weight, cell
number*, the proportion of pigmented cells and the extractable anthocyanin content were determined. The results are presented in Table 3.2.35. and Fig.3.2.39. (*In the two largest aggregate size ranges this was carried out after partial homogenisation by pumping the solutions into and out of a Pasteur pipette, a procedure which did not result in any detectable extra cell lysis.)

The great majority of the culture biomass was found to consist of cell aggregates within a size range of 64-500 μm. (Fig.3.2.39(a).) With the virtual absence of any aggregates >500 μm. and >75% of the culture biomass (cell no., cell volume or fresh wt.) consisting of aggregates of <200 μm. the very fine nature of these cell suspensions is clearly demonstrated. The greatest amount (ca. 40%) of the total culture biomass occurred in the 140-200 μm. size range.

The proportion of pigmented cells within these aggregate classes (Fig.3.2.39(e).) was generally found to increase with decreasing aggregate size. The values ranged from 2-3% in the largest aggregates to 17% in the smallest. A similar but less pronounced trend was found in the amount of anthocyanin/ g. fresh weight (Fig.3.2.39(f).) although in this case the greatest level was produced by cell aggregates in the 64-100 μm. range.

Using the values obtained it has also been possible to make an estimate of the amount of anthocyanin/pigmented cell. With the exception of the largest and smallest of the aggregate size classes, such estimates were found to be remarkably similar between the different aggregate groupings. In the two exceptional cases the >500 μm. aggregates, on average,
<table>
<thead>
<tr>
<th>Fr.wt. (g.)</th>
<th>PCV (ml.)</th>
<th>Total cell no. sample⁻¹</th>
<th>Anthocyanin OD/g./10ml.</th>
<th>Total a/c OD/sample/10 ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;500µm.</td>
<td>0.104</td>
<td>1.9x10⁶</td>
<td>0.316</td>
<td>0.033</td>
</tr>
<tr>
<td>500-200µm.</td>
<td>2.405</td>
<td>1.2x10⁸</td>
<td>0.505</td>
<td>1.215</td>
</tr>
<tr>
<td>200-140µm.</td>
<td>4.017</td>
<td>1.6x10⁸</td>
<td>0.499</td>
<td>2.005</td>
</tr>
<tr>
<td>140-100µm.</td>
<td>1.884</td>
<td>9.5x10⁷</td>
<td>0.592</td>
<td>1.115</td>
</tr>
<tr>
<td>100-64µm.</td>
<td>1.079</td>
<td>6.2x10⁷</td>
<td>1.106</td>
<td>1.193</td>
</tr>
<tr>
<td>&lt;64µm.</td>
<td>0.053</td>
<td>6.8x10⁶</td>
<td>0.760</td>
<td>0.040</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>% Pig. cells</th>
<th>Pig.cell no. sample⁻¹</th>
<th>A/c/pig. cell (OD/pig. cell/10ml.) (g.)</th>
<th>Cell fr.wt. [A/c] OD/pig.cell/10ml./g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;500µm.</td>
<td>3.4</td>
<td>6.6x10⁴</td>
<td>5x10⁻⁷</td>
</tr>
<tr>
<td>500-200µm.</td>
<td>4.6</td>
<td>5.5x10⁶</td>
<td>2.2x10⁻⁷</td>
</tr>
<tr>
<td>200-140µm.</td>
<td>7.1</td>
<td>1.1x10⁷</td>
<td>1.8x10⁻⁷</td>
</tr>
<tr>
<td>140-100µm.</td>
<td>5.7</td>
<td>5.4x10⁶</td>
<td>2.1x10⁻⁷</td>
</tr>
<tr>
<td>100-64µm.</td>
<td>10.0</td>
<td>6.2x10⁶</td>
<td>1.9x10⁻⁷</td>
</tr>
<tr>
<td>&lt;64µm.</td>
<td>17.0</td>
<td>1.2x10⁶</td>
<td>3.3x10⁻⁸</td>
</tr>
</tbody>
</table>

Table 3.2.35.

The contribution which cell aggregates, segregated into different size ranges, make to the biomass and pigmentation of a 14d. old light-grown *C.roseus* cell culture.
The biomass and pigment content of cell aggregates of differing size from a *C. roseus* cell culture (after 14d. growth in the light). The relative predominance of the (a) Fresh wt., (b) Cell no., (c) Pigmented cell no., and (d) Total anthocyanin yield of the different size categories. The proportion of pigmented cells (e), the anthocyanin yield/g.(fr.wt.) (f), the total anthocyanin yield/size category/culture (g) and the estimated anthocyanin content per pigmented cell (f).

[(f) in OD units/g.⁻¹/10ml., (g) in OD units/culture/10ml. and (h) in OD units (x10⁻⁷)/pigmented cell/10ml.]

**Figure 3.2.39.**
Figure 3.2.39.
contained >2 times the 'usual' value per pigmented cell and in contrast the pigmented cells within the <64 μm aggregates had <0.2 times this amount. However, when the mean cell size (estimated in g.fresh wt.) is taken into consideration, producing a value equivalent to the mean intracellular anthocyanin concentration an even smaller range of values is obtained. Once again the values for the four main aggregate groupings were in close agreement and as a result of the >2 times difference in the cell size between these groups and that of the largest cell aggregates the mean anthocyanin concentration of the pigmented cells in this group was reduced to a level comparable to the others. The value estimated for the cells from the smallest aggregates however, although proportionally increased due to their relatively smaller mean cell size, was still only ca.50% of the other estimates.

It is apparent therefore that in these cultures quite substantial differences occur between the arbitrary aggregate size classes chosen for this study as concerns both biomass distribution and anthocyanin productivity. Regarding the latter however, although certain trends are apparent when the results are expressed on a total culture basis, in cellular terms they become much more consistent, particularly with respect to the mean intracellular anthocyanin concentration.

The remaining part of this section briefly summarises the results of several experiments carried out to test the potential use of microculture techniques to study the accumulation of secondary metabolites in vivo in cultured cells of C. roseus.
vii. Microculture techniques using single cells of *C. roseus*

As it was possible in this investigation not only to identify secondary metabolite accumulating cells within the total culture cell population but also to quantify the actual cellular level of the compounds, it was considered that much valuable information could be obtained from monitoring the anthocyanin accumulation of a single cell as it proceeded through successive phases of growth and division. Such an investigation would allow us to examine in a very precise manner the nature of the differences between cells with regard to anthocyanin accumulation and also the changes which take place as cells pass through successive growth cycles.

Numerous microculture techniques were tested for suitability using slide plating procedures in solid or liquid media and using micro-manipulation (for apparatus see Patnaik *et al.*, 1982, p.108) or suspension culture (5 day old, light grown) filtration to obtain single cells. Despite the use of 100% conditioned CR medium, growing the cultures in low light or in total darkness and using varying population densities of 1-30 cells/10 µl drop none of the cells survived for more than 3-5 days and none were seen to divide.

Alternative methods were tested using visking tubing microchambers placed on top of *C. roseus* callus which was used as a 'nurse' culture. These were unfortunately also unsuccessful although in these experiments, the cells, in general, survived longer, often up to 14 days. Only one cell however (out of 100-200) divided when using this method, once, 3 days after isolation. Using KM medium (fresh or 100% conditioned)
which is frequently reported as a successful microculture medium (see Kao & Michayluk, (1975), Ellis, (1982) ) failed to make any improvement in the results in either of the two basic methods.

It appeared likely therefore, that only with a considerable concentration of effort which was not possible due to temporal reasons would a successful method of microculture be devised. As a result this line of investigation had unfortunately to be terminated.
Summary

(1) Using microdensitometric techniques it has been possible to quantify the intercellular differences in anthocyanin content of C. roseus cell populations.

(2) Considerable quantitative and qualitative differences were found within in vitro pigmented cell populations.

(3) The values obtained for cell populations from whole plants covered a similar overall range but were, within tissues very much less variable than those for in vitro cell populations.

(4) Both 8% aq. sucrose and enhanced irradiance levels increased the mean anthocyanin level/cell and modified the pattern of anthocyanin distribution within the pigmented cell population.

(5) The total anthocyanin content of cell aggregates was found to be dependent on aggregate size although the mean anthocyanin content/pigmented cell in these aggregates varied very little.
Chapter 4

DISCUSSION
The anthocyanins, although consisting of a relatively small number of water-soluble phenolic compounds, are the metabolites responsible for nearly all of the red to blue pigments found in higher plants. Consequently, they are one of the best-known and most widely distributed groups of secondary compounds within the plant kingdom (Harborne, 1967; Timberlake and Bridle, 1975; Markakis, 1982a). The occurrence of anthocyanins *in vitro* has been observed on several occasions (Table 1.1.1.) although the number of reports (involving <20 species) is perhaps unexpectedly small considering the frequency of their occurrence *in vivo*.

In the majority of previous *in vitro* investigations in which anthocyanin accumulation was demonstrated, research was centred upon the influence of culture conditions (light, nutrient availability, growth substance supplementation etc.) upon overall product yields (see Chapter 1). Surprisingly however, in none of these investigations was advantage taken of the visibility of these compounds *in situ* in order that the level of the investigation could be reduced to that of the cell as opposed to the whole culture. In this investigation considerable emphasis has been placed not only upon a study of the overall pattern of anthocyanin accumulation under standard and then modified conditions (inductive treatments, cell line selection procedures etc.), but also upon the influence which such modifications had, specifically upon the size of the productive cell population and the intercellular variation within it. By doing so, it has been possible to obtain more detailed information concerning the expression of and variation in secondary metabolite
(anthocyanin) accumulation in vitro which should lead us nearer to understanding its control and indicate the most appropriate mechanisms for its manipulation.

Anthocyanin accumulation under standard culture conditions; is there a relationship between anthocyanin accumulation and the culture growth cycle?

In the Catharanthus tissue cultures used in this investigation the accumulation of anthocyanins was entirely dependent upon the presence of an essential stimulus, light (Section 3.2.1.). This effect was observed to arise in the absence of any significant concomitant alteration to either the rate or duration of the culture growth phases although such effects have been observed elsewhere (Davies, 1972; Stickland and Sunderland, 1972a,b.).

The importance of light in anthocyanin synthesis has been demonstrated in numerous other in vivo and in vitro systems (e.g. see Hahlbrock and Grisebach, 1979; Barz and Koster, 1981; Hahlbrock, 1981) and its influence has been demonstrated to involve the increased activity of at least some of the enzymes of the phenylpropanoid and flavonoid pathways through increased enzyme synthesis (see Hahlbrock and Grisebach, 1979).

However, even in the continuous presence of the required stimulus, anthocyanin accumulation by these cultures was distinctly discontinuous. The results of those experiments where a time course analysis was performed (Sections 3.2.1(i-iv), 3.2.6(iii), 3.2.8(iii)) indicated unequivocally that pigment accumulation occurred only towards the end of each subculture period and was therefore in some way 'phase-dependent'. A very
similar discontinuous accumulation of secondary metabolites has been reported for numerous other in vitro systems; for example, phenolics accumulation in Acer suspension cultures (Westcott and Henshaw, 1976), anthocyanin accumulation in batch cultures of D. carota (Noé et al., 1980), terpenoid accumulation in Tripterygium suspension cultures (Hayashi et al., 1982), the accumulation of the steroid diosgenin in liquid cultures of Dioscorea (Yeoman et al., 1980) and anthraquinone accumulation in Morinda cell suspension cultures (Zenk et al., 1975) were all similarly observed to be associated specifically with the latter part of the subculture period. Analogous results have also been reported for larger-scale culture systems, e.g. the accumulation of serpentine by C. roseus cells in 3.5 and 22 litre fermenters (Vinas and Pareilleaux, 1982; and Zenk et al., 1977, respectively).

Such observations are not unexpected considering the pattern of secondary metabolite accumulation in vivo. It is often difficult to make conclusions concerning the kinetics of secondary product accumulation in whole-plants as plant tissue is generally quite heterogeneous with respect to the structure and function of its integrant cells. However, it is generally considered that the synthesis of these products in plants is expressed as a specific feature of certain cells during restricted periods (Luckner, 1972, 1980; Wiermann, 1981). This in vivo 'phase-dependence' (Luckner et al., 1977) has, on several occasions, clearly been associated with a phase-dependent synthesis of certain specific enzymes (see Hahlbrock and Grisebach, 1979; Luckner, 1980 for extensive reference lists).
Secondary metabolite accumulation in vivo is thus considered to be a facet of plant cell differentiation (Böhm, 1977; Luckner et al., 1977; Luckner, 1980; Wiermann, 1981).

The onset of the phase of anthocyanin accumulation in these cultures clearly did not occur at random but appeared specifically to be very closely dependent upon the culture growth cycle. Pigmentation did not appear (in cultures previously grown in darkness), or was not observed to become intensified (in preilluminated cultures) until the cell division phase was completed, or very nearly so (Section 3.2.1(i,iii)). An antithetical relationship between cell division, on the one hand and biochemical differentiation (anthocyanin accumulation), on the other was therefore a quite distinct feature of this system.

Such an apparent incompatibility between cell proliferation and cell differentiation has frequently been observed in both plant and animal tissues (Roberts, 1976). In the primary root, for example, the major region of cell expansion/differentiation is spatially separated from the major region of cell division (i.e. the root meristem) (Clowes, 1976). The appearance in in vitro systems of morphologically differentiated tissues arising from previously very poorly differentiated cultures has also been observed to be specifically associated with the end of the growth cycle. In 1970, Thomas and Street reported that morphogenesis in Atropa cultures occurred towards the end of the subculture period, at a time when growth (and presumably cell division) were at a minimum. Very similar observations were made by Lindsey, (1982) on embryoid formation in Solanum dulcamara.
cultures. Indeed, a standard procedure to induce tissue differentiation in vitro is to transfer the plant material onto a modified medium which characteristically decreases the generally unrestricted rate of growth and cell division of the cultures (Reinert and Yeoman, 1982; Flick, Evans and Sharp, 1983).

Consequently, from these observations it can be concluded that the apparent uncoupling of cell division and biochemical differentiation in the cultures used here would appear consistent with the observations from many other differentiating systems and was therefore not an artefact of the very abnormal growth conditions used but was merely a reflection of the usual pattern of organisation of biochemical events in plant tissues.

The transition from the cell division phase to the cell expansion phase of the culture growth cycle would thus appear pivotal to the accumulation of anthocyanins in these cultures. This system therefore provides yet another example of the inverse relationship which would appear to exist between primary metabolism (associated mainly with cell division) and secondary metabolism (generally associated with cell expansion/differentiation) as propounded by Davies (1972) and Phillips and Henshaw, (1977) and discussed by Kurz and Constabel, (1979) and Lindsey and Yeoman, (1983a,b.). Furthermore, these results, in being conducive to the proposal of there being two relatively distinct phases of cell metabolism, show remarkable similarity to the observations reported for a great many microbial systems which lead to the so-called trophophase (growth/cell division)/idiophase (production) concept as outlined by Bu'lock (1975),
Luckner et al., (1977) and Drew and Demain, (1977). The applicability of this concept to plant cell systems has indeed already been discussed (Mantell and Smith, 1983b). If such features of in vitro product accumulation as observed here are common to many culture systems (and the literature would indicate that they are) then they clearly have important implications for consideration not only when selecting suitable physical and chemical conditions for culture but also when designing the actual culture system itself (see Lindsey and Yeoman, 1983a).

The precise molecular mechanism by which the switch in the direction of cell metabolism comes about is, as yet, unknown. In the Catharanthus cultures used in this investigation in which a definite stimulus to anthocyanin accumulation was involved, it would appear that during the early part of the subculture period proceeding more or less up to the end of the cell division phase, the cells were either incapable of responding to the required stimulus (light) or were insensitive to it.

One factor which has been strongly argued to have a controlling influence in the expression of secondary metabolism in plant cells is the competition between primary and secondary metabolic pathways for common metabolites (Phillips and Henshaw, 1977). The cessation of cell division, generally considered to result from macronutrient limitation (which, in the system described here was unlikely to be either N or sucrose but may have involved phosphate (see Section 3.2.1(i) and 3.2.3(vi).), could bring about as a result of the concomitant downturn in primary metabolism, the increased availability of certain key metabolites (particularly amino acids) thus permitting or stimulating the expression of secondary metabolic pathways.
The position of phenylalanine, an important primary metabolite, at the beginning of the phenylpropanoid pathway makes its availability a possible critical factor in anthocyanin synthesis in *C.roseus* cells. The role of substrate supply in the regulation of phenylpropanoid accumulation in plant cells has been discussed extensively by Margna, (1977) who concludes, on the basis of a great number of *in vivo* and *in vitro* investigations, that substrate supply is the most likely limiting factor in this process. These ideas will be considered further in the following sections in which the influence of modified culture conditions upon *in vitro* secondary metabolism are discussed.

How did modified culture conditions influence the pattern of anthocyanin accumulation in *C.roseus* cultures and what does this tell us about the control of this process *in vitro*?

In Sections 3.2.2-3.2.8. many experiments have been described where *C.roseus* cultures had been grown under modified conditions. A substantial number of these treatments (increased irradiance, inoculum density etc.) resulted in a considerable alteration to the observed accumulation of anthocyanin. However, concerning the pattern of anthocyanin accumulation one feature was clear from all of these experiments; in those treatments where the modified conditions permitted further cell division it was invariably observed that anthocyanin accumulation was 'postponed' at least until the latter part of the experimental period (7-10d.).

However, if the modified conditions were not conducive to further cycles of cell division (e.g. P/N stress, 8% aq.sucrose) then anthocyanin accumulation was found to increase visibly...
shortly after culture initiation (see Section 3.2.6(iii)). Therefore it would appear that the uncoupling of secondary metabolite accumulation from cell proliferation remained, even under the modified conditions used. This is as might be expected if the nutrient competition hypothesis is correct. However, it is perhaps important to discuss at this point the occurrence in the literature of a small number of clear examples where there is no obvious growth phase/production phase delimitation.

Possibly two of the best known examples are those of Zenk et al., (1977) and Fowler, (1983), both of which coincidentally concern the accumulation of serpentine in *C. roseus* suspension cultures. One important feature links these two systems. In the former case, where alkaloid accumulation was observed to parallel culture growth, the medium used was Zenk's 'production medium' which was designed to enhance secondary metabolite yields albeit in conjunction with conditions being suboptional for growth (Zenk et al., 1977). Similarly, the example reported by Fowler was for a slow-growing system. In this instance the culture dry weight increased at a relatively steady rate but had not reached a maximum within the 32d. experimental period, by which time only a ca.4.5X increase had occurred. (In the *C. roseus* cultures described here a dry weight increase of 10-15X in ca.10-12d. was typical). In a further example, Stickland and Sunderland (1972a,b) observed the accumulation of anthocyanins in *Haplopappus* cultures during the linear phase of growth. However, such an accumulation was induced by light which, coincidentally brought about an extensive reduction in culture growth (dry wt., cell no.).
In such cases it could be proposed that the suboptimal growth conditions may have resulted in a smaller proportion of cells actively contributing to culture growth with the others remaining in a metabolic state characteristic of the 'stationary' phase throughout the subculture period. Such heterogeneity could, for example, also explain the general lack of distinct cell division/cell expansion phases in the culture growth cycle as observed by Nash and Davies, (1972) and Zenk et al., (1975).

Alternatively, under suboptimal growth conditions the full metabolic potential of the cells would be underexploited and thus the demand for precursors by primary metabolism would be submaximal. Consequently, the increase in secondary metabolite levels during the linear growth phase in the above examples may have come about as a result of this increased availability of the necessary (common) precursors. Such a system would require that the enzymes of the secondary metabolic pathways were present, even at low levels, during the early phases of the culture growth cycle in the cultures used. The presence of such (unexpressed) pathways in actively growing cultures has already been demonstrated in a number of culture systems (Banthorpe et al., (1976) and see Yeoman et al., (1982a)). These results would thus appear complementary rather than antagonistic to the ideas detailed previously.
How do the quantitative effects of modified culture conditions upon anthocyanin accumulation complement the proposed mechanisms of control of in vitro secondary metabolism?

Culture conditions, both physical and chemical, are known to be of paramount importance to the overall quantitative accumulation of secondary metabolites in vitro (see reviews by Kurz and Constabel, 1979; Mantell and Smith, 1983b). In both the *Daucus* and *Catharanthus* cultures used in this investigation modifications to the culture conditions were found to have a profound quantitative effect both upon culture growth and anthocyanin yield. In this section the possible reasons for these effects will be considered in relation to the overall mechanisms of control of secondary metabolite accumulation which are thought to operate in vitro.

In callus cultures of the carrot variety 'Takii's Winter Scarlet' a considerable enhancement in anthocyanin yield was induced by reduced 2,4-D supplementation of the medium (Section 3.1.2.). This effect came about in conjunction with a significant decrease in culture growth and also an increase in the overall level of tissue organisation/differentiation as indicated by increased greening, increased compactness/decreased friability and organogenesis. It is again possible therefore, to correlate other forms of tissue differentiation with secondary metabolite accumulation, thus providing further support for the consideration that the expression of the latter is a facet of plant cell differentiation.

The relevance of decreased growth to in vitro secondary
metabolite accumulation has been detailed above and in con-
junction with increased culture organisation has been discussed
extensively by Lindsey and a causal relationship has been
proposed (Lindsey, 1982; Lindsey and Yeoman, 1983b). This
carrot system had many of the characteristics of those cultures
described by Lindsey and indeed it was observed that in liquid
cultures, even in low auxin conditions, the failure to induce
increased tissue organisation also resulted in a failure to
enhance anthocyanin yield (Section 3.1.2(iii).).

In contrast however, it was clear that in the Catharanthus
cultures the accumulation of anthocyanins was not associated
with any other visible type of cell organisation or differentiation.
It must be concluded therefore, that although such features as
increased aggregation, compactness, organogenesis etc., con-
stituting an overall increase in the level of tissue organisation,
frequently accompany biochemical differentiation, these features
are clearly not an essential prerequisite for biochemical
differentiation, at least in every system. This increase in
tissue organisation likely instigates enhanced culture productivity
by providing conditions more conducive to secondary metabolite
synthesis, by increasing cell-cell contact, mechanical pressure,
instituting gradients etc., thus returning the cells to an
environment more reminiscent of the intact plant.

It was in fact observed in the last experiment reported
(Section 3.2.8(vi).) that increased aggregation actually appeared
to decrease anthocyanin accumulation in C. roseus cell suspensions.
This observation is not unique. Kinnersley and Dougall (1980a)
have reported that continued sieving of D. carota cultures to
retain finely dispersed suspensions enhanced product (anthocyanin) yields. These authors explained this result in terms of endogenous cytokinin levels which are reputed to be higher in larger cell aggregates (Szweykowska, 1974) and which were shown to be strongly inhibitory to anthocyanin accumulation when applied exogenously to their system. As cytokinins were found to be inhibitory to anthocyanin accumulation in the Catharanthus cultures used in this study (Section 3.2.3(i).) it would appear that a similar explanation for this result was applicable to this system also.

Before going on to discuss the implications of the results of the more detailed investigation using Catharanthus cultures one final point to arise from the carrot studies warrants attention. It was observed that anthocyanin accumulation in the cultures of the variety 'Takii's Winter Scarlet' was more pronounced under both standard and inductive conditions in comparison to cultures of the 'Autumn King' variety. This apparent differential capacity to accumulate these products in vitro mirrored the normal capacity of the source plants to produce anthocyanins in vivo. This might suggest that a direct correlation existed between the in vivo and in vitro potential to accumulate anthocyanins by these tissues. Such a correlation has been demonstrated elsewhere for nicotine production by (almost isogenic) tobacco lines (Kinnersley and Dougall, 1980b) and serpentine accumulation by C.roseus cultures (Zenk et al., 1977) and thus provides further support for the hypothesis that overall product yields in vitro can be strongly influenced by those normally attained in vivo. In this context Zenk (Zenk et al., 1977; Zenk and Deis, 1983) has proposed that the screening of
whole plants to ascertain the 'high-yielders' prior to culture initiation should be the first priority of anyone wishing to obtain the best yields of secondary metabolites from \textit{in vitro} systems.

The very pronounced effect of growth substance supplementation upon anthocyanin accumulation in the \textit{C.roseus} cultures, as reported in Section 3.2.3. indicates the importance of these compounds in this process. The inhibition both by cytokinins (kinetin, coconut milk) and the 'alternative' auxins tested (IAA,NAA) was quite distinct although it should be pointed out that only single concentrations of these compounds were used and as indicated by the 2,4-D treatments (Section 3.2.3(ii).), concentration was of considerable importance. Nevertheless, this system is somewhat unusual in that much better yields were observed in a 2,4-D supplemented medium in comparison to those supplemented with IAA or NAA to a similar level. In general 2,4-D has been found much less suitable for triggering secondary metabolism than other auxins (Furuya \textit{et al.}, 1971; Tabata \textit{et al.}, 1974; Zenk \textit{et al.}, 1975, 1977; Kamimura and Nishikawa, 1976).

Although the precise molecular basis of growth substance action still remains to be elucidated, it is generally believed that these compounds act upon secondary metabolism indirectly by altering cytological conditions rather than directly acting upon the biosynthetic pathways concerned (Kurz and Constabel, 1979). If the hypothesis of primary/secondary metabolic antagonism is correct it is possible that at least some of the growth substance effects observed could be explained in terms
of the influence of these compounds upon growth itself (Lindsey and Yeoman, 1983b; Tabata and Hiraoka, 1976). However, it is evident from the data presented that in these cultures many of the results are inconsistent with this theory thus suggesting an effect of growth substances independent of any growth-related response.

The indirect effect of growth substances on secondary metabolism through an effect upon primary metabolism (protein turnover etc.) remains a strong possibility. Both auxins and cytokinins can greatly influence primary metabolism. The latter are known antagonists of the natural ageing and senescent processes in plants, presumably by influencing primary metabolism (Fletcher, 1969) and 2,4-D has been demonstrated to increase protein turnover in Acer cells (Phillips and Henshaw, 1977). The influence of both growth substance types may thus arise through a stimulation of primary metabolism at the 'expense' of secondary metabolism. However, as is clear from Section 3.2.3(i) at least some 2,4-D was required by these cultures before anthocyanin accumulation could occur. Whether this was a direct effect upon cell metabolism or possibly an effect of the altered pattern of growth (the absence of cell division, for example) remains to be elucidated.

The doubling of secondary metabolite (anthocyanin) yield by nutrient (P/N) starvation in the C.roseus cultures closely agrees with results from other systems (Nettleship and Slaytor, 1974; Mantell and Smith, 1983b), although is somewhat disappointing in relation to the much more substantial increase observed by Knobloch and Berlin for a closely similar culture system, (Knobloch and Berlin, 1980; Knobloch, Bast and Berlin,
1982). However, the cultures used by these workers accumulated only negligible amounts of anthocyanin under non-inductive conditions. The final levels accumulated by both systems were in fact closely similar which may suggest that there is an upper limit to the ability to induce anthocyanin accumulation in these cultures by this method.

The failure to enhance anthocyanin yield through culture deprivation of a single macronutrient, despite the near or total absence of growth clearly emphasises the influence which basic macronutrients may have upon secondary metabolic processes. These results would indicate that limiting growth *per se* is only effective in enhancing secondary metabolism when other conditions are favourable.

P/N starvation was clearly not a *stimulator* of anthocyanin accumulation in this culture system. The results of the $^{13}$C-labelling experiment (Section 3.2.7(iv).) indicated that the rate of accumulation was actually considerably less in the treated cultures during the period of maximum accumulation. It would appear therefore that the enhanced accumulation was more simply the result of maintaining the cells in a 'stationary phase' metabolic state for a longer period than would normally have been possible in these cultures. By transferring the cells to the modified medium the normal cause of the rapid onset of culture senescence after 18-20d. (e.g. the build-up of toxic compounds or lethal nutrient stress) was removed while at the same time inhibiting a return to further cycles of growth and cell division by the exclusion of a critical nutrient.

The indication that the observed decrease in the rate of
anthocyanin accumulation may have been a consequence of a
decrease in the size of the intra-cellular phenylalanine
pool (see Section 3.2.7(iv).) has important and interesting
implications concerning the use of nutrient starvation as a
technique to enhance secondary metabolite yields *in vitro.*

If such an effect were to occur then it may therefore be
possible to increase anthocyanin yield further simply by the
exogenous supplementation of phenylalanine. Although, as
suggested by the precursor feeding experiments (Section 3.2.6.),
phenylalanine was unlikely to be a limiting factor under standard
culture conditions it is possible that it had become so under
the modified conditions, resulting perhaps from the extended
absence of a major N source. The combined use of nutrient
starvation and precursor feeding treatments has actually already
been used to great effect by Yeoman *et al.*, (1980) to enhance
capsaicin production by chilli pepper cells and such a technique
should prove of considerable value in other systems also.

A further possible improvement to these modified conditions
would be to combine the above treatments with the removal or
diminution of the growth substance supply. It has been
observed that 2,4-D enhances protein turnover (Phillips and
Henshaw, 1977) and also decreases the intracellular pool size
of certain amino acids (Kurz and Constabel, 1979). Both of
these effects could reduce the availability of precursors for
secondary metabolite synthesis and may therefore increase the
importance of precursor supply as a limiting factor in this
process.

The *ca.* 50% enhancement in anthocyanin yield induced by
8% sucrose was less than had been anticipated on the basis of previous findings (Knobloch and Berlin, 1980; Mantell and Smith, 1983b). Nevertheless, the absence of such a response in the 3% sucrose (+5% mannitol) treatment would indicate that the enhancement observed was a direct response by the cells to the conditions used, rather than merely being the result of an elongated stationary phase through a supplementation of the energy supply. This proposal is supported by the observations of Knobloch and Berlin (1980) who found that the sucrose-induced response was concentration-dependent and was maximal at ca. 8% sucrose.

The hypothesis that this effect may be the result of the increased osmotic potential of the medium (Mantell and Smith, 1983b) is not supported by the results reported here. The findings from those treatments where mannitol had been used as a (partial) substitute source of osmotic potential to sucrose, chosen for its known very slow rate of cellular uptake and for being relatively metabolically inert (Thomas and Davey, 1975; Evans and Cocking, 1977), have indicated that increased osmotic potential alone is insufficient to induce further anthocyanin accumulation. The failure of the combined sucrose/mannitol treatment to enhance product levels would suggest that the effect of high sucrose concentrations involved a direct effect upon cell metabolism and was likely independent of any change in the osmotic potential of the bathing medium.

However, perhaps the most interesting result to come out of this experiment was the observation that 8% sucrose-supplemented nutrient medium resulted in drastically reduced anthocyanin yields. The approximate halving of the culture fresh
weight by this treatment in the absence of any significant
effect on cell number or culture dry weight would strongly
suggest that cell expansion had also been severely reduced, if
not eliminated. A result complementary to this has been re-
ported by Knobloch and Berlin (1981) who found that Nicotiana
cultures subjected to an identical treatment exhibited a reduction
in the F.Wt./D.Wt. ratio of 50%. These effects are particularly
interesting as an almost identical result was achieved in this
study in quite a different way. By increasing the transfer of
spent medium at the time of culture inoculation (Section 3.2.2.),
a ca. 80% reduction in cell expansion in association with an
almost complete elimination of anthocyanin accumulation was
effectuated.

The apparent absence of any change in the extent of cell
division and of any observable change in the rate of cell density
increase in the treated cultures would indicate that a link may
exist between anthocyanin accumulation and the ability of the
cells to undergo expansion or the actual process of expansion
itself. The nature of this link and its relevance to the
expression and control of secondary metabolism in vitro will
now be considered.

The latter proposal, that the failure of the anthocyanin
accumulation response arose as a corollary of the absence of
cell expansion is an interesting but unlikely possibility. Such
an effect may have come about either through the inhibition of
secondary metabolic pathways as a result of the change in
nutrient status of the cultures arising due to the absence of
the final growth phase, or, as a direct consequence of the
absence of an increase in cell volume (in association with a dilution of cell contents). However, considering the former case, if nutrient conditions (see for example the importance of N and $\text{PO}_4^{3-}$ levels (Section 3.2.3(vi).) were inhibitory at the end of the division phase then it would not be expected that the onset of cell expansion and anthocyanin accumulation would have coincided under non-modified conditions but would have been subject to a temporal separation. Such a disjunction was clearly not present (Section 3.2.1.).

The physical increase in cell size almost certainly has important implications in the augmentation of anthocyanin accumulation and the data from the microdensitometric time course analysis (Section 3.2.8(iii).) and the experiments in which cellophane was used (Section 3.2.4.) (which will be discussed in the following section), would strongly support this. However, concerning the initiation of anthocyanin accumulation, if the physical increase in cell size was instigational to this effect through perhaps the dilution of a critical intracellular component (the potential importance of intracellular $\sigma$-phosphate levels has for example, been proposed to influence cell metabolism (Knobloch, Beutnagel and Berlin, 1981)) the concentration of this component would have to have fallen to almost subinhibitory levels at the time of the cessation of cell division in order to achieve a more or less simultaneous anthocyanin accumulation/cell expansion response. Furthermore, the factor involved would have to be very poorly utilized by non-dividing cells or the period during which secondary metabolite pathways may be initiated must be brief in order for anthocyanin accumulation not to occur after a (short) delay in the treated cultures.
Perhaps a more plausible explanation of the observed absence of pigment accumulation in these instances is that rather than being a corollary of the failure of cell expansion both effects arose coincidentally as a result of the failure of a common initiation mechanism caused by the modified culture conditions. The cessation of cell division results in substantial modifications to cell metabolism (Roberts, 1976; Wareing and Phillips, 1978) which permit or stimulate cell expansion, secondary metabolism etc. It is therefore possible that the modifications to the conditions of culture in some way interfered with or prevented some of these changes coming about, thus disrupting the normal series of events subsequent to cell division. What these changes are and what factors are instigational in their origin must unfortunately remain a subject for speculation.

The unknown active factor present in 12d. old medium interestingly, would appear to act, either directly or indirectly, specifically upon dividing cells. When present in stationary phase cultures, during which time it must be produced, no apparent detrimental effect appears despite the very much greater concentration of the factor which would be present. The 8% sucrose treatment, on the basis of the results presented above concerning anthocyanin accumulation under standard conditions and in 8% aqueous sucrose, would have been expected to have enhanced product yields rather than eliminated them. Although the utilization of sucrose was not studied it is likely that a considerable amount remained in the cultures after the growth phase (see for example Mantell and Smith, 1983b). This nevertheless failed to induce or enhance anthocyanin accumulation.
It could therefore be proposed that the high sucrose concentration present during the phase of cell division may have induced the premature accumulation of the active medium factor to inhibitory levels. Clearly a considerable amount of further work is necessary before any definite conclusions can be made regarding these extremely interesting and potentially very important effects.

The recurring trend in the results discussed so far in this thesis again draws attention to the complexity of the control of secondary metabolic processes in vitro. The results further emphasise the paramount importance of culture conditions in the accumulation of secondary metabolites in plant cultures and clearly indicate that culture modifications used to enhance product yield are only effective when the other conditions of culture are conducive to such a change. On the basis of the results described, some proposals have already been made as to how certain improvements to culture manipulation could be made. The interdependence of culture conditions concerning secondary metabolite accumulation in vitro can be explained on the basis of a competition between primary and secondary metabolic pathways for common metabolic precursors.

Increased culture productivity/unit biomass may theoretically come about in two ways. These are firstly, through an increase in the proportion of actively accumulating cells (in cultures in which not all cells are productive) and/or secondly by increasing the metabolite yield per productive cell. It now remains to discuss how the ability to identify the productive cell population in the cultures used in this investigation, thus
enabling us to relate overall culture yields to yield on a cellular basis, has added to our knowledge of secondary metabolite accumulation in plant cells, its control and possible modes of manipulation.

How has the use of detailed cellular studies using *C. roseus* cultures contributed to our knowledge concerning the possible factors which limit secondary metabolite accumulation in *vitro*?

If we consider the situation where a culture accumulates a particular secondary metabolite, the yield of this metabolite per unit of culture biomass essentially depends upon two critical factors. These are firstly, the mean concentration of product attained within the productive cells and secondly, the proportion of the total population which these cells make up. Any phenomenon which inhibits or limits one or both of these factors restricts the overall potential of the culture to accumulate the product. Let us now consider the possible factors which might be involved in this situation and by referring to the data collected in this investigation, ascertain the likely importance of each of these factors in the expression of *in vitro* secondary metabolism.

The following diagram is presented to indicate the main (non-exclusive) factors considered likely to be influential in the yield of secondary metabolites by plant tissues:
a. Intracellular concentration

In theory the length of time spent in the phase of active accumulation could prove of great importance to the final mean intracellular level of the metabolites attained. As observed in the first Catharanthus experiments reported (Section 3.2.1) the appearance of anthocyanin-containing cells was clearly asynchronous. It might therefore be expected that an increased period of accumulation would increase the mean intracellular anthocyanin concentration. However, one of the most interesting and perhaps surprising observations to emerge from the microdensitometric time course study (Section 3.2.8(ii).) was that under standard culture conditions an upper limit to the mean anthocyanin concentration of the pigmented cell population existed which was reached quite rapidly (ca. 3 days) after the onset of anthocyanin accumulation. An elongation of the
accumulation phase *per se* would therefore not have increased the final mean intracellular anthocyanin concentration in this system. In this respect it was observed that the two treatments used in this study which prolonged the period spent by the cells in a non-dividing state (8% aq. sucrose, Section 3.2.3(v); 3.2.8(iv); P/N starvation, Section 3.2.3(vi).) resulted in only a ca. 20% and 3% increase in intracellular anthocyanin concentration respectively.

Although the relationship between cell volume and the IOD value (total cellular anthocyanin content) for individual cells was not very clear (Section 3.2.8(i).) the data from the time course investigation (Section 3.2.8(ii).) emphasise the importance of cell size in the elaboration of anthocyanin accumulation in these cultures. In this experiment the plateau level of the intracellular anthocyanin concentration, reached on ca. day 10, consequently resulted in a dependence upon an increase in cell size and the proportion of pigmented cells to bring about a further increase in overall culture yield. The constant intracellular anthocyanin concentration during the latter part of the subculture period, at a time when the anthocyanin content/g. of cells was still increasing would suggest that cell size was limiting culture yield during this time. This would therefore imply that a feedback inhibition mechanism was operating in this system.

End-product inhibition through allosteric interaction or competition with the substrate for the enzyme binding sites is well known in biological systems (Cohen, 1976) and there are a number of examples of such effects involving enzymes of flavonoid pathways demonstrated both *in vivo* and *in vitro* (see refs. in
Luckner, 1980). However with respect to anthocyanin accumulation a distinct compartmentation exists which separates the enzymes from the final product. Anthocyanins, like many secondary metabolites are stored in a nonplasmic compartment (the vacuole) whereas the enzymes responsible for their synthesis are generally believed to be located in the cytoplasm (Hrazdina et al., 1978; Hahlbrock and Grisebach, 1979) although at least one preliminary report to the contrary exists (Pecket and Small, 1980). Such an inhibitory mechanism must therefore act across the tonoplast barrier. Most simply, this could come about through a blockage of the transfer mechanism responsible for the 'excretion' of the anthocyanin molecules into the vacuole. Such a mechanism could therefore terminate any further anthocyanin accumulation without the need to affect anthocyanin synthesis as any pigment subsequently produced would be rapidly degraded in the 'hostile' cytoplasmic environment. However, if an inhibition of anthocyanin synthesis was instigated by this process this would have to act either generally, upon the whole pathway or specifically, upon the last steps as precursor feeding (Section 3.2.6) was unable to override the inherent control of anthocyanin accumulation in this system.

It is interesting to note that in the cellophane treatments (Section 3.2.4) an apparent mean increase in cell expansion of ca. 30% was accompanied by only a slight (ca. 5%) alteration in the estimated intracellular anthocyanin concentration. No explanation as to the mode of influence of cellophane in this intriguing response can yet be forwarded although an effect through the extracellular degradation of the polymer or its
adsorptive properties remains possible. Similarly, from the results of the P/N starvation treatments it can be estimated that an increase in cell volume of ca. 100% occurred, once again with the estimated mean intracellular anthocyanin concentration remaining essentially unchanged. Nevertheless, as clearly indicated in Section 3.2.3(i), the maintenance of a more or less constant intracellular anthocyanin concentration does not automatically follow an increase in cell expansion. In this experiment the removal of growth substances from the nutrient medium resulted in a very substantial increase in mean cell volume without any further anthocyanin accumulation. This result, once again emphasises the dependence of in vitro secondary metabolism upon culture conditions which have an important, if not overriding influence upon metabolite yields.

The possible importance of 'synthetic capacity' and precursor availability in relation to the cellular yield of anthocyanins will be considered together as they are clearly very closely associated. By the former term is meant the ability of the anthocyanin biosynthetic pathway to act as an effective competitor for precursors common to more than one pathway. The conditions influential to these factors would be enzyme activity (enzyme levels), the relative predominance of primary pathways and also side branches of the anthocyanin pathway which compete for common precursors and if a system of enzyme compartmentation occurs, the rate of transfer of the product of one compartment to the next compartment for which it is the substrate. However, the use of precursor feeding (Section 3.2.6), despite the likelihood of precursor entry into the cells (Section 3.2.7), failed to induce any increase in the mean intracellular
anthocyanin concentration. Furthermore in the P/N starvation experiments (Section 3.2.7(iv)) a reduction in protein synthesis was apparent, as was an approximate doubling of anthocyanin yield although once again it can be estimated that the mean intracellular anthocyanin concentration remained unchanged.

It can be estimated from the data presented in Section 3.2.3(iv,v) that increasing the irradiance level from 30 to 130 \( \mu \text{mol.m}^{-2}.\text{sec}^{-1} \) increased the mean intracellular anthocyanin concentration by up to 60\%. This was the largest increase achieved by any treatment in this investigation. This effect was nevertheless, responsible for only a small fraction of the increase in overall culture yield which was generally elevated by 200-300\%. As illustrated in Fig.3.2.38, the influence of light was not to increase the range of concentration values but really to increase the relative proportion of cells in the upper part of the range.

How may light have induced this enhancement in intracellular anthocyanin concentration, a property of the cells which has, up to now, been observed to vary very little? The following mode of action of light is forwarded. The last step in the anthocyanin biosynthetic pathway is the glycosylation of the aglycone which is accompanied by the 'excretion' of the glycoside into the cell vacuole. The enzyme catalysing this reaction is considered to be located in the tonoplast (Fritsch and Grisebach, 1975; Harborne, 1980). As the anthocyanin is stored in the vacuole and the enzymes are located in the cytoplasm the apparent feedback inhibition mechanism is almost certainly instigated via this key enzyme. A simple competition between substrate and product
for these enzyme binding sites could explain the relatively constant final anthocyanin concentration achieved – a certain concentration of product being required to continually block all potential 'excretion' sites thus halting further accumulation. The inductive effect of light upon the synthesis of enzymes of the anthocyanin biosynthetic pathway is well known (see Hahlbrock and Grisebach, 1979 and refs. therein). If the synthesis of the key enzyme and presumably of the others also, is increased, then this entails not only that the cells have an increased capacity for synthesis but also and more importantly, for accumulation. An increase in the number of 'excretion sites' would require an increased concentration of product (anthocyanin) to inhibit accumulation fully.

The general conclusion which must therefore be drawn from the preceding discussion is that the mean intracellular anthocyanin concentration varied relatively little in these cultures and was likely under physiological control based upon a feedback inhibition mechanism. This was emphasised particularly by the data from the cell line analysis where it was found (Tables 3.2.16. and 3.2.18.) that despite a >30 fold variation in the anthocyanin content/g. (fr.wt.) the estimated (or measured) mean vacuolar concentration varied by <2 fold. Our generally poor ability to enhance this concentration probably reflects our poor ability to enhance the levels of certain key enzymes.

b. The proportion of productive cells.

Under standard culture conditions the Catharanthus cultures used in this study (either callus or suspension cultures)
contained, with almost monotonous regularity, 6-12% visibly pigmented cells at the end of each subculture period. Modifications to these culture conditions did however, result in a considerable variation to this proportion although the highest mean level attained using one or more inductive treatments, was still only 26% of the total cell population. Also, it was observed that all treatments which influenced culture yield likewise influenced the proportion of productive cells.

This feature was therefore of considerable importance in determining anthocyanin yield in this system. This was particularly emphasised by the data from the cell line analysis where it was observed that both the anthocyanin yield/g. fresh weight and the percentage of pigmented cells varied by >30 fold with a direct correlation existing between the two (r=0.95) (Section 3.2.5). Similarly, in the examination of anthocyanin distribution between the different aggregate size classes (Section 3.2.8(vi).) the variation in anthocyanin content/g. fresh weight was mirrored by a variation in the proportion of pigmented cells (with the estimated mean intracellular concentration remaining virtually unaltered in all but the smallest of the aggregate size categories). The microdensitometric time course experiment (Section 3.2.8(iii).) indicated that as the final mean intracellular concentration of anthocyanin was rapidly achieved under standard culture conditions, the increase in the proportion of anthocyanin-accumulating cells was a very important causal factor in the continued enhancement of anthocyanin yields in these cultures in the latter part of the subculture period. In the cultures exposed to high irradiance levels (Section 3.2.3(iv,v).) the 3-3.5 fold increase in anthocyanin yield was
in the main the result of a 2-2.5 fold increase in the percentage of pigmented cells.

The controlling factors which determined the proportion of anthocyanin-containing cells were therefore of enormous importance in determining the overall yield of this product in these cultures. Consequently, it would be of great value to identify those factors which governed whether a cell was to be productive or non-productive, an understanding of which should bring us considerably nearer to being able to control secondary metabolite yield more effectively in this and other systems.

One possible important influential factor in determining the proportion of anthocyanin accumulating cells was the degree of heterogeneity of the culture conditions. As was evident from many of the results presented in Part 3.2 of the results, and as emphasised extensively in the first part of the discussion, culture conditions play an extremely influential rôle in the control of the overall yield of secondary metabolites in vitro. Therefore, if the normal in vitro conditions were intrinsically heterogeneous then this may explain some of the metabolic heterogeneity observed within the culture cell population.

However, the proportion of pigmented cells observed in callus cultures, in which a substantial degree of heterogeneity of culture conditions would have been present (nutrient and light gradients, mechanical pressure differences etc.) was the same as that generally observed in liquid cultures in which conditions would have been considerably more homogeneous. Furthermore, the results of the experiments reported in Section 3.2.3(iv,v) would eliminate the possible involvement of a differential light effect. It could perhaps be proposed that the effects observed
under modified conditions resulted from an effect upon the
distribution of cells between the different cell aggregate
size classes (Section 3.2.8(vi)). However, in none of the
experiments performed was a visible difference in culture
aggregation ever observed. It would thus appear that intra-
cultural heterogeneity was not an important factor in this
process.

In the cultures used in this study, a known, essential
stimulus light was involved in anthocyanin synthesis. It is
therefore possible that different cells had different sensitivity
thresholds to this stimulus. Indeed in this respect, increasing
the irradiance level from 30-130μmol.m⁻².sec⁻¹ brought about a
2-2.5 fold increase in the proportion of anthocyanin accumulating
cells. However, this effect had clear upper limits both in
terms of the maximum effective stimulus and also the maximum
response obtained both of which were relatively low (Section
3.2.3(v)). Whether this difference in response was physio-
logically or genetically based is unknown although concerning the
latter possibility further mention will be made later. Clearly
however, stimulus intensity although partly limiting, was not a
major cause of the relatively poor numbers of anthocyanin con-
taining cells observed.

The possibility of nutrient inhibition, related to a form
of inter-cellular heterogeneity, as tentatively proposed by
Knobloch, Beunngel and Berlin, (1981) is a very interesting
concept worthy of consideration although little evidence for,
or against the hypothesis can be gleaned from the data reported
herein. These authors suggest that as in their system the
phosphate included in the nutrient medium was maximally taken up prior to cell division and as considerable amounts failed to be utilized by the cells during growth then as a result of the frequent inequality of many cell divisions (Yeoman and Street, 1977) the final cell population would be quite heterogeneous with regard to the intracellular phosphate concentration. The influence of intracellular phosphate concentration upon cell metabolism has already been demonstrated (Knobloch, Beutnagel and Berlin, 1981) and consequently such a heterogeneity may have been responsible for the subsequent variation in cell metabolism observed. This hypothesis could of course be extended to include other nutrients with uptake kinetics similar to phosphate or even compounds synthesised by the cells early on in the subculture period.

However, it is difficult to envisage how such a situation would regularly produce a relatively constant proportion of anthocyanin accumulating cells. Furthermore, the cells capable of anthocyanin synthesis would presumably arise at 'random' and it would therefore be expected that there would be a relatively random distribution of cells within the culture which was, for example, clearly not the case in C.roseus callus cultures in which approximately all of the coloured cells were located only in the uppermost cell layers. This hypothesis is nevertheless clearly worthy of further investigation and consideration particularly if the occurrence of productive/non-productive cell populations are a regular feature of other culture systems which, on the basis of the available evidence, would appear to be the case (see Chapter 1).

Finally, a factor of potentially critical importance in
determining the size of the pigmented cell population in these cultures was the possible occurrence of cell heterogeneity based upon epigenetic and/or genetic differences within the cultures. The possibility of there being two genetically distinct cell populations present, growing equally fast but only one of which, usually constituting ca. 10% of the total cell population was capable of accumulating anthocyanins under the conditions used has already been discounted upon the basis of the cell line analysis (Section 3.2.5). Nevertheless, although it was clear from this experiment that all of the cell lines which were isolated were capable of anthocyanin synthesis, it was immediately obvious that all exhibited reproducibly different capacities for accumulation. This feature of these cultures was found to be directly correlated with the final proportion of pigmented cells attained (Fig. 3.2.15.). It has been assumed that these differences arose as a result of variation inherent within the original stock callus and not as a consequence of the actual isolation procedure itself inducing such differences.

The ability to accumulate a certain percentage of pigmented cells under standard culture conditions appeared therefore to be fixed in these cultures as it had been in the original stock callus. However, the individual cells which went to make up this percentage appeared not to be determined by some specific feature of a certain proportion of the cell population, which may have been present or which may have arisen during culture development, but by some overall controlling mechanism in the cultures. As detailed previously, in the stock callus (as in those of the cell lines) the location of the anthocyanin accumulating cells was essentially restricted to one specific part
of the tissue (the uppermost cell layers) and thus the occurrence of pigmented cells was governed by the heterogeneity of the culture conditions but at no 'expense' to the final proportion of cells induced which was the same as that normally observed in liquid culture. In other words, which cells went to form the pigmented cell population was determined by culture conditions whereas the size of this population was determined by an inherent property of the culture. This entails that a cell which accumulated visible levels of anthocyanin at the end of one growth cycle would not necessarily have given rise to progeny which ultimately did so at the end of the next. One of the major aims of attempting studies on individual cells (Section 3.2.8(vii)) was to investigate this possibility.

As to the nature of this overall controlling mechanism which determined the proportion of productive cells, the following possibility is envisaged. It could be proposed that in this system the cells which became capable of responding to the stimulus first, upon doing so concomitantly instigated an inhibition of the initiation of anthocyanin accumulation in other cells. When ca. 10% of the cells had begun to accumulate anthocyanins the degree of this inhibition was sufficient to prevent all the remaining cells from producing these compounds. This inhibition may have been an active one in that the cells produced an inhibitory compound which acted in such a way as to suppress the expression of certain metabolic pathways in cells which were not already 'switched on'.

Alternatively, the inhibition may have been a passive one in that at the onset of anthocyanin synthesis the accumulating
cells became an active 'sink' for a certain component present in the cultures which was necessary for anthocyanin accumulation. This component may have been a remaining nutrient or perhaps a compound specifically produced by the cells - a type of 'cross-feeding' phenomenon where one cell population essentially supported aspects of metabolism in another. Once again in this case, a ca.10% proportion of cells being the necessary level to allow the accumulating cell population to become a 'sink' sufficiently powerful to prevent all of the remaining cells from beginning anthocyanin accumulation. These different mechanisms are of course not mutually exclusive.

Such phenomena as cross-feeding and the influence of 'sinks' upon cell metabolism have many precedents in whole-plant systems. After all, the whole basis of plant organisation is centred upon the production of compounds by one group of cells which have an influence on or are utilized in the metabolism of a second group. The involvement of sinks and intersink competition in plant development has been implicated for example, in the phenomenon of apical dominance and in determining the extent of flower and fruit development (Phillips, 1969; Wareing and Phillips, 1981).

The possible involvement of a specific inhibitor in this system is interesting in that a medium-induced inhibition of anthocyanin accumulation has already been demonstrated (Section 3.2.2.). The spent medium used was however from dark-grown cultures which may suggest either that a differential light/dark response in inhibitor production occurred in this system or perhaps that these effects were instigated by different mechanisms.

Modifications to the proportion of producive cells generally observed, such as those for the cell lines may reflect differences
within the cultures with respect to the cellular sensitivity to the factors involved or to their differing levels of production. Under altered culture conditions the increases in the red cell percentage observed may have arisen due to the more synchronous appearance of the pigmented cells. By modifying conditions in such a way as to make the metabolic state of the cells more conducive to anthocyanin accumulation (see earlier discussion) the anthocyanin accumulation response may have been more synchronous to the extent that a greater number of cells had begun to accumulate anthocyanins before the inhibitor had built up to fully active levels. Alternatively these conditions may have, as suggested above, influenced the cellular sensitivity to the factors involved (be they inhibitors or 'cross-feeders') or their level of production.

If we consider the accumulation of anthocyanin by intact plant tissues it is clearly possible to achieve 100% (or approximately) pigmented cells - as in the petal epidermis, for example. This does of course not only reflect the much greater degree of control of cell metabolism in vivo, as does the much greater consistency observed in the actual values observed for the intracellular anthocyanin content, (Section 3.2.8(ii)) but also serves to indicate that a much greater proportion of pigmented cells is theoretically possible in vitro. Whole-plant tissues, unlike cell cultures, are not closed systems however, and consequently they are subject to the influence of the rest of the plant. This outside influence may for example, provide an exit route for the putative inhibitor or, alternatively may provide a continual supply of the necessary 'cross-feeder'. It is of course possible that as a result of the greater degree
of internal control in these systems factors which limit accumulation *in vivo* are quite different from those which do so *in vitro*.

**Concluding remarks**

The accumulation of secondary metabolites by plant tissue and cell cultures is now a well known and frequently studied phenomenon. In the *in vitro* systems described here the accumulation of anthocyanin by cultures of *C. roseus* (and *D. carota*) exhibited many of the characteristics of secondary metabolite accumulation observed in other systems in that the enhancement of product levels was phasal and appeared to be associated solely with the latter part (post-division) of the culture growth cycle. The competition between primary and secondary metabolic pathways for common precursors almost certainly has an important determining influence in this process.

The importance of culture conditions to the accumulation of anthocyanins by these tissues was considerable and extensive alterations to metabolite yield were instigated through either physical or chemical modifications to the cellular environment. Through the ability to identify and examine the actual population of productive cells in these cultures it has been possible to determine that yield limitation was manifested in two ways. Firstly, a maximum mean level of the intracellular anthocyanin concentration existed, which appeared to vary relatively little, even under modified conditions. This has been proposed to result from a feedback inhibition mechanism. Secondly, the proportion of cells which accumulated visible levels of
anthocyanin appeared fixed under particular conditions with values rarely exceeding 20%. This factor was clearly a major limitation to culture yield and the available evidence would suggest that some type of metabolic interaction between the cells within the culture was responsible for the small proportion of productive cells regularly observed.

As the evidence available (see Chapter 1) would suggest that intercellular heterogeneity with respect to secondary metabolite accumulation was a common, if not ubiquitous, phenomenon in tissue culture systems it would clearly be of value to continue this work further to determine in particular the nature of the limitations to the productive cell percentage and to investigate the proposed existence and identity of the occurrent inhibitory factor(s). With this knowledge it should be possible to design much more effective secondary metabolite production systems.
PROPOSED FUTURE WORK

The work proposed as a continuation of this project is aimed towards developing a higher yielding cell culture system and would be directed along the following two, complementary lines of investigation.

(a) To determine the nature of the factors which influence the proportion of productive cells and ways to counteract their effects.

(b) To determine a means to remove the desired metabolite from the productive cells in order to prevent the elevation of its intracellular concentration to inhibitory levels.

Concerning line (a) suitable experiments would be designed to examine the influence of possible medium-borne factors upon metabolite yield by, for example: altering the cell/medium ratio of early stationary phase cultures by removing part of the cell population or by adding extra medium taken from a culture of the same age; by carrying out time course experiments to determine the effect of media in which cells had been grown for differing time periods; by exploiting the isolated cell lines to determine the nature of the differences between them through culture mixing, cross-feeding and cell transfer into media removed from a culture of a different cell line.

Concerning line (b) two approaches might be adopted. Firstly, to examine the possible existence of 'leaky' mutants which 'naturally' excrete metabolites into the medium by either active or passive mechanisms and secondly, to determine methods of cell permeabilization in either a continuous or intermittent (short,
sharp shock) manner. This might be achieved by using compounds which either increase membrane fluidity or which induce holes to form in the cell membranes e.g. anaesthetics, detergents, organic solvents (see Brodelius et al., 1979), DMSO (Brodelius et al., 1982) or by altering the Na/K/Ca ionic balance. Obviously an essential feature of any such method would be that the cell viability and metabolism would have to remain relatively unaffected by the treatment applied.
APPENDIX

"A preliminary study of the culture of saffron crocus, *Crocus sativus* L. tissues and its potential use as an *in vitro* source of the valuable spice, saffron."
Introduction

The work carried out for this thesis was originally directed along two parallel and complementary lines of investigation. The main line, concerning the use of cultures which accumulated anthocyanin pigments as tentative model systems with which to study secondary metabolism \textit{in vitro} has already been reported. The second line of research was concerned with investigating the potential use of an \textit{in vitro} method for the production of the 'active' components of the spice, 'saffron'. It was intended to use these two projects to exemplify both the academic and applied aspects of this important subject and it was also hoped that the results obtained from the former investigation would help to direct the approach in the latter.

"Saffron" is the general term given to the dried stigma + styles of \textit{C. sativus} and is a spice used both to colour and flavour foods (Ingram, 1969; Stobart, 1977; Grieve, 1980). The 'active' components are reputed to be derived from a single \textit{C}_{40}-carotenoid precursor (see Pfander and Schurtenberger, 1982 and references therein) and consist of crocetin (I) and its digentiobioside ester crocin (II), both of which are yellow

\begin{center}
\includegraphics[width=\textwidth]{crocetin_crocin}
\end{center}

\text{Gen} = \text{Gentiobiose}
in colour and also safranal (III, the aroma component) and its glucoside picrocrocin (IV, the flavour component), both of which are colourless.

The components of saffron were chosen for study as they are 'desirable' plant compounds, some of which are also coloured thus complementing the main research project. Naturally-produced saffron is the most expensive spice on the world market (Culpeper Ltd., pers.comm.) costing more than gold on a per unit weight basis. The causal factors which determine this high price are the considerable difficulties involved in growing the crop which in addition is very labour intensive, in combination with the very poor product yield/plant (Douglass, 1728; Ingram, 1969; Mathew, 1977). Notwithstanding these problems saffron is still a multi-million dollar industry. An in vitro method of "saffron" production is therefore theoretically a viable commercial proposition (see Yeoman et al., 1980; Fowler, 1983).

In this Appendix the work reported represents the results of a short investigation carried out to determine firstly, a method of culture for Crocus (which has not yet been reported) and secondly whether the cultures thus produced accumulated the coloured saffron components. No further work beyond this stage is reported as, upon the basis of the promising initial findings, this project was thereafter sponsored by an industrial concern and its continuation was carried out mainly by another researcher.

A photograph of C. sativus is presented in Figure A.1.1.
Figure A.1.1.

The saffron crocus, *Crocus sativus* L. at anthesis in September, 1981 (x0.5). Note the exceptionally long, pendulous "stigmas" which hang between the perianth segments (arrowed).
Section A.1.

"The initiation of callus cultures of four species of Crocus"
In this section are reported a small number of experiments which were carried out in a successful attempt to culture *Crocus* tissue. The prime aim was to isolate cultures of the saffron crocus - *C. sativus* for further experimentation. However, plant material of this species proved expensive and very difficult to obtain and consequently a number of exploratory 'test' experiments were initially carried out using material from other freely available *Crocus* spp. *C. vernus, C. tomassinianus* and *C. flavus*, three commonly grown spring crocuses, were chosen due to their ease of availability and very low cost. After testing various organs for their callusing ability and isolating suitable cultures, material from one species was selected for an exhaustive series of experiments to determine the most suitable growth substance supplementation for *Crocus* culture. The wealth of information gained from these introductory experiments was then successfully used to direct the line of research into the culture of *C. sativus* plant material, when this became available. The details of these experiments are now reported.
i. **Studies on the ability of different organs to produce callus using explants of C.vernus, C.tomassianianus, and C.flavus.**

Due to the considerable expense and very poor availability of *C.sativus* plant material in this country a preliminary survey was carried out, using three freely-available *Crocus* spp. to determine what were likely to be the most suitable organs, media and techniques to select for *C.sativus* callus induction.

Cultures of the above species (for variety see Table A.1.1.) were initiated as detailed in Chapter 2 using unplanted corms which had overcome their summer dormancy and had produced shoots *ca.1-1.5 cm. long*. At this stage all organs of the shoot were in a rapid phase of growth, with the exception of the anthers which surprisingly, had reached approximately their final size and constituted *ca.50%* of the total bud volume. Protocorm tissue was also used and this was obtained from greenhouse-grown plants immediately *post* anthesis at which time the protocorms (which become the storage organ for the following season) were undergoing a phase of rapid expansion and had reached *ca.5-8 mm.* in diameter. All material was plated (as detailed in Chapter 2) onto the Iridaceous culture medium of Hussey, (1977) using his recommended modifications (pers.comm.) and including the growth substance supplements listed in Table A.1.2.

The results are summarised in Table A.1.1. Irrespective of the level of growth substance supplementation, callus was successfully obtained from only a small number of the organs
<table>
<thead>
<tr>
<th>Organ</th>
<th>\textbf{C.tomassianus cv Whitewell's Purple}</th>
<th>\textbf{C. vernus cv. Remembrance}</th>
<th>\textbf{C.flavus cv.Golden Yellow Mammoth}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mature corm</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Protocorm</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cataphyll</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Leaf - basal third</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Leaf - medial &quot;</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Leaf - apical &quot;</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pedicel</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ovary</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Stigma/Style</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Perianth</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stamen</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flower sheath</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table A.1.1.

Callus production by explanted organs of 3 \textit{Crocus} spp. on a variety of culture media (see Table A.2.2.).

\(+\) = callus cultures \(-\) = callussing not observed \((+)\) = callussing isolated observed but failed to develop
<table>
<thead>
<tr>
<th>NAA/6-BAP</th>
<th>C. tomassianianus</th>
<th>C. vernus</th>
<th>C. flavus</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg. l(^{-1})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8/4</td>
<td>leaves, ++</td>
<td>roots(^2), +</td>
<td>roots(^1), +++</td>
</tr>
<tr>
<td>8/2</td>
<td>leaves, +++</td>
<td>roots(^2), +</td>
<td>roots(^1,2), +++</td>
</tr>
<tr>
<td>4/4</td>
<td>leaves, +++</td>
<td>leaves, +++</td>
<td>roots(^1,2), +++</td>
</tr>
<tr>
<td>4/2</td>
<td>leaves, +</td>
<td>roots(^1), ++</td>
<td>roots(^1), +++</td>
</tr>
<tr>
<td>4/0</td>
<td>NG</td>
<td>NG</td>
<td>roots(^2), +++</td>
</tr>
</tbody>
</table>

Table A.1.2.

Pattern and extent of regeneration in *Crocus* cultures grown on CS medium supplemented with the above-listed growth substances. Roots appeared in two forms: 

1 short, fat and often spherical, 2 elongated and thin resembling normal roots (see Fig.A.1.2.)

+ = isolated occurrence, ++ = frequent, +++ = extensive, often covering explant.

NG = no growth.
The forms of regeneration observed in *Crocus* callus cultures. See text and Table A.1.2. for details.

(a) Leaf and shoot formation, *C. tomassianus* cv. 'Whitewell's Purple'.

(b) Root formation of the thin type, *C. vernus* cv. 'Remembrance'.

(c) Root formation of the short, fat, bulbous type, *C. flavus* cv. 'Golden Yellow Mammoth'.

Bar = 0.5 cm.
tested. Protocorm, ovary, pedicel and basal leaf tissue routinely gave rise to callus, this having always been preceded by an often extensive (5-10 fold) increase in explant volume. Small areas of callusing were observed on segments of mature corm from all species but only with _C.tomassiananus_ segments (where the callus was exclusively associated with exposed vascular tissue) did this progress beyond the initial stages to give rise to actively growing cultures. All of those organs which were not observed to callus remained visibly unchanged, often for many months. In all cases however the tissues eventually became necrotic.

The timecourse of callus formation varied considerably between the different species. _C.tomassiananus_ produced visible callus within one week of initiation and production continued at a rate which permitted removal of all of the original explant tissue at the second subculture. Tissues of _C.flavus_ and _C.vernus_ were both considerably slower to respond with the latter taking _ca_.3 months to produce sufficient material for subculture in isolation of the original plant material.

The major problem encountered during the 'bulking up' phase of callus production was the extensive proliferation of organised structures, initially observed in virtually all of the cultures isolated. The findings have been summarised in Table A.1.2. and Fig.A.1.2. Irrespective of callus origin only leaves/shoots were observed in _C.tomassiananus_ cultures whereas only roots were observed in those of _C.flavus_. _C.vernus_ produced both roots and shoots, according to the growth substance supplementation of the medium. Attempts to
reduce this level of regeneration and also to decrease the length of time required for callus initiation through qualitative variation of the growth substance supplements added to the nutrient medium, using 2,4-D/6-BAP, 2,4-D/Kinetin and NAA/Kinetin, at the levels used previously, failed to bring about any improvement and those media containing kinetin actually failed to induce any callus formation from new explants. Nevertheless, by regular subculture every 2-3 weeks, selectively subculturing callus free from organised structures resulted in cultures free from visible signs of regeneration within 3 months (C.tomassianus) or 5 months (C.vernus, C.flavus). The growth rates of these cultures were however disappointingly low and consequently the following experiment was performed in an attempt to rectify this.


The aim of this experiment was to enhance the growth rate of C.tomassianus callus through modification of the growth substance supplementation of the nutrient medium. It was anticipated that information from this experiment would provide some information as to suitable media to select for C.sativus culture. Material from C.tomassianus cultures was chosen simply because, as a result of its initially faster growth, sufficient callus of this species became available for experimentation first.
Callus derived from *C. tomassianus* leaf bases, grown on 4mg/l NAA, 2mg/l 6-BAP-supplemented medium was used as the test material. Petri dishes (5 cm.) containing the media under test were each inoculated with a known weight (ca. 0.5 g.) of callus which had been grown on the same (test) medium for 3 weeks prior to experimentation. Initially three auxins, 2,4-D, IAA and NAA were tested in the presence of a single cytokinin, 6-BAP. The most productive of these combinations were then tested using kinetin as an alternative cytokinin. Three replicates/treatment were set up and fresh weight measurements were made after a three week period of incubation in darkness. As insufficient callus was available to enable all treatments to be run at the same time, each pair of growth substances was tested in separate experiments which were carried out over consecutive subculture periods. In all cases the callus used was taken from the *C. tomassianus* stock cultures grown on the above medium.

The results are presented in Table A.1.3. Increases in fresh weight were generally relatively low in the 2,4-D and IAA treated cultures with the greatest values arising from the 8 mg l⁻¹ auxin/4 mg l⁻¹ 6-BAP treatments. The values obtained for the NAA-treated cultures were, in all cases (where 6-BAP had been included) greater than the best values from the other treatments, irrespective of concentration. The greatest increases were once again found in the 8 mg l⁻¹/4 mg l⁻¹ treatment. Increasing the NAA concentration to 16 mg l⁻¹ and/or the 6-BAP concentration to 8 mg l⁻¹ resulted in reduced growth (16 mg l⁻¹/4 mg l⁻¹, 141%; 8 mg l⁻¹/8 mg l⁻¹, 132%; 16 mg l⁻¹
### Table A.1.3.

Fresh wt. increase in a 3 week subculture period of *C. tomassinianus* callus tissue grown on CS media supplemented with different growth substance combinations. Each value is a mean of 3 replicates and is presented as a percentage of the initial fresh wt./dish.

1 mg.l⁻¹.

<table>
<thead>
<tr>
<th></th>
<th>2</th>
<th>4</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-BAP : 2,4-D¹</td>
<td>4</td>
<td>48</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>35</td>
<td>46</td>
</tr>
<tr>
<td>6-BAP : 1AA¹</td>
<td>4</td>
<td>46</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>48</td>
<td>35</td>
</tr>
<tr>
<td>6-BAP : NAA¹</td>
<td>4</td>
<td>132</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>110</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>12</td>
<td>27</td>
</tr>
</tbody>
</table>
Each of the auxins tested, when used at a concentration of 8 mg l⁻¹ in the presence of the alternative cytokinin, kinetin (4 mg l⁻¹) sustained very little callus growth: 2,4-D, 26%; IAA, 12%; NAA, 33%. Consequently, the basal medium chosen for the initial isolation and subculture of *C. sativus* callus consisted of the basal CS medium supplemented with 8 mg l⁻¹ NAA plus 4 mg l⁻¹ 6-BAP.

iii. *The initiation and growth of callus obtained from tissues of the saffron crocus, Crocus sativus L.*

When *C. sativus* plant material became available immediate attempts were made to grow it *in vitro*. Corms were obtained from a British horticultural supply company and also from a research worker in one of the major commercial saffron-producing centres in Italy (see Cpt. 2.). Material from both these sources was used for *in vitro* experimentation.

All available organs were used to obtain sterile explants, but efforts were concentrated upon those which had proved successful with other species (Section A.1.i.). CS medium supplemented with 8 mg l⁻¹ NAA plus 4 mg l⁻¹ 6-BAP was used. All cultures were incubated in darkness and each was subcultured at 3 weekly intervals, irrespective as to whether they were producing callus or not. A summary of the results is given in Table A.1.4.

The results obtained are somewhat in contrast to those for the other species tested previously. In the variety 'Cashmirianus' (Avon Bulbs Ltd.) mature corm tissue was the
<table>
<thead>
<tr>
<th>Organ</th>
<th><em>C. sativus</em> cv. cashmirianus (British)</th>
<th><em>C. sativus</em> (Italian)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mature corm</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Protocorm</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>Cataphyll</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lead - basal section</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lead - medial section</td>
<td>-</td>
<td>½±/½⁻</td>
</tr>
<tr>
<td>Lead - upper section</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pedicel</td>
<td>NA</td>
<td>-</td>
</tr>
<tr>
<td>Ovary</td>
<td>NA</td>
<td>-</td>
</tr>
<tr>
<td>Stigma/style</td>
<td>NA</td>
<td>-</td>
</tr>
<tr>
<td>Perianth</td>
<td>NA</td>
<td>-</td>
</tr>
<tr>
<td>Stamen</td>
<td>NA</td>
<td>-</td>
</tr>
<tr>
<td>Flower sheath</td>
<td>NA</td>
<td>-</td>
</tr>
</tbody>
</table>

Table A.1.4.

Callus production by *C. sativus* organs explanted onto CS medium supplemented with 8 mg l⁻¹ NAA plus 4 mg l⁻¹ 6-BAP.

+ = callus culture  - = callussing not isolated  NT = not tested  NA = not available
most efficient callus producer. Callus appeared uniformly over the cut surfaces within 1-2 weeks of culture and continued to grow at a relatively slow but steady rate, permitting its isolation from the original explant at the 2nd/3rd sub-culture. Leaf-base tissue from these plants also gave rise to callus but at a much slower rate and that which arose appeared to be solely derived from the exposed vascular tissue.

In contrast, corm-derived callus tissue was never obtained from the Italian plants, despite several attempts to do so. However, leaf-base tissue from these plants was by far the most proliferative callus producer of all of the *C. sativus* tissues tested. Cultures from this source could be isolated within 3-4 weeks from initiation. Flower buds were also produced by these corms and, very much in contrast to all of the other species tested none of the floral tissues produced callus on the medium used. Although the explants appeared to survive for many months (often increasing in size), callusing was never observed.

The callus obtained, irrespective of its source of origin became bright yellow/orange in colour within one week of initiation. Thereafter all of the callus produced was of a similar colour (Fig.A.1.3.). All of the cultures were observed to grow in a distinctly nodular manner, very similar to that observed for onion (*Allium cepa*) callus (McKenzie, pers.comm.). Once again, much in contrast to the cultures of the other *Crocus* spp. which had been isolated, organ regeneration was only rarely observed in any of the *C. sativus* cultures obtained (Fig.A.1.3.).
Figure A.1.3.

The appearance of 3 week-old, dark-grown callus of *Crocus* spp.

(a) Left to right: *C. sativus* (Italian stock), *C. flavus*, *C. tomassianus*. (9 cm Petri dishes).

(b) *C. sativus* (Avon Bulbs Ltd.) callus showing rare plantlet formation. Bar = 1 cm.

(c) *C. sativus* cv. 'Cashmirianus' callus. (9 cm Petri dish).
The rate of growth of these cultures was unfortunately disappointingly low, especially of those derived from *C. sativus* var. 'Cashmirianus' corms. Shortly after initiation these cultures were found to undergo only a 20-30% increase in fresh weight in each 3 week subculture period (Fig.A.1.4.). Interestingly however, monitoring this growth over the three year period of this project revealed that on the standard medium the increase in culture fresh weight/subculture period improved at a remarkably steady rate. Unfortunately however, this rate was very low and only after ca.2 years had the doubling time (100% increase) been reduced to one subculture period.

The leaf-base callus cultures from the Italian stocks attained a 3 week doubling time soon after isolation. However, in contrast to the situation detailed above, this level of growth was more or less maintained throughout the project period (Fig.A.1.4.).
Figure A.1.4.

Fresh wt. increase of *C. sativus* cultures grown for 3 weeks on the standard CS medium plus 8mg.l\(^{-1}\) NAA, 4mg.l\(^{-1}\) 6-BAP, monitored over a 3 year period.

(*) *C. sativus* cv. 'Cashmirianus'; (O) *C. sativus* (Italian stock)
The following points have arisen from the work reported in this section.

1. Callus cultures of four *Crocus* spp. including the saffron crocus (*C. sativus*) have successfully been isolated.

2. Few organs proved successful callus producers under the range of conditions tested.

3. The problem of, often extensive, regeneration observed in some cultures was overcome through selective subculture.

4. Extensive tests revealed that 8 mg./l NAA plus 4 mg./l 6-BAP was the most productive growth substance supplement to the nutrient medium.

5. The cultures of *C. sativus*, irrespective of tissue origin, rapidly became orange-brown in colour.

6. This is believed to be the first official report of the growth of *Crocus* tissue *in vitro*.

Having obtained a suitable method for *Crocus* culture and having initiated cultures of the saffron crocus, *C. sativus*, analysis of the pigmentation of these cultures was performed in order to determine if any of the culture isolates were accumulating the saffron constituents, crocin and crocetin.
Section A.2.

"The pigmentation of *C. sativus* tissue grown in vitro"
As described in the introduction to this Appendix to the results chapter, two of the four components of saffron (crocin and crocetin) are deep yellow in colour, becoming orange/scarlet when highly concentrated, as for example, in *C. sativus* stigmas. Both of these compounds, but principally the water-soluble crocin constitute the major dye components of saffron and it was hoped to use these compounds as indicators of the intracellular presence of the taste/smell principles of saffron (picrocrocin/safranal) which are colourless aromatic compounds and require quite complex techniques for their detection.

It was indicated in Section A.1iii. that the callus cultures of *C. sativus* isolated from corm or leaf base tissue rapidly took on an orange/orange-brown colour which then became a permanent feature of the cultures. This therefore looked very promising with regard to the rationale behind this work and consequently when sufficient material became available the pigments present in these cultures were extracted in order to determine if they were the desired saffron constituents. This section contains the results of the analysis performed on these extracts.
i. TLC and spectrophotometric analysis of the pigment components of C. sativus stigma extracts.

Prior to examining the pigment content of C. sativus callus tissue, extracts of commercially-supplied C. sativus 'stigmas' (saffron) were used to determine suitable chromatographic and spectrophotometric procedures to employ and also to obtain the necessary 'standards' for comparison.

Whole, dried stigmas were extracted as detailed in Section 2.3.3v. using 80% acetone in distilled water. This proved to be an extremely efficient extraction solvent removing all but a trace of the yellow pigment from the tissue. The extract was then separated into ether-soluble and water soluble fractions which were concentrated in vacuo for chromatography. The great majority of the extracted pigment was found to enter the aqueous phase.

Initial chromatographic attempts proved very successful. Using thin layer silica gel chromatography plates in association with the solvent BAW (Butanol Acetic acid:Water, 5:1:4), which is generally recommended for water soluble phenolic compounds (Harborne, 1973) resulted in very good separation of the components of both extracts (Fig.A.2.1.). The aqueous phase was separated into a total of 9 visible yellow components and the ethereal phase, 4 yellow components. Examination of the plates under ultraviolet light revealed no further components with all but one of the visible pigments appearing dull-green. The exceptional spot, in the ethereal fraction (Rf, 76) appeared dull yellow under these conditions.

Attempts to improve this separation were unsuccessful.
Figure A.2.1.

TLC of water (A) and ether (E) -soluble fractions of a C.sativus stigma extract. All components were yellow in visible light. BAW solvent, silica gel-60 adsorbant.
The use of paper (Whatman, nos.1 or 3) or cellulose MN300 TLC plates resulted in considerable streaking of the individual components and gave very poor separation. Numerous other developing solvents were also tested (particularly those recommended by Harborne (1973)) but none improved upon the previous result and none revealed a greater number of visible components. Some of these solvents (Amyl alcohol:Acetic acid:water, 2:1:1, Methanol:water 4:1 and Butanol:chloroform:water, 5:1:4) were used as the second phase for 2 dimensional TLC and it was found that in no case were any of the separated components, visible after the first run, seen to consist of more than a single component when developed in the second dimension.

Butanol:water (5:4) was observed to give equally good separation to that of BAW and consequently when TLC was to be used as a means to isolate pigment components for spectrophotometry this solvent was chosen as the acetic acid, present in the latter solvent was found difficult to eliminate from eluted samples, prior to spectrophotometry.

The amount of pigment present in the ether fraction was very small and consequently a large amount of source tissue would have been required to yield sufficient amounts of the individual pigments for spectrophotometric analysis. As this was not available no further analysis could be performed on this fraction. However, in the aqueous extract a much greater quantity of pigment was present and it was therefore possible to examine 7 of the 9 components spectrophotometrically. After chromatography each component was scraped off the TLC
plate and eluted into double-distilled methanol. The silica gel was then removed by centrifugation/filtration and the eluant dried in vacuo at <30°C. Each component was then dissolved in methanol, ethanol and water and the absorption spectrum in each determined. The results of this analysis are presented in Table A.2.1. and Fig.A.2.2.

It is evident from the table that all of the components isolated had closely similar spectral properties. Components 2,3,4,5 and 7 exhibited spectral patterns which were almost identical in all three of the solvents used. Components 6 and 8 were also closely similar but had clearly different spectra to the others.

Although it was not possible to firmly identify any of these components their tripartite peak (usu. 2 peaks, 1 shoulder) in the 400-500 nm. range clearly distinguishes them as being carotenoids. Component 3 was the most abundant pigment in the extract and having spectral characteristics identical to those reported (Dhingra et al., 1975) was almost certainly \( \alpha \)-crocin, the \( \beta \)-digentiobioside ester of crocetin. Dhingra et al., (1975) report that they found two of the minor components in saffron methanol extracts to be the mono- and di-glucosides of crocetin and a third, crocetin monogentiobioside-D-monoglucoside. However, as to which components of the TLC pattern these compounds correspond to and as to the identity of the remaining components no further information can be given.

Details of the \( C. sativus \) callus pigmentation are now given.
<table>
<thead>
<tr>
<th>Component</th>
<th>MeOH</th>
<th>EtOH</th>
<th>H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>459, 434, 321</td>
<td>461, 435, 319</td>
<td>463, 444, 325</td>
</tr>
<tr>
<td>3</td>
<td>460, 435, 320</td>
<td>461, 435, 318</td>
<td>464, 445, 324</td>
</tr>
<tr>
<td>4</td>
<td>459, 434, 320</td>
<td>459, 434, 318</td>
<td>462, 441, 325</td>
</tr>
<tr>
<td>5</td>
<td>461, 435, 319</td>
<td>460, 435, 318</td>
<td>463, 444, 324</td>
</tr>
<tr>
<td>6</td>
<td>451, 428, 318</td>
<td>450, 430, 317</td>
<td>453, 433, 323</td>
</tr>
<tr>
<td>7</td>
<td>459, 434, 319</td>
<td>460, 434, 318</td>
<td>461, 442, 324</td>
</tr>
<tr>
<td>8</td>
<td>449, 427, 318</td>
<td>452, 430, 317</td>
<td>454, 434, 323</td>
</tr>
<tr>
<td>9</td>
<td>? 435 ?</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table A.2.1.

Spectral properties of water-soluble components in a *C. sativus* stigma extract. The values presented are the wavelengths (in nm.) of the spectral maxima (peaks) or the points of inflection (shoulders) between 300-500 nm. *From Fig. A.2.1.*
Figure A.2.2.

Absorption spectra of the 4th* (Rf=33 in BAW) and 6th* (Rf=52 in BAW) components of an aqueous C. sativus stigma extract in 3 different solvents. * labelled A and B respectively.
ii. The pigmentation of *C. sativus* callus cultures.

The first callus of *C. sativus* to be isolated, which had been derived from *C. sativus* cv. 'Cashmirianus' corm slices, was extracted when sufficient material could be spared for analysis. The cultures were 3-4 months old at this time. The extraction and chromatographic techniques employed were identical to those detailed in the previous subsection.

Thin layer chromatography of the ether-soluble fraction of this extract revealed the presence of 5 very pale yellow components (Fig.A.2.3(a).) all of which were visible in UV light as yellow/yellow-orange spots. Only one of these pigments (Rf, 76) appeared to correspond to a component of the ether-soluble stigma extract. As a result of the appearance of these spots under UV light this pigment could not have been the carotenoidal crocin precursor crocetin.

The water-soluble fraction of the callus extract was deep orange-brown in colour. Initial attempts to chromatograph this fraction failed due to interference by the brown (polyphenolic?) component(s) which remained on the origin and caused streaking. However, this problem was successfully resolved by carrying out a preliminary qualitative extraction of the aqueous extract with an equal volume of n-butanol. The brown pigments, which were found to be very poorly soluble in n-butanol remained in the aqueous phase whereas the yellow components passed into the alcohol. This procedure was not found to qualitatively modify the composition of the stigma extract and in those callus extracts which contained very low levels of the brown pigments (and could therefore be chromatographed without an initial butanol purification) no additional
Figure A.2.3.

(a) TLC of the ether-soluble fraction of *C. sativus* stigma (S) and callus (C) extracts. BAW solvent.

(b) TLC of the water-soluble fraction of *C. sativus* stigma and callus extracts. 1 = untreated callus extract, 2 = Butanol extract of aqueous stigma extract, 3 = Butanol extract of 1, 4 = untreated callus extract. See text for details.
yellow spots were ever observed (Figure A.2.3(b)).

TLC of *C. sativus* cv. 'Cashmirianus' aqueous extracts of callus taken from 3 week old cultures revealed the presence of 5 yellow components (Fig.A.2.4.), three very minor pale yellow pigments, a bright yellow pigment and a very abundant pale yellow pigment which was present in much greater quantities than any of the others. This component did not correspond to any of the stigma components and was intensely fluorescent (yellow) in UV light. The three minor components also failed to correspond to any of the pigments in the stigma extracts and appeared dull-yellow in UV light.

The remaining pigment was poorly visible in UV light, appearing dark green in colour and had an Rf.value (BAW solvent) identical to that of the major saffron component, presumed to be crocin. Although this pigment was present in very small amounts sufficient was isolated to enable its absorption spectrum to be determined. The results, presented in Table A.2.2. and Figure A.2.5(a). indicated that this compound had identical spectra to that of crocin in each of the solvents used. When spectrophotometric analysis was complete this pigment was cochromatographed with the major saffron component (No.3) and both were observed to co-run in the solvents Butanol:Chloroform:Water, 5:1:4 (Rf = 4), Butanol:Water, 5:4 (Rf = 15) and Butanol:Acetic acid:Water, 5:1:4 (Rf = 26). It was concluded therefore that these compounds were chemically identical. Quantification of the level of this pigment *in vitro* was possible only in the crudest of terms and it was estimated that the amount of crocin in 1 g. (fr.wt.) *C.sativus*
Figure A.2.4.

TLC of *C. sativus* stigma (S) and *C. sativus* cv. 'Cashmirianus' callus (C) aqueous extracts, the latter after butanol extraction (see text). BAW solvent.
Figure A.2.5a.

The absorption spectra of the major water-soluble saffron component (1) and the non fluorescent, bright yellow pigment isolated from a *C. sativus* cv. 'Cashmirianus' aqueous callus extract (2) in methanol.

Table A.2.2.

<table>
<thead>
<tr>
<th>Solvent:</th>
<th>MeOH</th>
<th>EtOH</th>
<th>H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Callus extract component (Rf=0.26)</td>
<td>460,435,413nm.</td>
<td>461,435,414nm.</td>
<td>464,445nm.</td>
</tr>
<tr>
<td>Stigma extract component (Rf=0.26)</td>
<td>460,435,413nm.</td>
<td>461,435,413nm.</td>
<td>464,445nm.</td>
</tr>
</tbody>
</table>

The spectral properties of the above mentioned pigments in three different solvents.
Figure A.2.5(b).

UV and visible light absorption spectrum of the major water soluble *C. sativus* callus pigment (Rf=30, BAW) in methanol.
callus (assuming ca. 90% water) was equivalent to <1% of a complete *C. sativus* stigma.

Further analysis of the major callus pigment revealed that its absorption spectrum was distinctly different from those of the carotenoids and consisted of a small, broad peak at ca. 380 nm. with 2 very intense peaks in the 200-300 nm. region (Fig.A.2.5(b).). The spectral pattern, in association with the colour and UV fluorescence of this pigment would suggest that this was a phenolic compound, possibly an aurone (see Mabry *et al.*, (1970); Markham (1982)). It has been reported that *C. sativus* stigmas are one of the richest known sources of Vitamin B$_2$ (Riboflavin) which is both yellow and intensely UV-fluorescent (Bhat & Broker, 1953). However, although the chromatographic properties of riboflavin were closely similar to that of the unknown callus pigment (Fig. A.2.6.) the absorption spectrum was entirely different (Fig. A.2.7.). Consequently the unknown pigment cannot have been riboflavin. No further attempt at identification was made.

Unfortunately, although these initial results looked promising in that the cultures obtained were observed to accumulate detectable levels of the major saffron component under standard cultural conditions this was found to be short-lived. Within 2 months of carrying out the first pigment analysis the crocin component was no longer detectable in these cultures. No change was however observed in any of the other visible components. Examination of all of the other isolates produced similar results. Initiating new cultures from *C. sativus* cv. 'Cashmirianus' corm tissue also resulted in callus which accumulated all of the pigments observed
Figure A.2.6.

TLC of an aqueous stigma extract (S), Riboflavin (R), C. sativus cv. 'Cashmirianus' aqueous callus extract (C) and a Butanol extract of 3 week-old C. sativus cv. 'Cashmirianus' suspension culture medium (M).

BAW solvent.
Figure A.2.7.
Absorption spectra of the major, water-soluble pigment component of *C. sativus* cv. 'Cashmirianus' callus extract (A) and Riboflavin (B). The latter from Sigma UK. Ltd. Both pigments were dissolved in methanol.
previously, excluding the crocin component.

It was observed that the medium upon which any of these isolates were grown, or especially the bathing medium of cell suspension cultures was always distinctly orange/brown in colour at the end of each subculture period. The possibility that these cultures might have been exporting crocin was thus considered. However, TLC of a concentrated Butanol extract of the suspension culture medium (3 weeks from culture initiation) revealed that the pigments concerned, which were present in substantial quantities, interestingly corresponded to the two most minor (UV fluorescent) components observed in callus tissue extracts (Fig.A.2.6.).

It was not known whether the loss of crocin accumulation in the original callus or its absence from the other isolates was total or whether levels simply failed to reach detectable proportions. However, certain microscopic observations revealed some interesting facts in this respect and these are now reported.

iii. Microspectrophotometric and fluorescence microscopic examinations of C. sativus cells derived from in vivo and in vitro-grown tissues.

As the major visible components of C. sativus stigmas (saffron) are deep yellow/orange in colour whereas the major component of C. sativus callus pigmentation was very pale yellow it was considered that it might be possible to distinguish between individual cells which were accumulating these pigments
using a light microscope. If this was so then this technique would provide a much more sensitive means to detect crocin accumulation in vitro.

Preliminary light microscope observations revealed that *C. sativus* callus, irrespective of source, consisted predominantly of cells with very pale yellow vacuoles (Fig. A.2.8., Table A.2.3.). The proportion of these cells varied between 50-70% of the total cell population. Approximately 1% (or generally less) of the cells were deep yellow/orange in colour. The hypothesis was therefore proposed that cells of the former type, present in very large numbers, were responsible for the presence of the unknown pigment in these cultures and that the latter cell type, present in very small amounts, was accumulating a different pigment which might be crocin. Two lines of investigation were followed to substantiate this hypothesis.

**Fluorescence microscopy**: Using a Vickers Photoplan UV microscope it was determined that the pale yellow cell vacuoles exhibited considerable yellow fluorescence when illuminated with light passed through a certain filter combination (wavelength unknown) (Fig. A.2.9a+b). This light was also found to be that necessary to induce fluorescence of the extracted unknown pigment as determined by microscopically scanning a silica gel TLC plate using epiillumination.

The deeply yellow/orange cells were observed to exhibit no/negligible levels of fluorescence under the illumination conditions used above. When fluorescence was observed this was always associated with the cell wall and not the vacuole
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Colourless</th>
<th>Pale Yellow</th>
<th>Deep Yellow/Orange</th>
<th>Brown/Plasmolysed</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 week old callus</td>
<td>28%</td>
<td>61%</td>
<td>1%</td>
<td>10%</td>
</tr>
<tr>
<td>3 week old suspension</td>
<td>33%</td>
<td>55%</td>
<td>0.6%</td>
<td>11%</td>
</tr>
</tbody>
</table>

Table A.2.3.
Predominance of cell types in *C. sativus* tissues grown *in vitro*.
All values were estimated by counting 1500-2000 cells from 3 cultures of each type.

Figure A.2.8.
A light micrograph of a squashed *C. sativus* (Avon Bulbs Ltd.) callus which had been grown in darkness for 3 weeks. The majority of cells were pale yellow in colour with only an occasional cell appearing orange/deep yellow (arrowed). Bar = 25 μm.
Figure A.2.9.

(a+b) The appearance of a pale yellow cell and an orange cell from a 3 week-old *C. sativus* cv, 'Cashmirianus' callus culture under visible (a) and UV. (b) light. Please note the considerable fluorescence of the vacuole of the pale yellow cell in comparison to that of the orange cell in which cell type fluorescence was generally associated with the cell wall. Bar = 25 μm.

(c+d) The appearance of *C. sativus* (Italian stock) stigma papillae cells under visible (c) or UV. (d) light. These cells retained most of their visible yellow pigments in chromoplasts [arrowed (c)] which appeared as dark absorbant spots under UV. light. In these cells most of the auto-fluorescence was distinctly associated with the sculptured cell wall. Bar = 10 μm.
The use of other filter combinations or completely unfiltered light failed to induce fluorescence of the vacuolar pigments in these cells.

Examination of *C. sativus* stigma cells revealed that in contrast to the callus cells, the majority of the yellow pigment was retained in the chromoplasts rather than the cell vacuole. However, exposure of these cells to UV light failed to induce fluorescence of the pigment present in either organelle. As is demonstrated in Fig.A.2.9(c+d), although these cells were observed to fluoresce, the light emitted was always associated with the cell wall. The cells illustrated are stigma papillae which actually accumulate very little crocin, nearly all of which, in this case, remains in the chromoplasts, and clearly these organelles are not associated with the fluorescence observed.

**Microspectrophotometry:** Figure A.2.5(b) clearly illustrates the differences between the absorption spectra of crocin and the unknown pigment in water. As the major difference occurred in the 400-500 nm. region the *in vivo* microspectrophotometric technique described in Chapter 2 should, despite its crudity, enable us to distinguish between cells accumulating these different compounds *in vivo*.

Cells of both types selected from 3 week old callus were examined in this way and a small number of typical results are presented in Fig.A.2.10(a). As anticipated the *in vivo* absorption spectrum of the deeply yellow cells clearly had an intense peak in the 440-445 nm. region. The pale yellow cells produced an absorption spectrum without any maxima in the
Fig. A.2.10(a).

Microspectrophotometric absorption spectra of orange (upper) and pale yellow (lower) *C. sativus* cv. 'Cashmirianus' callus cells.
Figure A.2.10(b).
Microspectrophotometric absorption spectra of *C. sativus* stigma cells.
wave-length range examined although the increase in absorption at the shortest wavelength region suggested the presence of a low peak immediately below 400 nm. Absorption spectra were also determined for *C. sativus* stigma cells (Fig.A.2.10(b)) and these proved to be identical to those for the deeply yellow callus cells.

All of these findings suggest that two distinct cell populations existed in these cultures which were accumulating different yellow pigments. The properties of the pale yellow cells were consistent with those of the unknown pigment detected by TLC and it would seem likely therefore that these cells were responsible for the accumulation of this compound in these cultures. The presence of a small number of deeply yellow/orange cells in the callus which had properties closely similar to crocin and crocin-accumulating cells would suggest that some saffron components were actually being accumulated by these cultures but at levels too low to detect by the extraction/chromatographic techniques used.

One important difference was however, observed between the *in vitro* cells and *C. sativus* stigma cells. As indicated above, in the latter cells only a small amount of pigment was observed in the cell vacuole with the majority remaining in the quite well developed chromoplasts (Fig.A.2.11). The chromoplasts in the *in vitro*-grown cells were extremely small and the majority of the pigment was accumulated in the vacuole. This might suggest that if these cells were indeed accumulating crocin then the greater export of pigment into the vacuole may result in a cell population with an increased capacity.
Figure A.2.11.

A light micrograph of a partly mascerated and squashed segment of a *C. sativus* (Italian stock) style. Note that in many cells the pigment is concentrated in well-developed chromoplasts although in some vacuoles pigmentation is clearly apparent (arrowed). Bar = 50 μm.
to accumulate this compound due to the more extensive use of the larger storage organelle and also the possible reduction in feedback inhibition which may occur within the synthesising chromoplasts. In support of this point the greatest IOD values were observed in cells from the *in vitro* grown tissues despite little difference in cell size.
The following represent the major points to emerge from the results of the work reported in this section.

1. Pigment analysis of *C. sativus* stigma extracts has been carried out and compared with those from *in vitro* grown tissues.

2. In the callus extracts only one ether-soluble yellow pigment was found to correspond to a component of the equivalent stigma extract. This compound is unlikely to have been a carotenoid.

3. In the water-soluble callus extract crocin was initially detected, in one isolate but this proved to be ephemeral.

4. Cellular investigations revealed that two yellow cell types were visible, one of which, present at very low density (<1%) was found to have properties closely similar to those of crocin and crocin-accumulating cells.

Having isolated *C. sativus* callus cultures and determined that some of the constituent cells were accumulating what appeared to be crocin* this line of investigation was terminated for the reasons stated in the introduction.

* It has since been confirmed using high performance liquid chromatography that the cultures which contained very small numbers of yellow/orange cells but which did not appear to be producing crocin, as determined using TLC, were indeed accumulating this compound but at very low levels as predicted.
REFERENCES


ALLEN, R.J.L. (1940) "The estimation of phosphorus" Biochemical Journal 34, 858-865.
ASEN, S., STEWART, R.N. and NORRIS, K.H. (1971)
"Co-pigmentation effect of quercetin glycosides on absorption characteristics of cyanidin glycosides and color of Red Wing Azalea"
*Phytochemistry* 10, 171-175.

ASEN, S., STEWART, R.N. and NORRIS, K.H. (1972)
"Co-pigmentation of anthocyanins in plant tissues and its effect on colour".
*Phytochemistry* 11, 1139-1144.

ASHWELL, G. (1957)
"Colorimetric analysis of sugars"

"The relationship of sugar concentration in the culture medium to anthocyanin accumulation in a plant callus culture"

BALL, E.A., HARBORNE, J.B. and ARDITTI, J. (1972)
"Anthocyanins of *Dimorphotheca* (Compositae) I. Identity of pigments in flowers, stems and callus cultures".

"Biosynthesis of geraniol and nerol in cell-free extracts of *Tanacetum vulgare*"
*Phytochemistry* 15, 91-100.


"Growth induction in cultures of Haplopappus gracilis I. The behaviour of cultured cells"

"Flavonoids of Parthenocissus tissue culture".
Experientia 30, 104-105.

BÖHM, H. (1977)
"Secondary metabolism in cell cultures of higher plants and problems of differentiation"
Mol.Biol.Biochem.Biophys. 23, 103-123.

"The formation of secondary metabolites in plant tissue and cell cultures"

BRODELIUS, P., DEUS, B., MOSBACH, K. and ZENK, M.H. (1979)
"Immobilized plant cells for the production and transformation of natural products"


BRUNET, G. and IBRAHIM, R.K. (1973)
"Tissue culture of Citrus peel and its potential for flavonoid synthesis"
Zeitschrift für Pflanzenphysiologie 69, 152-162.
BU'LOCK, J.D. (1975)
"The two-faced microbiologist: contributions of pure and applied microbiology to good research".
*Developments in Industrial Microbiology* 16, 11-19.

BUTCHER, D.N. and CONNOLLY, J.D. (1971)
"An investigation of factors which influence the production of abnormal terpenoids by callus cultures of *Andrographis paniculata*"

CAREW, D.P. and KRUEGER, R.J. (1976)
"Anthocyanidins of *Catharanthus roseus* callus cultures"
*Phytochemistry* 15, 442.

"The root apex"
Academic Press.

COHEN, P. (1976)
"Control of enzyme activity"

"Synthesis of malvidin and petunidin in pigmented tissue cultures of *Petunia hybrida*".
*Protoplasma* 107, 63-68.

"Flavor production in culture"
CONSTABEL, F., SHYLUK, J.P. and GAMBOG, O.L. (1971)
"The effect of hormones on anthocyanin accumulation in cell cultures of Haplopappus gracilis".

CORDUAN, G. and REINHARD, E. (1972)
"Synthesis of volatile oils in tissue cultures of Ruta graveolens"

CURTIN, M.E. (1983)
"Harvesting profitable products from plant tissue culture"

DAVIES, M.E. (1972)
"Polyphenol synthesis in cell suspension cultures of Paul's Scarlet Rose".

"Minor carotenoid glycosides from saffron (Crocus sativus)"

DOHOT, G.K. and HENSHAW, G.G. (1977)
"Organisation and alkaloid production in tissue cultures of Hyoscyamus niger".

"Hydroxylation of cinnamic acids and flavonoids during the biosynthesis of anthocyanins in Petunia hybrida Hort."
DOUGALL, D.K. (1978)
"Factors affecting the yields of secondary products in plant tissue cultures".
Ohio State University.

"A clonal analysis of anthocyanin accumulation by cell cultures of wild carrot".
Planta 149, 292-297.

DOUGLASS, J. (1728).
"An account of the culture and management of saffron in England"
Philosophical Transactions of the Royal Society 35, 566-574.

DREW, S.W. and DEMAIN, A.L. (1977)
"Effects of primary metabolites on secondary metabolism"
Annual Review of Microbiology 31, 343-356.

"Cell-to-cell variability in secondary metabolite production within cultured plant cell populations"

EVANS, P.K. and COCKING, E.C. (1977)
"Isolated plant protoplasts"
in: Street, H.E. (ed.) "Plant tissue and cell culture" 103-136.
Botanical Monographs 11, Blackwell Scientific Publications.

"Handbook of plant cell culture. Volume 1."
MacMillan.
"Retardation of leaf senescence by benzyladenine in intact bean plants".  

"Organogenesis"  

FOLCH, J., LEES, M. and SLOANE, S.G.H. (1957)  
"A simple method for the isolation and purification of total lipides from animal tissues".  

FORKMANN, G. (1977)  
"Precursors and genetic control of anthocyanin synthesis in *Matthiola incana*".  
*Planta* **137**, 159-163.

FORKMANN, G. (1980)  
"The B-ring hydroxylation pattern of intermediates of anthocyanin synthesis in pelargonidin and cyanidin producing lines of *Matthiola incana*".  

"Anthocyanin biosynthesis in flowers of *Matthiola incana*. Flavone 3- and flavonoid 3'-hydroxylases".  

FORREST, G.I. (1969)  
"Studies on the polyphenol metabolism of tissue cultures dervied from the tea plant, *Camellia sinensis* L."  
"Anthocyanidins of Lochnera rosea".  

"Commercial applications and economic aspects of mass plant cell culture".  
in: Mantell and Smith (1983a) 3-38.

"Flavour components in and cultures of onion (Allium cepa L.)"  
*Plant Science Letters* 3, 121-125.

FRITSCH, H. and GRISEBACH, H. (1975)  
"Biosynthesis of cyanidin in cell cultures of Haplopappus gracilis".  
*Phytochemistry* 14, 2437-2442.

FRITSCH, H., HAHLBROCK, K. and GRISEBACH, H. (1971)  
"Biosynthesis of cyanidin in cell suspension cultures of Haplopappus gracilis".  
*Zeitschrift für Naturforschung* 26, 581-585.

"Incorporation of \(^1\text{C}\)cinnamate into hydrolase-resistant components of the primary cell wall of spinach".  
*Phytochemistry* 23, 59-64.

"Plant tissue culture 1982".  
Japanese Association for Plant Tissue Culture, Tokyo.
FURUYA, T., KOJIMA, H. and SYONO, K. (1971)
"Regulation of nicotine biosynthesis by auxins in tobacco
 callus tissues".
Phytochemistry 10, 1529-1532.

GAMBORG, O.L. (1966)
"Aromatic metabolism in plants, II".

GERATS, A.G.M., de VLAMING, P., DOODEMAN, M., AL, B. and
"Genetic control of the conversion of dihydroflavonols into
 flavonols and anthocyanins in flowers of Petunia hybrida".
Planta 155, 364-368.

"Product cost analysis".

"A modern herbal".
Penguin.

"Flavonoids".
in: Conn, E.E. (ed.) "The biochemistry of plants, VII."
425-456.

HAHLBROCK, K. and GRISEBACH, H. (1979)
"Enzymic controls in the biosynthesis of lignin and flavonoids".
Annual Review of Plant Physiology 30, 105-130.
"Alkaloid production by plants regenerated from cultured cells of Datura innoxia".
*Phytochemistry* 13, 1671-1675.

"Subcellular localization of enzymes of anthocyanin biosynthesis in protoplasts".
*Phytochemistry* 17, 53-56.

HUSSEY, G. (1977)
"In vitro propagation of Gladiolus by precocious axillary shoot formation".
*Scientia Horticulturae* 6, 287-296.

IBRAHIM, R.K., THAKUR, M.L. and PERMANAND, B. (1971)
"Formation of anthocyanins in callus tissue cultures".
*Lloydia* 34, 175-182.

IKUTA, A., SYONO, K. and FURUYA, T. (1975)
"Alkaloids in plants regenerated from Coptis callus cultures".
*Phytochemistry* 14, 1209-1210.

INGRAM, J.S. (1969)
"Saffron (Crocus sativus)".
*Tropical Science* 11, 177-184.

ISHIKURA, N. (1975)
"A further survey of anthocyanins and other phenolics in *Ilex* and *Euonymus*"
*Phytochemistry* 14, 743-745.

ISHIKURA, N. (1978)
"Light absorption patterns of anthocyanin-containing cells".
*Plant and Cell Physiology* 19, 887-893.
"Anthocyanin production in cell cultures of Catharanthus roseus".

HARBORNE, J.B. (1967)
"Comparative biochemistry of the flavonoids".
Academic Press.

HARBORNE, J.B. (1973, 2nd ed. 1976)
"Phytochemical methods".
Chapman and Hall.

HARBORNE, J.B. (1980)
"Plant phenolics".
in: Bell, E.A. and Charlwood, B.V. (eds.) "The encyclopaedia
of plant physiology, NS 8", 329-402. Springer-Verlag.

(1977)
"A micro-colorimetric method for determination of ammonia in
Kjeldahl digests with a manual spectrophotometer".
Laboratory Practice 26, 545-547.

"Production of tripdiolide by a suspension culture of
Trypertygium wilfordii".

"The genetic controlled hydroxylation pattern of the anthocyanin
B-ring in Silene dioica is not determined at the p-coumaric
acid stage".
Phytochemistry 19, 2225-2226.
"Methylation of anthocyanins by cell-free extracts of flower  
buds of Petunia hybrida".  

"Growth and alkaloid production of the cultured cells of  
Papaver bracteatum".  
*Agricultural and Biological Chemistry* **40**, 907-911.  

KAMSTEEG, J., BREDERODE, J. van and NIGTEVECT, G. van (1980)  
"Genetical and biochemical evidence that the hydroxylation  
pattern of the anthocyanin B-ring in Silene dioica is determined  
at the p-coumaroyl coenzyme A stage".  

KAO, K.N. and MICHAYLUK, M.R. (1975)  
"Nutritional requirements for growth of Vicia hajastana cells  
and protoplasts at a very low population density in liquid media".  

"Anthocyanin synthesis in a white flowering mutant of P. hybrida  
by a complementation technique".  

KHO, K.F.F., BOLSMAN-LOUWEN, A.C., VUIK, J.C. and BENNINK, G.J.H.  
(1977)  
"Anthocyanin synthesis in a white flowering mutant of Petunia  
hybrida II. Accumulation of dihydroflavonol intermediates in  
white flowering mutants. Uptake of intermediates in isolated  
corollas and conversion into anthocyanins".  
"Increase in anthocyanin yield from wild carrot cell cultures by a selection system based on cell-aggregate size". 
*Planta* **149**, 200-204.

"Correlation between the nicotine content of tobacco plants and callus cultures". 

"Medium and light-induced formation of serpentine and anthocyanins in cell suspension cultures of *Catharanthus roseus*". 

"Influence of medium composition on the formation of secondary compounds in cell suspension cultures of *Catharanthus roseus* (L) G.Don." 

"Phosphate mediated regulation of cinnamoyl putrescine biosynthesis in cell suspension cultures of *Nicotiana tabacum*". 

"Influence of accumulated phosphate on culture growth and formation of cinnamoyl putrescines in medium-induced cell suspension cultures of *Nicotiana tabacum*". 


"Studies on the growth and metabolism of plant cells cultured on fixed bed reactors".

LINDSEY, K. and YEOMAN, M.M. (1983a)
"Novel experimental systems for studying the production of secondary metabolites by plant tissue cultures".

LINDSEY, K. and YEOMAN, M.M. (1983b)
"The relationship between growth rate, differentiation and alkaloid accumulation in cell cultures".
Journal of Experimental Botany 34, 1055-1065.

"Dynamics of plant cell cultures".

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. (1951)
"Protein measurement with the Folin-Phenol reagent".

LUCKNER, M. (1972)
"Secondary metabolism in plants and animals".
Chapman and Hall.

"Expression and control of secondary metabolism".
LUCKNER, M., NOVER, L. and BÖHM, H. (1977)
"Secondary metabolism and cell differentiation".
Springer-Verlag.

"The systematic identification of the flavonoids".
Springer-Verlag.

"The effect of nutrient medium composition on the growth cycle of Catharanthus roseus cells grown in batch culture".
Journal of Experimental Botany 31, 1315-1325.

"Cell culture mutants and their uses".
in: Vasil, I.K., Scowcroft, W.R. and Frey, K.J. (eds.)
"Plant improvement and somatic cell genetics". 221-237. Academic Press.

MANTELL, S.H. and SMITH, H. (1983a)
"Plant biotechnology".

MANTELL, S.H. and SMITH, H. (1983b)
"Cultural factors that influence secondary metabolite accumulation in plant cell and tissue cultures".
in: Mantell and Smith, (1983a), 75-110.

MARGNA, U. (1977)
"Control at the level of substrate supply - an alternative in the regulation of phenylpropanoid accumulation in plant cells".
Phytochemistry 16, 419-426.
MARKAKIS, P. (ed.) (1982a)  
"Anthocyanins as food colours".  
Academic Press.

MARKAKIS, P. (1982b)  
"Anthocyanins as food additives".  

"Techniques of flavonoid identification".  
Academic Press.

MATHEW, B.F. (1977)  
"Crocus sativus and its allies (Iridaceae)".  
Plant Systematics and Evolution 128, 89-103.

MATHEW, B.F. (1980)  
"Crocus"  
Cambridge University Press.

MATSUMOTO, T., NISHIDA, K., NOGUCHI, M. and TAMAKI, E. (1973)  
"Some factors affecting the anthocyanin formation by Populus cells in suspension culture".  
Agricultural and Biological Chemistry 37, 561-567.

"Analytical and quantitative methods in microscopy".  
Cambridge University Press.

MOK, M.C., GABELMAN, W.H. and SKOOG, F. (1976)  
"Carotenoid synthesis in tissue cultures of Daucus carota L."  
MURASHIGE, T. and SKOOG, F. (1962)  
"A revised medium for rapid growth and bioassays with tobacco tissue cultures".  

NAGEL, J. and REINHARD, E. (1975)  
"Das atherische öl der Calluskulturen von *Ruta graveolens* II".  

NASH, D.T. and DAVIES, M.E. (1972)  
"Some aspects of growth and metabolism of Paul's Scarlet Rose cell suspensions".  

"Adaptation of *Peganum harmala* callus to alkaloid production".  

"Formation of alkaloids in callus cultures of *Macleaya*".  

NOÉ, W., LANGEBARTELS, C. and SEITZ, H.U. (1980)  
"Anthocyanin accumulation and PAL activity in a suspension culture of *Daucus carota*".  

OGINO, T., HIRAOKA, N. and TABATA, M. (1978)  
"Selection of high nicotine-producing cell lines of Tobacco callus by single-cell cloning".  
PARKER, R.E. (1979)  
"Introductory statistics for biology".  
Ed. Arnold, Studies in Biology 43.

"A simple procedure for the manual isolation and identification of plant heterokaryons".  
*Plant Science Letters* 24, 105-110.

PECKET, R.C. and SMALL, C.J. (1980)  
"Occurrence, location and development of anthocyanoplasts".  
*Phytochemistry* 19, 2571-2576.

"Biosynthesis of C20-carotenoids in *Crocus sativus*".  
*Phytochemistry* 21, 1039-1042.

PHILLIPS, I.D.J. (1969)  
"Apical dominance".  

"The regulation of synthesis of phenolics in stationary phase cell cultures of *Acer pseudoplatanus* L."  

RANDEPATH, K. (1968)  
"Thin layer chromatography".  
Academic Press.

"Plant cell and tissue culture. A laboratory manual".  
Springer-Verlag.
REINHARD, E., CORDUAN, G. and VOCK, O.H. (1968)
"Uber Gewebekulturen von Ruta graveolens".
Planta medica 16, 8-16.

ROBERTS, L.W. (1976)
"Cytodifferentiation in plants. Xylogenesis as a model system".
Cambridge University Press.

RÖLLER, U. (1978)
"Selection of plants and plant tissue cultures of Catharanthus roseus with a high content of serpentine and ajmalicine".

SAITO, N. (1967)
"Light absorption of anthocyanin-containing tissue of fresh flowers by the use of the opal glass transmission method".
Phytochemistry 6, 1013-1018.

"High berberine-producing cultures of Coptis japonica cells".

SCHMITZ, M., and SEITZ, U. (1972)
"Hemmung der Anthocyansynthese durch Gibberellinsäure A₃ bei Kallustulturen von Daucus carota".
Zeitschrift für Pflanzenphysiologie 68, 259-265.

"Glucosylation of flavonoids in petals of Petunia hybrida".
Planta 153, 459-461.

"Environmental factors: A. Light."
SLABECKA-SZWEYKOWSKA, A. (1955)
"On the influence of the wavelength of light on the biogenesis of anthocyanin pigment in Vitis vinifera tissue in vitro".

"Anthocyanins of Strobilanthes dyeriana and their production in callus culture".
Journal of Natural Products 44, 609-610.

SMITH, P.M. (1976)
"The chemotaxonomy of plants".
Arnold.

"Chalcone synthesis and hydroxylation of flavonoids in the 3'-position with enzyme preparations from flowers of Dianthus caryophyllus L. (carnation)".
Planta 155, 176-182.

"Plant tissue culture as a source of biochemicals".
CRC Press.

STEINER, A.M. (1975)
"Cinnamate and shikimate incorporation into 3',4'– and 3',4',5'-hydroxy substituted anthocyanins: are there alternative pathways?"
Phytochemistry 14, 1993-1996.

STICKLAND, R.G. and SUNDERLAND, N. (1972a)
"Production of anthocyanins, flavonols and chlorogenic acids by cultured callus tissues of Haplopappus gracilis".
TABATA, M. and HIRAOKA, N. (1976) "Variation of alkaloid production in Nicotiana rustica callus cultures". 

*Phytochemistry* **13**, 927-932.

TABATA, M., YAMAMOTO, H. and HIRAOKA, N. (1971) "Alkaloid production in the tissue cultures of some Solanaceous plants". 

TABATA, M., YAMAMOTO, H., HIRAOKA, N. and KONOSHINA, M. (1972) "Organisation and alkaloid production in tissue cultures of Scopolia parviflora". 

THOMAS, E. and DAVEY, M.R. (1975) "From single cells to plants". 
Wykeham Publications.


TIMBERLAKE, C.F. and BRIDLE, P. (1975) "The anthocyanins". 
"Comparison of the onion plant (Allium cepa) and onion tissue
culture III. Feeding of 14C labelled precursors of the flavour
precursor compounds".

"Chromatography of anthocyanins on columns of insoluble
polyvinylpyrrolidone".

"Production d'alkaloïdes par des suspensions cellulaires de
Catharanthus roseus cultures *in vitro*".

WAGNER, F. and VOGELMANN, H. (1977)
"Cultivation of plant tissue cultures in bioreactors and
formation of secondary metabolites".

WAREING, P.F. and PHILLIPS, I.D.J. (1978)
"The control of growth and differentiation in plants".
Pergamon Press.

WESTCOTT, R.J. and HENSHAW, G.G. (1976)
"Phenolic synthesis and phenylalanine ammonia lyase activity
in suspension cultures of *Acer pseudoplatanus* L."

WIDHOLM, J.M. (1972)
"The use of fluorescein diacetate and phenosafranine for
determining viability of cultured cells".
*Stain Technology* **47**, 189-194.


"Formation of the indole alkaloids serpentine and ajmalicine in cell suspension cultures of Catharanthus roseus".  
in: Barz et al. (1977), 27-43.

ZENK, M.H., EL-SHAGI, H. and SCHULTE, U. (1975)  
"Anthraquinone production by cell suspension cultures of Morinda citrifolia". 
ANTHOCYANIN PRODUCTION IN CELL CULTURES OF CATHARANTHUS ROSEUS.

Robert D. Hall and Michael M. Yeoman.

Department of Botany, University of Edinburgh, Mayfield Road, Edinburgh, Scotland.

INTRODUCTION

Many plant tissue cultures which have been reported to produce anthocyanin (see 1 for Refs.) appear to have resulted from the selection of a few spontaneous cell mutations. Such cultures can give us little information about the normal control of synthesis and accumulation of secondary products, a knowledge of which is essential if we are to exploit fully plant tissue culture as a viable means of secondary metabolite production. In this investigation suspension cultures of Catharanthus roseus which produce anthocyanin as a normal concomitant of growth were used as a model system to study certain aspects of secondary metabolism in vitro.

MATERIALS AND METHODS

Stock cultures (50ml. liquid medium in 250ml Erlenmeyer flask) were grown in the dark or in continuous low intensity white light (25 μM m⁻² sec⁻¹. Osram, coolwhite fluorescent) at 23°C. Murashige and Skoog (1962) minimal organic medium was used, supplemented with 3% sucrose and 0.4mg.1⁻² 2,4-D. Routine subculture was performed every two weeks.

Experiments were performed using 20ml. of medium in each 100ml. flask inoculated with 1ml of cell suspension taken from a single stock culture in the late linear phase of growth. Three flasks were harvested per sample. Packed cell volume (PCV) was calculated as the percent cell volume of the total culture volume following centrifugation at 1000g for 5 min. Anthocyanin levels were estimated using 0.5g (fr.wt.) of cells. These were extracted in 10ml of 1% HCl in methanol at 4°C overnight on a rotary mixer, centrifuged and the absorbance at 535nm. was determined using a non-pigmented cell extract as the reference blank.

RESULTS AND DISCUSSION

In this study anthocyanin production by the cultures was light dependant. Cultures grown in continuous darkness produced either very small numbers of pigmented cells or none at all. If however, these cultures were transferred to low intensity white light intense pigmentation appeared before the next subculture. It was observed that a lag period existed between the onset of illumination and the appearance of red cells within the cultures. The duration of this period appeared to be associated with the length of time elapsed from the previous subculture.

As can be seen anthocyanin only begins to appear at, or just before the division phase is completed. This effect cannot simply be explained in terms of a net illumination response as cultures grown in darkness for either 5 or 10 days prior to exposure to light produced pigmentation in close
parallel with those which had received illumination throughout the culture period (results not presented). Such a link between in vitro secondary product production and the growth phase of the culture has been discussed elsewhere (Yeoman et al. 1982). Preliminary analysis of the medium has revealed that at the time of anthocyanin appearance both N(NH₄⁺) and sucrose concentrations had fallen to c50% of the original values whereas the phosphate level was negligible. A link between the cessation of division, production of phenolics and the intracellular phosphate concentration in Nicotiana cells has been suggested by Knobloch et al. (1981) and such a link may also occur in the situation reported here.

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>14</th>
<th>16</th>
<th>23</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell no.*</td>
<td>0.92</td>
<td>1.17</td>
<td>1.72</td>
<td>3.34</td>
<td>3.80</td>
<td>3.73</td>
<td>4.02</td>
<td>3.89</td>
<td>3.94</td>
<td>3.75</td>
</tr>
<tr>
<td>%PCV</td>
<td>3.88</td>
<td>5.07</td>
<td>8.19</td>
<td>12.44</td>
<td>16.01</td>
<td>17.66</td>
<td>24.96</td>
<td>25.15</td>
<td>26.69</td>
<td>28.82</td>
</tr>
<tr>
<td>%Red Cells</td>
<td>5.87</td>
<td>3.16</td>
<td>1.28</td>
<td>0.62</td>
<td>0.44</td>
<td>1.80</td>
<td>2.49</td>
<td>4.68</td>
<td>5.30</td>
<td>5.65</td>
</tr>
<tr>
<td>Total Red cells x 10^-5</td>
<td>0.55</td>
<td>0.37</td>
<td>0.22</td>
<td>0.21</td>
<td>0.17</td>
<td>0.66</td>
<td>0.91</td>
<td>1.80</td>
<td>2.08</td>
<td>2.12</td>
</tr>
<tr>
<td>Total a/c.</td>
<td>0.37</td>
<td>0.33</td>
<td>0.34</td>
<td>0.39</td>
<td>0.50</td>
<td>0.71</td>
<td>1.09</td>
<td>2.05</td>
<td>2.15</td>
<td>2.22</td>
</tr>
</tbody>
</table>

Table 2. Growth and pigmentation of light-grown C.roseus suspension cultures following subculture. *ml⁻¹ x 10⁻⁶.

The stock culture used in the second experiment (Table 2) had been grown in continuous white light for 3 months and regularly produced 6-8% pigmented cells at the end of each subculture period. In these cultures the percentage of red cells decreased following subculture until the period of cell division was over after which it returned to the original level. This decrease cannot simply be a dilution effect resulting from the proliferation of non-pigmented cells as the estimated number of red cells per culture decreased in a similar manner. Levels were reduced to 30% of the original by day 8. There was however, no statistically significant change in the total anthocyanin level per culture until day eight indicating that no net accumulation had occurred.

These results clearly indicate that pigmented cells were capable of division and the fall in the number of red cells resulted from pigment dilution to visibly undetectable levels through cell growth and division. The absence of an initial increase in red cell number as might be expected following early red cell divisions resulted from the large number of weakly coloured cells present in the initial pigmented cell population which, after one division would cease to be visibly coloured and would subsequently not be counted.

CONCLUSIONS

The appearance of secondary metabolites in plant tissue cultures results from their production by a population of biochemically differentiated cells. From the results reported here it is clear from direct observations that this population forms only a small part of the total cell population. Also, it would appear that for at least some secondary products this differentiated state is not an end-point in the cell life cycle but is quite reversible when growth conditions are altered in favour of cell division. This is consistent with the theory of competition for nutrients between primary and secondary metabolic pathways as discussed by Phillips and Henshaw (1977) and Yeoman et al. (1980).

REFERENCES

DIFFERENTIATION AS A PREREQUISITE FOR THE PRODUCTION OF SECONDARY PRODUCTS BY PLANT CELL CULTURES

Professor M.M. Yeoman, Dr. K. Lindsey, R.D. Hall.

UNIVERSITY OF EDINBURGH.
DIFFERENTIATION AS A PREREQUISITE FOR THE PRODUCTION OF SECONDARY METABOLITES

1. WHAT DO WE MEAN BY DIFFERENTIATION?

We will consider differentiation at two levels.

A. The first may be recognised as changes in the shape, structure and function of individual cells, i.e., cytodifferentiation. This involves changes in rate and type of cell division (from 'proliferative' to 'quantal'), resulting in changes in subcellular organisation and in the pattern of metabolism (such as lignin formation; Roberts, 1976).

B. The second level of differentiation may be recognised as changes in the organisation of (one or more) cells, to form more or less morphologically recognisable structures. This process involves organized cell divisions (and requires defined limitations to division) and may incorporate cytodifferentiation (as in 'A' above).

2. WHAT IS THE EVIDENCE FOR A RELATIONSHIP BETWEEN SECONDARY METABOLITE PRODUCTION AND BOTH 'TYPES' OF DIFFERENTIATION?

Most of the evidence suggests a positive correlation between the organisation of cells and metabolite production (Yeoman et al., 1980; Lindsey, 1982). Cell cultures which undergo organogenesis produce secondary compounds in amounts and proportions approaching those found in whole plants (Hagimori et al., 1982; Kamo et al., 1982). Only suspended cells which have undergone substantial expansion, organisation, or aggregation, at the stationary phase of growth, accumulate significant levels of secondary compounds, to much higher levels than fast-growing and friable suspended cells (Thomas and Street, 1970; Al-Abta et al., 1979; Takeda and Katoh, 1981). Anthocyanin accumulation in cell cultures is also maximal in the stationary phase (Stickland and Sunderland, 1972), although the role of aggregation in this case is not yet clearly defined.

There is some evidence for a correlation between cytodifferentiation and accumulation. Occasionally specialised cells are observed in callus (e.g., of Pimpinella sp., Morinda sp., Ruta graveoleus) which are the sites of accumulation,
but not synthesis, of secondary products (Reinhard et al., 1968; Becker, 1970; Corduan and Reinhard, 1972; Neuman and Mueller, 1974). There is as yet little or no evidence for cytodifferentiation in single cell cultures.

This implies that secondary metabolite production and cytodifferentiation are analogous facets of differentiation (Ozeki and Komamine, 1981; Yeoman et al., 1982) and are both only expressed maximally if cells are organised.

3. HOW DOES CELLULAR ORGANISATION AFFECT THE EXPRESSION OF SECONDARY METABOLIC PATHWAYS?

What are the characteristics of organised groups of cells which would contribute to the production of relatively high yields of secondary metabolites, and what is the supporting evidence?

A. CELL-CELL COMMUNICATION

The most obvious difference between an aggregate of cells, an embryoid etc., and a suspension culture of (ideally) single cells is the extent of contact and presumably communication between the cells. The metabolism of plant cells is not regulated in the relatively simple way that microbial metabolism is; cells of the whole plant are subjected to a variety of physical and chemical gradients (both from the external environment and from within the plant itself), a large number of which affect the expression of the genome. Examples of 'regulatory gradients' are oxygen, carbon dioxide, light, growth regulators, temperature, water. Changes in the concentration or amounts of each of these factors will have an effect on cell metabolism in one way or another, by regulating enzyme activity, the rates of DNA and RNA synthesis, membrane permeability etc. Therefore, an isolated plant cell is not subject to the same regulatory signals (ie differences in the level of a particular stimulus) as is a group of cells.

B. REDUCED GROWTH RATE

Freely suspended cells divide at a faster rate than callus cells, and the rate of increase in fresh and dry weight of organised structures is much lower than that of disorganized cultures. Most of the cells within an aggregate of embryoid, or in compact callus, are probably quiescent or dividing only very
slowly. Furthermore, cultured cells differentiate morphologically primarily when growth (increase in fresh and dry weight and cell number) slows down or stops (just prior to stationary phase), the time when secondary metabolite accumulation is at a maximum (Yeoman et al., 1980; Fowler, 1981). Experimental induction of slow culture growth rate is often associated with both organogenesis and increased secondary metabolite production (Neuman and Mueller, 1974; Meyer-Teuter and Reinert, 1973; Aitchison, 1977; Mizukami et al., 1977; Knobloch et al., 1981; Ozeki and Komamine, 1981).

How could the reduced growth rate of organised cells increase the yields of secondary products? Firstly, the organised structure, and hence cell-cell contact, is a function of, and is maintained by the controlled growth rate; should the cells of the structure proliferate rapidly, organisation would break down and the levels of secondary products would decrease (Lindsey, 1982). Thus, reduced growth rate may be important only in so far as it maintains the organisation of cells. On the other hand, there is evidence that cell growth rate directly regulates secondary metabolism by affecting the kinetic partitioning of precursors between primary and secondary metabolic pathways. If primary pathways (e.g. to protein synthesis) are blocked, then common precursors can be diverted to secondary pathways (Phillips and Henshaw, 1977; Lindsey, 1982; Yeoman et al., 1982). This implies that the interrelationship between cell organisation and growth rate is important only in that slow growth (as a result of organisation) allows a diversion of precursors between metabolic pathways.

These two views, i.e. 1) that slow growth is necessary for organisation (and hence secondary metabolite production), and 2) organisation results in slow growth (and hence secondary metabolite production) are not mutually exclusive, viz:

**FIG. 1**

Changes in membrane permeability, enzyme activation

Leading to switch in pathways

Kinetic control over production

Cytoplasmic control over production

(eg physical compartmentation of enzyme and substrate)

slow growth necessary corollary Aggregation
C. GREENING

Occasionally, aggregation or embryoids are distinct from the rest of the culture in having a higher chlorophyll content, and greening of cultured cells usually takes place at the stationary phase (Laetsch and Stetler, 1965; Lindsey, 1982). Although there is some evidence for a positive relationship between greening and high yields (Idrisova et al., 1978), and indeed it might be expected that green cells would have a more complete metabolism than white cells, it is likely that greening is not a primary determining factor in the production of high yields (Staba and Jindra, 1968), unless the desired secondary products are synthesised within the chloroplast (Wink and Hartmann, 1982). Rather, greening, like secondary metabolite production and other aspects of cytodifferentiation, may be a corollary of cell organisation; or, to put it another way, may occur to the greatest extent only if the cells are organised and in communication with each other, and supplied with appropriate physical and chemical stimuli from the environment (Fig. 2).

4. HOW CAN THESE CONCEPTS AID THE CELL CULTURIST TRYING TO INCREASE YIELDS?

A culture system should be developed which satisfies a number of requirements:

1) Cells should grow in close association with each other.
2) Cells should grow slowly, physically stationary.
3) The physical and chemical environments should be manipulated with ease.

Such a culture system has been developed in our department, and involves the immobilisation of plant cells, either on flatbeds or in columns, using a variety of substrata (Lindsey and Yeoman, 1983). Liquid nutrient medium is supplied from a reservoir and washed over the physically stationary immobilised cells.
before being pumped back to the reservoir in a closed loop system. Cells have been immobilised on polyester fabric, nylon netting, polyurethane foam, and in calcium alginate and agar gels. This technique encourages slow cell growth, cell organisation, and increased yields of secondary products including capsaicinoids, tropane alkaloids and steroidal glycoalkaloids. Further advantages of the closed loop culture system include 1) the facilitated application of chemical treatments designed to mimic the inductive processes of differentiation which obtain within the whole plant, 2) the ability to supply large quantities of possibly toxic precursors at low concentration and 3) facilitated extraction of the desired product from the medium.
REFERENCES


